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ANNOUNCEMENTS

American Institute of Nutrition

26th Annual Meeting — *Atlantic City, N. J.*

April 14 (9 AM) to April 19 (12 N), 1962

Abstracts of papers to be presented at the meeting must be in the hands of the Secretary, Arnold E. Schaefer, Building 16-A, National Institutes of Health, Bethesda 14, Maryland by December 30, 1961.

APPLICATIONS FOR AIN MEMBERSHIP

Forms for AIN membership application, along with the necessary requirements and instructions, may be obtained by writing to the Secretary.

American Society for Clinical Nutrition

Second Annual Meeting — *Colton Manor Hotel*

Atlantic City, April 28 — 1 to 5 PM

Investigators are encouraged to submit abstracts for consideration by the program committee. These abstracts should be limited to 300 words or less and 7 copies submitted on or before February 1, 1962 to the Secretary, Robert E. Hodges, M.D., University Hospitals, Iowa City, Iowa.

Eugene Floyd DuBois
— *A Biographical Sketch*

(June 4, 1882–February 12, 1959)

Eugene Floyd DuBois was born in West New Brighton, Staten Island, New York, on June 4, 1882, in a large rambling house surrounded by spacious grounds comprising vegetable gardens, an orchard, cow pasture and stable housing cows, horses and carriages. In this home he spent his first sixteen years.

His youth was a happy one. The household was typical of the period. A maternal grandmother and paternal grandfather were members until their death when DuBois was entering adolescence. Servants were plentiful and relatives and friends came for long visits. An intellectually stimulating atmosphere predominated.

The boy's grandfather was editor and publisher of the New York Mail and Express. He was also a member of the New York State Board of Health and an early trustee of Cornell University. Interested in scientific farming, he instructed Eugene in an approach to many biological problems.

DuBois's mother had an unusually active mind. Although not formally schooled, she had traveled extensively in Europe and was an incessant reader. She knew German and French fluently and studied Spanish after she was seventy years old. Her son regarded her as the "best educated woman" he had "ever met."

His father died when DuBois was nine—memories of him were faint, except that he remembered his father as a man of scholarly tastes who was very well-informed.

The DuBois ancestry was of Huguenot or Walloon origin, later mixed with Dutch in America. These people came from the region of the French-Belgian border near the town of LaBassée, migrating from France to Germany, on to Leyden and then to the virgin country near Kingston, New York, where they helped found the town of New Paltz. They were farmers until DuBois's great-grandfather, as a boy, walked to New York City where he remained and prospered as a wholesale grocer and importer. He was one of the founders of New York University and also served as a governor of the New York Hospital.

DuBois believed that he had received his best education at home before he was twelve years old. At nine, he began his formal schooling, walking eight miles daily to and from the Staten Island Academy. He was transferred to the Milton Academy near Boston when he was sixteen and studied there for two years in an atmosphere which he found stiff and cold.

His interest in medicine as a career arose during an unusual experience he had when tending desperately ill soldiers for two weeks; these were survivors of the Cuban campaign, ill with typhoid fever and dysentery. He had been spending a summer vacation after school with his family at Mastic, Long Island, near Camp Wyckoff on Montauk Point, and had volunteered with his brother as an orderly.

His decision to devote four years to medical college caused him to concentrate on the humanities at Harvard where he took only minimal amounts of chemistry and biology. He regretted this later, feeling himself handicapped by poor training in chemistry, physics and mathematics.

When in 1902 he entered Columbia College of Physicians and Surgeons, admission to medical schools did not require a premedical education. High school graduates entered without difficulty. The classes were large, standards were low, and instruction was didactic with very little bedside teaching.

The one bright spot in his medical school period was a special summer course he took with Theodore C. Janeway, who instituted the method of clinical clerkship.

It is believed that DuBois was the first such clerk in New York.

After graduation in June, 1906, he spent six months in Berlin working in pathology under Kenke at the Charlottenburg Krankenhaus where he learned techniques and performed autopsies. Many other American doctors were taking postgraduate courses in the city at the same time.

When DuBois returned to New York to intern at Presbyterian Hospital, the MacKenzie polygraph was being introduced in cardiac arrhythmias. X-rays were rarely employed except for the study of fractures. Laboratory tests were simple but not very informative. This was the era of careful physical examination, however, and DuBois's clinical approach for the balance of his life reflected it.

By the time he was finishing as house physician, a great change had taken place in New York's medical atmosphere. The influence of the Rockefeller Institute and Hospital was being felt. Men like Graham Lusk, Samuel Meltzer, Janeway, Christian Herter and others, had formed the Harvey Society and important reforms in medical training were being instituted by schools which were trying to raise medical standards and limit the size of classes. Many of DuBois's fellow interns at Presbyterian Hospital were then mapping out careers in scientific medicine.

Shortly before he left the hospital, DuBois became engaged, fortunately to a young lady with great understanding. When he was in conflict about accepting a proffered position as assistant pathologist at the hospital or returning to Europe for another six months of study, she insisted that his plans for his career come first. A few days before he was to leave for Europe he met John Howland by chance. Howland suggested that the best opportunity for research training lay in the field of metabolism. Instead of going to France as he had planned, he urged DuBois to go to Berlin. This DuBois did, taking several short courses in bacteriology and metabolism, subsequently working with Theodore Brugsch in Kraus's Clinic with the Pettenkofer-Voit apparatus. Given the problem of finding the calorie requirement in diabetes, he found the technique especially difficult because of his inexperience. He later said that nothing would have come of the work if it had not been for the recalculation of the data and rewriting by Graham Lusk. This was typical of Lusk, he said, who was always generous in helping young men.

When in 1909, he returned to Presbyterian Hospital, he found that the whole spirit of the institution had changed. Eugene Opie of the Rockefeller Institute had become pathologist. With him were Jonathan Meakins and Russell L. Cecil. In the pathological laboratory DuBois took charge of routine bacteriology which, at the time, was rather crude. He also did some autopsies, and was left with little time for research. During the year he made various efforts to have a metabolism ward established where studies similar to those he had done in Germany could be made. The hospital was unable to undertake it but Graham Lusk, whom he had recommended to lead the research, managed to do so at the Cornell Medical Division of Bellevue Hospital. Lusk was a director of the Russell Sage Institute which had recently been forced out of the City Hospital because of a political dispute and persuaded the Sage board to support the research and affiliation with Cornell. In 1911, DuBois was appointed medical director of the Russell Sage Institute of Pathology. He held this post until he retired.

His first assignment was working with Warren Coleman on their now classical studies on the value of high calorie diets in typhoid fever. They gradually developed an efficient metabolism ward which other institutions subsequently copied. A room for the respiration calorimeter was constructed where they conducted a series of calorimeter studies of many different diseases.

On June 4, 1910, Rebeckah Rutter of Irvington-on-Hudson and DuBois were married. Unable to support a household on his small salary, he opened an office in his apartment to supplement his income. He practiced medicine a few hours a day.

A few days before World War II was declared, Du Bois began active service in the Navy in the Bureau of Medicine and Surgery. At the time he was serving as chairman of the National Research Council Committee on Aviation Medicine and

was in charge of the Department of Physiology at Cornell Medical College. His commission had been reactivated as captain in the Medical Corps, U. S. Naval Reserve and he was given the unusual privilege of going on active duty for three-month periods when he would not be needed for teaching at the college.

During service he was concerned with gas warfare training and defense, aviation medicine, deep diving and submarine ventilation. He was fondest of the latter activity, making several cruises off the American coast. One 96-hour submergence remained the record until atomic or nuclear powered submarines were brought into duty.

An avid teacher and investigator, DuBois has said he was usually thought of as a physiologist by clinicians and classified as a clinician by the physiologists. He was involved in many things which were forerunners of our current day understanding of metabolic conditions. The mathematical formula for determining surface area was devised by him and his cousin, Delafield DuBois, an electrical engineer and an ingenious, skillful technician. Another significant contribution made by him was mapping out the curve for changes in basal metabolism with age. This was published in 1916 and later incorporated into the Aub and DuBois standards which are in current use.

DuBois was proudest of the concept he and David P. Barr had worked out that the body could give off as much heat through a cool skin as a warm one. Severely challenged for a long time, it was finally proven true when Hardy developed his radiometer. Barr and DuBois stated that the body could change the skin and subcutaneous tissues into "a suit of clothes" by vasoconstriction. This is universally accepted today and is one of the important parts of the mechanism of heat regulation.

As a teacher, DuBois was particularly significant in applying to clinical medicine the principles of heat dissipation, metabolic factors and the life processes. He felt his "chief contribution was popularizing the simple, fundamental principles of metabolism in disease so that they eventually found their way into the textbooks and habits of thought."

In clinical medicine, one of DuBois's original observations was the recognition of the first case of hyperparathyroidism in America. In the field of therapeutics, his work was of considerable importance in the management of diabetes, thyroid diseases and particularly fever. His work on the physiology of fever and heat production is classical and is the basis for much of our thinking on this function of the body.

When DuBois, in 1939, transferred from the Department of Medicine at Cornell Medical College to become Professor of Physiology, he again had the opportunity to do research which he had been deprived of by the tremendous load of administrative work in the clinical fields.

Collecting antiques, old medical books and early American paintings were his hobbies. He was always an interested athlete but never a medal winner. In college he had rowed on the crew. He played squash until late in life and ran about a mile in Central Park before breakfast almost every morning of his life. This kept him in wonderful physical condition.

DuBois died February 12, 1959. His comment on the kind of life he led expressed the rare, modest man he was. To quote: "I have had an unusually placid uneventful life, fortunate in a sense of security. My family life has been all that a man could desire. The world has treated me better than any man deserves."

There are many stories that are told about Eugene DuBois. His students and residents admired and respected him at all times. Dr. Deitrick tells the story that at one time he was walking along one of the cross streets in New York and saw one of his residents moving himself into a new apartment. Dr. DuBois saw that the resident needed help. He pulled off his coat and helped to carry the furniture up one flight of steps to the new apartment. The resident was so embarrassed that he tripped and fell down the steps and the Professor had to help him to his feet.

On one occasion, when Dr. DuBois was making rounds with the House Staff, he asked them if they knew the best type of fireman's lift for carrying an unconscious patient down a flight of steps. The House Staff were either embarrassed or ignorant.

In any event, Dr. DuBois proceeded to toss one of the residents over his shoulder and carried him up and down the hall to demonstrate the proper method.

These examples demonstrate his direct approach to teaching and very practical problems.

Dr. DuBois, while himself conservative in his approach to life, recognized that the orthodox approach in medicine was not always the best. When one of his men, Edward Tolstoi, wrote the classical paper on the harmlessness of glycosuria in the treatment of diabetes, the criticism that was directed at Dr. Tolstoi was vituperative from many people. Dr. DuBois rose to his defense and wrote to many critics that he had seen the orthodox treatment of diabetes change at least every decade, and the novel approach of Dr. Tolstoi was important for the progress of medicine.

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Algae Feeding in Humans [1,2]

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The use of microalgae as a food source for humans has been considered for overpopulated countries (Spoehr, '51; Burlew, '53), and for space travel (Haldane, '51). There are several suggested advantages. If algae is grown under proper environmental conditions, the protein yield from it may be quite high (Spoehr and Milner, '49). Animal studies indicate that this protein, although sometimes deficient in methionine, compares favorably with cereal protein in quality (Fisher and Burlew, '53; Geoghegan, '53; Fink and Herold, '57; Tamura et al., '58a). The potential growing season is unlimited and the possible yield per acre is excellent when compared with higher plant life (Tamiya, '57, '59; Ryther, '59). Because of its photosynthetic capabilities, algae may be useful as a respiratory gas exchanger as well as a food source in closed ecological systems (Myers, '54; Gaume, '58; Brockman et al., '58).

In this paper the experience gained from feeding algae to human volunteers will be discussed. Both acceptability and acute toxicity data are presented.

METHODS

The algae used in this study was kindly furnished by the Japan Nutrition Association.[3] It was a homogenous mixture of *Chlorella* and *Scenedesmus* strains cultured by the "open circulation method" (Kanazawa et al., '58). After harvesting, centrifugation, washing, heat treatment at 100°C for two minutes, and vacuum drying, the final product was a green powder appearing amorphous and without definite cellular structure under the microscope (fig. 1). Proximate analysis is shown in table 1.

Cultures from the powdered algae for bacteria and fungi contained no pathogens. Preliminary tests in rats revealed no histologic evidence of toxicity when the algae was fed as the only food source for three weeks. As a further precaution, the algae was autoclaved at 160°C for two hours.

TABLE 1
Analysis of powdered algae

	gm/100 gm
Protein[1]	59
Fat	19
Carbohydrate	13
Moisture	3
Ash	6
Bomb calorimetry	5.5 Cal./gm

[1] Total nitrogen × 6.25.

The subjects were 5 healthy young men, 18 to 23 years of age, who weighed on the average 74.1 kg (range 63.0 to 90.5 kg). They were housed on a metabolic ward but were permitted off the ward for supervised activities. One subject, because of prior commitments, was dropped from the study at the end of the third week.

The study was divided into 7 periods in which all subjects completely consumed diets supplemented with algae in increasing amounts. In the first period (control) a basic diet containing no algae was fed for 5 days. This diet contained a total of 3190 Cal., 91 gm of protein, 315 gm of carbohydrate, and 167 gm of fat for each man daily. In succeeding periods,

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[1] Presented at the 24th Annual Meeting of the American Institute of Nutrition, Chicago, 1960.

[2] The volunteer subjects in this study were provided through the cooperation of Dr. Norman E. Witt, Professor of Chemistry, University of Colorado, and the Surgeon General, U. S. Army, (contract no. DA-49-007-MD-549).

[3] Courtesy of Dr. H. Tamiya of the Japan Microalgae Research Institute, Dr. H. Nakamura of the Japan Nutrition Association, and the United States Army 406th Medical General Laboratory, Japan.

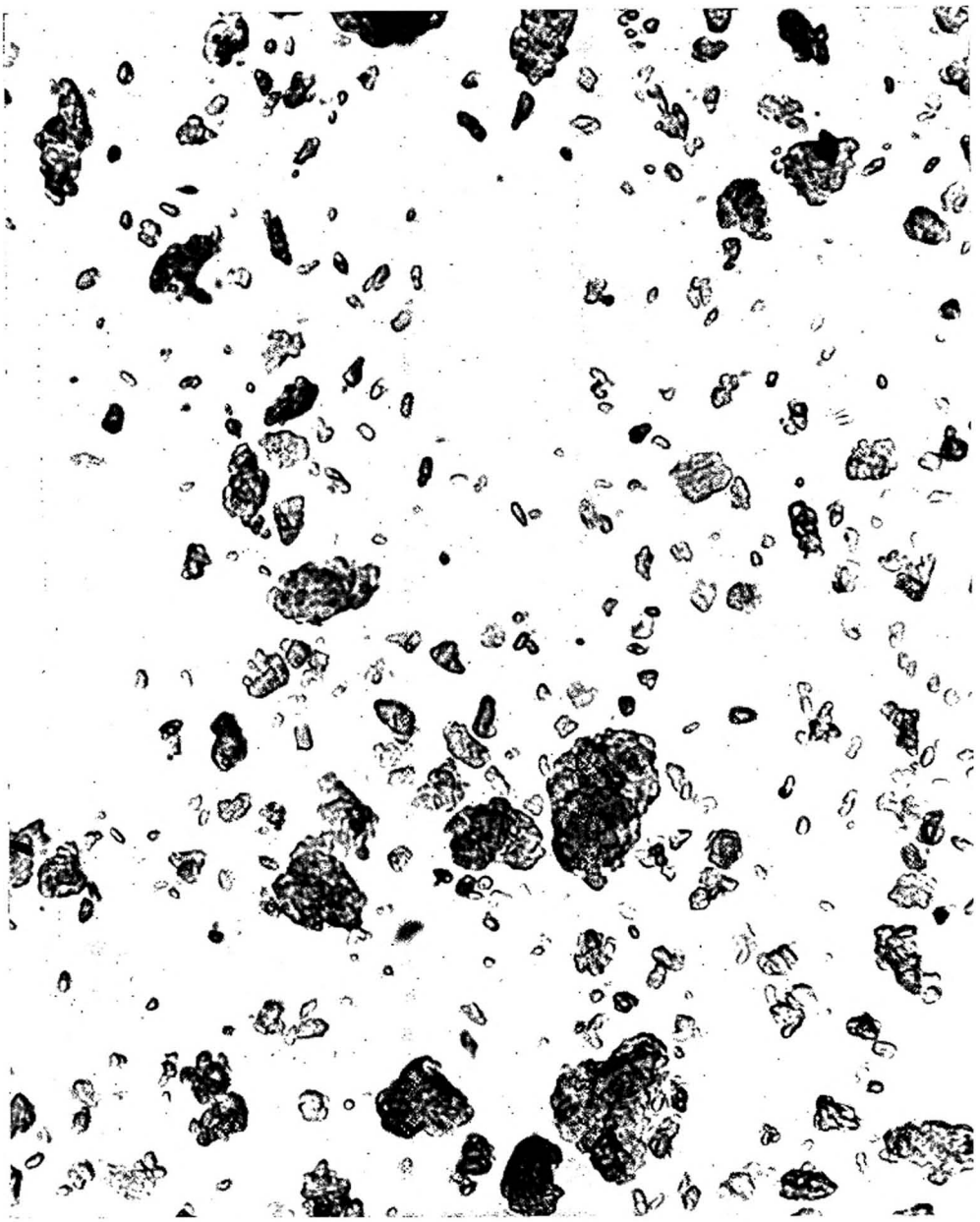

Fig. 1 A microscopic preparation (water suspension) of the heat treated, dried algae. × 270.

algae was added to this diet at levels of 10 gm, then 20, 50, 100, 200, and 500 gm per man per day (fig. 2). As algae was added, the basic diet was adjusted to keep the total calorie and protein content in each period relatively constant. In the 500-gm algae feeding period, protein intake was by necessity increased because of the high-protein content of algae. The second, third, and 4th periods (10, 20 and 50-gm algae diets) were 6-day periods. The 5th and 6th periods (100- and 200-gm algae diets) were three-day periods, and the 7th period (500-gm algae diet) was

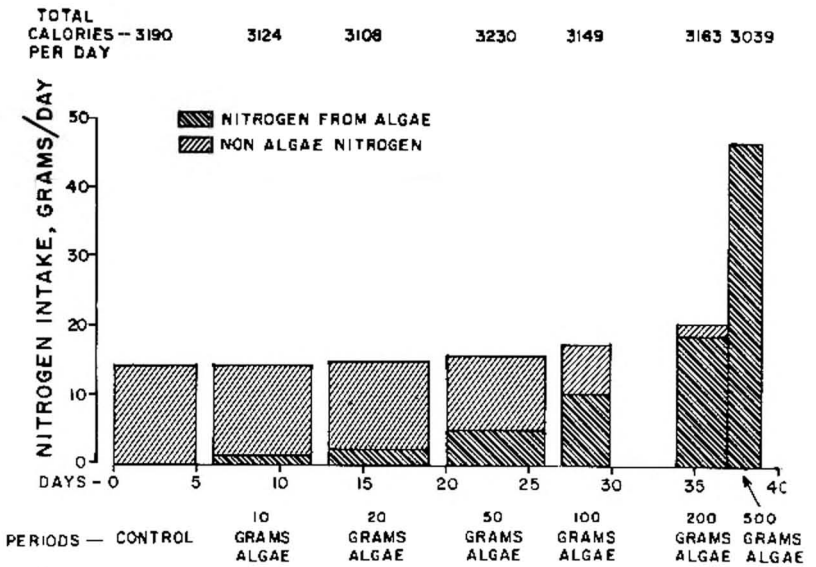

Fig. 2 Algae feeding schedule showing the daily total caloric and nitrogen intake for each man.

two days in duration. All periods except the last two were separated by one day free of dietary restrictions. Four days were permitted between the 5th and 6th periods (fig. 2).

Algae was added, before baking, to gingerbread, chocolate cookies, and chocolate cake in amounts up to 25 gm per normal serving (100 gm). Algae was also added to cold milk and water in amounts up to 75 gm per glass (200 cm^3). After vigorous stirring, this was consumed directly.

Throughout the study frequent physical examinations were performed. In addition, the following clinical laboratory procedures were obtained: complete blood counts, reticulocyte counts, urinalyses including urobilinogen and benzidine tests, and liver function tests including total and direct bilirubin, thymol turbidity, prothrombin time, bromsulfalein retention, and serum glutamic oxaloacetic transaminase. These tests were performed twice weekly, using standard methods (Hepler, '49; Simmons and Gentskow, '55).[4] Daily urine volumes were recorded and 24-hour stool collections were obtained for proximate analysis (AOAC, '55) and bomb calorimetry (Cleland and Harding, '57). Quantitative estimates of the bacterial content of stool homogenates were made by diluting fresh stool with sterile saline and making pour plates. Violet Red Bile Agar was used to estimate the number of coliform colonies and Trypticase Soy Agar was used to determine the total bacterial count.

RESULTS

All subjects tolerated diets supplemented with algae in amounts up to 100 gm per man per day. Even at the 10- and 20-gm feeding levels, however, certain symptoms were noted. The algae taste was strong and somewhat disagreeable. It was dominant in all foods and was compared to bitter spinach or strong green tea. This taste tended to linger several hours. After several days, however, the subjects grew more accustomed to the taste of algae and soon tolerated it quite well. Algae discolored food to which it was added. Chocolate cake, for example, turned olive drab in color. Abdominal distention, associated with increased eructation and flatulence, was noted early in the study. During the 4th period (50-gm algae level) bowel movements increased in quantity. The stools were not loose or watery; on the contrary, they were bulky and dry.

At the 200-gm feeding level, all the above mentioned symptoms were more severe. In addition, nausea was noted. One subject suffered diffuse lower abdominal cramping pains, increased flatulence,

[4] Sigma Technical Bulletin 505-5-60 1960 Sigma Chemical Company, St. Louis, Missouri.

TABLE 2

Representative fecal excretion data for subject HY

Period	Wet stool weight	Dry stool weight	Ash	Fat	Nitrogen	Carbohydrate	Total
	gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*Cal./day*
Control	195	49	6.3	6.2	5.6	1.8	257
Algae, 50 gm	284	74	8.3	7.5	7.1	13.8	401
Algae, 100 gm	282	85	8.6	6.7	8.8	14.6	476
Algae, 200 gm	393	136	12.5	15.3	15.2	22.2	806
Algae, 500 gm	529	217	19.6	20.8	22.4	36.4	1272

nausea, and persistent vomiting. He was forced to discontinue the study. At the 500-gm level, another subject could not tolerate the algae because of similar complaints. Two subjects, however, completed this two-day period. Even they noted nausea, mild abdominal cramping pain, headache, malaise, and hard bulky stools. Bowel evacuation caused some rectal pain. One of the authors (M.E.M.) attempted the 500-gm feeding level but was able to consume only 350 gm the first day and 270 gm the second day because of malaise, headache, and gastrointestinal symptoms. Except for headache and malaise (in the last period) all symptoms were confined to the gastrointestinal tract and disappeared within 48 hours after the algae was discontinued. It was further noted that symptoms were most marked at the beginning of each period, and for this reason, free days were eliminated between the last two periods. Physical examinations failed to reveal any abnormalities other than those associated with the gastrointestinal tract. The subjects lost from one to two kilograms in body weight during this study.

There was no change in water intake or urine excretion throughout the study. Because all laboratory tests (blood counts, urinalyses, and liver function studies) remained within normal limits, they are not listed in detail here.

Bowel excreta turned dark green in color by the second day of the second period (10-gm of algae) and remained green until algae was discontinued from the diet. The average daily stool weight increased more than twofold in the 6th and 7th periods. Moisture content decreased from 75 to 65% so that the dry weight of the stool was also increased. In addition, proximate stool analysis showed an absolute increase in ash, fat, nitrogen, carbohydrate and calorie content during the higher algae feeding periods. Representative fecal excretion data for one of the subjects who tolerated the 500-gm feeding level are shown in table 2. Coliform counts and total bacterial counts on stool homogenates did not change significantly.

DISCUSSION

Despite a great deal of work on the growth, physiology, and composition of algae, and much speculation about the potential uses of algae as a food, relatively few human feeding studies have been reported. Most of these have been limited to taste tests and rough estimates of nutritive value. For many years, Orientals have eaten seaweed (macroscopic algae) and some varieties are considered a delicacy. "Plankton soup," consisting largely of green algae, was fed in amounts up to 35 gm per man daily to patients in a Venezuelan leper colony for one to three years (Jorgensen and Convit, '53). In many patients acceptability was good, and increased energy, weight gain, and improved general health was noted. Toxic effects were not described. Morimura and Tamiya ('54) reported that *Chlorella* cells taste similar to powdered green tea, or to dry "aonori," a seaweed commonly used as a seasoning in Japanese cookery. The *Chlorella* was added to soups, French bread, noodles, cookies, and ice cream, though only in small amounts. These foods were acceptable, having an agreeable taste and appearance. In later reports from Japan (Tamura et al., '58b; Hayami and Shino, '58), adverse factors were mentioned. An algae-supplemented diet (30 gm) was poorly accepted by 5 humans because of its odor, flavor, and color.

To our knowledge, human feeding studies with more than about 35 gm of algae per man per day have not been reported. It was therefore elected to vary over a wide range the quantity of algae used to supplement our diets in order to determine whether the higher algae levels were tolerable. Unexpectedly, levels up to 100 gm per man daily were tolerated quite well. In preparation for this study, algae was added to a variety of recipes including white and rye bread, tomato aspic, a spinach casserole, a variety of soups, pizza, and ice cream. It was most acceptable, however, when added to gingerbread, chocolate cookies, and chocolate cake. Amounts up to 25 gm per normal serving were achieved. It should be emphasized that, although acceptable, the taste of algae was quite dominant and the cake and cookies turned olive drab in color. It was also found that algae added to cold milk in amounts up to 75 gm per glass was tolerable though distasteful. Vigorous stirring was required immediately before consumption. At the 500-gm level we attempted to feed algae as the only food source. Thus, algae suspended in cold water was consumed, allowing a small amount of cherry or strawberry syrup as a flavoring agent when necessary. This was not well tolerated.

With respect to acceptability, the authors feel that algae in amounts up to 100 gm per man per day can be well tolerated at least for a short time. Although the taste and odor were moderately unpleasant at first, all volunteers soon grew accustomed to them. The greenish discoloration of foods was not a real problem. Gastrointestinal symptoms were not a serious problem at these lower levels. That abdominal bloating and flatulence were most pronounced at the beginning of each period suggests the possibility of acquiring a tolerance to algae. Longer feeding periods will be required to settle this issue. At the 200- and 500-gm feeding levels, algae was not acceptable and gastrointestinal symptoms were quite disagreeable. Certainly, then, in the form tested, algae would not be suitable as a sole food source even if nutritionally adequate.

Nevertheless, algae may have value as a food supplement for populations where dietary protein is limited. Proximate analysis of the algae used in this study suggested a relatively high-protein content. Assuming most algae nitrogen to be protein nitrogen and assuming good digestibility, a supplement of approximately 50 to 60 gm of protein could be provided by adding 100 gm of this algae to the daily ration. Our results would not permit a generalization for all microalgae since the protein content is variable, depending on species and the production methods employed.

The results of our acute toxicity studies can be stated simply. Physical examinations failed to reveal any abnormalities other than those associated with the gastrointestinal tract. Even these were transient and disappeared soon after algae was discontinued. All laboratory tests remained within normal limits. The subjects were followed from one to 6 months after this study and no evidence of toxicity was found.

Diarrhea was not encountered. On the contrary, bowel evacuations were difficult because of hardness and increased bulk. Accurate digestibility data were, of course, not obtained in this study because of the short periods and the marked gastrointestinal symptoms encountered at the higher feeding levels. Stool excretion data (Table 2), however, certainly suggest a decrease in digestibility for all macronutrients at the higher algae feeding levels.

It should be emphasized that improved methods for processing algae may render the final product more acceptable in terms of taste, odor, color, and digestibility. Minami ('59) pointed out that *Chlorella* has a hard, indigestible (cellulose) membrane and this may account in part for decreased digestibility. Electron microscopy studies of fecal specimens from *Chlorella*-fed men and rats have shown that the cellular membrane remains intact when dried algae is consumed (Tamura et al., '58c). For this reason a variety of processing methods are currently under investigation. It is also possible that other species of algae will be more acceptable and digestible. It will, therefore, be necessary to test further algae feeding in humans, using several species processed in

various ways. Only when this information is available, can we properly evaluate the use of microalgae as a food source for humans.

SUMMARY

Human feeding of algae was studied in volunteers by adding algae as a supplement to the diet in amounts varying from 10 to 500 gm per man per day. Although the bitter, strong, spinach-like flavor predominated in all foods supplemented with algae, the most acceptable preparations were cookies, chocolate cake, gingerbread, and cold milk. Amounts up to 100 gm per man daily were tolerated by all.

When larger amounts were added, gastrointestinal symptoms were more prominent. These included nausea, vomiting, abdominal distention, flatulence, lower abdominal cramping pains, and bulky hard stools. No other evidence of toxicity was found and the gastrointestinal symptoms disappeared shortly after the algae was discontinued. It was concluded that algae in this form (heat treated, dried algae) can be tolerated as a food supplement but further processing will be necessary if algae it to be useful as a major food source. Methods to improve both acceptability and digestibility are needed.

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Effect of Trypsin Supplementation on the Utilization by the Chick of Diets Containing Raw Soybean Oil Meal[1]

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The detrimental effects that result from including raw soybean oil meal in the diet of the chick and other monogastric animals have long been recognized. The growth-inhibiting materials present in the raw meal are heat labile, since moist heat treatment will greatly improve the nutritive quality of extracted soybeans for these animals (Wilgus et al., '36).

The presence of potent trypsin inhibitors in raw soybean oil meal was first discovered by Bowman ('44) and by Ham and Sandstedt ('44). A soybean trypsin inhibitor was isolated, crystallized and characterized in extensive investigations by Kunitz ('45, '46, '47). Ham and others ('45) and Borchers et al. ('48) were able to inhibit the growth of chicks and rats by supplementing their diets with crude preparations of trypsin inhibitor. Westfall and Hauge ('48) found that the protein efficiency of soybean flour for the mouse was inversely proportional to the trypsin inhibitor content of the flour. Westfall et al. ('48) fed mice with protein hydrolysates supplemented with crude preparations of soybean trypsin inhibitor and observed a decreased growth rate and protein efficiency. These authors suggested that the action of the trypsin inhibitor was not necessarily associated with poor hydrolysis of dietary protein. Several attempts have been made to overcome the growth-depressing properties of raw soybean oil meal by trypsin supplementation. Borchers and Ackerson ('51) reported that 5% of trypsin powder improved the growth of rats fed diets containing 25% of raw soybean oil meal. Autoclaving the trypsin powder, however, did not destroy its beneficial effect on growth even though its proteolytic activity was destroyed. Almquist and Merritt ('52a), using a diet in which only ¼th of the soybean protein was in the raw form, were able to overcome the growth depression caused by the raw meal with relatively small amounts of a crude trypsin preparation. Later work by these same workers showed that a crystalline trypsin preparation was effective in improving growth of chickens receiving raw soybean oil meal (Almquist and Merritt, '53). Renner and Hill ('60) reported that the metabolizable energy of extracted raw soybean flakes for the chick was considerably lower than the metabolizable energy for heated flakes. These workers suggested that this indicated there was impaired digestion or absorption of raw soybean oil meal by the chick. In view of the work by Renner and Hill in which heat treatment had such a marked effect on metabolizable energy of raw soybean oil meal, it was considered that measurements of metabolizable energy might be a useful tool to evaluate other factors than heat treatment that have been reported to affect nutritive value of raw soybean oil meal. The experiments described in this paper were designed to determine whether trypsin supplementation of diets containing raw soybean oil meal would improve their metabolizable energy yield for the chick.

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EXPERIMENTAL

Male Rhode Island Red × Barred Plymouth Rock chicks purchased from a commercial hatchery were used in all experiments. Chicks were housed in battery brooders provided with raised wire floors and thermostatically controlled heating units. In experiments 1, 2 and 3, the chicks were raised for one week with a preliminary diet, after which they were individually weighed and allotted to the experimental groups on the basis of body weights. In experiment 4 the experimental diets were fed from one day of age. Feed and water were supplied ad libitum. The composition of the diets used is given in table 1. All experimental diets were mixed on a dry matter basis and the trypsin supplementations were made at the expense of glucose. Chromic oxide was incorporated at a level of approximately 0.3% as an index material in order to eliminate the need for quantitative collection of excreta (Dansky and Hill, '52). Body weight and feed consumption of experimental groups were recorded weekly, and chicks were weighed individually when the experiment was completed. Excreta were collected on 4 consecutive days during the last experimental week. The metabolizable energy of the experimental diets was determined by methods described by Hill and Anderson ('58). At the end of each experiment a random sample of chicks from each group was sacrificed for determination of pancreas weight. The pancreas was removed immediately after the chick was killed, and weighed in the fresh state. In each experiment, pancreas weights were adjusted by analysis of covariance (Steel and Torrie, '60) to eliminate effects due to differences in body weight in comparisons of treatment effects. Pancreas weights adjusted to the same body weight are given in all tables.

TABLE 1

Composition of experimental diets

Ingredients	Preliminary diet	Experimental diet
	%	%
Glucose[1]	52.74	32.41
Soybean oil meal (50% protein)	16.00	50.00
Soybean oil, degummed	—	10.00
Crude casein	11.00	—
Menhaden fish meal	4.00	—
Gelatin	2.50	—
Fish solubles	2.00	—
Brewers' dried yeast	2.50	—
Dried whey	2.00	—
Corn oil	2.50	—
Glycine	—	0.50
DL-Methionine	—	0.13
Dicalcium phosphate	1.00	2.50
Calcium carbonate	2.00	1.00
Iodized salt	0.64	0.50
Mineral mixture	0.37[2]	1.14[3]
Vitamin mixture	0.50[2]	0.50[3]
Manganese sulfate	0.03	0.02
Chromic oxide "bread"[4]	—	1.00
Antioxidants (BHT + DPPD)	0.02	0.02

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

[2] Vitamin and mineral mixture supplies in milligrams per 100 gm of diet: choline Cl, 900; niacin, 3.0; Ca pantothenate, 2.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 0.5; folic acid, 0.2; menadione, 0.1; biotin, 0.04; vitamin B_{12}, 0.001; vitamin A, 1000 I.U.; vitamin D_3, 150 I.C.U.; *d*-α-tocopheryl acetate, 2.4; K_2HPO_4, 220; $MgSO_4$, 120; $Fe_2SO_4 \cdot 7H_2O$, 28; $CuSO_4 \cdot 5H_2O$, 0.8; ZnO, 6; $NaMoO_4 \cdot 2H_2O$, 0.8; NaI, 0.3; Na_2SeO_3, 0.02; $CoCl_2 \cdot 6H_2O$, 0.2.

[3] Renner and Hill, ('60).

[4] Contains 30% Cr_2O_3.

The levels of trypsin used were based on amounts reported by Almquist and Merritt ('52a) to be sufficient to improve chick growth with diets containing raw soybean oil meal. None of the trypsin levels used, 0.1 to 0.8%, had any effect on chick growth rate or metabolizable energy value of the experimental diet.

The lack of effect of supplemental trypsin in this experiment did not confirm the report by Almquist and Merritt ('52a). In an attempt to explain the difference in results, the *in vitro* relationship of the added dietary trypsin to the amount of trypsin inhibitor present in the soybean oil meal was determined. Trypsin inhibitor assays were carried out on the raw meal by a slight modification of the technique described by Borchers et al. ('47). A comparison of the trypsin inhibitor content of the meal and the ability of the trypsin preparation to neutralize the trypsin inhibitor activity *in vitro* indicated that the

highest level of supplemental trypsin used in the first experiment was able to neutralize only ¼th of the total inhibitor content of the diet. Therefore, it seemed likely that the lack of response to trypsin supplementation in the above experiment may be attributed to the excess of the inhibitor present in the raw soybean oil meal.

A second experiment was conducted to determine whether a lower level of raw soybean oil meal in the diet would give the same effect as higher levels so that the use of excessively high levels of the crude trypsin preparation could be avoided. Almquist and Merritt ('52b) reported that 5% of protein from raw meal was enough to give nearly maximum growth depression in a diet containing 20% of protein from soybean oil meal. Therefore, the soybean meal component of the diet was varied from all raw meal to all heated meal in ratios of 0/5, 1/4, 2/3, 3/2, 4/1 and 5/0. Three groups of 8 chicks each were fed the experimental diets; the experimental period lasted three weeks, the chicks starting to receive the experimental diet when one week of age. Data obtained from the various criteria of measurement are given in table 2. Body weight data and metabolizable energy values were subjected to analysis of variance and the linear and quadratic components of treatment effects were determined. The body weight data indicated that each increment of raw soybean oil meal replacing heated soybean oil meal resulted in a further depression of chick growth. Contrary to the results of Almquist and Merritt ('52a) the response to levels of raw soybean oil meal in the diet was essentially linear ($P < 0.01$) with only a slight suggestion of less growth depression after the ratio of raw soybean oil meal to heated meal had reached 3/2. The metabolizable energy and nitrogen utilization figures also show a step-wise decrease as the raw soybean oil meal content of the diet increases. By the time the raw soybean meal content of the diet reached 4/5ths, however, most of the depression in nitrogen utilization and in metabolizable energy had occurred. The pancreas weight of the chicks was also increased by the addition of raw soybean oil meal but by the time the raw soybean oil meal content of the diet reached 3/5ths, by far the largest increase in pancreas size had already occurred. The increase in pancreatic size in response to raw meal confirmed the observations of Chernick et al. ('48).

The results of this experiment did not reveal any one dietary level of raw soybean meal that could be used to give the maximum effect from raw meal. The proportion of 3/5ths raw meal to 2/5ths heated meal was chosen for future experimental diets to study trypsin supplementation since, beyond this level of raw soybean meal in the diet, the effect on the criteria measured was relatively less than with lower levels of raw soybean meal.

In vitro trypsin inhibitor assays of the sample of raw soybean oil meal indicated that 2.4% of the trypsin 1:300 preparation would be required to neutralize all of the trypsin inhibitor present in a basal diet containing 30% of the raw soybean oil meal. Therefore an experiment was designed to test the effect of levels of 1.2, 2.4 and 3.6% of the trypsin 1:300 concentrate on the response of chicks receiv-

TABLE 2

Proportion of raw to heated soybean oil meal and chick response

Treatment	Body weight, 4 weeks	Feed consumption	Feed/gain	Adjusted pancreas weight	Nitrogen retention	Metabolizable energy
	gm	*gm*		*gm*	*% of intake*	*Cal./gm dry matter*
5/5 Raw soybean oil meal[1]	367	642	2.39	2.86	32	3.19
4/5 RSOM + 1/5 HSOM[2]	391	657	2.25	2.75	32	3.20
3/5 RSOM + 2/5 HSOM	402	665	2.19	2.62	37	3.31
2/5 RSOM + 3/5 HSOM	440	667	1.95	2.05	40	3.40
1/5 RSOM + 4/5 HSOM	461	656	1.81	1.56	45	3.54
5/5 Heated soybean oil meal	507	608	1.49	0.90	51	3.65

[1] RSOM.

[2] Heated soybean oil meal.

ing the basal diet in which 3/5ths of the soybean oil meal was in the raw form. Additional treatments included 2.4% of inactivated trypsin added to the diets containing raw meal and the diet with all heated meal. Inactivated trypsin was prepared by autoclaving the trypsin at 121°C for 30 minutes. The resulting material had no proteolytic activity as determined by the method of Anson ('38). Each treatment was given to three groups of 8 chicks each and the experimental period lasted from one to three weeks of age. The results of this experiment are given in table 3. Growth data, metabolizable energy values and nitrogen retention data were analyzed statistically by analysis of variance (Steel and Torrie, '60) and treatment differences were evaluated by Duncan's multiple range test (Federer, '55). Again, chicks receiving the heated meal basal diet grew considerably faster than those receiving the raw meal basal ($P < 0.01$). The addition of either active or inactivated trypsin did not improve the growth of chicks receiving the diets containing raw soybean oil meal. When an active trypsin preparation was included in the diet containing all heated soybean oil meal, however, the growth rate and feed consumption of chicks receiving this diet were severely depressed. This depression was not observed when the inactivated trypsin preparation was added to the heated meal basal diet. Although the addition of trypsin to the basal diet did not improve growth of chicks receiving the raw meal, the feed consumption of groups fed the diets containing 2.4 or 3.6% of the trypsin preparation was depressed, and this was reflected in improved feed efficiency of those receiving the higher levels of trypsin. The inactivated trypsin did not improve feed efficiency. The metabolizable energy of diets containing raw soybean oil meal was significantly improved ($P < 0.01$) by the addition of 2.4 or 3.6% of the active trypsin preparation but not by inactivated trypsin. There was no effect on metabolizable energy of the diet by either active or inactivated trypsin when the diet contained all heated soybean oil meal. This was true even though the active trypsin preparation considerably reduced the growth rate of chicks receiving the heated meal.

The feces of the chicks receiving the raw meal basal diet were observed to be quite high in gross energy and appeared to be high in fat. Therefore, the absorbability of the soybean oil in the diet was determined (table 3). Absorbability of the added soybean oil in the basal diet was considerably depressed by the raw soybean oil meal. Supplementing the raw meal basal diet with 2.4 or 3.6% of trypsin markedly improved the absorbability of this added soybean oil, whereas the trypsin did not affect the absorbability of the soybean oil in the diet containing heated meal. The inactivated trypsin appeared to improve the absorbability of the soybean

TABLE 3

Response of chicks to levels of a trypsin 1:300 concentrate

Treatment	Body weight, 3 weeks	Feed consumption	Feed/gain	Adjusted pancreas weight	Nitrogen retention	Metabolizable energy	Apparent fat absorbability	Adjusted metabolizable energy[1]
	gm	*gm*		*gm*	*% of intake*	*Cal./gm dry matter*	*%*	*Cal./gm dry matter*
Basal raw	236	340	2.46	1.96	34	3.05	73	3.30
+1.2% trypsin 1:300[2]	241	345	2.38	1.96	39	3.10	74	3.36
+2.4% trypsin 1:300	239	306	2.17	1.72	39	3.30	88	3.38
+3.6% trypsin 1:300	235	285	2.06	1.72	39	3.28	91	3.34
+2.4% inactivated trypsin	247	352	2.34	2.07	28	3.04	83	3.18
Basal heated	316	332	1.52	0.77	48	3.64	96	3.64
+2.4% trypsin 1:300	209	192	1.71	1.02	46	3.73	96	3.72
+2.4% inactivated trypsin	328	354	1.54	0.82	48	3.64	96	3.64

[1] Adjusted to 96% fat absorbability.

[2] Purchased from Nutritional Biochemicals Corporation, Cleveland.

oil in the diet containing raw soybean oil meal to an intermediate level.

Since the fat portion of the diet seemed to be affected by the trypsin supplementation, it seemed desirable to compare the metabolizable energy value of the diets at the same fat absorbability in order to determine whether trypsin supplementation improved the utilization of the nonfat components of the diet. Therefore, all of the metabolizable energy values were adjusted to 96% fat absorbability, and these figures are also included in table 3. These data show that there is relatively little effect on the metabolizable energy of the diets containing raw soybean oil meal by added trypsin, when differences in fat absorbability are removed. The metabolizable energy of the diet containing the inactivated trypsin is significantly less ($P > 0.05$) than the other diets containing raw meal when the fat absorbability is adjusted to 96%. This difference cannot be readily explained.

The active trypsin preparation caused an improvement in percentage of absorbed nitrogen retained by chicks fed the raw meal basal diet, whereas inactivated trypsin did not. The adjusted pancreas weights were significantly lowered by the addition of 2.4 or 3.6% of an active trypsin preparation ($P < 0.05$) as determined by analysis of covariance, and the application of Duncan's multiple range test. The effect of trypsin on the pancreas weight was relatively minor compared with the effect of raw meal on pancreas weight.

Because of the results obtained in the previous experiment, an experiment was conducted in which 0.75% of a crystalline trypsin preparation was added to a basal diet containing a ratio of 3/5ths raw to 2/5ths heated meal or all heated soybean oil meal. These diets were fed to three groups of 6 chicks each from one day to two weeks of age. Based on *in vitro* assay, the levels of crystalline trypsin fed were equivalent in trypsin activity to the 2.4% of the trypsin concentrate fed in the previous experiment.

The results of this experiment are included in table 4. The crystalline trypsin preparation markedly depressed growth and feed consumption of chicks receiving either raw or heated soybean oil meal. Crystalline trypsin depressed growth rate slightly less when chicks received diets containing raw soybean oil meal as compared with heated soybean oil meal. The metabolizable energy of the diet containing raw soybean oil meal was improved by the addition of the crystalline trypsin preparation, whereas the metabolizable energy of the heated meal basal diet was not affected. The absorbability of the soybean oil included in the basal diet was again drastically reduced by the raw soybean oil meal, however, and the crystalline trypsin preparation markedly improved the absorbability of the soybean oil. When the metabolizable energy values were adjusted to a uniform 96% fat absorbability, the effect of trypsin supplementation on metabolizable energy again disappeared. The efficiency of nitrogen utilization by chicks receiving the raw meal basal diet plus crystalline trypsin was improved, although when the crystalline trypsin was included in the heated meal basal diet, nitrogen utilization was slightly decreased. The

TABLE 4

Response of chicks to crystalline trypsin

Treatment	Body weight, 2 weeks	Feed consumption	Feed/gain	Adjusted pancreas weight	Nitrogen retention	Metabolizable energy	Apparent fat absorbability	Adjusted metabolizable energy[1]
	gm	*gm*		*gm*	*% of intake*	*Cal./gm dry matter*	*%*	*Cal./gm dry matter*
Basal raw	123	169	2.07	0.66	37	2.72	49	3.23
+0.75% crystalline trypsin[2]	78	79	2.02	0.66	43	3.13	82	3.29
Basal heated	168	168	1.33	0.32	56	3.58	91	3.63
+0.75% crystalline trypsin	59	50	2.28	0.80	46	3.54	94	3.57

[1] Adjusted to 96% fat absorbability.

[2] Trypsin, 2X cryst. (salt free) purchased from Nutritional Biochemicals Corporation, Cleveland.

pancreas weights were unaffected by the addition of crystalline trypsin to the diet containing raw soybean oil meal, and appeared to be considerably increased by the addition of crystalline trypsin to the heated meal basal diet.

DISCUSSION

These experiments indicate that trypsin is not effective in reversing the growth inhibition of chicks receiving raw soybean oil meal. In this study, addition of trypsin to diets containing raw soybean oil meal resulted in improved efficiency of utilization of nitrogen and slightly lowered pancreas weight. The effect of trypsin on growth is complicated by the growth-depressing action of trypsin observed when a crude preparation was added to a diet containing heated soybean oil meal and when crystalline trypsin was added to either raw or heated meal diets.

These observations are in contrast with reports by Almquist and Merritt ('52a, '53) that either crude or crystalline trypsin would overcome the growth inhibition of chicks fed raw soybean oil meal. Active trypsin was not added to the heated meal control diet in the above authors' experiments, and hence no observations were made as to the detrimental properties of the trypsin as observed in the present study.

It is difficult to explain the contrast between the results of our experiments and those of Almquist and Merritt. The trypsin inhibitor content of the raw soybean meal used may have differed greatly from that used in the present experiments. Perhaps the relationship between trypsin inhibitor content of the raw meal and added trypsin is very important and the proper levels were not used in our experiments. In the present study the basal diet contained a much higher level of fat than that used by the above workers. This may have affected the response to trypsin.

The detrimental effects of dietary trypsin supplementation are difficult to explain. The most marked observation was the lowered feed consumption of chicks receiving the trypsin. The effect of trypsin on growth and feed consumption was less severe in diets containing raw meal. This suggests that the trypsin inhibitor was inactivating the exogenous trypsin. Heat-inactivated trypsin concentrate was without effect on growth or feed consumption.

The raw soybean oil meal lowered the utilization of the added soybean oil contained in the basal diet, and this effect of raw soybean oil meal on fat utilization appeared to be more marked in the younger chick. In experiment 4, the chicks were two weeks old at the end of the experiment and the absorbability of the soybean oil was only 49%. In three-week-old chicks in experiment 3 the absorbability of the soybean oil was 73% and analysis of the diet and feces of chicks receiving the diet in experiment 2, which contained the 3/5ths level of raw soybean oil meal, indicated that the soybean oil was digested to 92% in a 4-week-old chick. The mechanism of action of raw soybean oil meal on fat absorption in the young chick is not clear. No anti-lipase activities have been reported for raw soybean oil meal that could correspond to the effects from an anti-trypsin. Lyman and Lepkovsky ('57) have reported that the lipase secretion of the pancreas of the rat is greatly increased by including raw soybean oil meal in the diet. Perhaps in the presence of raw soybean oil meal too much lipase is present, causing nearly complete hydrolysis of the triglycerides present so that the proper emulsification mixture for absorption is not formed.

The effect of trypsin on soybean oil absorption is probably not due to contamination from a lipase or other enzymes, because the crystalline trypsin preparation also affected fat absorption as much as the crude preparation. It is possible that the trypsin preparation is inhibiting the secretion of certain pancreatic enzymes which might be interfering with fat absorbability. The data on pancreas weight indicate that in at least one experiment trypsin significantly depressed pancreas size, even though the depression was relatively small when compared with the enlargement of the pancreas brought about by feeding raw soybean oil meal.

It is not likely that the fat in feces of chicks receiving raw soybean oil meal is of endogenous origin since analysis of fecal material from chicks receiving such a diet with no added oil did not show

elevated fecal fat levels.[4] The active trypsin preparation substantially improved efficiency of nitrogen utilization of chicks receiving raw soybean oil meal, whereas the inactivated trypsin preparation did not. This improvement in nitrogen utilization may be due to an increase in digestibility of the dietary nitrogen, or inhibition of pancreatic secretion. Lyman and Lepkovsky ('57) have suggested that the nitrogen in the feces of rats receiving raw soybean oil meal may be largely from excess pancreatic secretion.

Aside from the effect on fat digestibility, trypsin had no apparent effect on the metabolizable energy of the diet. If one can assume that the exogenous trypsin did, indeed, combine with the trypsin inhibitor present in the raw meal in the digestive tract of the chick, then it would appear that the trypsin inhibitor does not affect the digestibility of the protein of raw soybean oil meal by the chick. We have not determined in this study the extent of the neutralization of the anti-trypsin activity in the intestinal tract by the exogenous trypsin.

SUMMARY

Four experiments were conducted in an attempt to determine the effect of trypsin supplementation on the metabolizable energy of diets containing raw soybean oil meal for the chick. The results indicated that trypsin supplementation would not overcome the growth-depressing properties of raw soybean oil meal. Supplementing diets containing raw soybean oil meal with a crude or crystalline trypsin preparation did not improve the metabolizable energy of the nonfat components of these diets. A level of 2.4% of a crude trypsin 1:300 concentrate was markedly growth-depressing when added to a diet containing heated soybean oil meal. A level of 0.75% of crystalline trypsin was markedly growth-depressing when added to diets containing either raw or heated meal. The trypsin preparations were more detrimental in diets containing heated soybean oil meal than in diets containing raw soybean oil meal.

The digestibility of soybean oil in the basal diet used in these studies was markedly depressed when raw soybean oil meal was included in the diet. Trypsin supplementation of the diets improved digestibility of the dietary soybean oil. The effect of raw soybeans on fat digestibility appeared to depend upon the age of the chick.

Chicks fed diets containing raw soybean oil meal showed a marked hypertrophy of the pancreas compared with chicks receiving heated meal. Supplementing the diet with the crude trypsin preparation appeared to slightly reduce the pancreatic hypertrophy but crystalline trypsin was without effect.

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Toxic Effects of Stable Strontium in Young Pigs[1]

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In recent years, great interest has been shown in radioactive strontium due to its presence in "fallout" (Kulp et al., '57), its rapid absorption and retention by the living organism (Comar et al., '57) and its association with an increased incidence of neoplastic changes (Brues et al., '47). The effects of feeding strontium were first described by Lehnerdt ('09, '10). Jones ('38) concluded that when strontium was included in the ration it formed insoluble phosphate in the gut to produce a phosphorus deficiency. MacDonald et al. ('52) studied dietary factors affecting deposition of strontium in bone.

Swine are an important source of food for man and have a digestive system similar to that of man, but swine have been used in only two studies involving strontium. A progress report from Oak Ridge National Laboratories (Anonymous, '55) outlined a study on the absorption of strontium-89 in pigs, and Comar et al. ('52) investigated bone growth in pigs by means of strontium-90 autoradiographs. Neither of the reports presents a complete study of strontium metabolism in swine. The study reported here was designed to clarify and extend the knowledge of stable strontium metabolism in swine.

MATERIALS AND METHODS

The composition of the rations is given in table 1, and that of the basic salt mixtures in table 2. The salt mixture in experiment 1 was prepared by adding either calcium or strontium salts to the basic salt mixture so that the final concentration in the ration was either 0.55% of calcium and no strontium or 0.48% of strontium and 0.16% of calcium. Calcium was added as the lactate and carbonate. Strontium was added as strontium chloride.

TABLE 1
Percentage composition of the rations

	Experiment	
	1	2
	%	%
Casein	35.0	35.0
Lard	20.0	20.0
Dextrose	19.0	25.0
Lactose	7.0	6.0
Non-nutritive cellulose[1]	10.0	3.0
Salt mixture	5.0	7.0
Vitamin mixture[2]	2.5	2.5
Wheat germ oil (2 I.U. vitamin E/gm)	1.0	1.0
Cod liver oil (1000 U.S.P. vitamin A and 100 U.S.P. vitamin D/gm)	0.5	0.5

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[2] The following vitamins in the quantities given were made to 5 gm and added to 195 gm of ration: (in milligrams) cyanocobalamin, 0.01; biotin, 0.025; folic acid, 0.13; menadione, 0.29; thiamine, 1.10; riboflavin, 1.80; pyridoxine, 2.00; *p*-aminobenzoic acid, 5.00; Ca pantothenate, 7.10; nicotinic acid, 10.1; inositol, 26.8; ascorbic acid, 130; and choline chloride, 260.

Nine Hampshire pigs from two litters, born two days apart, were used in experiment 1. Six pigs from the first litter were divided equally at random, into two experimental groups. The pigs were 27 days old at the start of the experiment. One pig from the second litter was placed in the group fed 0.55% of calcium and no strontium. Two pigs from the second lit-

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TABLE 2

Composition of the basic salt mixture

	Experiment	
	1	2
	gm	gm
Sodium fluoride	2.03	2.03
Potassium iodide	0.16	0.16
Ammonium alum	0.37	0.37
Manganese sulfate	0.80	0.80
Cupric sulfate·$5H_2O$	0.31	0.31
Ferric ammonium citrate	61.21	61.21
Magnesium oxide	80.00	
Magnesium sulfate	179.15	
Sodium chloride	616.80	616.80
Potassium chloride	362.70	362.70
Potassium phosphate (monobasic)	932.00	
Potassium phosphate (dibasic)		596.60
Sodium phosphate·H_2O		716.00
Total	2235.53	2356.98

ter were placed in the group fed 0.48% of strontium and 0.16% of calcium. The pigs from the second litter were 25 days old at the start of the experimental period.

A factorial design with calcium and strontium in the ration as variables was used in experiment 2. Sixteen Hampshire pigs from three litters were used. The pigs from each litter were divided at random and equally into 4 treatment groups. All pigs were 21 days old at the start of the trial. The ration designated Ca contained 0.89% of calcium and no strontium and had a calcium phosphorus ratio of 1.5:1. The ration designated Sr contained 0.67% of strontium and 0.16% of calcium and had a strontium-phosphorus ratio of 1.5:1. The ration designated Ca-Sr contained 0.67% of strontium and 0.89% of calcium and had a calcium plus strontium-phosphorus ratio of 3:1. The ration designated "neither" contained 0.16% of calcium and no strontium and the molar ratio of calcium and strontium to phosphorus was 0.8:1.

Pigs were fed a gruel of equal parts water and ration twice daily in experiment 1. The ration was fed dry in experiment 2. Distilled water was offered ad libitum. The food intake was ascertained by weighing the food refused after each feeding. The animals were weighed at the start of each experimental period and at weekly intervals. The pigs were kept in metabolism cages throughout the 35-day experimental period. An attempt was made to maintain the animals on the same food intake, but when the toxic effects became severe late in the study this could not be done.

The pigs were killed by electrocution and examined for gross lesions at the end of the experimental period. The costochondral junction of the 7th rib was taken from each pig and fixed in 10% formalin. The samples were demineralized electrolytically, embedded in paraffin and sectioned. The sections were defatted and stained with hematoxylin and eosin as described by the Armed Forces Institute of Pathology (Anonymous, '57).

The left fore and hind limbs were removed at necropsy and two radiographs were taken of each limb. The right femur was taken from each pig and the loose connective tissue and muscle were removed. The bones were measured and frozen until it was convenient to make the chemical determinations. Samples of ground bone and of each ration were weighed, dried for 48 hours at 110°C and reweighed. The fat was extracted for 24 hours in the Goldfisch apparatus with a solution of equal parts of diethyl ether and Skelly B petroleum ether. The samples were dried, weighed, and ashed at 600°C for 72 hours. After a final weighing, the ash was dissolved in concentrated hydrochloric acid. Samples were made to a known volume, and aliquots were taken for calcium and strontium determinations.

Calcium and the strontium in the aliquots were precipitated by the addition of 10 ml of 4% ammonium oxalate. Two drops of methyl red were added to each sample. The samples were made pale yellow by adding concentrated ammonium hydroxide (Ballaczo and Doppler, '56). The supernatant was removed by means of filter sticks after 30 minutes at room temperature. The precipitate was first washed with 3 ml of 1% ammonium oxalate and finally with 3 ml of distilled water.

The precipitate was taken up in 5 ml of approximately 1 N hydrochloric acid and made to a known volume. The acid concentration was well below that reported

by Bianchi ('56) to interfere with the flame photometric analysis of calcium. Lithium (100 ppm) was added as an internal standard to the samples and the standards. The concentration of calcium and strontium was determined by comparison of the samples with appropriate calcium and strontium standards on the flame photometer.[3] Strontium was read at 465 mμ and calcium at 425 mμ. No interference was observed between the two ions at these wave lengths (Taylor and Paige, '55).

RESULTS AND DISCUSSION

Studies with stable strontium have been hampered by lack of an analytical method for strontium and calcium in the same solution. The ions are almost inseparable due to their close chemical similarity. The flame photometer has been used successfully to determine the concentration of calcium and strontium in a solution free of interfering ions (Ray et al., '56). A modification of the procedure used by Ballaczo and Doppler ('56) was combined with flame photometric analysis to obtain the data reported here. The range of the percentage recovery of strontium was 100.0 to 93.8, and 94.9 to 90.0 for calcium from standard solutions which contained ratios of calcium and strontium similar to those found in the bone samples.

Aubel et al. ('41) reported that 0.41% of calcium in the ration would allow normal growth and development of pigs. Since Beeson et al. ('53) recommended 0.89% of calcium in the ration for pigs, the calcium content of the ration was increased in experiment 2.

The daily weight gain and the feed efficiencies are summarized in table 3. The two sexes were allotted at random. The sex was taken into account in the statistical analysis and found to have no significant effect. The difference between the mean daily weight gain of the two groups in experiment 1 was significant ($P < 0.01$). The increased gain, when 0.89% of calcium was fed, was significant ($P < 0.05$) in experiment 2. A joint analysis of the two experiments indicated that pigs fed 0.55 or 0.89% of calcium utilized their feed significantly ($P < 0.01$) more efficiently than those fed 0.16% of calcium. There was a significant ($P = 0.05$) interaction of strontium and calcium. It appears that strontium had a deleterious effect when the calcium content of the ration was low. Pigs that were fed the ration containing 0.16% of calcium and strontium gained less weight per day and utilized their feed less efficiently than pigs fed the ration containing 0.16% of calcium.

The significant reduction of daily weight gain and feed efficiency due to calcium deficiency agrees with the results of other studies (Aubel et al., '41; Boelter and Greenberg, '41). Shipley et al. ('22) reported that rats fed a diet containing strontium and inadequate calcium gained more weight than those fed inadequate

[3] Perkin-Elmer Flame Photometer, Model 52C. The Perkin-Elmer Corporation, Glenbrook, Connecticut.

TABLE 3

Summary of weight gain and food efficiency data

Exp. no.	Means of the feeding groups				Treatment standard errors		
	Neither	Ca	Sr	Ca and Sr	Ca	Sr	I[1]
			Weight gain (pounds/day)				
1		0.48 (4)[2]	0.36 (5)			0.022**	
2	0.36 (4)	0.38 (4)	0.26 (4)	0.40 (4)	0.050*	0.054	0.056
			Food efficiency (gain/intake)				
1		1.01 (4)	0.79 (5)			0.041**	
2	0.76 (4)	0.80 (4)	0.65 (4)	0.83 (4)	0.026**	0.027	0.028*

[1] I is the designation for the interaction of calcium and strontium.
[2] Numbers in parentheses indicate the number of pigs in that mean.
* Statistically significant: $P < 0.05$.
** Statistically significant: $P < 0.01$.

calcium. There appeared to be little effect on the daily weight gain or feed efficiency when up to 0.1% of strontium in the presence of adequate calcium was fed to rats (Forbes and Mitchell, '57).

The development of signs of toxic effects in the pigs fed strontium and 0.16% of calcium were similar in both experiments. The first signs were noted after the third experimental week in experiment 1 and after the second experimental week in experiment 2. The earlier onset in experiment 2 was probably due to the higher concentration of strontium in the diet.

The first signs of strontium toxicity noticed were a decreased activity and an increased time for eating. One or two days later the affected pigs showed incoordination and weakness in the rear limbs. They stood with humped backs and braced legs. The pigs sat while eating and preferred to pull themselves around even though they were able to stand. The toxic effects progressed until the affected pigs were unable or unwilling to stand unaided. They could stand by bracing the legs and supporting some weight with the nose when lifted to their feet. One week after the first signs were seen the pigs became paralyzed in all 4 limbs and eventually lay on their sides continually. Food intake was impaired by the inability to stand.

Bone abnormalities were noted grossly, radiographically and microscopically. The lesions were of the same type in the pigs fed 0.16% of calcium, in those fed strontium and 0.16% of calcium and in those fed strontium and 0.89% of calcium. The pigs fed strontium and 0.16% of calcium were the most severely affected in all cases. The bones of pigs fed either 0.55 or 0.89% of calcium appeared normal.

Gross appearance of bone. Beading of the ribs and bending of the long bones of the limbs were the most evident deformities. The average femoral length was significantly ($P < 0.01$) greater in those pigs fed 0.55 or 0.89% of calcium, regardless of the presence or absence of strontium (table 4). Feeding strontium and

TABLE 4

Length of femurs, percentage composition of bone and calcium and strontium concentration of bone

Exp. no.	Means of the groups				Standard errors		
	Neither	Ca	Sr	Ca and Sr	Ca	Sr	I[1]
			Length of femur, cm				
1		10.6 (4)[2]	9.6 (5)			0.12**	
2	10.3 (4)	11.0 (4)	9.6 (4)	10.8 (4)	0.17**	0.18*	0.18
			Total solid content of whole femur, % of wet weight				
1		41.3 (4)	36.8 (5)			2.26	
2	38.6 (3)	44.4 (3)	39.8 (3)	43.4 (3)	0.84**	0.81	0.93
			Fat content of whole femur, % of dry weight				
1		13.7 (4)	8.7 (5)			1.02*	
2	9.8 (3)	9.8 (3)	8.9 (3)	5.7 (3)	0.57*	0.56**	0.64
			Ash content of whole femur, % of dry weight				
1		35.2 (4)	31.2 (5)			1.02*	
2	37.1 (3)	41.6 (3)	34.7 (3)	43.7 (3)	1.12**	1.09	1.24
			Calcium concentration in whole femur, mEq/kg dry tissue				
1		6213 (4)	4392 (5)			70.2**	
2	5640 (3)	6727 (3)	4579 (3)	5794 (3)	218.5**	210.9**	242.5
			Strontium concentration in whole femur, mEq/kg dry tissue				
1		0 (4)	466 (5)			28.8**	
2	0 (3)	0 (3)	519 (3)	705 (3)	33.6*	32.8**	37.6*

[1] I is the designation for the interaction of calcium and strontium.
[2] Numbers in parentheses indicate the number of pigs in that mean.
* Statistically significant: $P < 0.05$.
** Statistically significant: $P < 0.01$.

0.16% of calcium significantly ($P < 0.05$) decreased the average length of the femur.

Radiographic appearance of bone. The epiphyseal plates and the joint surfaces in pigs fed strontium and 0.16% of calcium were very irregular. Cupping of the metaphyses was common. The epiphyseal plates were widened. The borders between the epiphyses and the metaphyses were less opaque than normally and poorly delineated. Close examination of the radiographs of pigs fed 0.16% of calcium and those of pigs fed strontium and 0.89% of calcium revealed slight widening and irregularity of the epiphyseal plates. Cupping of the metaphyses was slight and the borders of the epiphyses were slightly irregular and hazy.

Radiographs by Roberts[4] of 100-pound pigs experiencing a calcium and phosphorus deficiency revealed that the radiolucidity was greater in the diaphyses than in the epiphyses. According to Stein et al. ('55) radiographic changes in advanced human rickets were similar to those seen in the pigs fed strontium and 0.16% calcium.

Microscopic appearance of bone. The chondro-osseous junction was markedly widened and disorganized. The zone of proliferating cartilage was widened and cartilage persisted in the metaphysis. Some trabeculae appeared calcified, but the majority had extensive areas of pink uncalcified osteoid tissue around a small calcified area. The microscopic changes of the bones of the pigs fed 0.16% of calcium were similar to the pigs fed strontium and 0.16% of calcium except that margins of osteoid tissue were observed less often in the bones from the former pigs. The bones of the pigs fed the higher levels of both ions had microscopic lesions similar to those described, but not as marked. The proliferative zone was slightly widened but not disorganized. The presence of cartilage in the metaphysis and of trabeculae with borders of osteoid tissue were less common than in the groups fed 0.16% of calcium. Little difference was seen in the microscopic structure of bones of the pigs fed 0.55 or 0.89% of calcium. The zone of proliferative cartilage was of normal width and highly organized. Active calcification was evident, and a normal amount of uncalcified osteoid tissue was seen.

Shipley et al. ('22) summed up the histopathologic changes induced by strontium as "exaggerated rickets." The changes appeared to be similar to those observed when a diet low in phosphorus and high in calcium was fed. The most striking lesion was the persistence of cartilage in the metaphyses. Similar changes were described by Sobel et al. ('35) in rats fed strontium. Their description of the histopathologic changes was similar to that noted in pigs fed strontium and 0.16% of calcium.

The signs observed were similar to those reported in older pigs fed rations deficient in calcium (Aubel et al., '41; Bohstedt et al., '26) or calcium and phosphorus.[5] The paralysis has been attributed to vertebral fractures (Bohstedt et al., '26) and to fractures of the femora and ilia.[6] No fractures were noted in the pigs in the present study. Shipley et al. ('22) attributed the signs observed in rats fed strontium and inadequate calcium to a direct action of strontium. Boelter and Greenberg ('41), however, reported similar paralysis in growing rats severely deficient in calcium. They attributed the paralysis to massive hemorrhages in the spinal cord and brain. No such hemorrhages were observed in the present study. The specific cause of the paralysis in the present study is unknown.

The percentages of total solids, fat, and ash of the femurs are shown in table 4. The pigs fed 0.55% of calcium had significantly ($P < 0.01$) lower concentrations of ash in bone than pigs fed 0.89% of calcium. This decrease in bone ash was probably due to the lower dietary intake of calcium and phosphorus. There was no significant difference in total solids or ash content of femurs of pigs in experiment 1. The pigs in experiment 2 fed 0.89% of calcium had significantly ($P < 0.01$) higher percentages of total solids and ash

[4] Roberts, C. Y. 1953 Effects of calcium and phosphate supplementation on growth performance and skeletal composition of growing-fattening swine. Thesis. Iowa State College, Ames.

[5] See footnote 4.

[6] See footnote 4.

of bone than pigs fed 0.16% of calcium. The effect of feeding strontium was not significant.

The bones of pigs fed 0.55% of calcium contained a significantly ($P < 0.01$) higher average percentage of fat than the bones of pigs fed strontium and 0.16% of calcium. The difference between the average fat content of the femurs of pigs receiving 0.16% of calcium and the average of those fed 0.89% of calcium was significant ($P < 0.05$). Strontium had a significant ($P < 0.01$) effect in reducing the fat content of the bone. The reduction in the fat content appeared to be due to a direct effect of strontium on bone. The increase in osteoid tissue and in trabecular size in the pigs fed strontium may have reduced the bone lumen so that there was less room for bone marrow fat.

The calcium and strontium content of the femurs on a dry weight basis are shown in table 4. The level of either calcium or strontium in the diet affected the bone concentration of calcium. The calcium content of the femurs of pigs fed 0.48% of strontium and 0.16% of calcium was significantly ($P < 0.01$) reduced. The pigs fed 0.89% of calcium had significantly ($P < 0.01$) higher concentrations of bone calcium. Strontium in the diet significantly ($P < 0.01$) depressed the calcium content of bone.

The total concentration of strontium and calcium in bones of pigs fed both elements in experfiment 1 was significantly ($P < 0.01$) below that of the calcium concentration of bone of the pigs fed adequate calcium. The sum of the strontium and calcium milliequivalents in the dried femurs of pigs fed 0.67% of strontium and 0.16% of calcium was significantly ($P < 0.01$) lower than the milliequivalents of calcium in femurs of pigs fed 0.89% of calcium. This result verified that found in experiment 1. The histopathologic changes observed in the matching femurs and the reduced ash content confirmed the reduced calcium and strontium content of these femurs. The sum of the strontium and calcium milliequivalents in a kilogram of bone from pigs fed 0.89% of calcium and 0.67% of strontium was not significantly different from the milliequivalents of calcium found in pigs fed 0.89% of calcium. The high level of calcium and strontium in the bones of pigs fed 0.67% of strontium and 0.89% of calcium was reflected in the high ash content of the femur.

The calcium in the diet of the pigs receiving 0.67% of strontium and 0.89% of calcium was high enough to support some calcification and new bone growth. This conclusion was confirmed by the chemical and histopathological studies of bone. The bones were able to adsorb more strontium than the severely damaged bones of pigs fed 0.67% of strontium and 0.16% of calcium ($P = 0.05$). Calcium made up a smaller proportion of the ash of bones of pigs fed 0.67% of strontium and 0.89% of calcium due to the strontium content.

Strontium in the bones of pigs fed 0.48% of strontium and 0.16% of calcium constituted 9.6% of the total strontium and calcium content on a molecular basis. Strontium constituted 10.2% of the strontium and calcium content of the bones of the pigs fed 0.67% of strontium and 0.16% of calcium. Strontium constituted 10.8% of the strontium and calcium content of bones of pigs fed 0.89% of calcium and 0.67% of strontium.

Various estimates have been made of the proportion of calcium in the bone crystal that is exchangeable. Three per cent of the calcium was exchangeable in the rat tibia (Bauer et al., '55). About 10% of the bone calcium was replaced by strontium in the pigs. If strontium can replace calcium in the exchangeable fraction and adsorb to bone as well (MacDonald et al., '51; Tutt et al., '52), then the percentage of strontium substituting for calcium can be explained by these two mechanisms. If strontium had been taking part in calcification, the percentage of strontium substituting for calcium should have been higher.

The reduction in the bone calcium attributed to strontium could be an indirect effect due to loss of phosphate through precipitation in the gut. Those pigs fed both calcium and strontium should have been the most severely affected if phosphate precipitation was the cause of the

signs of the bone abnormalities and of the reduced bone calcium resulting from feeding strontium. The pigs fed 0.67% of strontium and 0.89% of calcium received a greater proportion of ions that would precipitate phosphorus than the other pigs since both calcium and strontium form insoluble precipitates with phosphorus. The toxic effects, the decrease in bone calcium and the bone changes observed radiographically and microscopically, however, were more marked in pigs fed strontium and 0.16% of calcium. There was an excess of phosphorus in these rations. Shorr and Carter ('47) stated that the histologic changes observed in strontium rickets were not the same as changes in bones of phosphorus-deficient animals.

SUMMARY

The metabolism and toxic effects of strontium in pigs, three to 8 weeks old, were investigated. In experiment 1 Hampshire pigs were fed a synthetic ration containing 0.55% of calcium or 0.16% of calcium and 0.47% of strontium. In experiment 2 a factorial design was used in which 0.89 or 0.16% of calcium and zero or 0.67% of strontium were included in the rations.

The pigs fed strontium and 0.16% of calcium were the most severely affected by incoordination and weakness followed by posterior paralysis. Occasionally mild toxic effects were observed in the pigs fed 0.89 or 0.55% of calcium and strontium. One pig fed 0.16% of calcium was mildly affected by the treatment.

The bone deformities seen grossly, radiographically, and microscopically were most marked in pigs fed strontium and 0.16% of calcium. There was widening of the epiphyseal plates and cupping of the metaphyses. Radiographically, the margins of the epiphyses were poorly delineated and radiolucidity was marked. The microscopic examination revealed cartilage remaining in the metaphyses and a marked increase in osteoid tissue.

When 0.55 or 0.89% of calcium was included in the ration the weight gain, feed efficiency, bone ash, bone total solids, bone calcium and bone strontium were significantly increased as compared with pigs fed 0.16% of calcium. Feeding strontium depressed calcium and fat content of bone.

The results were discussed in respect to present knowledge of strontium, calcium, and bone metabolism.

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Effects of Feeding X-Irradiated Pork to Rats, on their Thiamine Nutrition as Reflected in the Activity of Erythrocyte Transketolase[1]

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Extensive investigations have been made to determine the feasibility of using x-irradiation for the preservation of foods for human consumption. Investigations of safety, both toxicological and nutritional, have been supported in large part by the Research and Development Division, Office of the Surgeon General, Department of the Army, and the results of these investigations have been published in their Contractors' Annual Reports. Summary articles of these results have appeared in the literature and it has been the responsibility of individual investigators to report their results (Kraybill, '60). Presented in this paper are studies of the effects on erythrocyte transketolase of feeding x-irradiated pork unsupplemented with thiamine to rats. Reported in other papers of this series are studies on the effects of feeding x-irradiated pork, unsupplemented with pyridoxine to rats, to serum transaminases (Brin et al., '61); also the effects on 5 blood enzyme systems (Brin and Ostashever, '61) of feeding x-irradiated pork, bread, green beans and shrimp, each with complete vitamin supplement, to young rats.

EXPERIMENTAL

Albino rats of both sexes from the Food and Drug Research Laboratories' colony were assigned to this study when they were about 21 days old. The animals were housed in raised-bottom wire cages with food and water available ad libitum.

Lean ground pork was used in this study. It was canned, x-irradiated and supplied by the Quartermaster Food and Container Institute (Chicago). Control unirradiated pork was kept frozen during shipment and storage. Experimental material was exposed to two levels of ionizing radiation, namely 3×10^6 r (2.79 megarad) or at 6×10^6 r (5.58 megarad). These are referred to as the 1 × and 2 × levels, respectively. X-irradiated pork was maintained at ambient temperature for at least 30 days before it was fed to rats. All pork whether irradiated or control was cooked before feeding by being maintained at a temperature of 90°C for 45 minutes. Proximate analysis of the pork was made with the results shown in table 1.

Composition of the diets is presented in table 2. Pork was incorporated at 35% of the diet on a dry weight basis. The remainder of the diet was composed of corn starch, glucose,[6] a balanced salt mixture and additional vitamins as described. The diet coded DPO contained control pork to which was added all vitamins including thiamine as shown in the table. The code DPO-T indicated that thiamine was excluded from the vitamin mixture. The diets coded DP3-T and DP6-T contained irradiated pork at their respective 1 × and 2 × levels plus all of the other ingredients

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TABLE 1

Proximate analyses of pork (uncooked)

Sample and treatment[1]	Lot no.	Total solids	Water	Protein (N × 6.25)	Fat	Ash	Crude fiber	Carbohydrate	Calories
		%	%	%	%	%	%	%	*per 100 gm*
Pork, unirradiated	1	39.98	60.02	18.56	17.74	0.89	0.31	2.48	244
	2	39.99	60.01	20.75	17.95	0.93	0.21	0.15	245
Pork, 1×	1	41.39	58.61	18.50	20.56	0.89	0.33	1.11	263
	2	35.91	64.09	18.69	14.68	0.96	0.27	0.89	210
Pork, 2×	1	39.89	60.11	18.38	20.33	0.94	0.24	0	256
	2	37.06	62.94	21.00	14.84	0.96	0.26	0	218

[1] The sign 1× signifies radiation exposure of 3.0×10^6 r or 2.79 megarad; 2× denotes twice the 1× exposure.

of the DPO diet except that thiamine was omitted.

Two lots of the three samples of pork were submitted for thiamine analysis (table 3). Appreciable thiamine had been destroyed by the ionizing radiation; more was destroyed at the 2 × level than at the 1 × level.

The rats were weighed initially, and weekly thereafter. After feeding the diets for certain periods, animals were removed from the test groups for the assay of erythrocyte transketolase by the method of Brin et al. ('60).

RESULTS

The effects of feeding irradiated pork on the growth rate of rats are summarized in table 4. Body weights of rats fed control pork without supplemental thiamine (DPO-T) did not differ from body weights of rats of the same sex which were fed the control pork with added thiamine (DPO). Body weights of rats fed the irradiated pork diet were slightly depressed by the first week, but markedly depressed by the second week of the diet. The growth of rats fed pork irradiated at the 2 × level (DP6-T) was retarded more than that of rats fed pork irradiated at the 1 × level (DP3-T). The growth of rats fed irradiated pork ceased after three weeks on test.

On the basis of the retarded growth of rats fed irradiated pork it appeared appropriate to submit erythrocytes for the transketolase assay at the end of the 4th week. These analyses were made on the blood of 6 rats from each group chosen at random (table 5). Two measures for the activity of transketolase are presented as previously described (Brin et al., '60), namely the utilization of pentose and the formation of hexose. The erythrocyte transketolase activity of rats fed the control material without added thiamine was as high as that of animals fed diets to which thiamine was added (DPO-T and DPO, respectively). Erythrocyte transketolase of rats fed diets containing irradiated pork without thiamine supplement was markedly depressed. The depression was greater in rats fed diets containing pork irradiated at the 2 × level. The addition of thiamine pyrophosphate to the erythrocyte hemolysates *in vitro* had no effect on the trans-

TABLE 2

Composition of pork diets

	Diet codes			
	DPO	DPO-T	DP3-T	DP6-T
	%	%	%	%
Pork, unirradiated	35	35	—	—
Pork, 1×[1]	—	—	35	—
Pork, 2×[1]	—	—	- -	35
Corn starch	29.5	29.5	29.5	29.5
Glucose[2]	29.5	29.5	29.5	29.5
Salt mixture[3]	4	4	4	4
B-Complex vitamins[4]	1	—	—	—
Thiamine-free vitamin mixture[5]	—	1	1	1
Vitamins in oil[6]	1	1	1	1

[1] 35% in diet on dry weight basis. Pork was cooked as described in text.
[2] Cerelose, Corn Products Refining Company, New York.
[3] Hawk et al. ('47).
[4] Supplied per 100 gm of diet: (in micrograms) thiamine·HCl, 600; riboflavin, 1200; pyridoxine, 400; biotin, 1; pteroylglutamic acid, 1; cyanocobalamin, 1; and (in milligrams) Ca pantothenate, 4; niacin, 5; choline chloride, 200; inositol, 100; *p*-aminobenzoic acid, 2.5; liver concentrate 1:20, 25.
[5] Supplied per 100 gm of diet: (in micrograms) riboflavin, 1200; pyridoxine, 400; biotin, 1; pteroylglutamic acid, 1; cyanocobalamin, 1; and (in milligrams) Ca pantothenate, 4; niacin, 5; choline chloride, 200; inositol 100; *p*-aminobenzoic acid, 2.5; liver concentrate 1:20, 25.
[6] Supplied per 100 gm of diet: vitamin A, 200 USP units; vitamin D, 20 USP units; α-tocopherol, 12 mg; and vitamin K, 100 μg.

TABLE 3

Thiamine content[1] of pork samples

Lot no.	Treatment	Control pork	Irradiated pork 1×	Irradiated pork 2×
		μg/gm	μg/gm	μg/gm
1	Before cooking[2]	9.85	0.63	0.35
	After cooking	6.65	0.63	0.40
3	Before cooking	6.83	0.71	0.40
	After cooking	5.17	0.63	0.33

[1] AOAC ('55).
[2] As described in text.

ketolase activity of any of the 4 groups as measured by the utilization of pentose. As measured by the formation of hexose the addition of thiamine pyrophosphate to the erythrocytes of rats fed control pork had no effect on the activity of the enzyme, whereas when the coenzyme was added to hemolysates of rats fed irradiated pork an enhancement of enzyme activity was observed.

To determine whether the adverse effects of feeding x-irradiated pork on the growth of rats and on the transketolase activity of their erythrocytes were caused specifically by a lack of adequate thiamine in the diets, 10 animals per group were chosen at random and injected with 15 mg of thiamine hydrochloride by the intraperitoneal route three times per week for approximately 4 weeks. The growth of rats in groups DP3-T and DP6-T resumed immediately as shown in table 4. No effect was seen on the growth of rats fed unirradiated pork.

Erythrocyte transketolase was assayed on the blood of 10 animals of each group after 4 weeks of continued treatment with thiamine hydrochloride. Data for these assays are presented in table 6. Injection of thiamine into rats fed control pork with or without added thiamine had little effect on their transketolase levels (compare with table 5). Injection of thiamine into rats fed irradiated pork resulted in mark-

TABLE 4

Body weights of rats fed irradiated pork

Diet code	Modification	No. rats and sex	Weeks on test								
			0	1	2	3	4	5	6	8	10
			gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
DPO		20M	44.9	75.4	116.5	159.4	191.6	223.6	250.7	289.5	317.7
		20F	44.0	71.6	103.2	130.9	150.4	163.5	185.8	202.1	216.2
DPO-T		20M	44.8	74.4	113.2	150.8	190.8	228.0	252.1	290.4	327.2
		20F	45.2	74.1	103.8	133.4	149.0	161.2	179.0	194.6	201.6
DP3-T		20M	44.6	66.8	88.8	94.5	91.4				
		20F	44.9	69.6	91.4	94.3	—				
	Thiamine injected	14M					—	124.3	146.2	214.4	252.5
		13F					108.3	138.6	156.2	184.7	194.3
DP6-T		20M	45.0	69.4	78.0	72.7	72.2				
		20F	44.7	69.8	77.1	70.8	65.7				
	Thiamine injected	9M						95.0	148.5	203.0	262.0
		12F						91.6	129.5	155.3	173.5

edly increased erythrocyte transketolase activity, although not to normal levels (compare with table 5). The addition of thiamine pyrophosphate to the hemolysates *in vitro* of rats fed diets coded DP3-T and DP6-T had an even further stimulating effect on the transketolase activity of these samples.

DISCUSSION

Data presented in this report confirm the lability of thiamine in foods to ionizing radiations (Sheffner and Spector, '57), as measured by the thiochrome method. Thiamine content of the 4 diets, calculated on the basis of the data presented in table 3, was 10.1, 4.1, 0.44, and 0.25 μg of thiamine hydrochloride per gm of diet, on a dry weight basis for the diets coded DPO, DPO-T, DP3-T, and DP6-T, respectively. With a minimum daily requirement for the rat of about 1.0 μg of thiamine per gm of diet (Arnold and Elvehjem, '38), it would appear that the thiamine content of diets DP3-T and DP6-T was within the limiting range. A graded effect was observed both in the rate of growth and in the activity of erythrocyte transketolase for rats in these groups. A more recent study[7] has demonstrated a dose response curve of erythrocyte transketolase in the rat to graded levels of thiamine in the diets suggesting that this enzyme may be used as a biological assay tool for the thiamine content of foodstuffs. That the adverse effects on growth and erythrocyte transketolase activity of rats fed x-irradiated pork increased with the level of treatment indicated that the transketolase response was not an all-or-none effect but may have been directly related to the level of thiamine in the diet.

The feeding of x-irradiated pork which was exposed to high levels of ionizing radiations to rats resulted in markedly depressed rate of growth and erythrocyte transketolase activity. These adverse effects were lessened by injecting the affected animals with thiamine hydrochloride.

[7] Brin, M., and B. A. Owens 1960 The relationship between vitamin deficiency and certain blood enzymes in rats. Federation Proc., *19:* 321 (abstract).

TABLE 5

Effects on transketolase activity in rat hemolysates of feeding pork unsupplemented with thiamine[1]

Diet	Unmodified[2]			
	Pentose		Hexose	
	(−)	(+)	(−)	(+)
	μg pentose/ml blood/hour		*μg hexose/ml blood/hour*	
DPO	1351 ± 29[3]	1231 ± 43	771 ± 12	732 ± 31
DPO-T	1326 ± 28	1280 ± 48	798 ± 24	744 ± 61
DP3-T	579 ± 32	567 ± 69	134 ± 19	181 ± 16
DP6-T	461 ± 26	453 ± 40	74 ± 25	131 ± 26

[1] The minus (−) sign denotes no additions. The (+) sign denotes the addition of thiamine pyrophosphate to each hemolysate.

[2] Fed experimental diets for 3 to 4 weeks; 6 animals/group.

[3] Mean ± standard error.

TABLE 6

Transketolase activity in rat erythrocytes after repletion in vivo with thiamine hydrochloride[1,2]

Diet	Pentose		Hexose	
	(−)	(+)	(−)	(+)
	μg pentose/ml blood/hour		*μg hexose/ml blood/hour*	
DPO	1399 ± 52[3]	1478 ± 64	863 ± 41	889 ± 35
DPO-T	1378 ± 47	1379 ± 60	822 ± 23	875 ± 25
DP3-T	925 ± 43	1037 ± 20	416 ± 22	502 ± 26
DP6-T	900 ± 40	1017 ± 19	344 ± 31	419 ± 37

[1] The minus (−) sign denotes no additions. The (+) sign denotes the addition of thiamine pyrophosphate to each hemolysate.

[2] Injected with 15 mg thiamine·HCl, three times/week for approximately 4 weeks; 10 animals/group.

[3] Mean ± standard error.

In this study, and as reported previously (Brin et al., '60), the formation of hexose in the transketolase assay has been affected more adversely by thiamine deficiency than the disappearance of pentose. It has also been our experience, that the stimulation in enzyme activity observed upon adding thiamine pyrophosphate to deficient hemolysates has been greater on the formation of hexose than on the disappearance of pentose. Such variations in magnitude of effect are probably due to the complexity of the hemolysate enzyme system. This has been discussed previously (Brin et al., '60).

These data are in confirmation of the sensitivity of erythrocyte transketolase to thiamine in the diets of rats and man as reported previously with another transketolase assay (Brin et al., '58; Wolfe et al., '58).

SUMMARY

Chemical analysis showed that thiamine was destroyed to the extent of 92 and 98% in pork which was exposed to ionizing radiations at levels of 2.79 and 5.58 megarad, respectively. Unirradiated pork, when incorporated into diets at 35% on a dry weight basis contained sufficient thiamine to support normal growth and normal erythrocyte transketolase activity in rats. X-irradiated pork, unsupplemented with thiamine, resulted in growth retardation of rats to the extent of 53 and 63% and a depression of erythrocyte transketolase to the extent of 83 and 94% (on a hexose basis) when fed to rats at 35% of the diet on a dry weight basis for a 4-week feeding period. Treatment of the rats fed the diet containing irradiated pork with thiamine hydrochloride resulted in a rapid resumption of the normal growth

rate and a concomitant increase in erythrocyte transketolase activity.

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Effects of Feeding X-Irradiated Pork to Rats on their Pyridoxine Nutrition as Reflected in the Activity of Plasma Transaminases[1]

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This report is one of three on the effects of feeding x-irradiated foods on growth and on a group of blood enzymes in the rat (Brin et al., '61; Brin and Ostashever, '61).

Several investigators have reported that the activities of serum and tissue transaminases were depressed in pyridoxine-deficient animals (Schlenk and Fisher, '47; Brin et al., '54; Marsh et al., '55), often with conflicting results. A more recent study has confirmed this effect in rat plasma (Brin et al., '60). It was considered appropriate, therefore, to study the effects on two plasma transaminases of feeding pork which had been exposed to ionizing radiations at the 1 × and 2 × levels[5] to rats.

EXPERIMENTAL

Pork was procured from the Quartermaster Food and Container Institute, and handled as previously described (Brin et al., '61). Albino rats of both sexes from the Food and Drug Research Laboratories' colony were assigned to this experiment at about 21 days of age. They were maintained in wire-bottom cages with food and water ad libitum.

As shown in table 1, cooked pork was incorporated into all diets at 35% on a dry-weight basis. The remainder of the diet contained cornstarch, glucose,[6] salt mixture, B-complex vitamins and vitamins in oil. The diet coded DPO contained control pork plus a complete vitamin mixture, whereas for diet DPO-P pyridoxine was omitted. Diets coded DP3-P and DP6-P contained pork which was exposed to ionizing radiation at the 1 × and 2 × levels, respectively,[7] plus all of the additional ingredients with the exception of pyridoxine. Plasma alanine and aspartic transaminases were assayed according to the method described previously (Brin et al., '60). The pyridoxine content of the pork samples is shown in table 2.

RESULTS

As measured by microbiological assay there was little destruction of pyridoxine in the pork irradiated at the 1 × level. At the 2 × level, however, approximately 60% of the pyridoxine was destroyed. No further losses occurred in cooking the pork for feeding.

The rats were maintained with the test diets for 12 weeks during which growth, appearance and behavior were normal for all groups (table 3).

Data obtained on the activity of the alanine and the aspartic transaminases in the plasmas of these rats at the end of 12 weeks are presented in table 4. There was no significant difference in the activity of the plasma aspartic transaminase between any of the experimental groups.

Marked differences between groups were observed, however, in the activity of the plasma alanine transaminase. The alanine

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[5] The 1 × signifies an exposure of 3×10^5 r or 2.7 megarad. The 2 × signifies twice this exposure.

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

[7] See footnote 5.

TABLE 1

Composition of pork diets

Ingredients	Diet codes			
	DPO	DPO P	DP3-P	DP6 P
	%	%	%	%
Pork, unirradiated[1]	35	35	—	
Pork, 1×[1]	—	—	35	—
Pork, 2×[1]	—	—	—	35
Corn starch	29.5	29.5	29.5	29.5
Glucose[2]	29.5	29.5	29.5	29.5
Salt mixture[3]	4	4	4	4
B-complex vitamins[4]	1	-	—	—
Pyridoxine-free vitamin mixture[5]	—	1	1	1
Vitamins in oil[6]	1	1	1	1

[1] 35% in diet on dry-weight basis; assumed 50% total solids. Pork was cooked as described in text.

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

[3] Hawk et al. ('47).

[4] Supplied per 100 gm of diet: (in micrograms) thiamine·HCl, 600; riboflavin, 1200; pyridoxine, 400; biotin, 1; pteroylglutamic acid, 1; cyanocobalamin, 1; and (in milligrams) Ca pantothenate, 4; niacin, 5; choline chloride, 200; inositol, 100; *p*-aminobenzoic acid, 2.5; liver concentrate 1:20, 25.

[5] Supplied per 100 gm of diet: (in micrograms) riboflavin, 1200; pyridoxine, 400; biotin, 1; pteroylglutamic acid, 1; cyanocobalamin, 1; and (in milligrams) Ca pantothenate, 4; niacin, 5; choline chloride, 200; inositol, 100; *p*-aminobenzoic acid, 2.5; liver concentrate 1:20, 25.

[6] Supplied per 100 gm of diet: vitamin A, 200 USP units; vitamin D, 20 USP units; α-tocopherol, 12 mg; and vitamin K, 11 μg.

TABLE 2

Pyridoxine content of pork[1]

Code	Uncooked	Cooked
	μg/gm	μg/gm
DPO	3.3	3.2
DP3	3.3	3.6
DP6	1.5	1.8

[1] Assayed microbiologically in accordance with the method described by Parrish et al. ('55).

enzyme activity of the DPO-P group was significantly depressed below that of the DPO group ($P = 0.05$). When compared with the activity of the DPO group, the activities of the DP3-P group and the DP6-P group were each significantly depressed ($P = 0.001$). Furthermore, the activity of this enzyme from rats fed pork exposed at the 2 × level was lower than that for rats fed pork irradiated at the 1 × level, although this was not statistically significant.

DISCUSSION

Growth was not adversely affected by feeding x-irradiated pork to rats in the manner described, nor was the activity of the plasma alanine transaminase in these animals. These data are in support of the pyridoxine content of the pork samples (table 2) in which it was shown, as determined by microbiological assay, that pyridoxine was not destroyed at the 1 × level, although partially at the 2 × level. They also confirm a previous report (Anonymous, '60) that pyridoxine was not destroyed by radiation sterilization more more than by the canning process.

Data on the activity of plasma alanine transaminase of rats fed irradiated pork are in conflict with these observations. That little effect was seen in the plasma aspartic enzyme of these rats might be reconciled with the data presented on the alanine enzyme on the basis of previous work (Brin and McKee, '56; Awapara, '53; Brin et al., '54), in which it was demonstrated that the aspartic enzyme was far more inert to physiological and nutritional change than was the alanine enzyme. In a previous study (Brin et al., '60), it was demonstrated that in heavier rats maintained with pyridoxine-deficient diets, serum transaminases were markedly depressed before the maintenance of body weight was affected. Therefore, the data on normal growth may be reconciled with that on depressed plasma alanine trans-

TABLE 3

Body weights of rats fed irradiated pork

Diet	No. rats and sex	Average body weight by weeks							
		0	2	4	6	8	9	10	11	12
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
DPO	20M	44.6	110.4	197.6	268.5	315.5	328.1	337.5	353.3	365.4
	20F	43.5	119.0	160.1	199.5	226.2	231.7	237.3	243.4	243.0
DPO-P	20M	44.5	104.5	184.4	248.6	305.1	317.9	341.4	349.3	360.8
	20F	43.7	101.7	160.6	186.9	209.1	223.6	231.6	244.7	265.6
DP3-P	20M	44.2	103.7	175.6	243.3	288.5	305.0	320.4	333.5	363.7
	20F	44.2	97.9	153.6	186.9	209.1	215.8	222.8	226.7	250.5
DP6-P	20M	43.5	100.9	170.7	240.6	282.9	295.3	319.9	321.3	347.5
	20F	43.9	94.6	144.9	171.1	196.6	203.4	209.3	213.6	220.0

TABLE 4

Effect on plasma transaminases in rats of feeding control and irradiated pork[1]

	Glutamic-aspartic[2] transaminase	Glutamic-alanine[3] transaminase
	mg	*mg*
	Rats depleted for 12 weeks	
(a) DPO	583 ± 40[4]	246 ± 24
(b) DPO-P	610 ± 43	172 ± 17
(c) DP3-P	593 ± 50	142 ± 16
(d) DP6-P	574 ± 76	109 ± 11

[1] Eighteen to 20 rats (males plus females) per group. Na pyruvate produced per milliliter of plasma per hour.

[2] Statistical significance of differences: aspartic enzyme; no significant differences between any groupings.

[3] Statistical significance of differences: alanine enzyme; (1) not significant c–d, b–c; (2) $P = 0.05$ a–b; (3) $P = 0.01$ b–d; (4) $P = 0.001$ a–c, a–d.

[4] Mean ± standard error.

aminase, on the basis that the enzyme may be more sensitive to a depletion of pyridoxine than the process of growth or the maintenance of body weight. When all diets were supplemented with pyridoxine initially, both normal growth and normal plasma aspartic and alanine transaminases were observed in all groups and no other toxic signs were noted (Brin and Ostashever, '61). It was concluded that the rats fed unsupplemented control pork or irradiated pork in this study may have been subject to a marginal deficiency of pyridoxine.

SUMMARY

Pork samples which were x-irradiated at the 1 × (2.79 megarad) and at the 2 × (5.58 megarad) levels and unirradiated pork were incorporated into diets at 35% on a dry-weight basis and fed to rats in the following 4 groups: control pork with added pyridoxine (DPO), control pork without added pyridoxine (DPO-P), 1 × pork without added pyridoxine (DP3-P), and 2 × pork without added pyridoxine (DP-6). Growth was followed for 12 weeks at which time plasmas were submitted for the determination of the alanine and the aspartic transaminase enzymes. No differences in growth were noted. Although no effect was seen on the activity of the aspartic transaminase, the plasma alanine enzyme was depressed

significantly and increasingly in the DPO-P, DP3-P and DP6-P groups, respectively. It was concluded that the rats fed the x-irradiated pork were subject to marginal deficiency of pyridoxine.

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Weight Gains of Overnourished and Undernourished Preweanling Rats[1]

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Recent observations have emphasized the significance of nutritional status during infancy in the determination of adult body size and composition. First generation Nisei children in America approach Caucasian American children in rate of growth and development (Greulich, '58). Rats of large size at weaning attain the highest adult weight, but here the relative roles of early nutritional status and heredity cannot be distinguished (Berg, '60). Kennedy ('57) and Widdowson and McCance ('60) have shown that rats from relatively large litters (15 to 18) were small at weaning and, during extended observations, remained small in comparison with animals from small litters (3 to 4 animals) that were large at weaning.

The purpose of the present study was to determine the maximum differences in the weaning weights and body composition that could be obtained by varying the milk supply in the preweaning period. Postpartum rats readily accept infants, even of other mothers, in numbers up to 18. An abundant supply of milk was provided one group by rotating a litter of 6 animals among three lactating females. Successively decreasing milk supplies were provided other preweanling animals by establishing litters of 6, 12 and 18 that remained with one mother until weaning. Weight gains of small-, intermediate- and large-sized male animals at weaning were followed for an additional 60 days and the effect of feeding diets high in fat or in carbohydrate on weight gain determined.

METHODS

Newborn animals were delivered of Rochester Colony females of unknown parity. Females before and after delivery were housed individually; pellets[2] and water were available ad libitum. A maximum milk supply was provided one group of young by a method described by Cox et al.[3] Within 24 hours of birth, the newborn of 6 units of three females were combined and reduced to three litters of 6 animals each of undetermined sex. Litters were then randomly assigned as group 1, 2 and 3, and rotated every 8 ± 1 hours between three mothers and nursed in turn by each of them. The newborn of group 3 were used to replace dead animals in groups 1 and 2 and remaining animals of this group were discarded during the first 48 hours. Three lactating females and two litters of 6 animals each remained. From two days to weaning at 21 days, group 1 animals were transferred three times daily to a mother that had been without young for 8 hours and group 2 to the lactating female that had been suckling group 1 animals.

Progressively decreasing milk supplies were provided additional groups of newborn by establishing 5 litters each of 6 (group 4), 12 (group 5) and 18 (group 6) animals. Animals of these groups were not rotated but remained with one mother throughout the preweaning period. Litter sizes were maintained by the addition, if necessary, of marked, preweanling animals of comparable age and weight. Replacements were not included in the experiment and were discarded at weaning. Infant rats were weighed daily. At 21 days, some young of each group were sacrificed for carcass analysis.

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[1] This work was supported in part by a grant from the National Institutes of Health.

[2] Big Red Dog Food, Cooperative G.L.F. Exchange, Inc., Canandaigua, New York.

[3] Cox, G. J., D. V. Morgan and J. Nathans 1954 Maximum growth of suckling rats. Federation Proc., 13: 454 (abstract).

At weaning, group 1 animals were significantly heavier than rats of all other groups and were continued postweaning as group 1. The body weights and body composition of weanling animals of groups 2 and 4 and groups 5 and 6 were similar and these 4 groups were reduced to two: group 2 + 4 and group 5 + 6. All weaned animals were housed in individual cages and fed ad libitum a diet high in either fat or carbohydrate. Female young of all groups were sacrificed at 30 days; males at 81 days of life. Body weights were determined daily and food intake of male animals for the first 24 postweaning days.

Mothers ate the pellet diet throughout the period of lactation; this was also available to the young animals until the time of weaning. The percentage composition of the high-carbohydrate diet fed postweaning was as follows: glucose, 60; casein, 20; fat,[4] 15; salt mixture, 4; plus a complete vitamin supplement. The diet high in fat contained (in per cent): glucose, 10; casein, 20; fat,[4] 40; salt mixture, 4; non-nutritive bulk,[5] 20; plus the vitamin supplement. The caloric density of the high-fat diet was, by calculation, 105% of that of the 60% carbohydrate diet. Food efficiency was calculated as grams of body weight gain per gram of food ingested.

Animals were killed with ether. Following removal of gut content, animals were reweighed and lyophilized to constant weight. The dry carcass was ground and duplicate aliquots taken for analysis. Ash was determined following heating at 550°C for 48 hours. Protein content was calculated as 6.25 times the carcass nitrogen determined by micro-Kjeldahl. For fat analysis, aliquots of dry carcass were extracted twice with ethanol-ether (3:1) and two times with methanol-chloroform (3:1). Extractions were pooled, evaporated to dryness and re-extracted with petroleum-ether chloroform (6:1). Fat was measured gravimetrically following evaporation of solvent. Body water content was determined from live weight minus gut content and dry weight following lyophilization.

[4] Crisco, Procter and Gamble, Cincinnati.

[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Body composition of newborn rats and rats of groups 1, 2, 4, 5 and 6 at 21 days (weaning)

	Newborns[1]	Group 1[2]	Group 2[3]	Group 4[3]	Group 5[4]	Group 6[4]
No. animals	6	6	6	6	5	5
Body weight,[5] gm	6.3 ± 0.20[6]	52.8 ± 2.70	36.8 ± 2.80	35.4 ± 1.80	20.5 ± 1.30	19.5 ± 0.20
Water, gm	5.40 ± 0.11	36.8 ± 2.3	27.35 ± 1.42	27.24 ± 0.10	18.27 ± 1.17	14.87 ± 0.50
Ash, gm	0.10 ± 0.02	1.73 ± 0.02	0.95 ± 0.05	1.01 ± 0.02	0.78 ± 0.14	0.72 ± 0.05
Protein, gm	0.59 ± 0.02	6.39 ± 0.32	4.38 ± 0.30	4.09 ± 0.07	3.10 ± 0.50	2.57 ± 0.15
Fat, gm	0.17 ± 0.03	8.01 ± 0.51	2.62 ± 0.26	2.96 ± 0.30	1.52 ± 0.26	0.75 ± 0.13
% H_2O/fat-free body weight	88.5 ± 0.2	79.1 ± 0.5	82.1 ± 1.6	82.8 ± 0.6	81.9 ± 0.7	80.9 ± 0.6

[1] Newborns are significantly different, ($P < 0.05$) from all other groups for all determinations.

[2] Group 1 animals differ ($P < 0.05$) from all other groups in all determinations except percentage body water, from all other groups.

[3] Group 2 and 4 animals do not differ in any determinations. Both groups differ significantly from group 5 and 6 animals only in respect to fat content.

[4] Body composition of group 5 + 6 animals are not significantly different.

[5] Body weight following removal of gut content.

[6] Mean ± standard error.

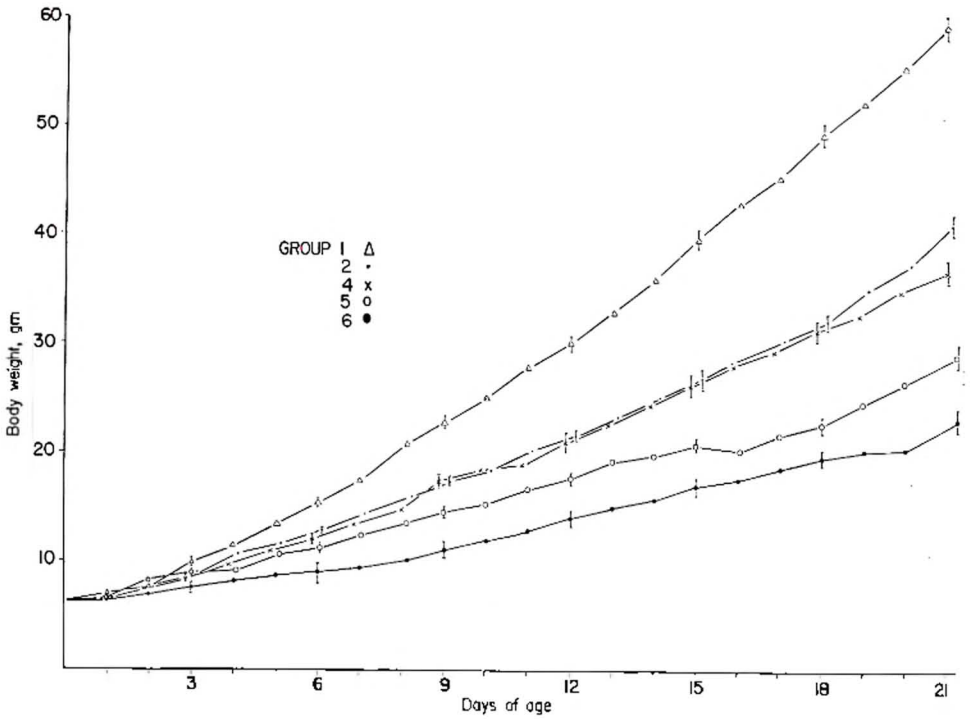

Fig. 1 Weight gains of albino rats from birth to 21 days (weaning).

RESULTS

Preweaning weight gains. Litters, before groups were established, averaged 12.1 ± 1.1 animals. Newborns, after initial suckling, but following removal of stomach contents, weighed 6.3 ± 0.2 gm; body composition is shown in table 1. After the initial 48 hours, mortality in groups 1 through 5 was zero; group 6 had an average mortality of 3.4 ± 0.8 animals per litter for the entire preweaning period.

Group 1 animals gained weight most rapidly and by day 5 were significantly heavier than rats of all other groups (fig. 1). Animals of groups 2, 4 and 5 gained weight similarly for the first 9 days. Young of group 6 were significantly lighter than those of group 2 and 4 by day 6. The rate of growth of all groups accelerated between days 6 to 9; greatest change in growth rate occurred in group 1 animals. Total 21-day preweaning weight gains of groups 1, 2, 4, 5 and 6 averaged 2.51 ± 0.05, 1.53 ± 0.04, 1.48 ± 0.04, 0.91 ± 0.05 and 0.70 ± 0.12 gm per day, respectively. Time of appearance of hair and opening of eyes was the same for all groups.

Animals of all groups increased in total amount of fat, protein and ash in the initial 21 days (table 1). Group 1 animals contained significantly more of all constituents measured than weanlings of all other groups but the greatest difference was in fat content. Group 2 and 4 animals differed from group 5 and 6 animals only in amount of fat present (table 1). Body composition of animals of groups 2 and 4 and groups 5 and 6 were alike. The percentage of water in the fat-free body weight decreased from birth to approximately the same value for animals of fast, intermediate and slowly gaining groups.

Postweaning weight gains. In the immediate postweaning period, all animals, regardless of diet, grew at approximately the same absolute rate (fig. 2). Female animals, fed the high-carbohydrate diet, gained approximately 1 gm of dry solids per day until sacrifice at 30 days (table 2). Females in group 1 and group 2 + 4 ingested significantly more diet than group 5 + 6 animals during this interval. Fat content of animals in group 1 was 12.3 ± 0.7 and that of group 2 animals, 8.4 ±

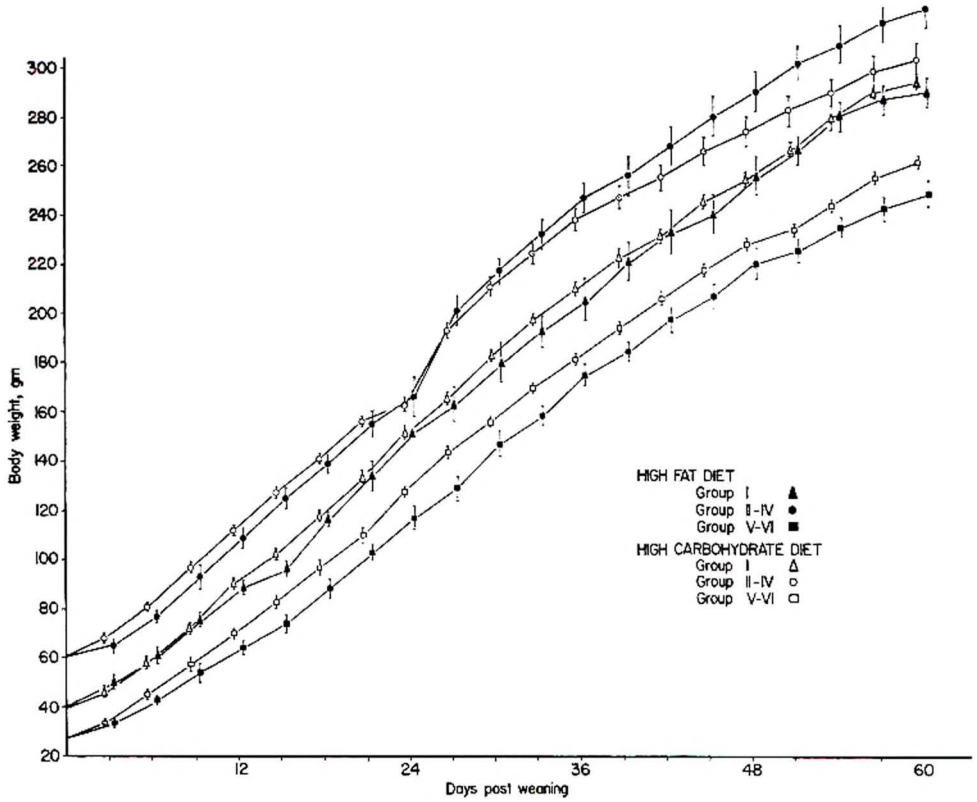

Fig. 2 Weight gains of rats small, intermediate and large at weaning.

TABLE 2

Intake and weight gain of female animals fed 60% carbohydrate diet at 30 days of age

Group	No. of animals	Food intake[1]	Total solids accumulated[2] in 9 days	Body composition	
				Solids[3]	Water[4]
		gm	*gm*	*gm*	*%*
1	6	74.3 ± 4.4[5]	11.0 ± 2.3	30.3 ± 1.6	63.6 ± 1.2
2+4	12	70.0 ± 2.5	11.0 ± 1.4	21.7 ± 1.3	69.1 ± 1.0
5+6	12	57.3 ± 2.0	10.4 ± 1.0	17.0 ± 0.8	69.3 ± 0.8

[1] Food intake of group 1 and 2+4 animals are not significantly different; both are significantly above intake of group 5+6 animals ($P < 0.05$).

[2] Calculated from food intake and weight gain from weaning (21 days) to 30 days. Differences between groups are not significant.

[3] Body solids of all groups are significantly different from each other ($P < 0.05$).

[4] Percentage of water of group 1 animals significantly lower than group 2+4 and 5+6 ($P < 0.05$).

[5] Mean ± standard error.

0.1 gm. At 30 days the percentage of water in the group 1 animals was significantly less than that of all other groups. The water content of the fat-free carcass decreased in 9 days to the same extent in females in groups 1, and 2 + 4, and was 74.5 ± 0.9 and 75.0 ± 0.6%, respectively. Food intake and rate of gain for the initial 9 days of postweaning of all groups fed the high-fat diet was not different from those reported for animals receiving the 60% carbohydrate diet.

Food intake of male animals was determined for the first 24 days of the postwean-

TABLE 3

Food intake and efficiency and weight gains of male animals during first 24 postweaning days

Diet	Group	No. of animals	Food efficiency[1]	Food intake[2]	Weight gain[3]
			gm weight gain/gm food	*gm*	*gm*
60% Carbohydrate	1	6	0.392 ± 0.015[4]	288.0 ± 8.6	117.0 ± 4.6
60% Carbohydrate	2+4	12	0.381 ± 0.011	282.9 ± 4.6	111.2 ± 3.5
60% Carbohydrate	5+6	12	0.415 ± 0.019	241.3 ± 9.9	106.0 ± 3.1
40% Fat	1	8	0.427 ± 0.011	300.0 ± 11.7	118.9 ± 4.6
40% Fat	2+4	12	0.422 ± 0.005	263.3 ± 9.6	106.6 ± 4.8
40% Fat	5+6	12	0.385 ± 0.010	231.7 ± 10.8	94.4 ± 5.2

[1] Food efficiency figures for animals ingesting 40% fat diet corrected for 5% greater caloric density. There are no significant differences.

[2] Food intake of group 5+6 animals fed either diet significantly less than that of groups 1, and 2+4 animals ($P < 0.05$).

[3] Weight gain of group 5+6 fed high-fat diet significantly less than group 1 animals ingesting 60% carbohydrate or 40% fat diet and group 2+4 animals fed 60% carbohydrate diet.

[4] Mean ± standard error.

TABLE 4

Body weight, carcass solids and percentage of water of male animals at 81 days

Diet	Group	No. of animals	Body weight[1]	Carcass solids[2]	H_2O[3]
			gm	*gm*	%
60% Carbohydrate	1	6	294.5 ± 11.8[4]	119.1 ± 6.5	59.6 ± 0.6
60% Carbohydrate	2+4	12	271.6 ± 12.3	107.0 ± 4.8	59.6 ± 1.0
60% Carbohydrate	5+6	12	249.9 ± 5.4	93.8 ± 3.5	61.0 ± 0.8
40% Fat	1	8	311.7 ± 10.4	121.3 ± 6.0	60.4 ± 0.5
40% Fat	2+4	12	275.2 ± 9.2	109.4 ± 4.7	60.4 ± 0.8
40% Fat	5+6	12	239.5 ± 8.9	90.7 ± 4.4	62.3 ± 1.0

[1] Body weights of group 1 animals fed either high-fat or high-carbohydrate diets are significantly different ($P < 0.05$) from group 5+6 animals. Body weights of group 2+4 animals fed either diet are not different from group 1 or 5+6 animals.

[2] Carcass solids of animals of group 1 and group 2+4 fed either the high-fat or carbohydrate diet are significantly greater ($P < 0.05$) than group 5+6 animals.

[3] The percentage of body water of three groups is not significantly different for any group fed either diet.

[4] Mean ± standard error.

ing period (table 3). Intake of animals in groups 1 and 2 + 4 continued to be significantly greater than that of group 5 + 6 animals, regardless of type of diet ingested. Efficiency of the two diets was the same for all groups and was not modified by diet. In the first 24 postweaning days, weight gain of group 1 animals was significantly greater than that of group 5 + 6 animals only.

At sacrifice, 81 days from birth, group 1 animals were significantly heavier in both total and dry solid content than group 5 + 6 but not group 2 + 4 animals (table 4). Carcass solids of animals in groups 1 and 2 + 4 were significantly greater than those of group 5 + 6 animals. The percentage of water in the carcass of heavier- and lighter-weight groups was not different. At sacrifice the largest animal was from group 2 + 4 and weighed 349 gm; the smallest animal from group 5 + 6 weighed 197 gm. Two deaths occurred postweaning: one animal from group 6 died 48 hours and another 48 days after weaning. No cause of death was determined.

DISCUSSION

The relative availability of the milk supply markedly influenced rate of weight gain in preweaning animals. Weight differences between groups were significant by days 6 to 9. The observation that animals in group 2, which followed group 1 animals to each successive lactating female, gained weight as rapidly and attained the same body composition at weaning as group 4 animals, suggests that group 1

young were eating to appetite without consuming all available milk.

Group 1 animals may have gained weight at, or close to, the maximally achievable rate. The postweaning acceleration in rate of gain of group 1 animals, when a high-caloric-density diet was fed, suggests that preweaning growth may be limited by gut capacity and the relatively low caloric density of milk, and not reflect the achievable potential for weight gain. All body constituents, but primarily fat, were increased by overnourishment during the preweanling period. Average rate of weight gains was greatest for group 1, next greatest in group 2 and 4 animals, and least in group 5 and 6 animals.

At weaning, body weights of animals within litters of all groups showed little variation, suggesting that caloric supply may be the main determinant of rate of increase during this period. The nutritional state of the animal did not influence rate of maturation as measured by eye opening, hair appearance and percentage of water in the fat-free carcass. At 81 days group 1 animals still maintained the weight advantage over group 5 + 6 but not group 2 + 4. By this time, perhaps as a reflection of other factors, greater variability was present in the range in weights present in each group. The significance of early nutritional status on eventual body size is indicated by the observation that in 60 postweanling days none of the smallest-weight weanling animals achieved the body weight of the overnourished preweanling animals. Smaller weight gains of group 5 + 6 animals appear to be attributable to a proportionally smaller caloric intake, as the efficiency of utilization is not different in large and small animals.

Widdowson and McCance ('60) reported that animals from small litters gained weight more rapidly in the preweaning period than animals of large litters and that the weight advantage present at weaning was maintained for an extended period. They found that animals from the small litters initially accumulated significantly more fat than smaller animals but following weaning, differences in body composition were not maintained.

Miller and Bender ('55) reported that the ratio of body nitrogen to water in rats is constant at any given age. In our experiment, the total water, as well as dry weights, increased similarly in animals of all groups suggesting that luxus consumption of calories during the preweaning period results in an eventual accumulation of nonfat solids as well as fat solids. Studies on the effect of weanling weights on eventual composition, morbidity and mortality are currently being carried out.

SUMMARY

1. The preweaning weight gain of albino rats varied directly with the available milk supply. With an abundant supply, average weaning weight was 60.0 gm; animals from litters of 18 weighed 25 gm.

2. Carcass of animals heavy at weaning contained more ash, protein and fat than smaller animals. The greatest difference was in total body fat.

3. Time of opening of the eyes, initial appearance of hair and percentage of water in fat-free body at weaning was not influenced by rate of weight gain.

4. In the immediate postweaning period rate of growth of all animals accelerated to approximately twice that of the fastest gaining group during the preweaning period.

5. At 60 days postweaning, animals large at weaning contained significantly more dry solids than the small animals, but not intermediate-sized animals at weaning.

ACKNOWLEDGMENT

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Etiology of Muscular Dystrophy in the Lamb and Chick[1]

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For the experimental production of muscular dystrophy in lambs and calves, large amounts of unsaturated fats have been considered a dietary essential. Under these conditions vitamin E (Blaxter et al., '53) and the synthetic antioxidant, diphenyl-*p*-phenylenediamine (Draper, '56) have been found to be effective in preventing myopathy. Since the muscle lesions produced under experimental conditions simulated those that occur in lambs and calves in enzootic areas, most investigators assumed that the field condition was an uncomplicated vitamin E deficiency. The occurrence of myopathy in the lamb, however, has been found to be prevented by selenium rather than vitamin E administration to the ewe (Muth et al., '58).

Vitamin E deficiency and the nutrients that modify the various dyscrasias have been more thoroughly studied in the chick (Machlin and Gordon, '60). In the vitamin E- or antioxidant-deficient chick, myopathy is associated with a deficiency of sulfur amino acids; the production of encephalomalacia is dependent upon dietary linoleic acid; and exudative diathesis can be prevented by dietary selenium. Vitamin E deficiency alone will not result in appearance of any of these dyscrasias. Encephalomalacia and exudative diathesis have not been reported in the lamb or calf and no studies are reported concerning the effect of the sulfur amino acids on the appearance of dystrophy in ruminant animals.

The present study was designed to separate the dietary ingredients that contribute to the production of muscle degeneration in the young lamb and at the same time to compare the origins of the dyscrasias in lambs and chicks. The same dietary variables (antioxidant, selenium, and sulfur amino acids) which affect the chicken dyscrasias were studied factorially. Torula yeast was used as the only source of protein since this yeast is deficient in tocopherols, selenium, and sulfur amino acids. Coconut oil was fed as the only source of added fat because its composition simulates the composition of milk fat better than other available sources of fat.

PROCEDURE

To a Torula yeast-coconut oil basal ration (table 1) were added, in a 2^3 factorial study, sodium selenite (1.4 ppm of Se), Santoquin[2] (0.1%) and MHA[3] (0.6%). All lambs (46) were allowed to suckle their dams for 24 hours. Thereafter, the dry rations were mixed with water (1:4), homogenized, and fed to lambs twice daily for 60 days. To insure adequacy, all lambs were injected with 100,000 I.U. of vitamin A intramuscularly at birth. Serum glutamic oxaloacetic transaminase (SGO-T) was determined[4] weekly in all lambs until the serum levels were elevated or until the animals were sacrificed. Muscular dystrophy was evaluated by gross pathology, and by histological examination of the biceps femoris. Skeletal muscle, heart muscle, liver, and cerebellum of dystrophic and normal lambs were sampled and relative levels of fatty acids were determined by gas-liquid chromatography

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[1] A preliminary report was presented at the American Society of Animal Production, November 25, 1960, Chicago, Illinois (Abstract no. 273).

[2] Registered trademark of the Monsanto Chemical Company for 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline.

[3] Registered trademark of the Monsanto Chemical Comapny for calcium *dl*-2-hydroxy,4-methylthio butyrate, the hydroxy analogue of methionine.

[4] Sigma Chemical Company 1958 Technical Bulletin no. 508.

(Marco et al., '61). While the lambs were fed synthetic milk, chicks were fed the same diets as a dry meal for 28 days.

TABLE 1

Composition of basal diet

	%
Torula yeast	37.81
$Ca_3(PO_4)_2$	4.43
Iodized salt	0.50
Trace minerals	0.50
Saccharin	0.07
Choline chloride	0.20
Chlorotetracycline, 25 gm/pound	0.10
Vitamin A, 50,000 I.U./gm	0.04
Vitamin D_3, 7,500 I.U./gm	0.02
Vitamin mixture[1]	0.50
Lecithin	2.00
Coconut oil	24.84
Glucose[2]	28.99

[1] Vitamin mix supplied the following in mg/kg of final mix: vitamin B_{12}, 0.03; biotin, 0.30; menadione, 1.0; pyridoxine·HCl, 8.0; folic acid, 4.0; riboflavin, 16.0; Ca pantothenate, 20.0; thiamine·HCl, 24.0; nicotinic acid, 100.0.

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

RESULTS

All of the lambs fed coconut oil-Torula yeast diets without supplemental Santoquin or selenium developed symptoms characteristic of myopathy (table 2). The diagnosis was verified by necrospy and histopathology (see addendum). Dietary MHA did not alter the incidence of muscular dystrophy in lambs, whereas in chicks methionine was the primary nutrient governing the incidence of muscular degeneration. The antioxidant completely prevented muscular dystrophy in the lambs, whereas selenium only delayed the onset of the disease. With lambs fed selenium and no antioxidant, three out of 12 exhibited clinical symptoms; 4 out of 12 showed lesions at necrospy, and 9 out of 10 were classified dystrophic by histopathology. In all lambs fed diets devoid of Santoquin, SGO-T was markedly elevated after 35 days regardless of clinical symptoms of dystrophy. At necrospy nearly all of these lambs, regardless of

TABLE 2

Selenium, antioxidant, and sulfur amino acid deficiency in lambs and chicks

	Dietary supplements							
	0.1% Santoquin				None			
	1.4 ppm Selenium		None		1.4 ppm Selenium		None	
	0.6% MHA[1]	None	0.6% MHA	None	0.6% MHA	None	0.6% MHA	None
No. lambs	4	6	6	6	6	6	6	6
Incidence of dystrophy:								
Clinical	0	0	0	0	3	0	6	6
Age of symptoms, days	—	—	—	—	24–42	—	35–48	24–50
Gross pathology	0	0	0	0	4	0	6	6
Other abnormalities[2]					3	1,4	1,2,4	1,2,3,4
SGO-Transaminase, units/ml	138	80	104	61	1221	1670	755	1600
Av. days for transaminase change					36	35	—	25
No. chicks	40	40	40	22	29	39	39	38
Encephalomalacia, %	0	0	0	0	41	10	19	13
Muscular dystrophy, %	0	0	2.5	45	0	72	5	79
Exudative diathesis, %	0	0	2.5	0	0	0	47	5

[1] MHA indicates registered trademark of the Monsanto Chemical Company for calcium *dl*-2-hydroxy, 4-methylthio butyrate, the hydroxy analogue of methionine.

[2] Number 1 indicates hydropericardium; 2, kidney and liver degeneration; 3, fatty liver and brownish fat; 4, petechial hemorrhage, subcapular hematoma of kidneys.

TABLE 3

Fatty acid composition of various tissues from normal and dystrophic lambs[1]

	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Dietary fatty acids, %	4.3	50.4	22.9	9.8	0.5	0.8	6.8	3.6	0.3	—
Skeletal muscle:										
Normal[2]		8.1	13.1	17.4	1.8	9.6	16.4	21.3	1.1	6.4
Dystrophic[3]		6.4	10.6	19.7	2.4	14.2	19.9	16.3	0.5	6.7
Cerebrum[4]										
Normal		0.6	4.0	30.6	2.5	21.5	26.3	2.8	0.3	6.5
Dystrophic		0.5	4.0	29.6	2.4	22.0	23.1	2.3	0.6	6.5
Cerebellum[4]										
Normal		1.5	4.9	30.2	2.6	25.5	29.0	2.7	0.9	7.4
Dystrophic		0.7	3.9	26.5	2.7	23.1	25.3	3.5	2.1	8.6
Heart muscle[4]										
Normal		4.9	11.1	19.8	6.6	10.6	11.8	25.4	—	8.4
Dystrophic		4.8	10.6	20.7	2.4	12.5	10.8	25.2	—	9.9

[1] The first number in the ratio heading refers to the carbon chain length, and the second number refers to number of double bonds in the fatty acids.
[2] Means of 6 lambs.
[3] Means of 5 lambs.
[4] Means of three lambs.

myopathy, exhibited syndromes (hydropericardium and petechial hemorrhage) indicating a vascular disturbance which may be analogous to exudative diathesis in the chick. In chicks fed the same diets, both selenium and Santoquin prevented exudative diathesis. Fatty liver and brownish fat were found in the negative control lambs. Although the dietary fat contained only 3.6% of linoleic acid, the lambs possessed a large proportion of linoleic acid in the heart (25%) and skeletal muscle (16%) fatty acids (table 3). Linoleic acid levels in dystrophic tissues appeared lower than in normal skeletal muscles; however, the difference was not statistically significant ($P < 0.05$).

The same diets that produced muscular dystrophy in lambs (devoid in Santoquin) produced encephalomalacia in the chick (table 2). MHA did not alter the incidence of myopathy in the lamb, although sulfur amino acids was the principle factor that decreased the incidence of dystrophy in the chick (table 2).

DISCUSSION

Whenever synthetic rations have been fed to lambs and calves, highly unsaturated fat has always been used as a stress factor since it was thought to be necessary for the production of myopathy. In this study coconut oil was used as the only fat source since it simulated milk fat in composition. Even though only 3.6% of the total dietary fatty acids was linoleic acid, heart and skeletal muscles contained high levels of this dienoic acid. These results are not unexpected since Peifer and Holman ('59) found that by increasing the hydrogenated coconut oil in the diets of rats, the unsaturated fatty acid level in heart muscle was elevated. The deposition of linoleic acid in tissues, and consequently the need for an antioxidant, can be affected by one or all of the following factors: (1) level of dietary linoleic acid (Marco et al., '61); (2) proportion of saturated fatty acids to linoleic acid in the diet as suggested by Peifer and Holman ('59); and (3) the proportion of linoleic acid to the level of dietary fatty acids that are deposited or incorporated *per se* intracellularly. Since milk contains essentially similar combinations of the same fatty acids fed to lambs in this study, one would expect suckling animals to have tissue compositions similar to those reported here, except that the lower levels of linoleic acid in the myopathic tissues in the lambs in this study suggest possible autoxidation. The prophylactic effect

of the antioxidant, Santoquin, lends further support to this postulate.

Vitamin E deficiency in rabbits (Oppenheimer et al., '58), myopathy in humans (Oppenheimer et al., '59a), as well as muscular dystrophy in mice (Oppenheimer et al., '59b) elicit altered serum lipoprotein fractions. McCormick et al. ('60) showed that the vitamin is associated with the albumin lipoprotein. When no antioxidant is present, possibly the transport system is damaged by peroxides formed by autoxidation of easily oxidizable lipids bound to circulating proteins. Nishida and Kummerow ('60) found that the hydroperoxide of methyl linoleate possessed specific affinity for the low density serum lipoprotein and upon *in vitro* incubation, the hydroperoxide specifically destroyed the beta fraction without affecting the other types of serum lipoproteins. Since the hydroperoxide did not affect the serum proteins *per se*, the hydroperoxide must affect the affinity of the specific globulin for lipids.

The role of selenium remains obscure but it is of interest to speculate that since the properties of selenium are similar to those of sulfur, and since selenium analogues of amino acids have been found in plants (Schwartz, '60), selenium may be associated with specific proteins, functioning therein as a biological antioxidant, as suggested by Machlin and Gordon ('60), and/or to maintain the integrity and affinity of proteins for the transportation of antioxidants, lipids, and other nonaqueous soluble materials. If selenium functions only as a biological antioxidant, is must possess specificity since selenium does not alter the occurrence of encephalomalacia (a specific antioxidant deficiency disease). Furthermore, it has been recognized that antioxidants of widely different chemical structure can prevent this dyscrasia in the chick.

Generally, the need for serum proteins to transport most dietary nutrients can be overcome by increasing the dietary level of those nutrients. In chicks, as well as in the lambs in this study, high dietary levels of the antioxidant, Santoquin, completely prevented all recognized selenium deficiency symptoms. Probably, various tissues require different amounts of an antioxidant to maintain homeostasis. Possibly the minimum level of Santoquin that would prevent encephalomalacia in the chick would not be effective in preventing myopathy in selenium-deficient lambs. Selenium functioning in the transport system would also explain the higher antioxidant activity found by Zalkin et al. ('60) in tissues from animals fed selenium. Other data from the Oregon Experiment Station suggest that selenium may aid in the metabolic transportation of vitamin E in sheep. When forages were fed from enzootic muscular dystrophy areas, vitamin E administration to the ewe was not effective in preventing myopathy, whereas selenium administration to the ewe completely prevented the dyscrasia (Muth et al., '58). Either vitamin E or the selenium prevented muscular dystrophy when they were administered to the newborn lambs from ewes fed the "dystrophy producing forage" (Oldfield et al., '60). The vitamin E content of the milk from these ewes was not reported, but, undoubtedly, this milk contained some vitamin E. The effectiveness of selenium in the prevention of myopathy in lambs has always been associated with the feeding of crude diets containing varying amounts of vitamin E. In our study, with better defined diets, selenium at 1.4 ppm without an antioxidant did not prevent myopathy but only modified the severity of symptoms. Possibly, with selenium-deficient rations more antioxidant is required to prevent the various dyscrasias associated with vitamin E-selenium deficiency. If this were the case, it would be easy to reconcile the different results obtained by Oldfield et al. ('60) and by us.

Although sulfur amino acid supplementation did not influence the incidence of dystrophy in lambs, in chicks fed the same diets sulfur amino acid deficiency was required in order to produce myopathy. With the diets used in this study, sulfur amino acids may not have been a limiting nutrient for the lambs. Again sulfur amino acids almost certainly are necessary to maintain the transport system of nonaqueous soluble nutrients including antioxidants and hormones as well as

lipids. In this respect, serum lipoprotein fractions in rats have been altered by varying the quality of dietary proteins (Erwin, '60). Since sulfur amino acids will prevent dystrophy in the absence of antioxidants in the chick, probably the antioxidant transport system is not altered in this species. The transport of other nonaqueous soluble materials to tissue sites may be changed, however. It is of interest that the same diet that produced muscular dystrophy in lambs resulted in encephalomalacia in chicks. Apparently, the tissue site in which the antioxidant level becomes critical differs between species. In chicks, selenium appears to be the principle factor that prevents exudative diathesis, although Santoquin, when fed at a high dietary level, also exhibited a protective effect. Syndromes such as petechial hemorrhage and hydropericardium that simulate exudative diathesis in chicks, were produced in lambs deficient only in the antioxidant and selenium feeding did not alter the occurrence of this dyscrasia.

SUMMARY

Using a coconut oil-Torula yeast basal ration, Santoquin, selenium and a sulfur amino acid analog were used as dietary supplements for lambs and chicks. All lambs fed without Santoquin and selenium developed muscular dystrophy. The antioxidant prevented the dyscrasia, whereas selenium only delayed the onset and modified the syndromes of myopathy. These same diets (antioxidant-deficient) that resulted in dystrophy in lambs produced encephalomalacia in the chick. Antioxidant deficiency in lambs resulted in symptoms analogous to exudative diathesis in chicks. Both selenium (1.4 ppm) and Santoquin (0.1%) prevented exudative diathesis in chicks. A supplemental source of a sulfur amino acid analog did not alter the development of muscular dystrophy in lambs but prevented the dyscrasia in chicks.

ADDENDUM

Microscopic examination of biceps femoris of the lamb showing severe degree of myopathy. Under low power the section shows an increased cellularity due to infiltration of muscle fibers, interstitial spaces, and blood vessels by lymphocytes and phagocytes. There is no excess of fat cells or areolar tissue. The number of larger and smaller blood vessels is normal.

Clumps of cells are gathered around vessels, invading the adventitia, muscularis, and appear within the lumen of the vessel, adherent in some places to the endothelial cells of the intima. In many places a hyperplasia of intimal endothelial cells has occurred.

Under higher power, cross section of muscle fibers shows a marked variation in size of the fibers. Some are larger than normal, others considerably smaller. Sarcolemmal nuclei where present are superficially placed, but invading cells are present within some sarcolemmal sheaths. Abundant round cells lie between muscle fibers, in some areas separating them. Longitudinal section of the muscle shows a general loss of cross striations except in a few isolated fibers. The sacroplasm is homogeneous, having the appearance of hyalin necrosis. Vacuoles appear within the areas of necrosis in some portions of some fibers. Boundaries between some fibers are lost. A few fibers appear swollen to twice the size of others. Intraluminar cells are abundant in some muscle fibers, absent in others. These are small cells, appearing to be lymphocytes; a few have polymorphous nuclei. In some areas of necrosis of fibers, the invading dense cell masses extend from within the fiber to interstitial space and invade the adjacent fiber. A single fiber may lose its uniform caliber, only a portion being swollen, another portion narrower than normal, still another portion of normal size.

Sarcolemmal nuclei are in some areas elongated and vesicular; in others narrow and pycnotic where they form rows of closely placed nuclei. In some regions they appear to be altogether absent.

The total picture is one of necrosis of muscle fibers, with phagocytosis, lymphorrhagia, and vascular lymphocytic reaction.

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Fatty Acid Components of Rat Liver Lipids: Effect of Composition of the Diet and of Restricted Access to Food[1]

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Methods for controlling food intake have assumed additional importance in view of the postulation that obese individuals are most prone to develop atherosclerotic lesions. Attention has been centered on the effects of food intake on levels of serum cholesterol. Few comparisons of the component fatty acids of the blood and tissue lipids, however, have been made under conditions of controlled feeding of diets of known and different lipid content.

A recent paper from this laboratory (Lis and Okey, '61) reported plasma lipid fatty acid values of groups of male and female rats fed both ad libitum and with access to food restricted to two hours daily. "Meal feeding" of female rats with diets furnishing either 10% of cottonseed or 10% of coconut oil with 1% of cholesterol resulted in higher plasma cholesterol values and lower percentages of arachidonic acid in plasma cholesterol ester than those found either in females fed ad libitum or in males on either regimen. Differences were greater when the animals were fed the linoleate-rich diet.

A second group of workers in our laboratory undertook the silicic acid column fractionation of the liver lipids of the same rats, followed by gas-liquid chromatographic measurements of the fatty acid components of the 4 principal fractions. The effects due to the variation in the composition of the diets proved to be greater than those ascribable to restriction of access to food. Emphasis in presentation of the data has been altered accordingly.

METHODS

Detailed descriptions of the composition of the diets, methods of feeding, growth rates and plasma lipid analyses are to be found in previous reports (Lis and Okey, '61; Lis et al., '61).

For present purposes it is pertinent to note that the two basal diets were semisynthetic, supported normal growth, and differed only in that one contained 10% of cottonseed oil (CSO) and the other 10% of coconut oil (CN). Subgroups of rats (5 to 12 of each sex, table 1) received the diets either with or without the addition of 1% of cholesterol. The diets containing cholesterol are designated CSO+C and CN+C, respectively. Part of each group were fed ad libitum, and part had access to food only from 8 to 9 A.M. and from 4 to 5 P.M. daily. Rats were supplied with the diet at weaning and sacrificed 7 weeks thereafter. Food cups were removed from all cages about 15 hours before sacrifice. Rats were anesthetized with sodium pentothal, and as much blood as possible was removed by heart puncture. Liver samples were therefore both postabsorptive and relatively free of blood.

Livers were weighed and 2- to 3-gm samples were taken from approximately the same areas in each liver, weighed separately, and immediately homogenized in redistilled 95% ethyl alcohol. The whole procedure was usually completed within 5 minutes.

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TABLE 1

Liver lipids

Diet group[1]	No./ group	Total lipids		Total cholesterol		"Free" cholesterol[2]		Phospholipids	
		moist weight %	*mg/liver*	*moist weight %*	*mg/liver*	*moist weight %*	*mg/liver*	*moist weight %*	*mg/liver*
Males									
CSO ad libitum	7	5.5 ± 0.3[3]	519 ± 20	0.33 ± 0.02	31 ± 3	0.23 ± 0.004	22 ± 0.4	3.7 ± 0.06	357 ± 6
CSO restricted	9	4.4 ± 0.2	381 ± 9	0.26 ± 0.01	23 ± 2	0.22 ± 0.007	19 ± 0.6	3.7 ± 0.08	321 ± 7
CN ad libitum	6	4.5 ± 0.3	460 ± 32	0.23 ± 0.02	24 ± 0.6	0.22 ± 0.007	22 ± 0.7	3.9 ± 0.08	398 ± 8
CN restricted	8	4.6 ± 0.1	385 ± 24	0.26 ± 0.01	22 ± 2	0.23 ± 0.006	19 ± 0.5	3.8 ± 0.07	312 ± 6
CSO + C ad libitum	11	18.6 ± 1.0	2524 ± 167	4.6 ± 0.4	625 ± 10	0.32 ± 0.002	43 ± 2.7	3.3 ± 0.09	438 ± 12
CSO + C restricted	12	15.3 ± 0.7	1590 ± 80	3.8 ± 0.2	396 ± 25	0.31 ± 0.015	32 ± 2.0	3.5 ± 0.09	367 ± 9
CN + C ad libitum	10	8.8 ± 0.6	968 ± 88	1.5 ± 0.2	168 ± 25	0.26 ± 0.006	28 ± 0.4	3.5 ± 0.10	385 ± 11
CN + C restricted	12	9.3 ± 0.6	885 ± 75	1.5 ± 0.2	140 ± 19	0.25 ± 0.005	23 ± 0.5	3.7 ± 0.07	339 ± 6
Females									
CSO ad libitum	5	5.4 ± 0.2	320 ± 32	0.27 ± 0.04	11 ± 3	0.25 ± 0.004	15 ± 0.2	3.5 ± 0.1	208 ± 7
CSO restricted	5	3.6 ± 0.2	221 ± 36	0.20 ± 0.02	12 ± 1	0.22 ± 0.020	14 ± 1.0	3.5 ± 0.1	214 ± 5
CN ad libitum	5	4.4 ± 0.1	272 ± 16	0.22 ± 0.02	14 ± 0.8	0.24 ± 0.005	15 ± 0.3	3.5 ± 0.1	218 ± 4
CN restricted	5	4.2 ± 0.2	262 ± 20	0.20 ± 0.01	12 ± 0.4	0.23 ± 0.004	15 ± 0.3	3.5 ± 0.1	244 ± 7
CSO + C ad libitum	10	13.3 ± 1.0	1017 ± 106	3.5 ± 0.3	279 ± 33	0.37 ± 0.03	29 ± 1.9	3.4 ± 0.06	263 ± 5
CSO + C restricted	10	10.0 ± 1.4	854 ± 92	3.1 ± 0.3	240 ± 28	0.32 ± 0.01	25 ± 0.8	3.5 ± 0.06	266 ± 5
CN + C ad libitum	11	6.8 ± 0.4	476 ± 26	1.2 ± 0.2	83 ± 13	0.31 ± 0.02	27 ± 1.0	3.5 ± 0.06	248 ± 4
CN + C restricted	11	7.3 ± 0.6	456 ± 34	1.5 ± 0.3	92 ± 15	0.30 ± 0.01	19 ± 0.9	3.5 ± 0.07	221 ± 4

[1] Diet code: CSO indicates 10% of cottonseed oil; CN indicates 10% of coconut oil; +C indicates with addition of 1% of cholesterol.

[2] Free cholesterol determined on alcohol ether extract.

[3] Means and standard errors, S.E. $= \frac{\Sigma d^2}{n(n-1)}$.

The liver homogenates were extracted for one hour each, at room temperature, with three successive 150-ml portions of redistilled ethanol. The residue was extracted 16 to 18 hours, with freshly redistilled ethyl ether, in a Soxhlet extractor. The combined alcohol-ether extracts were evaporated *in vacuo*, the last of the solvent was removed in a stream of nitrogen, and the residue was dissolved at once in redistilled petroleum ether. The petroleum ether extracts were stored at 0°F until they could be analyzed. Appropriate aliquots were measured for analysis. Total lipids were determined by the method of Bloor ('28). Total cholesterol was determined by the method of Sperry and Webb ('50) with modifications as previously described (Okey and Lyman, '57). "Free cholesterol" determinations (Sperry-Webb) were included in order to rule out the possibility of major changes due to "meal feeding."[2]

An aliquot of each extract was fractionated on freshly prepared silicic acid columns by a procedure modified from that of Fillerup and Mead ('53). Larger samples, larger columns, and proportionately more eluate were used for liver extracts than for plasma. Solvents for elution were: fraction I, *cholesterol* ester, 1% ethyl ether in petroleum ether; fraction II, *triglyceride*, 4% ethyl ether in petroleum ether; fraction III, *free cholesterol, free fatty acid, mono- and diglycerides*, 50% ethyl ether in petroleum ether; fractions IV and V, *phospholipids*, 25% methanol in ethyl ether, and pure methanol. The latter fractions were combined for analyses.

Solvents were removed from the eluent fractions containing ether. They were transferred to petroleum ether and stored at 0° F and made to volume at 20° C just before analysis. The phospholipid fractions were concentrated and stored in methanol. Methylation was accomplished by controlled heating with 1% sulfuric acid and absolute methanol. Cholesterol was removed from fractions I and III before methylation. Iso-octane solutions of the methyl esters were chromatographed for determination of the fatty acid constituents of the various lipid fractions. (Wilkins aerograph, columns packed with diethylene glycol succinate polyester on chromasorb W.) Areas were measured with a planimeter. Area factors for the individual fatty acids were determined and checked at least daily against standards made up of known amounts of the most nearly pure fatty acids obtainable commercially. Data for individual fatty acids are calculated as percentages of the total fatty acid moieties of the fractions concerned, using the area factors so determined.

RESULTS

Gross liver lipid and cholesterol data are summarized in table 1. The differences in percentage of total liver cholesterol due to the kind of fat in the diet consistently exceeded the differences due to restricted access to food. Despite a very considerable variation between values for individual animals, these differences were statistically significant ($P < 0.05$) for cholesterol-fed males of both the restricted and the ad libitum groups. Total liver lipid differences between the males fed ad libitum the CSO+C diet and those fed the CN+C diet were of somewhat greater significance ($P < 0.02$). Restricted access to food brought the lipid differences below the point of significance.

The only groups in which significant ($P < 0.05$) lowering of total liver cholesterol could be ascribed to restricted access to food were the males fed cottonseed oil and cholesterol (CSO + C). The lack of significant change in the liver lipids of females due to restriction of access to food was in sharp contrast with the large increases in plasma cholesterol levels observed in the time-restricted females (Lis and Okey, '61). Moreover, when values for total liver cholesterol were plotted against those for plasma cholesterol for individual rats of the various groups, there was no indication of correlation be-

[2] It should be noted that, while the Sperry-Webb procedure is probably the most accurate of the reasonable rapid methods for "free" cholesterol, it gives liver values which are 10 to 15% high due to the effect of chromagenic contaminants which are eliminated by saponification in the procedure for total cholesterol. Williams, in our laboratory, has determined by a series of chromatographic measurements (unpublished) that at least 10% of the cholesterol in the livers of rats fed cholesterol-free diets is esterified.

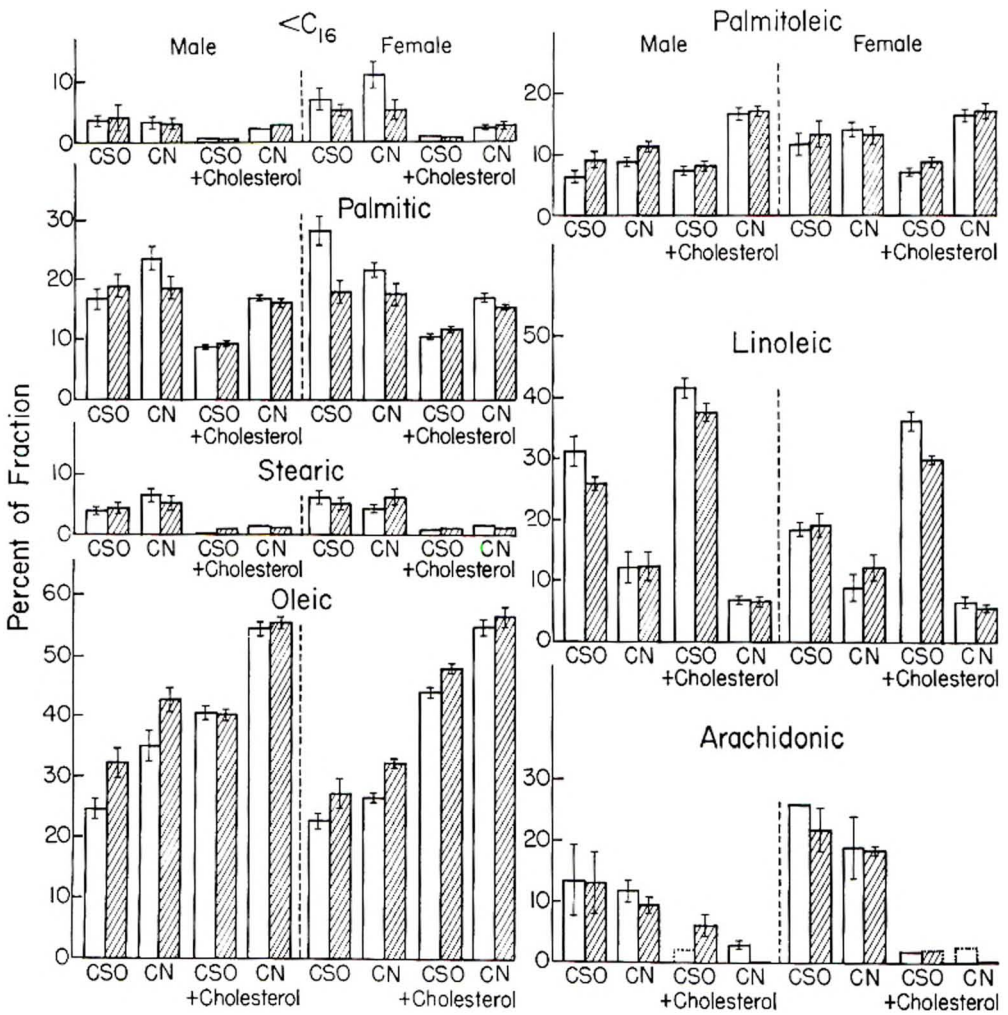

Fig. 1 Fatty acids of liver cholesterol ester (as weight percentages of the total fatty acids in the ester fraction). Tops of columns represent means, and height of bar above or below the top, one standard error of the mean. Shaded bars represent values for animals with restricted access to food, unshaded, for those fed ad libitum. The first set of bars in each group represents the values for animals fed the diets without cholesterol, the second those for the animals fed cholesterol.

tween the liver and plasma lipid and cholesterol values regardless of sex, diet or method of feeding.

"Free" cholesterol values showed only the usual variation noted in rats fed ad libitum when cholesterol was added to the diet. Cholesterol ester was found in all liver extracts when the lipids were separated chromatographically.[3]

Fatty acid components of the liver lipids: cholesterol ester fraction (fig. 1). Proportions of the fatty acid components of liver cholesterol ester varied with the composition of the fat in the diet. There was more linoleic acid in the ester when cottonseed oil was fed, more palmitoleic acid when coconut oil was fed. Cholesterol feeding led to retention of large proportions of linoleic acid in the rats fed cottonseed oil, but decreased the proportion of ester linoleic acid in the rats fed the linoleate-low coconut oil diet. The

[3] See footnote 2.

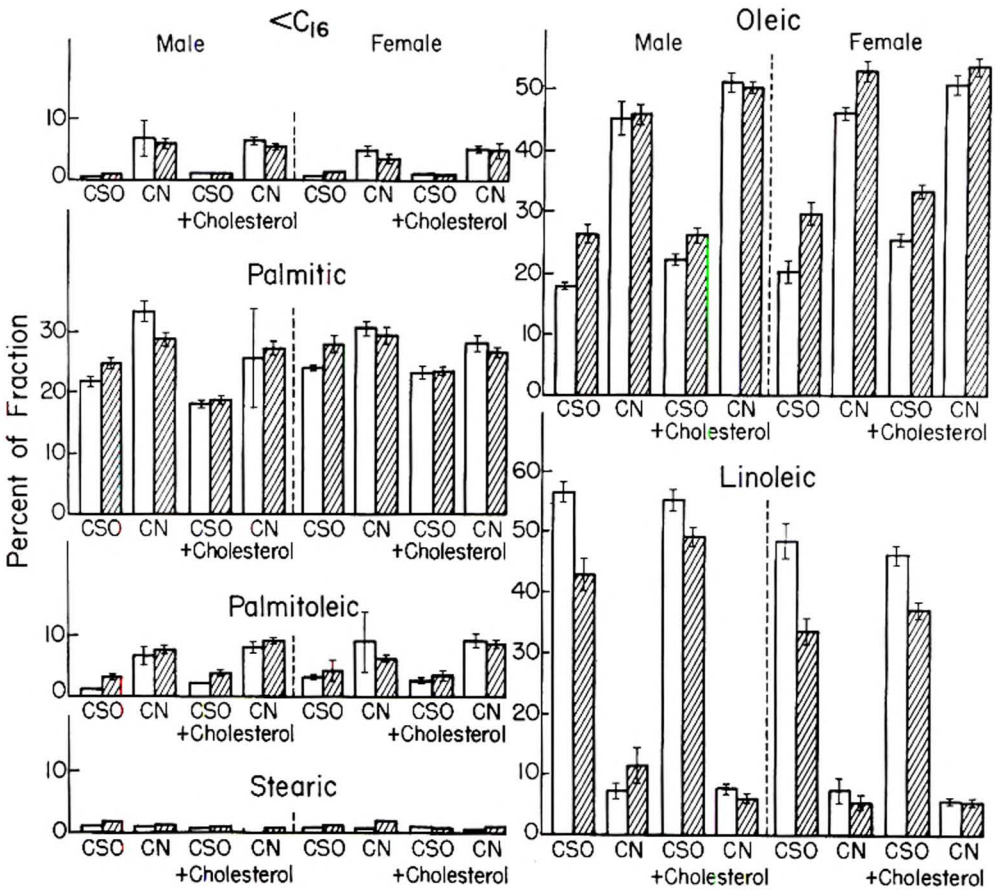

Fig. 2 Fatty acids of liver triglycerides. Data are arranged as in the previous figure.

latter evidently used higher proportions of oleic and palmitoleic acids in the esterification of cholesterol. Since the concentration of liver cholesterol ester was lower when the rats were fed coconut oil rather than cottonseed oil (table 1), perhaps the monoenoic acid was less effective in holding back cholesterol in the liver. Restriction of time of access to food had a tendency to lower the percentage of the ester linoleic acid of the rats fed cottonseed oil. Oleic and palmitoleic acids were, if anything, increased.

Changes in the fatty acid moieties of liver cholesterol ester followed much the same pattern as in the cholesterol esters of plasma (Lis and Okey, '61). No very striking differences were attributable to restriction of time of access to food in either case. The largest difference between liver and plasma was due to the very much lower content of arachidonic acid in the liver cholesterol ester, namely, 10 to 20% for the rats fed no cholesterol, decreasing to percentages too low to measure for the cholesterol-fed rats. This was in contrast with 50 to 60% for plasma cholesterol arachidonate in rats fed no cholesterol and 15 to 30% for those fed cholesterol.

Triglycerides (fig. 2). The fatty acid components of the liver triglycerides resembled those of the dietary fat to a greater extent than did the fatty acids of cholesterol esters. In the case of the males fed the CSO diet ad libitum, the percentage of linoleic acid reached that of the cottonseed oil. As in plasma, restriction of food intake had a tendency to lower percentages of linoleic acid in the liver

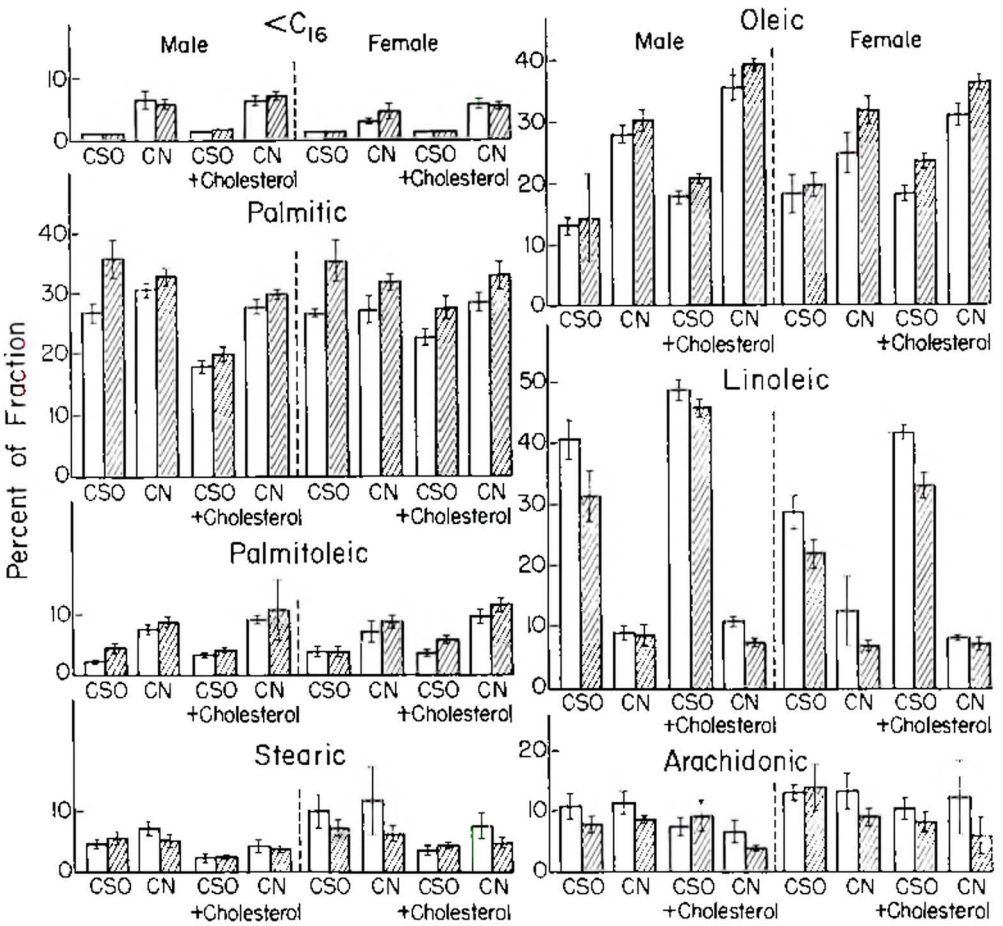

Fig. 3 Fatty acids of fraction III from silicic acid columns—mono- and diglycerides and free fatty acids. Data are arranged as in the previous figure.

triglycerides of rats fed cottonseed oil, whereas the percentage of oleic acid was increased. Also, lauric and myristic acid concentrations in liver triglyceride of the rats fed the coconut oil diet never approached those in coconut oil.

Mono- and diglycerides and free fatty acids (fig. 3). Linoleic acid content of this fraction of the liver lipids was much higher in the rats fed cottonseed oil than in those fed coconut oil. In contrast with plasma, arachidonic acid was observed consistently in proportions varying from 6 to 10% of the total fatty acid of the liver fraction, regardless of diet or method of feeding. Restriction of access to food resulted in lowered percentages of linoleic acid and increased percentages of oleic, palmitic and palmitoleic acids. The relatively large samples of liver lipid available from the silicic acid columns, however, probably made for more nearly accurate determinations of arachidonic acid than were possible for plasma.

Phospholipids (fig. 4). Percentages of the individual fatty acids for both liver and plasma were found to be more nearly constant in the phospholipids than in the other lipid fractions. Effects of restriction of time of access to food were minimal. In males fed coconut oil with cholesterol, the percentages of liver phospholipid arachidonic acid were slightly lowered—more so in the restricted rats than in the animals fed ad libitum. Percentages of stearic acid were slightly but consistently

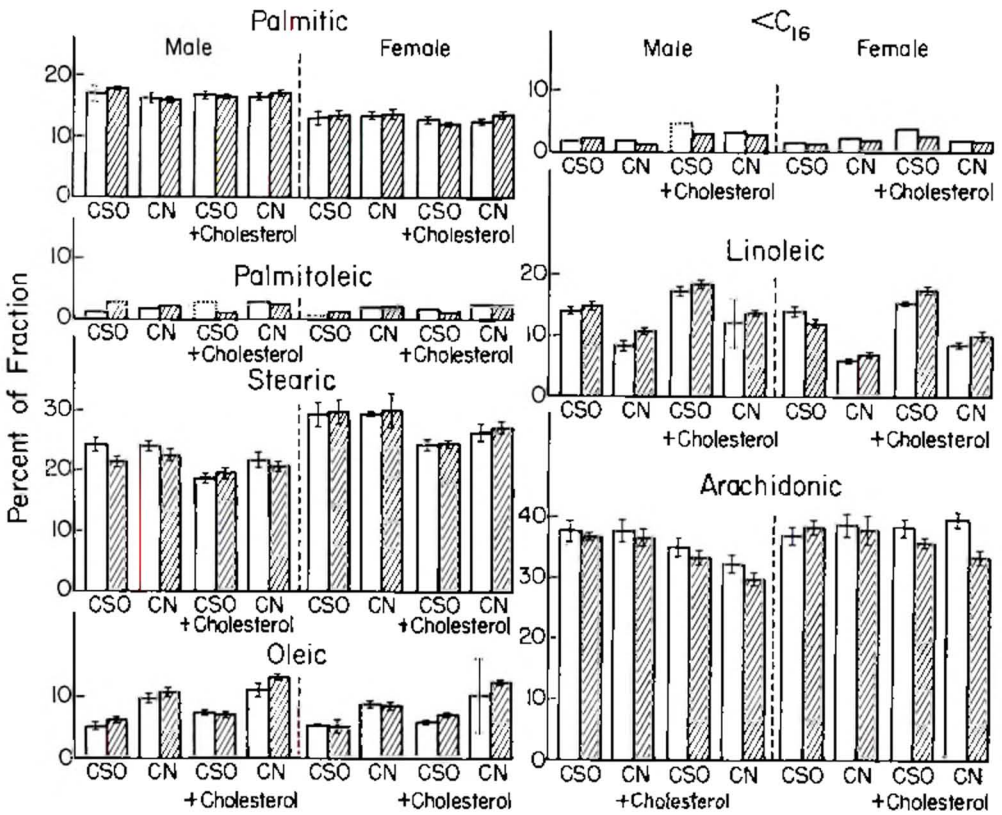

Fig. 4 Fatty acids of liver phospholipids. Data are arranged as in the previous figures.

higher and the percentages of palmitic acid lower in the liver phospholipids of females than in those of males.

In general there were greater variations in the percentages of total saturated and total unsaturated fatty acids in the liver lipid fractions than in those of plasma. It could not be concluded, as for plasma, that any one fraction of the liver lipids tended to consist of a definite percentage of saturated or of unsaturated acid.

DISCUSSION

The liver is generally considered to be the chief site of synthesis of both cholesterol and arachidonic acid. There is unmistakable evidence that fatty acids of longer chain length than about 10 carbons, as well as cholesterol, are absorbed through the lymph and so bypass the portal circulation (Kiyasu et al., '52; Chaikoff et al., '52). In a recent report, Swell et al. ('60) indicated that diversion of lymph by a fistula lowered the proportion of polyunsaturated acids in cholesterol ester of both liver and serum and, at the same time, decreased the total amount of cholesterol ester in the liver. Their data are based on observations for rats which had been fasted 48 hours before sacrifice and had previously been fed a pelleted chow containing about 5% of a fat of unknown composition. In other words, the animals were in a state in which the content of cholesterol ester of liver could be expected to be low and under which liver cholesterol synthesis might be at a minimum. The data raise the question of the influence of supply of lipids to plasma from sources other than the liver.

Data here reported and observations presented by Lis and Okey ('61) make it possible to compare liver and plasma lipid components of rats fed cholesterol-free and cholesterol-rich diets, each made

with a predominantly saturated fat and with one rich in linoleate. Since the animals were sacrificed about 15 hours after removal of food cups, they can be considered to have been in early postabsorptive rather than starved status. The rats with restricted access to food show the added effect of changed feeding patterns.

The extent to which different fatty acids are retained in liver differs considerably. The sample of coconut oil that we fed assayed about 50% laurate and 15 to 18% myristate. Neither of these acids was observed in any comparable proportions in any of the liver or plasma lipids. The obvious implications would be that they are either quickly metabolized or stored in fat depots such as adipose tissue. Linoleic acid, on the other hand, seemed to be selectively retained in liver and used in esterification of cholesterol. In coconut oil-fed rats, the percentages of linoleic acid in the cholesterol ester, glyceride and phospholipid of both plasma and liver exceeded those in the coconut oil itself. On the other hand, stresses such as restriction of food intake and feeding of excess cholesterol tended to lower the linoleic acid content of all the liver lipid fractions except the phospholipid. Linoleic acid of plasma lipid fractions varied in the same directions as those of the liver, except that the proportion of cholesterol ester linoleic acid was always lower, in plasma.

Linoleic acid is considered an essential fatty acid, necessary for the synthesis of arachidonic acid in animal tissue. Its effect in lowering serum cholesterol levels in man has been emphasized in recent clinical literature, with the implication that dietary linoleate helps prevent formation of atherosclerotic plaques. On the other hand, it is fairly well established that without a high intake of vitamin E or some equally potent antioxidant, it favors formation of lipoperoxides (Horvitt, '60). Nishida and Kummerow's ('60) observations that the hydroperoxides of methyl linoleate degrade β-lipoproteins *in vitro* possibly implies that formation of hydroperoxides may facilitate breakdown of lipoproteins, with subsequent deposition of lipid in blood vessels. The type of combination in which polyunsaturated fatty acid may occur in tissue is therefore likely to be important, as is the vitamin E content of the diet which carries the polyunsaturated fat.

Swell et al. ('60) have postulated that when the characteristic proportion of arachidonic acid in the plasma cholesterol ester of a given species of animal is high the formation of atherosclerotic plaques is less likely to occur than when it is low. The rat, with its very high percentage of plasma ester arachidonic acid, is one of the least prone to develop atherosclerosis.

The fact that the percentage of arachidonic acid in the plasma ester in this species is very much higher than that in liver may be of metabolic importance, the more so if liver is the principal site of formation of arachidonic acid from linoleic acid. We found the proportion of arachidonic acid in fraction III (probably free fatty acid) consistently much higher in liver than in plasma. Also, the percentage of this acid was higher in liver than in plasma phospholipid. Rats under the stresses imposed by cholesterol feeding or restricted access to food had lowered percentages of arachidonic acid in liver cholesterol ester, but showed comparatively little change in the arachidonic acid of phospholipid or fraction III. This may indicate that arachidonic acid formation may be limited and to some degree adapted to normal need to maintain a high arachidonic acid content of plasma cholesterol ester. Females under the stress of restricted access to a cholesterol-rich diet sometimes had too little arachidonic acid in the liver cholesterol ester to measure accurately. These were the rats with high plasma cholesterol ester values and somewhat decreased percentages of arachidonic acid in the plasma cholesterol ester. The present data might be interpreted as indicating either that liver phospholipid is the site of transformation of linoleic to arachidonic acid which is then broken off and enters plasma cholesterol ester as free fatty acid—or vice versa—that the free acid is formed and then held in phospholipid.

The proportions of *monoenoic* and the longer chain *saturated* acids in the choles-

terol ester of liver were always much higher than in the ester fraction of plasma, whereas the opposite was true of the glyceride fractions. Variations with diet and method of feeding were greater in liver than in plasma. The smaller variations in plasma may be related to the comparatively short period (15 to 18 hours) between the last access to food and sacrifice of the animals—namely, the plasma was not actively transporting dietary fat, but the composition of the liver lipids was still affected by it. Proportions of liver monoenoic acid were increased by cholesterol feeding, by lack of dietary linoleate, and by restricted access to food. Apparently the common factor in the above conditions was the need for a substitute for polyunsaturated acid as a means of maintaining the physical properties of the lipid or the lipoprotein of which it forms a part. Percentages of saturated acids in liver cholesterol ester were decreased by cholesterol feeding at the time that the percentages of monoenoic acids were relatively high (as compared with polyenoic acids). The high percentages of short-chain acids in the glyceride fractions (II and III) of the rats fed coconut oil would allow for increased proportions of saturated acid without great alterations of the melting points.

The data as a whole suggest that, under the conditions of the experiment, fatty acid components of the liver lipids show a greater capacity for variation with diet than do those of plasma. It is unexpected that even though cholesterol feeding results in more than a tenfold increase in the liver cholesterol ester of males, neither the total cholesterol ester of plasma nor its arachidonic acid content is very materially affected; also that a two- to threefold increase in the plasma cholesterol ester of females with restricted access to food may take place without changes in liver lipid composition significantly different from those for the males that show little increase in plasma cholesterol on the same dietary regimen. The data suggest the possibility of sex differences in the use of unsaturated fat.

SUMMARY

The compositions of the fatty acid moieties of the principal liver lipid fractions were determined for groups of young rats fed ad libitum and with access to food for only two one-hour periods daily. Plasma lipid data for the same rats were reported previously.

Diets were semisynthetic, and differed only in respect to fat and cholesterol content. One contained 10% of cottonseed oil, the other, 10% of coconut oil. Each fat was fed with and without 1% of cholesterol. Animals were supplied with the diet at weaning and sacrificed 7 weeks thereafter. Variations in the proportions of the fatty acids of the principal liver lipid fractions with diet and method of feeding are reported.

It was evident that linoleic acid must be selectively retained in liver. Linoleic acid percentages in the liver cholesterol ester of coconut oil-fed animals were higher than in coconut oil itself even when time of access to food was limited and cholesterol was fed. The percentages of arachidonic acid in liver cholesterol ester were much lower than in plasma ester, particularly so in animals with restricted access to cholesterol-rich diets. On the other hand, the percentage of arachidonic acid of the mono- and diglyceride and free fatty acid fraction was maintained between 6 and 10% in the livers—and was too small to measure in plasma.

Lauric and myristic acids, which constitute over 60% of the fatty acids in coconut oil, were present only in small amounts either in liver or plasma. Although percentages of monoenoic acids in liver lipids were increased when the polyunsaturated acids were decreased, percentages of saturated acids of some of the lipid fractions were decreased at the same time. Variations in the fatty acid components of the liver lipids with the stresses imposed by dietary fat, by restricted access to food, and by addition of cholesterol to the diet, suggest that there is a tendency toward maintenance of physical properties of each lipid within a characteristic range, rather than maintenance of a relatively constant ratio of saturated to unsaturated acid such as that found in the

plasma lipids. Composition of the phospholipids was more nearly independent of diet and method of feeding than was that of the other lipids. The latter was true for both liver and plasma.

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Studies on the Toxicity and Antagonism of Amino Acids for Weanling Rats[1,2]

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With the increasing use of amino acids as dietary supplements or replacements for protein, it is of importance to recognize those amino acids which may prove to be potentially toxic and the conditions under which such toxicities would most likely be encountered. From the several reports that have appeared in the literature, it is recognized that toxic or detrimental effects may result from the addition of an excess of individual amino acids to certain diets for experimental animals (Wretlind, '49, '50; Graham et al., '50; Grau and Kamei, '50; Anderson et al., '51; Hsu and Combs, '52; Russell et al., '52; Harper et al., '55). In many instances, however, the toxic effects of amino acids have been associated with studies using diets low in niacin and tryptophan (Salmon, '54; Elvehjem and Krehl, '55; Harper, '56) or with attempts to devise synthetic amino acid mixtures to replace protein in the diet (Wretlind, '49; Phillips and Berg, '54). Additional related observations on the toxicity of amino acids have been summarized by Harper et al. ('55), by Harper ('58) and by Salmon ('58).

Retardation of growth and other detrimental effects that result from feeding excessive amounts of single amino acids have usually been attributed to amino acid toxicities. More recent evidence indicates, however, that at least some of these effects may be due instead to amino acid imbalances or to antagonisms between two or more amino acids (Harper et al., '55; Elvehjem and Krehl, '55; Benton et al., '56a, b; Sauberlich, '56, '61; Harper, '58).

For the most part, the toxicities of amino acids have not been studied methodically. In numerous instances contradictory reports may be found in the literature as to the toxicity of certain amino acids or of the isomers of amino acids (Graham et al., '50; Harper et al., '55). Some of these reported discrepancies in toxicity may, perhaps, be explained by differences in the diet or experimental conditions employed. Since the individual amino acids were tested under varying conditions in the several laboratories, true comparative toxicities cannot be ascribed.

The present report is on a comparative study of the toxicity or antagonism of 19 amino acids when fed in excess under several conditions to weanling rats.

METHODS

Groups of male weanling Sprague-Dawley rats were kept singly in screen-bottom cages in an air-conditioned room. In a few instances, somewhat older animals were used. Food and water were given ad libitum, and the animals were weighed at weekly intervals. Food consumption records were kept for part of the studies. The composition of the basal diets is presented in table 1. Supplements of amino acids or protein to the basal diets were added at the expense of the sucrose in the

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TABLE 1

Composition of basal diets

Ingredients	Diet no.[1]						
	I	II	III	IV	V	VI	VII
	gm per kg of diet						
Casein (extracted)[2]	60	100	—	—	—	—	—
Corn grits[3]	400	—	—	—	—	—	—
Blood fibrin	—	—	100	—	—	—	—
Egg albumin	—	—	—	100	—	—	—
Lactalbumin	—	—	—	—	100	—	—
Soy protein[4]	—	—	—	—	—	100	—
Yellow corn (ground)	—	—	—	—	—	—	680
Corn oil	40	40	40	40	40	40	10
Cod liver oil	10	10	10	10	10	10	10
Salts[5]	40	40	40	40	40	40	40
Sucrose	445	805	805	805	805	805	242
Choline	2	2	2	2	2	2	2
L-Cystine	3	3	3	3	3	3	3
DL-Isoleucine	—	—	—	—	—	—	2
L-Lysine·HCl	—	—	—	—	—	—	6
DL-Threonine	—	—	—	—	—	—	2
DL-Tryptophan	—	—	—	—	—	—	1
DL-Valine	—	—	—	—	—	—	2

[1] All diets were supplemented with the following vitamins (mg per kg of diet): α-tocopherol, 100; α-tocopheryl acetate, 100; 2-methyl-1,4-naphthoquinone, 5; inositol, 1000; niacin, 25; Ca pantothenate, 30; thiamine, 6; riboflavin, 6; pyridoxine, 6; biotin, 0.5; folacin, 2.0; and vitamin B_{12}, 0.05.

[2] Schaefer and Knowles ('51).

[3] Nonfortified corn grits, Western Milling Company, Birmingham, Alabama.

[4] Water-washed and alcohol-extracted alpha soy protein, Drackett Company, Cincinnati, Ohio.

[5] Salmon ('47).

TABLE 2

Growth-depressing effect of amino acids when fed in low-protein diets to weanling rats

Group and basal diet no.	Supplements added to basal diet	No. of rats[1]	Av. daily food intake	Av. weight gain/week[2]	Depression in growth
			gm	*gm*	%
	Experiment 1				
1-I	None (6% casein; control)	12	9.1	21.5 ± 1.1	0
2-I	5% Glycine	8	8.1	10.9 ± 0.7	50
3-I	5% DL-Valine	8	8.4	10.0 ± 0.6	54
4-I	5% DL-Phenylalanine	12	7.6	7.6 ± 0.4	65
5-I	5% L-Tyrosine	8	7.1	5.9 ± 0.3	73
6-I	5% DL-Methionine	8	3.7	−5.0 ± 0.3	123
	Experiment 2				
7-I	None (6% casein; control)	8	7.1	15.5 ± 1.1	0
8-I	5% DL-Alanine	4	6.9	15.5 ± 1.0	0
9-I	5% L-Glutamic acid	8	6.5	14.2 ± 0.9	8
10-I	5% L-Proline	3	7.9	13.0 ± 0.6	16
11-I	5% DL-Serine	3	6.4	13.0 ± 0.7	16
12-I	5% DL-Threonine	3	6.4	11.3 ± 0.6	27
13-I	5% L-Lysine	4	6.0	9.7 ± 0.3	37
14-I	5% L-Arginine	4	5.3	8.7 ± 0.4	44
15-I	5% L-Asparagine	3	5.5	8.3 ± 0.4	47
16-I	5% DL-Isoleucine	4	6.5	7.8 ± 0.3	50
17-I	5% L-Leucine	4	6.9	7.0 ± 0.5	55
18-I	5% L-Cystine	(6/8)[3]	5.6	5.7 ± 0.3	63
19-I	5% L-Histidine	8	4.6	4.2 ± 0.2	73
20-I	5% DL-Aspartic acid	4	4.6	3.7 ± 0.3	76
21-I	5% DL-Tryptophan	8	3.6	3.2 ± 0.1	79

[1] Average initial weight of rats was 45 gm.

[2] Average ± standard error of the mean.

[3] Six rats survived out of the 8 initially started.

diets. The amino acid supplements fed were of C.P. grade or higher purity.

Animals were fed the experimental diets for 4 weeks. The majority of the studies were repeated one or more times. At the termination of most of the experiments, blood samples were obtained by heart puncture. Protein-free filtrates were prepared (Hier and Bergeim, '45) and analyzed microbiologically for various amino acids (Steele et al., '49).

RESULTS

The effects of feeding single excess amino acids in a low-protein diet to weanling rats are presented in table 2. A 6% casein—40% corn grits diet (diet 1; table 1) was used in these studies. This diet permits relatively good growth despite its low level of protein (average of 20 gm per week gain). Under such conditions less excess of amino acids would be present in the diet to complicate the study than might occur if higher levels of protein were used. The amino acids were fed at a level of 5% of the diet and in the most frequently used and readily available form (L- or DL-).

Of the amino acids tested, DL-methionine caused the most severe depression in growth; animals lost weight and occasionally died. DL-Tryptophan was also quite toxic and produced approximately an 80% depression in growth. Other amino acids producing a marked growth inhibition were, in decreasing order (76 to 27% growth-depression), DL-aspartic acid, L-histidine, L-tyrosine, DL-phenylalanine, L-cystine, L-leucine, DL-valine, DL-isoleucine, glycine, L-asparagine, L-arginine, L-lysine and DL-threonine. DL-Alanine produced no inhibition in growth and the effects of excess L-glutamic acid, L-proline or DL-serine were only slight. Deaths also occurred among the animals receiving the excess L-cystine in the diet. Although the growth inhibitions were usually accompanied by a corresponding reduction in food intakes, exceptions were noted. Rats receiving the high levels of leucine, valine, isoleucine or glycine maintained relatively high food intakes despite a growth reduction of 50% or more, but the efficiency of gain was lowered.

The toxicity of the essential amino acids appeared to be related somewhat to the dietary needs of the rat for the individual amino acids and to their relative abundance in tissues and balanced proteins. Thus, methionine, tryptophan and histidine, essential amino acids that occur normally in the least amounts in tissues and

TABLE 3

Comparative growth-depressing effect of the isomers of several amino acids for the weanling rat

Group and basal diet no.	Supplements added to basal diet	No. of rats[1]	Av. daily food intake	Av. gain/gram of food intake	Av. weight gain/week[2]	Depression in growth
			gm	*gm*	*gm*	*%*
		Experiment 1				
1-I	None (6% casein; control)	5	—	—	25.4 ± 1.3	0
2-I	5% L-Lysine	4	—	—	15.6 ± 1.1	39
3-I	5% DL-Lysine	4	—	—	17.8 ± 0.9	30
4-I	5% L-Aspartic acid	8	—	—	15.1 ± 1.0	41
5-I	5% DL-Aspartic acid	8	—	—	8.3 ± 0.3	68
		Experiment 2				
6-I	None (6% casein; control)	4	9.2	0.36	22.6 ± 1.2	0
7-I	2.5% D-Valine	4	8.6	0.32	20.9 ± 1.1	7
8-I	2.5% DL-Valine	4	8.4	0.29	18.9 ± 0.9	16
9-I	2.5% L-Leucine	4	8.2	0.19	10.3 ± 0.5	54
10-I	2.5% DL-Leucine	4	8.8	0.26	16.9 ± 0.9	25
11-I	1.0% L-Tryptophan	4	8.4	0.27	18.7 ± 1.0	17
12-I	1.0% D-Tryptophan	4	8.6	0.27	19.4 ± 0.9	14

[1] Average initial weight of rats was 45 gm in experiment 1 and 55 gm in experiment 2.
[2] Average ± standard error of the mean.

proteins, were more toxic when fed in excess than the other more abundant essential amino acids. A similar order of toxicity applied to some extent for the "nonessential" amino acids, with aspartic acid being the greatest exception.

Several amino acids were tested as to the toxicity of their isomers (table 3). The results indicated that the D-isomers of lysine, valine and leucine were less toxic than the corresponding L-form. DL-Aspartic acid, however, produced considerably more depression in growth than did a comparable level of L-aspartic acid. D-Tryptophan and L-tryptophan, when tested at a level of 1% of the diet, produced essentially the same degree of depression in growth. In another study, D-methionine was observed to be somewhat less toxic than L-methionine or calcium α-hydroxy-γ-methyl-mercaptobutyric acid (hydroxymethionine).

When the casein level in the diet was increased from 6 to 40%, the depression in growth induced by feeding excess amino acids was partially or completely counteracted (table 4). For example, the addition of 5% of L-tyrosine to the 6% casein basal diet depressed the gain in weight from 19.8 gm per week to that of only 5.5 gm per week. Increasing the casein content in the diet to 40% completely reversed the inhibition and permitted growth of 46 gm per week, which was equal to that of control animals (table 5; groups 1, 13 and 16). With some amino acids, the high level of casein reversed the inhibition to the extent that it permitted growth comparable to or better than that of animals receiving the 6% casein basal diet, but less than that obtained with the 40% casein control diet (namely, groups 1, 2, 3, and 16; table 4), indicating that some toxic effects still remained.

Supplements of DL-isoleucine (1%) and DL-valine (1%) were only slightly effective in reversing the effects produced by a 5% level of DL-leucine in the diet. However, a combined supplement of DL-methionine (0.5%), DL-isoleucine (0.5%), DL-tryptophan (0.2%), and DL-threonine (0.5%) was quite effective in reversing the growth inhibition produced by excess DL-phenylalanine in the diet. This supplement permitted 70% of the growth obtained with the 6% casein basal diet. Supplements of L-glutamic acid (1%)

TABLE 4

Influence of level of protein in the diet on the growth-depressing effect of amino acids in the weanling rat

Group and basal diet no.	Supplements added to basal diet[1]	No. of rats	Av. weight gain/ week
			gm
1-I	None (6% casein; control)	8	19.8
2-I	5% DL-Aspartic acid	4	4.0
3-I	5% DL-Aspartic acid + 34% casein	4	29.0
4-I	5% DL-Leucine	4	1.2
5-I	5% DL-Leucine + 1% DL-isoleucine + 1% DL-valine	4	4.5
6-I	5% DL-Leucine + 34% casein	4	44.0
7-I	2% DL-Methionine	8	11.6
8-I	2% DL-Methionine + 34% casein	8	20.0
9-I	5% DL-Phenylalanine	4	2.8
10-I	5% DL-Phenylalanine + 34% casein	4	43.0
11-I	5% L-Tyrosine	4	5.5
12-I	5% L-Tyrosine + 1% DL-phenylalanine	4	1.0
13-I	5% L-Tyrosine + 34% casein	4	46.0
14-I	5% DL-Tryptophan	4	3.8
15-I	5% DL-Tryptophan + 34% casein	4	28.0
16-I	34% Casein	4	44.0

[1] Average initial weight of the rats was 46 gm; the percentages of amino acids or protein indicated were added at the expense of the sucrose in the diets.

TABLE 5

Influence of various proteins on the growth-depressing effect of certain amino acids in weanling rats[1]

Group and basal diet no.	Supplements added to basal diets	Av. weight gain in 4 weeks	Growth as % of controls
		gm	
1-II	None (10% casein; control)	126.0	100
2-II	5% L-Leucine	13.3	11
3-II	Same as group no. 1-II, but food intake restricted to that of group no. 2-II	49.0	39
4-II	5% L-Aspartic acid	112.0	89
5-II	5% Glycine	106.7	85
6-II	2% DL-Methionine	76.3	61
7-III	None (10% blood fibrin; control)	115.3	100
8-III	5% L-Leucine	8.7	7
9-III	5% L-Aspartic acid	82.6	72
10-III	5% Glycine	103.4	90
11-III	2% DL-Methionine	56.7	49
12-IV	None (10% egg albumin)	108.6	100
13-IV	5% L-Leucine	48.3	44
14-IV	5% L-Aspartic acid	87.4	80
15-IV	5% Glycine	68.0	63
16-IV	2% DL-Methionine	51.7	48
17-V	None (10% lactalbumin; control)	102.4	100
18-V	5% L-Leucine	42.0	41
19-V	5% L-Aspartic acid	57.0	56
20-V	5% Glycine	54.3	53
21-V	2% DL-Methionine	46.7	46
22-VI	None (10% soy protein; control)	36.7	100
23-VI	5% L-Leucine	7.6	21
24-VI	5% L-Aspartic acid	29.0	79
25-VI	5% Glycine	32.0	87
26-VI	2% DL-Methionine	51.3	140

[1] Four rats used/group; average initial weight was 68 gm.

were ineffective in reducing the inhibitory effects of DL-aspartic acid.

In table 5 are presented results obtained when excess amounts of leucine, aspartic acid, glycine, or methionine were fed in diets containing a 10% level of either casein, blood fibrin, egg albumin, lactalbumin, or soy protein as the source of protein. The degree of inhibition in growth produced by the addition of the excess amino acids to the diets was dependent not only upon the level of protein in the diet, but also upon the specific protein used. Thus, for instance, a level of 5% of L-aspartic acid in the diet produced a greater inhibition in growth when fed in the presence of lactalbumin than when fed with the other proteins. Moreover, DL-methionine, at a 2% level of the diet, produced a growth depression when fed with casein, blood fibrin, egg albumin or lactalbumin, but improved growth when fed in the presence of soy protein (namely, groups 7 and 11 vs. 22 and 26; table 5). The reduction in food intake that usually accompanied the feeding of an excess amount of an amino acid appeared, based on pair-feeding studies, to account for only part of the reduction in growth (groups 1, 2, and 3; table 5). As noted earlier, the efficiency of gain was reduced by the presence of excess amounts of the individual amino acids in the diets (tables 2 and 3).

The response observed from the feeding of excess cystine in the diet appeared to be different from that of the other amino acids studied (table 6). Excess L-cystine (5% level) when fed in the 6% casein basal diet produced some deaths,

whereas the growth of the survivors was considerably inhibited. When the casein in the high-cystine diet was increased to a level of 36%, the number of deaths was greatly increased (groups 1, 2 and 3; table 6). Growth of the animals, however, prior to death and of the survivors, was greatly enhanced by the additional amount of casein in the diet. The deaths occurred very suddenly and were accompanied by an apparent massive hemorrhage of the liver. In contrast, supplements of 30% of lactalbumin to the low-protein, high-cystine diet prevented the deaths and permitted good growth of the animals.

Although excess methionine in the diet inhibited growth very markedly, no deaths were noted when it was fed in the presence of supplements of casein or other proteins. It was noted, however, that proteins low in the sulfur amino acids, such as peanut meal or soybean meal, were more effective than casein or lactalbumin in preventing the depression in growth (table 6). In other studies (data not shown), it was noted that the inhibition in growth resulting from an excess of DL-methionine (3% level) in the 6% casein diet was not influenced appreciably by dietary supplements of cystine (1% of the diet), glycine (0.5%) and arginine (0.5%), guanidoacetic acid (1%), niacin (1%) or choline (1%), or by the omission of choline from the diet.

Previously it had been noted that the growth of rats fed a corn grain diet was severely suppressed by additions of hemoglobin to the diet (Sauberlich, '56). The effect of the hemoglobin could be prevented by further supplementation of the diet with isoleucine. Studies summarized in table 7 were designed to determine whether the high levels of leucine in the hemoglobin were responsible for the inhibition in growth. The basal diet (no. VII, table 1) used contained 68% of ground yellow corn supplemented with those amino acids previously demonstrated to be deficient in corn (Sauberlich et al., '53) in order to obtain a reasonable rate of growth (25 gm gain per week). Again it was observed that 10 or 20% supplements of hemoglobin to the basal diet completely inhibited growth of weanling rats. Dietary supplements of 0.5% of DL-isoleucine fully prevented the inhibitory effects of the hemoglobin.

When supplements of L-leucine were added to the basal diet comparable to the amounts contributed by a 10 or 20% level of hemoglobin (1.6 or 3.2% level of leucine supplement to the diet), inhibition in growth was also noted. The inhibition in growth was not as severe as that produced by the hemoglobin supplements. Dietary

TABLE 6

Cystine and methionine growth-depressing effect in the weanling rat

Group and basal diet no.	Supplements added to basal diets	No. of rats and survival[1]	Av. weight gain/ week
			gm
1-I	None (6% casein; control)	4/4	24.5
2-I	5% L-Cystine	6/8	7.7
3-I	5% L-Cystine + 30% casein	2/10	33.2
4-I	5% L-Cystine + 30% lactalbumin	8/8	37.7
5-I	30% Casein	4/4	44.0
6-II	None (10% casein; control)	4/4	29.3
7-II	3% DL-Methionine	4/4	8.6
8-II	3% DL-Methionine + 30% lactalbumin	4/4	24.2
9-II	3% DL-Methionine + 30% casein	4/4	23.0
10-II	3% DL-Methionine + 60% peanut meal[2]	4/4	30.5
11-II	3% DL-Methionine + 60% soybean meal[2]	4/4	31.5
12-II	30% Casein	4/4	43.0

[1] Four out of 4 rats started survived the experiment, etc.; average initial weight was 59 gm.
[2] The peanut meal and soybean meal each contained 50% of protein and were extracted with methanol in a continuous extractor prior to use.

TABLE 7

Leucine growth-depressing effect in the weanling rat

Group and basal diet no.	Supplements added to basal diet	Av. weight gain/ week[1]
		gm
1-VII	None (68% corn; control)	25.3
2-VII	10% Hemoglobin	0.3
3-VII	20% Hemoglobin	0.1
4-VII	10% Hemoglobin + 0.5% DL-isoleucine	31.0
5-VII	20% Hemoglobin + 0.5% DL-isoleucine	39.0
6-VII	1.6% L-Leucine[2]	17.2
7-VII	3.2% L-Leucine[2]	10.0
8-VII	1.6% L-Leucine + 0.5% DL-isoleucine	31.2
9-VII	3.2% L-Leucine + 0.5% DL-isoleucine	18.9

[1] Four rats used/group; average initial weight was 48 gm. Supplements to the basal diet were added at the expense of sucrose.

[2] These levels of L-leucine correspond to the amounts contributed by the hemoglobin added to the diets for groups 4-VII and 5-VII, respectively.

additions of 0.5% DL-isoleucine completely reversed the effects of the 1.6% level of L-leucine and partially that of 3.2% level. The results indicated that the inhibition in growth produced by the hemoglobin was related largely to its high leucine content. The excess leucine in the diet appeared to produce an imbalance or antagonism which was primarily associated with the availability and metabolism of isoleucine.

Summarized in table 8 are the results of analyses for free amino acids in plasma obtained from rats fed diets containing excess amounts of various amino acids. As expected, the plasma free amino acid level increased for the specific amino acid fed in excess in the diet. Marked differences were noted, however, as to the degree of the increase among the various amino acids fed. The increase was the least for glutamic acid and the highest for tyrosine and methionine when these amino acids were fed in excess. Feeding of the individual amino acids in excess in the diet was not reflected in any constant relationship between toxicity and the free amino acid level in the plasma. Those amino acids fed in excess, however, and found to produce the greatest relative increase in the plasma free amino acid level over that of control animals, were frequently the more toxic amino acids. Thus, for example, the plasma free amino acid level of methionine was very markedly increased by excess amounts of this amino acid in the diet (group 1 vs. 14 and 15; table 8). Similar observations were noted for tryptophan, DL-aspartic acid, histidine and tyrosine. Each of these amino acids, when fed in excess at the same level, also produced marked depressions in growth. Excess leucine, isolucine or glycine in the diet did not produce as marked an increase in the plasma level of these amino acids, respectively, but did inhibit growth considerably. This may indicate a form of antagonism or toxicity peculiar to these particular amino acids. Nevertheless, increases of two- to threefold in the plasma were noted for these amino acids.

Excess alanine in the diet produced high plasma free levels of this amino acid without any inhibitory effects on growth. Similarly it was observed that excess proline or serine in the diet produced high free levels of the corresponding amino acid in the plasma, but with only slight effects on growth. Excess glutamic acid in the diet produced little or no inhibition in growth and only a 50% increase in the free amounts of this amino acid in the plasma. Dietary supplements of DL-threonine (5% of the diet) increased considerably the level of free L-threonine in the plasma (11 μg per ml to 158 μg), but with only a 27% inhibition in growth. The free glycine level of the plasma was also increased by the addition of threonine to the diet as a probable reflection of threonine metabolism. The free arginine con-

TABLE 8

Plasma free amino acid levels of weanling rats fed diets containing high amounts of amino acids

Group no.	Supplement added to basal diet[1]	Amino acid content (μg/ml plasma)[2]													
		Alanine	Arginine	Aspartic acid	Cystine	Glutamic acid	Glycine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Tyrosine
1	Non (6% casein; control)	17	15	7	6	55	22	20	7	15	37	9	10	11	5	12
2	+5% DL-Alanine	*114*	—	—	—	—	21	16	—	—	—	—	—	—	5	—
3	+5% L-Arginine	—	*84*	—	—	—	19	16	—	—	—	—	—	—	2	—
4	+5% L-Aspartic acid	—	3	*46*	—	—	26	18	—	—	—	—	—	—	2	—
5	+5% DL-Aspartic acid	—	4	*120*	—	—	25	23	—	—	—	—	—	—	3	—
6	+5% L-Cystine	—	—	—	*58*	—	21	24	—	—	—	—	—	—	4	—
7	+5% L-Glutamic acid	—	20	—	—	*76*	16	22	—	—	—	—	—	—	4	—
8	+5% Glycine	—	6	—	—	—	*68*	18	—	—	—	—	—	—	5	—
9	+5% L-Histidine	—	16	—	—	—	25	*268*	—	—	—	—		—	6	—
10	+5% DL-Isoleucine	—	10	—	—	—	21	22	*23*	—	—	—		—	4	—
11	+5% L-Leucine	—	19	—	—	—	21	18	—	*34*	—	—	—	—	5	—
12	+5% L-Lysine	—	23	—	—	—	18	19	—	—	*272*	—	—	—	2	—
13	+5% DL-Lysine	—	—	—	—	—	—	—	—	—	*114*	—	—	—	—	—
14	+2% DL-Methionine	—	27	—	—	—	15	14	—	—	—	*143*	—	—	5	15
15	+3% DL-Methionine	—	—	—	—	—	12	—	—	—	—	*260*	—	—	—	—
16	+5% DL-Phenylalanine	—	—	—	—	—	21	18	—	—	—	—	*52*	—	6	—
17	+5% DL-Threonine	—	17	—	—	—	42	22	—	—	—	—	—	*158*	5	—
18	+5% DL-Tryptophan	—	7	—	—	—	22	13	—	—	—	—	—	—	*60*	—
19	+5% L-Tyrosine	—	12	—	—	—	17	14	—	—	—	—	—	—	—	*740*
20	+Casein (34%)	—	39	13	—	—	16	17			54	29	19	—	23	20
21	+Casein+2% DL-methionine	—	—	—	—	—	16	13	—	—	—	*288*	—	—	12	21
22	+Casein+3% DL-methionine	—	—	—	—	—	15	14	—	—	—	*324*	—	—	—	—
23	+Casein+5% DL-leucine	—	39	—	—	—	16	14	—	*51*	—	—	—	—	21	—
24	+Casein+5% L-lysine	—	—	—	—	—	—	—	—	—	*136*	—	—	—	—	—
25	+Casein+5% DL-phenylalanine	—	35	—	—	—	14	17	—	—	—	—	*33*	—	21	—
26	+Casein+5% DL-tryptophan	—	—	—	—	—	15	17	—	—	—	—	—	—	*53*	—
27	+Casein+5% L-tyrosine	—	46	—	—	—	9	17	—	—	—	—	—	—	20	*135*
28	+Casein+5% DL-aspartic acid	—	—	*164*	—	—	23	24	—	—	—	—	—	—	19	—

[1] Basal diet no. 1 (6% casein) used in these studies.

[2] Average of individual analyses on 3 to 12 rats per group; protein-free plasma filtrates assayed by microbiological methods.

tent of plasma appeared to be reduced below normal levels by the presence of excess aspartic acid, glycine or tryptophan in the low-protein diet (groups 1, 5, and 18; table 8). The free aspartic acid level of the plasma was enhanced considerably more by the dietary supplements of DL-aspartic acid than by L-aspartic acid.

Increasing the casein level in the control diet from 6 to 40% (groups 1 and 20) also increased the plasma free amino acid levels, except for glycine and histidine. When excess amino acids were fed in the presence of the higher level of casein, the relative increase of the respective amino acid free in the plasma was reduced from that observed at the lower level of casein.

DISCUSSION

Based on the toxic effects observed in the present study, there appears to be little likelihood that amino acid toxicities as such would occur under ordinary feeding practices with normal levels of dietary protein. Nevertheless, these studies indicate which amino acids could be suspect. Moreover, the investigations emphasize again the importance of maintaining a proper balance among the amino acids in a diet.

It is apparent from the present study that nearly all amino acids can be demonstrated to produce deleterious effects under certain conditions; but no common basis is apparent to explain all of the amino acid toxicities. The deleterious effects appear to be the result of several possible actions associated to a large extent with specific individual amino acids. Russell et al. ('52) have suggested that the toxic effects are the result of inherent properties of the individual amino acid molecules or their breakdown products.

Although the effects resulting from feeding excess amounts of individual amino acids in the diet have usually been attributed to amino acid toxicities, indications are that this may not always be true. It would appear that amino acid imbalances may also be created by the presence of excess amino acids in the diet. When the excess amino acids were fed singly in a high-protein diet containing an adequate supply of all amino acids rather than in a low-protein diet, the deleterious effects were prevented or reduced. Excess amino acids would have less effect in disrupting the normal balance of amino acids when added to a high-protein diet.

The high level of the individual amino acids in the diet in itself did not appear to explain the toxic effects, since diets containing 80 or 90% of protein may be fed to rats with no appreciable resultant depression in growth. Yet these same diets will contain a number of amino acids in excess of the levels fed in these toxicity studies. Similarly, the effects do not appear to be associated with the use of free amino acids in the diet since properly balanced amino acid mixtures can be used to replace protein completely in diets, with no reduction in growth (Sauberlich, '61).

The alterations in the balance or ratio of the free amino acids in the plasma as a result of feeding the excess amino acids in the diet may be responsible for some of the effects. Such changes in plasma amino acid balance may interfere with the passage of amino acids into the cells and with enzymatic ability and equilibrium for tissue protein synthesis (Sauberlich, '56, '58; Harper, '58).

Earlier studies have indicated that the D-isomers of amino acids were more toxic than the corresponding L-forms (Graham et al., '50; Berg, '53; Phillips and Berg, '54) although in a few instances the reverse has been found to occur (Wretlind and Rose, '50; Harper et al., '55; Berg, '53). As indicated above, when balanced amino acid mixtures were used as substitutes for protein in diets, the D-isomers of amino acids produced little or no effect on the growth of rats (Sauberlich, '61). The present studies indicate that the D-isomers of those amino acids that are converted readily to the L-form by the rat exhibited inhibitory effects when fed in excess. The effect of the D-isomer, however, did not exceed that of the corresponding L-form. Aspartic acid appeared to be an exception, since DL-aspartic acid was more inhibitory than L-aspartic acid. Graham et al. ('50) observed a similar effect, while noting high plasma levels of free aspartic acid when excess DL-aspartic acid was fed. The difference in inhibitory effects exhibited may be related to difficulties in the metabolism of D-aspartic acid or to an antago-

nism by this isomer. In this respect, Graham et al. ('50) also noted that feeding DL-aspartic acid increased considerably the urinary excretion of glutamic acid. In the present study, however, it was noted that supplements of glutamic acid in the diet did not reduce the inhibitory effects of DL-aspartic acid. Anderson et al. ('51) observed that high amounts of DL-aspartic acid in the diet did not inhibit the growth of chicks, indicating specie differences in the toxicity of amino acids.

The results noted with DL-aspartic acid, leucine and phenylalanine would indicate that antagonistic effects exist among certain amino acids. Similar effects have been reported by several investigators (Harper et al., '54, '55; Benton et al., '56a, b; Harper, '58; Elvehjem and Krehl, '55). When a more normal balance of ratio exists between the amino acids in the diet, these antagonistic effects are reduced or eliminated. Wretlind ('56) also noted that D-leucine antagonized the utilization of D-valine for growth of the rat. Undoubtedly additional examples will be revealed in the future.

Excess dietary amino acids may also be inhibitory as a result of the high plasma free amino acids levels interfering in the reabsorption of the filtered essential or limiting amino acids by the kidney (Beyer et al., '47; Kamin and Handler, '51; Salmon, '54; Sauberlich and Salmon, '55). Such effects could be counteracted by additional protein in the diet or by supplements of the appropriate amino acids. Antagonism of amino acids in their absorption from the intestine is possible, but little evidence has been found thus far to indicate this to be a major factor (Kamin and Handler, '52; Sauberlich and Salmon, '55; Jervis and Smyth, '59).

The rate of detoxification, metabolism and removal of excess amino acids or their breakdown products in the plasma may be a limiting factor and thereby produce detrimental effects. Thus the high levels of methionine and tyrosine noted in the plasma may indicate difficulty in the metabolism of these amino acids. Limitations in the availability of compounds necessary in the removal or detoxification of certain amino acids may also account for some of the effects. Such a condition may be associated with the toxic manifestations of excess dietary cystine and methionine. It has been observed that the toxic effects of excess dietary methionine can be counteracted by increasing the intake of arginine and glycine (Roth and Allison, '49; Hardin and Hove, '51) or of glycocyamine (McKittrick, '47). Although these compounds had little influence in the present study, it is possible that their use in higher amounts or with lower levels of methionine would have demonstrated such protective effects.

Gullino et al. ('55a) reported on the toxicity of the L- and D-forms of the 10 essential amino acids as determined by single intraperitoneal injections of the individual amino acids into adult rats. Considerable differences were noted in the toxicity of the L-amino acids, whereas the corresponding D- forms were reported to be uniformly less toxic, except for arginine whose D- and L-forms were nearly equally toxic. The toxicity of the individual amino acids when injected differed considerably from that observed when amino acids are fed in the diet. In another communication, Gullino et al. ('55b) reported that L-arginine demonstrated a protective effect when injected along with the amino acids studied. In the present study it was noted that the plasma level of free arginine was reduced by dietary excesses of certain individual amino acids. These results indicate further the complexity of factors that may influence the toxicity, antagonism and metabolism of amino acids.

SUMMARY

The toxicity of 19 amino acids when fed individually in excess (5% level) in a low-protein diet was studied with weanling rats. With the exception of alanine, all amino acids tested produced depressions in growth to varying degrees. Methionine produced the severest growth depressions followed in decreasing order by tryptophan, DL-aspartic acid, histidine, tyrosine, phenylalanine, cystine, leucine, valine, isoleucine, glycine, asparagine, arginine, L-aspartic acid, lysine and threonine. Glutamic acid, proline and serine produced only slight growth depressions.

Results of studies on the isomers of several amino acids indicated that the D-iso-

mers usually produced less inhibition in growth than the corresponding L-form. DL-Aspartic acid, however, produced greater detrimental effects than L-aspartic acid.

The growth depression of the several amino acids studied could be partially or completely prevented by supplements of protein to the diet. The degree of toxicity of the amino acids was dependent also upon the specific protein fed the animals.

L-Cystine at a level of 5% in a low-protein diet depressed growth and produced some deaths. When the casein in the high-cystine diet was increased to a level of 36%, the number of deaths was greatly increased. When lactalbumin was fed in place of casein, however, no deaths occurred and growth was nearly normal.

Supplements of leucine or of hemoglobin to a corn grain basal diet produced a severe depression in growth. The effect was partially-to-completely reversed by supplements of isoleucine.

Analyses of the plasma usually demonstrated high values for free amino acid content of the amino acid supplemented in excess in the diet. However, the toxicity of several amino acids did not appear to be directly related to their plasma concentration.

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Food Intake and Energy Expenditure of Elderly Women with Varying-Sized Families[1]

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The Food and Agriculture Organization of the United Nations has summoned two Committees on Calorie Requirements and, in each of the two reports ('50, '57), attention has been called to the dearth of information on energy requirements; the reports noted especially how little is known about the effects of aging on energy needs. It is now trite to state that elderly people are becoming a larger and larger proportion of the population in many countries of the world. In time of food shortage, they, and adolescents, are peculiarly vulnerable groups and there is little indication that, in the near future, the available food will be more than sufficient for the world's population. It is therefore of paramount importance to assemble knowledge about the nutritional requirements of elderly people.

As part of a large-scale survey on various population groups of elderly people living in Scotland, we have recently published the results of studies on the food intake and energy expenditure of elderly women living alone in their separate households (Durnin et al., '61a) and of elderly men working in heavy and light engineering (Durnin et al., '61b; Durnin and Blake, '61). Previously we have reported similar measurements on young and on middle-aged women (Durnin et al., '57), and so we are particularly interested in obtaining any relevant data on changes in these measurements with aging. The present paper describes an investigation, during a period of one week, of the total food intake and expenditure of energy of a group of elderly housewives living with their various families.

EXPERIMENTAL

Twenty-one women took part in the study and all lived in the Glasgow area. Their mean age was about 60 years and mean body weight was about 60 kg. Precise details of the age, height and weight of the women are given in table 1.

The subjects were studied in two groups, 9 women taking part in a survey in February and the other 12 in September, 1959. The separate results for the two groups did not differ significantly (e.g., mean daily intake of calories: group 1, 1962 Cal.; group 2, 1931 Cal.), nor did the ages, heights and weights; they have thus been considered as one group of 21 women.

The living conditions of the women and their social groups differed considerably. Some of them were middle-middle class, some lower-middle and some were

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TABLE 1

Mean ages, heights and weights, with standard deviations and ranges, of 21 elderly women

	Mean	S.D.	Range
Age	60	3.7	54–66
Height, feet, inches	5′ 1″		4′ 7⅞″–5′ 6½″
Height, cm	155.3	6.4	142–169
Weight, pounds	133		103–172
Weight, kg	60.4	9.6	47–78

from the lower social groups. Their household accommodation also varied accordingly. All of them had separate kitchens and either bathroom or toilet. Excluding the kitchen and bathroom, 7 women lived in two-room flats, three in three-room flats and one in a 4-room flat. Two women inhabited three-room semi-detached houses, two were in 4-room semi-detached houses and one had a 5-room semi-detached house. Two women lived in 4-room detached houses; and one owned a small hotel.

Two of the subjects lived alone, 6 lived with their husbands only, two with their respective sons only, two with husband and son, two with husband and daughter, one with husband, son and daughter, one with daughter and son-in-law, one with two daughters, one with husband and two daughters, one with two sons, one with her brother and one looked after her hotel guests. Thus the social groups, the dwellings and the family size were typical of those of large numbers of elderly women living in towns and cities in Britain.

The women were all involved in the routine duties of running a household but 6 of them did other work as well; one supervised the running of her small hotel, one was a seamstress who had her own small shop, one acted as a chemist's assistant for 3½ hours each evening, one worked full time in a lawyer's office, one spent about two hours weekly in her husband's gown shop and another served for 6 hours a day in a canteen in British Railways. All of the subjects were healthy. They were a very cooperative group and had volunteered to participate in the study in response to a newspaper report. Their leisure activities were wide-ranging and included golf, dancing, visits to the cinema, amateur dramatics, and so on, as well as visiting and being visited by friends and looking after grandchildren.

Measurements of energy expenditure. These were obtained by the factorial method, namely, the subjects kept, in special diary cards, records of their activities throughout the whole 24-hours of each day during the week's survey. The energy expenditure in most typical "activities" was measured, in the individual homes, by indirect calorimetry using the Max-Planck respirometer (Müller and Franz, '52). Expired air was collected in butyl-rubber bladders stored in canisters, also containing expired air so as to minimize diffusion of gases across the rubber. The bladders were then transported to the laboratory and the oxygen and carbon dioxide measured by means of a Godart analyzer, calibrated against a Haldane gas-analysis apparatus. The total daily energy expenditure was estimated by multiplying the calorie cost of each activity by the length of time spent in it and summing the separate totals. This method is described in some detail by Garry et al. ('55) and its relative accuracy has been assessed by Durnin and Brockway ('59).

Measurements of food intake. Food intake was measured by having the subject weigh and record each item of food immediately prior to eating. Plate wastage after the meal was also weighed and noted and deducted from the original quantities. The nutrient value of the foods was calculated using the tables of McCance and Widdowson ('60); net calorie values of 4, 9 and 3.75 were applied to protein, fat and carbohydrate, respectively.

Each subject was visited at least once daily by a dietitian from the survey team in order to elicit possible error and assist with difficulties. The dietary procedures are explained in more detail by Durnin et al. ('57).

RESULTS AND DISCUSSION

The mean daily intake and expenditure of calories for the 21 women and the proportion of the calorie intake derived from protein, fat and carbohydrate are shown in table 2. The agreement between the mean values for intake and output of energy (about 8 to 9% difference) is reasonable for such measurements, but again the discrepancy between individual intakes and expenditures is considerable (Durnin, '61). The mean value of about 1950 Cal. per day is very similar both to the FAO recommendation ('57) to that of the National Research Council ('58), and to our own previously published observations for women of the same age who

TABLE 2

Mean daily intake and expenditure of calories and contribution of protein, fat, carbohydrate and alcohol to the energy value of the diets of 21 elderly women

	Cal./person/day	% of total
Protein	247	12.7
Fat	833	42.8
Carbohydrate	854	43.9
Alcohol	10	0.6
Total energy intake	1944	
Mean energy expenditure	2133 (S.D. ± 298)	

lived alone (Durnin et al., '61a). Few experimental studies give comparable results; Gsell ('58) tabulates some of the few investigations on the dietary intakes of elderly people and most of the results for women show lower values than ours.

The intake of protein provides about 13% of the total calories; some 40% of this was of animal origin and 60% vegetable, which is the reverse of the proportions in our previous group of elderly women (Durnin et al., '61b).

The proportion of fat (43%) seems large, and is higher than the 41% found for elderly (Durnin et al., '61a) and young and middle-aged women (Durnin et al., '57), the 39% for elderly men (Durnin et al., '61b) and the 35% described for coal miners (Garry et al., '55).

In table 3 are given the actual intakes of protein, fat, carbohydrate, calories, calcium and iron. One quarter of the protein came from meat and meat products and about 1/5th from milk, cheese and eggs. Of the fat, butter provided just over 20%, cakes and biscuits 20%, meat about 16% and milk, cheese and eggs about 15%. The important contributions to the calorific value of the food were cakes and biscuits (20%), meat (12%), bread and rolls (12%), milk, eggs and cheese (11%) and butter (9%). Since the flour in Britain is fortified by the addition of calcium, most of the intake of calcium was derived from bread, rolls, cakes, and biscuits and from milk, both of which contributed about 250 mg daily per person; about one third of a pint of milk was taken per day on the average. Cheese supplied about 100 mg of calcium. Some of the individual intakes of calcium were quite low, 5 of the women having about 500 mg per day or less (one had less than 400 mg daily).

In table 4 are shown the mean times spent in the different activities of the day by the 21 women, together with the energy cost of these activities. The 8½ hours spent daily in bed is almost 1½ hours less than that recorded by the elderly women living alone (Durnin et al., '61a). "Sitting," as with all groups, is an "activity" which occupies a large amount of the day including as it did meals, visits to the

TABLE 4

Average length of time spent per day in various activities, with energy expenditures, by 21 elderly women

Activity	Time	Cal./day
	minutes	
Bed	509	443
Personal	55	103
Sitting	405	457
Standing	48	58
Housework	285	662
Heavy housework	9	37
Walking	66	206
Shopping	26	60
Dancing	3	10
Miscellaneous[1]	34	77
Total	1440	2113

[1] Includes working in shop, working in canteen, working as seamstress, playing golf, and other activities.

TABLE 3

Mean daily intake of protein, fat, carbohydrate, calories, calcium and iron for 21 elderly women

	Mean	S.D.	Range
Protein, gm	61.7	12.3	41.7–87.3
Fat, gm	92.5	23.2	54.5–152.4
Carbohydrate, gm	227.6	54.5	142.0–326.6
Calories	1944	404	1243–2886
Calcium, mg	758	237	388–1412
Iron, mg	11.1	2.8	8.3–17.8

TABLE 5

Energy expenditures in various household activities: means from 56 determinations on two groups of women of varying body weight

No. of subjects	Mean weight	Range	Washing dishes	Preparing food	Sweeping and dusting	Polishing furniture
	kg	*kg*	*Calories per minute*		*Calories per minute*	
9	52	47–57	2.3	2.1	3.3	4.1
8	69	62–76	2.6	2.5	3.2	4.5

Miscellaneous activities

	Cal./minute
Sitting knitting	1.7 (76)[1]
Pushing baby in pram	2.8 (52)
Cleaning gas cooker	2.5 (50)
Sitting ironing	2.4 (67)
Washing floor by hand	3.4 (75)
Serving and tidying up in canteen	3.7 (75)

[1] Numbers in parentheses indicate weight of subject.

hairdresser, the cinema, office work and sewing. "Housework" took up somewhat less than 5 hours, and "walking" a little over one hour. "Personal" includes washing, dressing, using the toilet, and so on, and "shopping" includes only the time actually spent in and between shops.

In table 5 are shown some of the measurements of the energy expenditures in different forms of housework. The various headings are simplified; "washing dishes" often included drying them also, "preparing food" embodies such things as peeling potatoes, preparing vegetables, cooking, and similar activity.

The apparent energy requirements of this group of housewives, who looked after families of varying size, are very similar to those of women of the same age who lived alone; in this instance, therefore, the size of the families (which did not have any young children) seems to have little measurable effect on the degree of physical energy required to care for the household.

SUMMARY

The total food intake and energy expenditure were measured, during a period of one week, on 21 elderly women living with their families of varying sizes. They were aged about 60 and weighed about 60 kg. The mean intake and expenditure of energy were about 1950 and 2100 Cal. per day, respectively. Protein accounted for about 13% of the calories, fat about 43% and carbohydrate, 44%. The exact intakes of protein, fat, carbohydrate, calcium and iron are tabulated.

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Magnesium Deficiency in the Guinea Pig. Mineral Composition of Tissues and Distribution of Acid-Soluble Phosphorus[1,2]

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House and Hogan ('55) reported a high incidence of mineral deposits in guinea pigs reared with diets which contained excessive phosphorus and relatively low levels of potassium and magnesium. When magnesium and potassium were added so as to supply approximately 1.5% of potassium and 0.35% of magnesium, deposits were rare. Soft tissue calcification has since been shown to be due primarily to magnesium deficiency (Maynard et al., '58; O'Dell et al., '60). Maynard et al. ('58) reported that the kidneys of deficient animals contained a large excess of calcium and that other organs such as liver, heart and muscle were affected to a lesser degree.

A calcinosis syndrome similar to that observed in magnesium-deficient guinea pigs was described by Wulzen and Bahrs ('41) and attributed to lack of an anti-stiffness factor. Later, van Wagtendonk ('44) investigated the distribution of the acid-soluble phosphorus in tissues of guinea pigs deficient in this factor and found an increase in the total acid-soluble and inorganic phosphorus and a decrease in the easily hydrolyzable phosphorus. Vitale et al. ('57) reported that magnesium deficiency in young rats caused uncoupling of oxidative phosphorylation in heart mitochondria. One would expect a decreased concentration of labile and easily hydrolyzable phosphorus compounds if oxidative phosphorylation is impaired and it seemed worthwhile to investigate the distribution of acid-soluble phosphorus in guinea pigs with unequivocal magnesium deficiency.

This communication deals with the ash, calcium, phosphorus and magnesium composition of both osseous and soft tissues from magnesium-deficient guinea pigs and with the concentration and distribution of acid-soluble phosphorus in kidney, heart and skeletal muscle.

EXPERIMENTAL

The experimental conditions and the composition of the magnesium-deficient basal diet were the same as previously described (O'Dell et al., '60).[3] The diet contained from 0.003 to 0.005% of magnesium, 0.9% of calcium and 0.4% of phosphorus. Phosphorus was varied by the addition of $NaH_2PO_4 \cdot H_2O$ and magnesium by the addition of MgO, both at the expense of sucrose. The basic experimental design included two levels of phosphorus, 0.4 and 1.7%, and two levels of magnesium, the basal level and 0.3%, but some results are reported for two other magnesium levels. A magnesium level of 0.3% is more than adequate for growth, and diets that contain this level will be considered control diets. The dietary magnesium levels given in the tables are analytical values. Groups of 4 animals were caged together and commonly not more than this number were supplied with a given diet at one time. The usual

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experimental period was 12 to 14 weeks but some animals receiving the high-phosphorus diets were maintained only 4 weeks. Feed and distilled water were supplied ad libitum.

Tissues to be analyzed for ash were removed immediately after the animal was killed, rinsed with water, blotted free of excess moisture, and dried in an oven at 65°C. The dried samples were ground, extracted with ether and redried before analysis. A portion was ashed at 450°C overnight, the ash dissolved in dilute hydrochloric acid, and aliquots removed for the various determinations. Calcium was precipitated as the oxalate and determined by permanganate titration. Phosphorus was determined by the method of Fiske and Subbarow ('25). Magnesium was precipitated as magnesium ammonium phosphate and finally determined by the thiazol yellow method of Mitchell ('54). The ash and phosphorus of bone and teeth were determined by standard AOAC methods ('50) and the calcium and magnesium by a spark-in-flame spectrophotometric method.[4]

Tissues for determination of the acid-soluble phosphorus were removed from animals under pentobarbital anesthesia. The tissues were quick-frozen by use of a clamp precooled to liquid-air temperature (Wollenberg et al., '58). The frozen tissues were held in liquid air until finely ground in a steel mortar which was precooled with liquid air. The homogenized sample was placed in a tared centrifuge tube containing cold perchloric acid (0.4 M), quickly weighed and centrifuged. The residue was washed with cold 0.2 M perchloric acid, and the supernatants combined, filtered and made to volume. The above operations and the precipitation of the inorganic phosphate were carried out in the cold room, 3 to 5°C. The total acid-soluble, inorganic and labile phosphorus fractions were determined as described by LePage ('57). The easily hydrolyzable phosphorus was determined as the inorganic phosphorus released by hydrolysis for 7 minutes in N HCl at 100°C. Calcium was precipitated from the perchloric acid extracts as the oxalate and determined by permanganate titration.

RESULTS

Rate of growth was the chief criterion of magnesium deficiency, and the average daily gain of the animals from which tissues were taken are shown in table 1. Animals fed the magnesium-deficient diets grew at a significantly lower rate than the controls. At least 50% of the animals that consumed magnesium-deficient diets died during the 12-week experimental period and an equal percentage developed visible mineral deposits. No mortality or visible calcification occurred among the control groups.

Since the consumption of excess phosphate increases the magnesium requirement and causes deposition of excess calcium in soft tissues even when the diet contains 0.34% of magnesium, it was deemed desirable to feed a still higher level of magnesium. A total of 18 animals fed a diet containing 1.7% of phosphorus and 0.6% of magnesium gained at a rate of 2.1 gm per day which is approximately one-half the rate of animals fed 0.34% of magnesium. The 4 animals of this group from which tissues were analyzed gained at the rate of 1.0 gm per day (table 1).

Mineral composition of soft tissues. The ash, calcium, phosphorus and magnesium analyses of dry, fat-free tissues from guinea pigs fed variable levels of magnesium and two levels of phosphorus are summarized in table 2. All tissues from guinea pigs reared with the low-magnesium diets contained a higher percentage of total ash, calcium and phosphorus than did tissues from the control animals. When the diet contained 0.4% of phosphorus and 0.005% of magnesium, the calcium content of kidney and muscle was approximately 10 times, and that of heart almost 20 times, that of control animals consuming 0.34% of magnesium. Statistical analysis proved these differences to be highly significant except in the case of heart. Kidney tissue from the deficient animals contained about 35%

[4] The authors wish to express their appreciation to Dr. E. E. Pickett who did the calcium and magnesium analyses on the bones and teeth and to Dr. C. W. Gehrke who did the ash and phosphorus analyses on these samples.

TABLE 1

Response of guinea pigs fed variable levels of magnesium and phosphorus

Diet composition[1]		No. of animals	Average daily gain[2]
P	Mg		
%	%		gm
0.4	0.005	12	2.7 ± 0.3[4]
0.4	0.06	9	5.5 ± 0.5
0.4	0.34	8	5.3 ± 0.3
0.6	0.003	10	3.4 ± 0.3[4]
0.6	0.30	10	5.5 ± 0.3
1.7	0.01	16[3]	−0.3 ± 0.2[4]
1.7	0.06	9	1.9 ± 0.3[4]
1.7	0.34	16[3]	3.9 ± 0.3
1.7	0.34	8	4.1 ± 0.4
1.7	0.60	4	1.0 ± 0.3[4]

[1] All diets calculated to contain 0.9% of calcium and the phosphorus values indicated; magnesium figures are analytical values.

[2] Average daily gain for 8 weeks and standard error of the mean.

[3] Experimental period was 4 weeks.

[4] Significantly different from the group fed 0.3% of magnesium and the same level of phosphorus, $P < 0.01$.

more phosphorus, and muscle and heart slightly more, than similar tissues from control animals. A dietary magnesium level of 0.06% maintained nearly normal values for ash, calcium and phosphorus.

As reported previously (O'Dell et al., '60), excess phosphorus accentuates the symptoms of magnesium deficiency and greatly decreases survival. Because of early mortality among animals fed magnesium-deficient diets containing a high level of phosphorus, tissues were taken after feeding this diet for 4 weeks. Part of the control tissues were taken after 4 weeks and the remainder after 12 to 14 weeks. Kidney tissue from animals maintained for 4 weeks with diets that contained 1.7% of phosphorus and 0.01% of magnesium had 50% more calcium but only slightly more total ash and phosphorus than the controls that received 0.34% of magnesium. The values were lower than for comparable animals receiving the 0.4% phosphorus diet, but no doubt this was due to the age of the animals. Kidneys of animals that received 0.06% of magnesium and 1.7% of phosphorus for 12 to 14 weeks had nearly the same composition as those from animals that received 0.005% of magnesium and 0.4% of phosphorus. The ash and calcium content of kidneys from control guinea pigs fed 1.7% of phosphorus were greater than those of kidneys from control animals fed 0.4% of phosphorus. Thus 0.34% of magnesium did not afford complete protection against kidney calcification in animals fed a high level of phosphate, but it did maintain calcium values appreciably lower than those of animals that received 0.06% of magnesium. Both the 0.06 and 0.34% levels of magnesium maintained near normal mineral composition of muscle. Therefore, the level of magnesium required in the diet to prevent calcification varies for different organs of the body.

Even though growth rate was depressed by feeding a diet that contained 0.6% of magnesium and 1.7% of phosphorus (table 1) it was of interest to determine the effect of this level of magnesium on tissue composition. Tissues from 4 animals reared with this diet were analyzed and the results are presented in table 2. Although the phosphorus of the tissue was not changed, the ash was decreased slightly and kidney calcium was decreased to about one-third that of the 0.34% magnesium level. Whereas the higher level of magnesium decreased soft tissue calcification, it depressed growth rate.

In general, soft tissues from animals fed the basal diets contained less magnesium than when adequate magnesium was consumed. These differences failed to reach statistical significance only in the case of kidney and muscle from animals fed the low-phosphorus diet.

Mineral composition of osseous tissue. One of the symptoms of magnesium deficiency in the guinea pig is defective teeth, particularly the incisors (O'Dell et al., '60). In an attempt to ascertain the nature of this defect, bones and teeth were analyzed and the results are summarized in table 3.

Magnesium deprivation caused no significant difference in the percentage of ash, phosphorus or calcium in bone. For the reasons described above, animals that received the high-phosphorus diet were analyzed after 4 weeks. The older animals had a higher bone ash, but their bones contained less ash than the low-phosphorus groups. Bone magnesium con-

TABLE 2

Dietary magnesium and mineral composition of soft tissues (analyses based on dry weight of fat-free tissues from animals fed diet 12 to 14 weeks unless otherwise noted)

Dietary Mg	0.4% P in diet					1.7% P in diet				
	No. animals	Ash	P	Ca	Mg	No. animals	Ash	P	Ca	Mg
%		%	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*		%	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
					A. Kidney					
0.005[1]	7	8.56[6]	1580[6]	659[6]	103	3[2]	7.24	1460	300	83.6[6]
		(0.57)[3]	(99)	(53)	(5.2)		(0.71)	(84)	(89)	(2)
0.06	4	6.06	1170	60.0	91.9[6]	5	8.30	1530	629	105
		(0.09)	(55)	(6.8)	(0.9)		(0.39)	(58)	(364)	(4.3)
0.34	5	6.20	1160	52.8	104	4[2]	6.78	1360	196	104
		(0.12)	(35)	(2.0)	(2.4)		(0.71)	(25)	(34)	(1.2)
0.34						8	7.23	1420	370	110
							(0.19)	(33)	(98)	(2.3)
0.60						4	7.04	1410	124	143[6]
							(0.21)	(21)	(27)	(8.6)
					B. Muscle					
0.005[1]	7	6.34[4]	1030	250[6]	106	7[2]	6.85	1130	297[5]	110[6]
		(0.21)	(37)	(93)	(4.0)		(0.34)	(42)	(94)	(2.1)
0.06	4	6.24	941	48.3	121	8	6.06	1040	39.0	128
		(0.16)	(9.1)	(7.9)	(2.0)		(0.16)	(21)	(7.0)	(3.0)
0.34	4	5.56	991	26.8	120	8[2]	6.10	1070	34.7	125
		(0.16)	(16)	(3.7)	(3.5)		(0.19)	(15)	(4.9)	(2.0)
0.34						9	5.71	1050	46.8	128
							(0.06)	(11)	(11)	(8.7)
0.60						4	5.64	1040	34.4	129
							(0.05)	(11)	(2.9)	(1.3)
					C. Heart					
0.005[1]	4	6.28	1040	435	92.1[4]					
		(0.87)	(135)	(350)	(2.9)					
0.06	4	5.35	958	31.4	105					
		(0.20)	(29)	(7.5)	(4.1)					
0.34	7	5.30	969	23.7	103					
		(0.17)	(21)	(5.8)	(2.7)					

[1] The 1.7% P basal diet contained 0.01% of Mg.

[2] Animals received diet 4 weeks.

[3] Standard error of the mean.

[4] Significantly different from the 0.34% magnesium level, $P < 0.05$.

[5] Significantly different from the 0.34% magnesium level, $P < 0.02$.

[6] Significantly different from the 0.34% magnesium level, $P < 0.01$.

TABLE 3

Dietary magnesium level and composition of guinea pig bones and teeth (expressed as percentage of dry, fat-free weight)

Diet composition[1]		No. animals	Bone				Molars				Incisors			
P	Mg		Ash	P	Ca	Mg	Ash	P	Ca	Mg	Ash	P	Ca	Mg
%	%													
0.4	0.005	5	65.0 (0.67)[2]	11.5 (0.12)	24.3 (0.48)	0.20[5] (0.03)	79.0[5] (0.22)	14.6[4] (0.0)	28.8 (0.47)	0.38 (0.03)	76.5 (0.22)	14.6[5] (0.07)	27.3 (0.33)	0.36[5] (0.10)
0.4	0.06	5	64.7 (0.63)	11.7 (0.14)	23.9 (0.39)	0.40[5] (0.08)	77.9 (0.39)	15.1 (0.07)	27.7 (0.34)	1.17 (0.05)	77.3 (0.29)	15.1 (0.09)	26.4 (0.46)	0.93[4] (0.03)
0.4	0.34	5	65.5 (0.22)	12.0 (0.24)	24.4 (0.55)	0.60 (0.04)	77.8 (0.22)	15.2 (0.07)	27.0 (0.92)	1.33 (0.08)	76.8 (0.17)	15.1 (0.07)	26.3 (0.74)	1.18 (0.07)
1.7	0.01[3]	5	60.0[4] (0.55)	11.3 (0.10)	23.1 (0.77)	0.34[5] (0.05)	77.2[4] (0.39)	15.4 (0.22)	29.0[4] (0.22)	0.76[5] (0.06)	74.3 (0.55)	14.8 (0.10)	27.8 (0.81)	0.67 (0.06)
1.7	0.34[3]	4	60.7 (2.02)	11.5 (0.05)	22.8 (0.29)	0.60[5] (0.03)	76.0 (0.13)	15.0 (0.13)	28.1 (0.29)	1.22 (0.02)	74.4 (0.29)	15.0 (0.06)	28.0 (0.41)	1.23 (0.07)
1.7	0.34	4	62.2 (0.82)	11.7 (0.11)	21.8 (0.22)	0.80 (0.02)	76.9 (0.09)	15.0 (0.05)	27.0 (0.56)	1.23 (0.04)	76.4 (0.29)	15.1 (0.29)	26.0 (0.58)	1.24 (0.06)

[1] All diets contained approximately 0.9% of calcium.

[2] Standard error of the mean.

[3] Guinea pigs had received the diets only 4 weeks; all others 12 to 14 weeks.

[4] Significantly different from the 0.34% magnesium level, $P < 0.05$.

[5] Significantly different from the 0.34% magnesium level, $P < 0.01$.

centrations were one-third to one-half those of the controls. A level of 0.06% of magnesium was not sufficient to maintain maximum bone magnesium.

The molars from magnesium-deficient animals contained a significantly higher percentage of ash than those of the controls. On the other hand the percentage of ash of the incisors was slightly depressed by the deficiency. These incisors were normal in appearance. The phosphorus content of both the molars and incisors was significantly less in the magnesium-deficient animals that received the low-phosphorus diet, but this difference did not occur with the high-phosphorus diets. Although small in magnitude, the teeth of magnesium-deficient animals contained a higher percentage of calcium than those of the controls. Magnesium deficiency decreased the percentage of magnesium in teeth in a manner parallel to its effect on bone.

Distribution of acid-soluble phosphorus. In this phase of the study the low-phosphorus diet contained 0.6% of phosphorus and by analysis 0.003% of magnesium. The total acid-soluble phosphorus content of the kidney and heart was increased almost twofold by feeding a diet low in magnesium regardless of the level of dietary phosphorus, but the values for muscle were only slightly affected (table 4). The concentrations of labile and easily hydrolyzable phosphorus were slightly higher in all tissues from deficient animals but the differences approached statistical significance only in the case of

TABLE 4

Dietary level of magnesium and distribution of acid-soluble phosphorus in kidney, heart and skeletal muscle (values in mg per 100 gm of fresh tissue)

Tissue analyzed	Dietary Mg	Acid-soluble phosphorus					Acid-soluble calcium
		Total	Inorganic	Labile	Easily hydrolyzed	Undetermined[1]	
	%						
		A. 0.6% dietary phosphorus (fed diet 12 weeks)					
Kidney (10)[2]	0.003	139[6]	79.8[6]	4.1	12.0[4]	43.8	163[6]
		(17)[3]	(14)	(1.1)	(1.5)	(2.6)	(31)
Kidney (10)	0.30	68.3	18.0	2.6	7.4	40.9	11.9
		(6.5)	(5.0)	(1.0)	(1.2)	(5.6)	(2.3)
Heart (10)	0.003	135	66.8	2.4	15.3	50.9	133
		(48)	(33)	(0.7)	(2.1)	(13)	(83)
Heart (10)	0.30	71.2	24.9	1.7	14.8	30.2	6.3
		(6.7)	(2.6)	(0.7)	(2.3)	(5.4)	(1.4)
Muscle (10)	0.003	155	34.1	45.9	35.2	40.0	17.6
		(5.9)	(2.2)	(2.9)	(2.4)	(4.0)	(3.4)
Muscle (10)	0.30	143	28.3	47.7	29.8	36.8	8.2
		(7.1)	(3.6)	(4.8)	(5.0)	(4.3)	(3.1)
		B. 1.7% dietary phosphorus (fed diet 4 weeks)					
Kidney (8)	0.002	167[5]	107[5]	7.0	17.8	35.5	226[5]
		(29)	(26)	(2.1)	(2.9)	(4.4)	(60)
Kidney (9)	0.30	91.4	34.8	3.3	12.2	42.2	33.6
		(4.3)	(3.4)	(0.5)	(1.1)	(2.0)	(7.4)
Heart (8)	0.002	144[4]	94.6[4]	7.4	16.4	25.2[5]	204[4]
		(15)	(15)	(0.7)	(0.1)	(0.1)	(37)
Heart (9)	0.30	87.9	32.8	4.5	16.3	36.8	25.9
		(6.0)	(5.0)	(2.1)	(1.2)	(4.4)	(16)
Muscle (8)	0.002	170	38.2[4]	64.8	37.0	29.6	53.7[4]
		(7.3)	(7.1)	(2.8)	(1.0)	(2.8)	(14.0)
Muscle (9)	0.30	156	26.8	55.7	34.7	38.8	5.2
		(2.6)	(2.0)	(4.5)	(1.8)	(5.2)	(1.1)

[1] Undetermined phosphorus is the total minus the sum of inorganic, labile and easily hydrolyzable.
[2] Number of samples analyzed.
[3] Standard error of the mean.
[4] Significantly different from the 0.3% Mg, $P < 0.05$.
[5] Significantly different from the 0.3% Mg, $P < 0.02$.
[6] Significantly different from the 0.3% Mg, $P < 0.01$.

kidney. The high variability of the undetermined fraction probably reflects accumulated analytical errors and no biological significance is attributed to the differences.

The higher concentration of acid-soluble phosphorus in the tissues of the magnesium-deficient animals was almost entirely accounted for by the inorganic phosphorus fraction. The concentrations of inorganic phosphorus in the deficient kidney and heart tissues were three to 4 times those of the respective control tissues and for the most part these differences were statistically significant.

If the calcinosis observed in magnesium deficiency results from the precipitation of calcium phosphate, the acid extract should contain quantities of calcium in stoichiometric relation to the inorganic phosphorus. As shown in table 4, this was approximately the case. For both levels of phosphorus, all tissues from deficient animals contained a higher level of acid-soluble calcium than the control tissues. If one calculates the excess calcium and inorganic phosphorus in kidney and heart tissue of deficient animals as the total minus that found in the control tissues, the average ratio of calcium to phosphorus is approximately 2.5. This is a wider ratio than normally found in bone and suggests that some calcium is bound to protein or held in some unknown manner. Nevertheless, it seems probable that the calcium and phosphorus accumulates in tissues primarily as calcium phosphate.

DISCUSSION

The results of this investigation confirm earlier observations made on the guinea pig (Maynard et al., '58), as well as those on the rat (Watchorn and McCance, '37; Tufts and Greenberg, '38; MacIntyre and Davidson, '58; McAleese and Forbes, '61), that magnesium deficiency results in marked accumulation of calcium in soft tissues. Cotton rats fed diets low in magnesium also develop a calcinosis characterized by an elevated ash in heart tissue (Constant and Phillips, '52). MacIntyre and Davidson ('58) observed a significant decrease in the concentration of both magnesium and potassium in muscle tissue from rats fed magnesium-deficient diets.

As observed in the rat (Orent et al., '34; Watchorn and McCance, '37; Duckworth et al., '40; McAleese and Forbes, '61) and in the calf (Smith, '59) magnesium deficiency in the guinea pig lowers bone magnesium to about one-third the normal value. In contrast with some of the earlier reports on other species there were no significant changes in the ash, calcium or phosphorus composition of bone. In agreement with the observations of Watchorn and McCance, teeth from deficient animals contained slightly more calcium and slightly less phosphorus than controls. The slight increase in calcium may have resulted from replacement of magnesium by calcium on the crystal surfaces. A calculus of unknown composition was frequently observed on the molars and this possibly contributed to this calcium content. The incisors analyzed did not show gross damage such as frequently occurs during an extended period of magnesium deficiency. If magnesium were directly concerned with the integrity of the mineral component of the tooth, one would expect progressive change with the degree of depletion. Since this was not the case, it appears to be more concerned with cell function and development of the organic matrix of the tooth rather than with mineralization *per se*. In this connection it should be pointed out that Becks and Furata ('39) observed partial degeneration of the enamel epithelium in the magnesium-deficient rat. A functional failure of this tissue would result in defective enamel and might well explain the erosion and decay observed in the guinea pig incisors.

There is reason to believe that magnesium was among the limiting factors in the diet which van Wagtendonk ('44) used to study the anti-stiffness factor. In agreement with the present study, he observed elevated values for inorganic phosphate in kidney and liver, but in contrast, noted a marked decrease in easily hydrolyzable phosphorus. Dietary deficiencies other than magnesium may account for the latter discrepancy.

Labile phosphorus is believed to arise largely from creatine phosphate and the easily hydrolyzable phosphorus from adenosine di- and triphosphates. These high-energy compounds are formed by phosphorylation coupled, for the most part, with oxidative processes. The fact that there was no decrease in the concentration of high-energy phosphate compounds in the tissues leads to the conclusion that oxidative phosphorylation was not seriously impaired in the intact magnesium-deficient guinea pig. The *in vitro* studies of Beechey et al. ('59) showed no diminution in the ability of heart sarcosomes from magnesium-deficient rats to perform oxidative phosphorylation. Magnesium ion is a cofactor required by many enzymes, particularly those which are involved in phosphate transfer. The slightly elevated concentrations of the high-energy phosphate compounds in tissues from magnesium-deficient animals suggests that phosphate utilization is impaired.

It is reasonable to believe that magnesium is more directly concerned with phosphorus than with calcium metabolism. That is to say, calcium accumulation in soft tissue may be secondary to the high phosphate content of body fluids which occurs in magnesium deficiency. Excess dietary phosphate accentuates the calcinosis syndrome primarily because it decreases magnesium absorption, but it may also increase the magnesium requirement by a direct metabolic effect. Excess dietary phosphate increases serum phosphate and may thus increase the amount of magnesium required in its metabolism. Although adequate magnesium prevents metastatic calcification when dietary phosphorus is within the normal range, levels of magnesium sufficient to support the maximal growth rate do not entirely prevent calcium and phosphorus accumulation when excessive phosphate is consumed.

SUMMARY

Osseous and soft tissues from magnesium-deficient and control guinea pigs fed two levels of phosphorus, 0.4 and 1.7%, were analyzed for ash, calcium, phosphorus and magnesium. The distribution of the acid-soluble phosphorus in kidney, heart and muscle was also investigated.

The concentrations of ash, calcium and phosphorus in kidney, muscle and heart were greatly increased, whereas that of magnesium was slightly decreased by the deficiency. A dietary magnesium level of 0.06% maintained near normal tissue composition with the low-phosphorus diet, whereas even 0.34% of magnesium did not maintain normal tissue composition in animals fed the high-phosphorus diet. It may be concluded that a higher magnesium level is required to maintain tissue composition than to support maximal growth and that the requirement for maintenance of normal tissue composition varies with the tissue.

Magnesium deficiency caused no major change in the ash, calcium or phosphorus content of bones and teeth, but caused a marked lowering of magnesium.

The increase in total acid-soluble phosphorus of soft tissues from magnesium-deficient animals was accounted for almost entirely as inorganic phosphorus. The excess inorganic phosphorus and calcium were in approximately the proportions found in tricalcium phosphate.

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Some Effects of Feeding Frequency on the Utilization of Isocaloric Diets by Young and Adult Sheep[1]

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In 1952, Gordon and Tribe reported that growing Cheviot ewes fed a mixed ration in 8 meals per day gained 260% more weight than those fed the same ration in one large feeding daily. The rates of body weight gain of dairy heifers fed a mixed concentrate-forage diet in 4 meals per day were 60 and 52%, respectively, greater than those of heifers given the same amount of feed in one and two meals per day. Heifers fed an all-hay ration in 10 meals gained 92% more body weight than their mates receiving the same amount of feed in two meals (Rakes et al., '57). Subsequent studies with growing heifers[4] and fattening steers (Mohrman et al., '59) have confirmed these findings.

On the other hand, Mochrie et al. ('56) observed no difference in the yield of 4% fat-corrected milk or in the body weight changes of first-lactation cows fed the same amount of a mixed ration in 8 and two meals per day. Although Campbell and Merilan ('60) reported data which purport to demonstrate that milking cows fed 4 times per day produced more milk than those fed two times per day, the greater amount of feed ingested by the more frequently fed cows could wholly account for the difference in yield obtained. In addition the rations consumed by the two groups of cows were different qualitatively. Leffel and Komarek ('59) noted no effect of feeding frequency on the weight gain of sheep fed either a high-forage or a low-forage diet. Also, the rates of gain in pigs fed two and 4 meals per day were not different (Melnikov and Struk, '56).

Experiments conducted with ruminants to determine the causes underlying the benefits of frequent feeding have yielded conflicting results. Those observed in several studies (Moir and Somers, '57; Rakes et al., '57; Mochrie et al., '56; Blaxter et al., '56)[5] indicate that feeding frequency has either no, or very little, effect upon the apparent digestibility of the ration. Although Campbell and Merilan ('60) associated a high digestibility with frequent feeding, the composition and amounts of the diets ingested at the various frequencies were different. Small increases in the digestibility of both nitrogen and energy were associated with frequent feeding, by Mohrman et al. ('59). Mochrie et al. ('56) noted no significant effect of feeding frequency on the retention of nitrogen by steers. Moir and Somers ('57), however, reported that sheep retained more nitrogen when fed 4 times per day than when fed one or two times daily. No difference in the production of heat by mature sheep was effected by feeding the same daily quantity of feed at 6-, 12-, and 24-hour intervals (Blaxter et al., '56). The ruminal ingesta of steers fed 8 times per day contained a higher concentration of propionic acid and a lower concentration of acetic acid than that of steers fed the same ration in two meals per day. On the other hand, Leffel and Komarek ('59) reported that the frequency with which lambs were

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[4] Clark, B. and H. A. Keener, University of New Hampshire, 1958, personal communication.
[5] Mochrie, R. D. 1958 Relationship of frequency of feeding to feed utilization and other physiological activities of the bovine. Ph.D. thesis, North Carolina State College, Clemson.

fed had no significant effect on the concentration of these acids in the ruminal ingesta.

It was the purpose of the experiments to be reported here to study further the effects of frequency of feeding, with the hope of obtaining information on the mechanism involved.

EXPERIMENTAL

Experiment 1. Three experiments were conducted. In the first, 12 young wethers (6 months old) and 4 adult wethers (2.5 years old) were used. The effects upon several physiological functions of feeding the same quantity of a ration (consisting of 50% of chopped hay and 50% of mixed concentrates) in one and 8 meals per day were compared in a single-reversal experiment. The criteria examined were bodyweight gain, the digestibility of energy and nitrogen, the excretion of urinary energy and nitrogen, the total output of heat and the pH of the ruminal ingesta. During the first experimental period of 49 days, one half of the animals received their daily allowance of feed in one meal at 8:30 A.M. and the remaining animals were fed the same daily amount of feed relative to metabolic size (body $\text{weight}_{kg}^{0.73}$) divided into 8 equal portions at 65-minute intervals between 8:30 A.M. and 4:10 P.M. At the end of the first period the treatments were reversed and the experiment was continued another 49 days.

The initial feeding level was determined during a 14-day standardization period and represented the amount of feed which all sheep would consume within one hour without an appreciable waste when provided in two meals per day. During the two experimental periods, the feed intake of each animal was adjusted weekly to the same level per unit of metabolic size as that established during the standardization period.

During the last week of each experimental period, conventional digestion trials and nitrogen-balance experiments were conducted. Measurements of the respiratory exchange of oxygen and carbon dioxide from which heat production was computed were effected using the method introduced by Zuntz and Lehmann (1889) and revised recently by Flatt et al. ('58) and Lister.[6] The measurements of oxygen and carbon dioxide exchange were made during 4 nonconsecutive 24-hour periods with the growing animals and during three such periods with the older animals. During the same periods the pH of ruminal ingesta was determined in the older wethers.

Experiment 2. During the first experiment it was observed that noticeable increases in oxygen consumption by the sheep receiving one meal occurred at the times that the sheep receiving 8 meals were being fed in their presence. Although there appeared to be considerable variation, the infrequently fed sheep fretted and became excited at the times when their frequently fed mates were given meals. As a consequence experiment 2 was conducted to determine the extent of the effect of fretting on the production of heat. After experiment 1 was completed, 4 young wethers and two adult wethers which had received one meal per day previously were removed to quarters isolated from other animals and activity. These wethers were continued on the same frequency (one meal) of feeding for three weeks during which measurements of the respiratory exchange were made.

Experiment 3. This experiment was undertaken to determine the effects of feeding the same quantity of feed in one, 4 and 8 meals per day on the excretion of total nitrogen, urea and creatinine in the urine and on the diurnal variation in oxygen consumption. Five wethers (about 14 months old) were fed a diet containing equal parts of alfalfa-orchardgrass hay and mixed concentrates during three 21-day experimental periods. During the first period all animals were fed once daily at 7:30 A.M.; during the second period, 4 times daily at 7:30 A.M., 10:30 A.M., 1:30 P.M. and 4:30 P.M.; and during the third period, 8 times daily at 90-minute intervals between 7:30 A.M. and 6:00 P.M. During the last 7 days of each period the urine was collected totally and total nitrogen, urea (xanthydrol method described

[6] Lister, E. E. 1960 Effect of short-chain fatty acids on the heat production of cattle. Ph.D. thesis, Cornell University, Ithaca, New York.

by Engel and Engel, '47) and creatinine (modified Jaffé reaction as outlined by Owen et al., '54) were determined. Oxygen consumption was determined at two-hour intervals during three 24-hour periods in each of three experimental periods.

RESULTS

Experiment 1. Data obtained in experiment 1 are summarized in table 1. Young sheep gained an average of 65% more body weight ($P < 0.01$) when fed 8 meals than when fed the same isocaloric ration in one meal per day. A difference of similar magnitude in the efficiency of feed utilization existed between the two feeding frequencies imposed. The apparent absorption of energy or nitrogen was not significantly different for the feeding treatments. On the other hand, the young wethers excreted approximately 20% less nitrogen ($P < 0.01$) in the urine when they were fed 8 times per day than when fed only once daily. As compared with the output of heat above energy equilibrium on the one-meal regimen, that of the sheep under the 8-meal per day regimen was about 20% less ($0.1 > P > 0.05$).

The frequency with which the group of older sheep was fed did not affect significantly the rate of body gain, the efficiency of feed utilization, the digestibility of nitrogen and energy or the rate of excretion of nitrogen in the urine. The influence of feeding frequency on heat production by these wethers was not consistent. Although the average pH value (6.61 ± 0.05) of the rumen contents of sheep fed once a day was not different from that (6.74 ± 0.04) of the ingesta of sheep fed 8 times per day, the reduction in pH after feeding was much more marked on the one-meal regimen.

Experiment 2. The apparent excitement and fretting of the infrequently fed sheep did not affect significantly their daily expenditure of energy. When sheep fed once a day were kept in the immediate presence of the animals fed 8 times per day, they consumed 24.5 l of oxygen per day per unit of metabolic size. When they were isolated and continued to be fed one meal per day, they consumed 23.6 l of oxygen per day per unit of metabolic size.

TABLE 1

Effect of feeding frequency on responses of young and adult sheep

Meals/day	Body weight gain	Efficiency of gain	Apparent absorption		Urinary nitrogen output[1]	Heat production	
			Nitrogen	Energy		Total[2]	Above energy equilibrium[3]
	pounds/day	*gm/pound feed*	%	%			
			Young sheep, 6 months old				
1	0.22 + 0.05[4]	34 ± 8.8	73 ± 1.2	71 ± 2.0	0.54 ± 0.08	127 ± 2.0	45 ± 23.6
8	0.36 + 0.07[5]	61 ± 10.7[5]	73 ± 0.9[5]	72 ± 1.3[5]	0.43 ± 0.04[5]	119 ± 1.9[5]	36 ± 18.0[5]
			Adult sheep, 2.5 years old				
1	0.17 ± 0.06	24 ± 8.0	74 ± 0.7	73 ± 0.3	0.52 ± 0.28	111 ± 11.4	27 ± 13.0
8	0.18 ± 0.06	26 ± 8.3	74 ± 0.7	73 ± 1.0	0.51 ± 0.43	104 ± 13.5	18 ± 15.6

[1] Expressed as gm/body weight$_{kg}^{0.73}$/day.
[2] Expressed as Cal./body weight$_{kg}^{0.73}$/day.
[3] Expressed as Cal./body weight$_{kg}^{.073}$/day above energy equilibrium (Marston, '48).
[4] Mean and standard deviation of the mean.
[5] One animal died; missing plot calculated.

Between 8:00 A.M. and 4.00 P.M., however, the sheep receiving one meal consumed 10.0 l of oxygen when housed with the frequently fed sheep and 8.8 l of oxygen when they were isolated. For each individual sheep in isolation, oxygen consumption between 8:00 A.M. and 4:00 P.M. was lower than that in the presence of frequently fed sheep and the difference was highly significant ($P < 0.01$). Therefore, the energy expenditure during the period of 4:00 P.M. to 8:00 A.M. (when no meals were given to any sheep) for the sheep fed one meal was somewhat less when they were housed in the presence of frequently fed sheep than when they were isolated.

Experiment 3. The urinary creatinine, urea and total nitrogen output of five 14-month-old sheep fed one, 4 and 8 times per day are summarized in table 2. The feeding frequencies imposed had no significant effect on any of these criteria; however, the manner by which this experiment was conducted confounded to some degree the frequency of feeding with age of sheep. Obviously these observations are much different from those made in experiment 1.

There appear to be two possible explanations for the difference in results between these two experiments. In experiment 3 the feed intake was lower than that for the young animals in experiment

TABLE 2

Effect of feeding frequency on the excretion of creatinine, urea, and total nitrogen in the urine of 14-month-old sheep

Meals/day	Urinary nitrogen/body weight$_{kg}^{0.73}$/day		
	Total	Urea	Creatinine
	gm	*gm*	*mg*
1	0.58 ± 0.03[1]	0.46 ± 0.02	1.85 ± 0.57
4	0.56 ± 0.03	0.45 ± 0.02	1.87 ± 0.56
8	0.56 ± 0.02	0.44 ± 0.04	1.81 ± 0.55

[1] Mean and standard deviation of the mean.

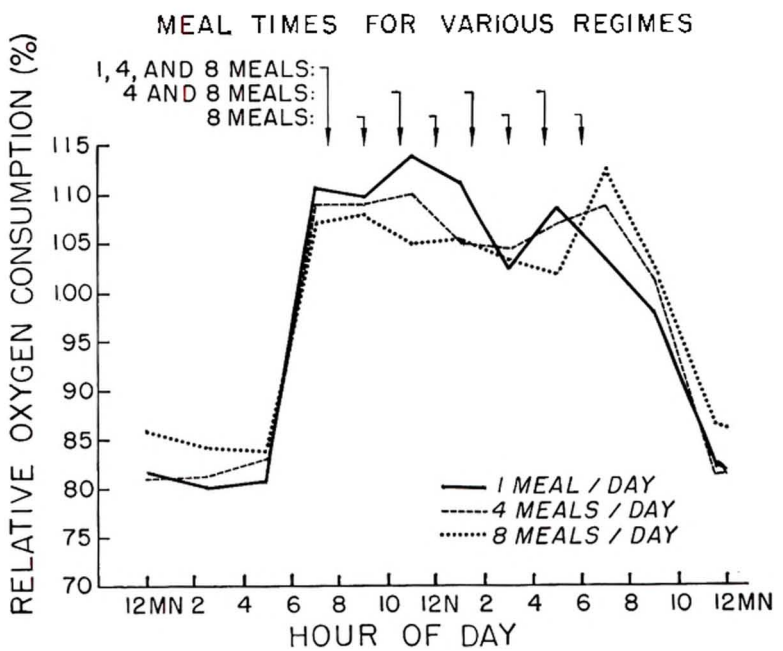

Fig. 1 Effect of feeding frequency on the diurnal consumption of oxygen by sheep.

1, chiefly because of differences in the acceptability of the hays. The animals used in experiment 3 were older and more nearly mature than those used in experiment 1.

In figure 1 are shown the diurnal variations in oxygen consumption of the 5 wethers fed the same amounts of feed per unit of metabolic size in one, 4 and 8 meals per day. The values plotted are expressed as percentages of the mean rate of oxygen consumption per 24-hour period. In all three treatment periods a marked increase in the oxygen consumption rate occurred during the period of 6:00 to 8:00 A.M. During this period, the animals under all feeding regimens were given a meal, and it was noted that all animals were more active during this period than during any other two-hour period. When the wethers were fed one meal (at 7:30 A.M.), a pronounced peak was obtained between 10:00 A.M. and 12:00 N. Although the environmental conditions (other than feeding) were not controlled and definite conclusions cannot be drawn, it seems reasonable that such a peak in oxygen consumption may be the result of the absorption of a large quantity of metabolites. Maximum rates of oxygen consumption occurring until about 8 P.M. for the more frequently fed sheep might be a reflection of the same effect. Between 10:00 P.M. and 6 A.M. the oxygen consumption rates were markedly lower than during the part of the day when meals were provided. Some of the reduction in oxygen consumption might be the result of a diminished physical activity and a decrease in environmental temperature, both of which were noted. But, in addition, a reduction of the level of metabolites available could account for a part of the reduction in heat production during this period.

DISCUSSION

Data obtained in these experiments showing that the feeding of an isocaloric diet in frequent meals effects a more rapid rate of body gain in young sheep than the same diet offered in one meal per day are in agreement with data obtained previously with growing ruminants by Gordon and Tribe ('52), Thomas and Mochrie ('56), Rakes et al. ('57) and Mochrie et al. ('56). Though Mohrman et al. ('59) observed that 6 meals per day produced a higher rate of gain in steers than did two meals, the frequently fed steers consumed more feed. Leffel and Komarek ('59), however, were not able to affect the rate of gain by varying the feeding frequency.

In the present experiments, as well as in other experiments (Blaxter et al., '56; Mochrie et al., '56), frequent feeding failed to effect a body gain response in mature animals or in those approaching adulthood. The animals used in one of Leffel and Komarek's experiments ('59) were more mature than the animals used in the experiments of others in which responses were obtained, and this may be the reason these workers did not obtain a response.

There is no evidence that the efficiency of absorption of energy or nitrogen is influenced by the frequency of meals. This agrees with the observations of Rakes et al. ('57), Mochrie ('58), Blaxter et al. ('56) and Moir and Somers ('57) though the latter two groups of workers did not report an effect of feeding frequency on the rate of body gain. Thus, the more rapid rate of body gain does not appear to be attributable to an improvement in absorption.

It seems possible that although the total quantity of nutrients apparently absorbed is not affected by feeding frequency, the nutrients absorbed might be chemically different for rations fed at different frequencies. Rakes et al. ('57) noted higher rumination times for heifers fed 10 times per day than for those fed twice a day. As Mochrie et al. ('56) pointed out, this might be the reflection of a less complete mechanical degradation of the ingesta, which in turn might result in the absorption of a greater proportion of unfermented carbohydrates from the lower part of the gastrointestinal tract than would be absorbed with a two-meal-per-day regimen. It has been shown that the absorption by ruminants of unfermented carbohydrates results in a lower heat increment than does the absorption of the usual end products (volatile fatty acids) of carbohydrate fermentation (Armstrong and Blaxter, '56, '57). The same workers dem-

onstrated further that the heat increment of fatty acid mixtures high in acetic acid is greater than that of mixtures high in propionic acid. Recently Knox and Ward ('60) reported that the concentration of propionic acid is higher, and that of acetic acid is lower, in the ruminal ingesta of cows fed 8 times per day than in that of cows fed two meals. The frequency of feeding had no effect on the concentrations of fatty acids in ruminal ingesta in the studies of Leffel and Komarek ('59).

Rakes et al. ('57) suggested that animals fed once or twice a day probably produce more heat subsequently than those fed the same amount per day but in frequent meals. Since the peak of heat production exceeds the needs of the body for temperature maintenance, an expenditure of energy by the body may be required to dispose of the surplus heat (Brody, '45). On the other hand, division of the ration into small, frequent meals would tend to produce heat at a lower level at a given time and more uniformly throughout the day. In support of this, Richardson and Mason ('23) observed that large rations divided into small meals provided at two-hour intervals resulted in the same output of heat as that produced by feeding a smaller amount of feed in fewer meals.

In the present experiments, the feeding of growing sheep one meal per day resulted in a greater loss of nitrogen and energy in the urine than the feeding of the same amount of feed in 8 meals. Frequency of feeding, however, did not produce this effect in more mature sheep. Although the relation of urinary nitrogen and energy excretion to the body-gain response is not known, the efficiency with which the ammonia liberated in the rumen is used to synthesize protein might be involved. It is well established that large quantities of ammonia are produced in the rumen after a normal meal and that at least a part of it is absorbed directly into the ruminal veins (Annison and Lewis, '59). If the utilization of the liberated ammonia depends upon the amount of substrate available, it is conceivable that the level of ammonia in the rumen might exceed the capacity of the bacterial flora to convert it into amino acids and microbial protein. On a regimen of more frequent meals the liberation and utilization of ammonia might be more uniform and the synthesis of protein more efficient. In addition, it is known that the formation of urea is accompanied by a heat loss of 1 to 2 Cal. per gm of nitrogen involved and that the excretion of urea is accompanied by a further heat loss equivalent to 1 to 2 Cal. per gm of nitrogen (Brody, '45). In mature animals this effect might be masked by the nitrogen losses resulting from the deamination of amino acids to provide carbon chains for the synthesis of fat. In young animals lipogenesis would be occurring to a much lesser degree than in mature animals. Also, it seems possible that the mature ruminant might have a greater ability than the young ruminant to recycle and use some of the urea formed from the excess ammonia absorbed from the rumen (Schmidt-Nielsen and Osaki, '58; Houpt, '59).

These studies indicate that the lower energy economy of infrequently fed animals is not caused by a greater physical activity of these animals when more frequently fed animals are fed in their presence.

SUMMARY

Three experiments were conducted to determine the effects of the frequency of feeding on the utilization of isocaloric rations by sheep of different ages. Growing sheep (6 months old) fed 8 times per day gained 65% more weight ($P < 0.01$), excreted 20% less of nitrogen in the urine ($P < 0.01$) and tended to produce less heat than when fed the same daily quantity of feed in one meal. The same feeding frequencies had no effect on the body gain or urinary nitrogen excretion by more mature sheep (2.5 years old). Feeding frequency also had no effect upon the apparent absorption of energy or nitrogen by either growing or adult sheep. Although the average pH values of the ruminal ingesta were not different for the two feeding frequencies, those for the animals fed once daily fluctuated more drastically.

The total output of heat per day by animals on the one meal regimen housed in isolation was not different from that produced when they were housed in the same quarters with the sheep fed 8 times per day. During the daylight hours (when sheep were fed), however, the output of heat by the sheep fed one meal was 13.4% greater ($P < 0.01$) in the presence of the frequently fed sheep than it was under isolation. The animal's output of heat during the remainder of the 24-hour period was compensatory.

The amount of creatinine, urea or total nitrogen excreted in the urine of 14-month-old sheep was not different when isocaloric diets were ingested in one, 4 and 8 meals per day. A study of the diurnal output of heat showed that the production of heat by sheep fed one meal per day reached a higher peak after eating and decreased more abruptly during the day than that of sheep fed more frequently. During the nonfeeding hours for the frequently fed sheep, the output of heat was lowest for all sheep irrespective of their feeding-frequency regimen.

The results of these studies and those reported by others suggest an interaction of age and frequency of feeding on the response of ruminants. Further study is needed to determine the fundamental reasons for this apparent relationship.

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Pathologic Changes Induced by β-2-Thienylalanine[1,2]

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It has been shown that feeding β-2-thienylalanine to experimental animals will produce a marked inhibition of growth (Ferger and du Vigneaud, '49). The loss of weight can be prevented by feeding phenylalanine concurrently (Garst et al., '49). This effect has been ascribed to its antagonistic action to phenylalanine, an essential amino acid. No systematic study has been made, however, of the tissue changes brought about by feeding β-2-thienylalanine. The present report deals with the effect of β-2-thienylalanine on the tissues of the albino rat.

MATERIAL AND METHODS

Six-week-old male albino rats of the Sprague-Dawley strain weighing an average of 147 gm each were fed a synthetic diet of the following composition per 100 gm of diet: glucose, 67; vitamin-free casein, 18; salt mixture, 4 (Hegsted et al., '41); corn oil, 11 (containing 0.001 cm^3 of halibut liver oil).[3] Crystalline vitamins were added to supply the following amounts per 100 gm of diet: thiamine chloride, 400 μg; pyridoxine hydrochloride, 400 μg; riboflavin, 800 μg; Ca pantothenate, 1.5 mg; and nicotinic acid, 2.5 mg. Eight experimental animals were fed this basal diet to which was added 1% of β-2-thienyl-DL-alanine. Control animals were pair-fed. Under this regimen, the animals gained weight after the first week. To prevent this weight gain, the thienylalanine supplement was increased to 2% on the 17th day. At this time the expected loss of weight occurred. The entire experiment lasted 4 weeks. In addition, 4 animals, 5 weeks old, with an average weight of 126 gm were fed the diet supplemented with 2% of thienylalanine for the duration of the 4-week experiment. The pair-fed control technique was used throughout.

Autopsies were performed and tissues were fixed in 10% buffered formalin and stained with hematoxylin and eosin. The liver, kidney, and heart were stained for fat using Sudan IV. The following tissues were removed for histologic examination: brain, heart, aorta, lung, trachea, esophagus, pyloric portion of stomach, duodenum, proximal jejunum, ileum, colon, liver, pancreas, parotid, submaxillary and sublinguinal salivary glands, kidney, urinary bladder, prostate, seminal vesicles, testis, epididymis, thymus, cervical lymph nodes, spleen, pituitary, thyroid, adrenal, bone (tibia), and skin.

RESULTS

The rats receiving 1% thienylalanine lost weight during the first week but during the second week gained similarly to the pair-fed controls. At 17 days the thienylalanine supplement was increased to 2% and the animals lost weight for one week. Weight loss leveled off and the weight remained stationary during the 4th and final week of the experiment (fig. 1). The animals that were fed the 2% thienylalanine supplement for the entire 4-week experiment showed weight loss during the first week and their weight remained at this low level for the duration of the experiment (fig. 1).

Histologic changes were seen only in those organs described below. The changes in the various organs were identical in the animals fed 1% of thienylalanine for 17 days and the 2% dose for the remainder

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[2] Presented in part at the 45th Annual Meeting of the American Society for Experimental Pathology, Atlantic City, New Jersey, April, 1961.
[3] Parke-Davis Haliver oil containing no more than 60,000 U.S.P. units of vitamin A and no more than 1,000 U.S.P. units of vitamin D/gm.

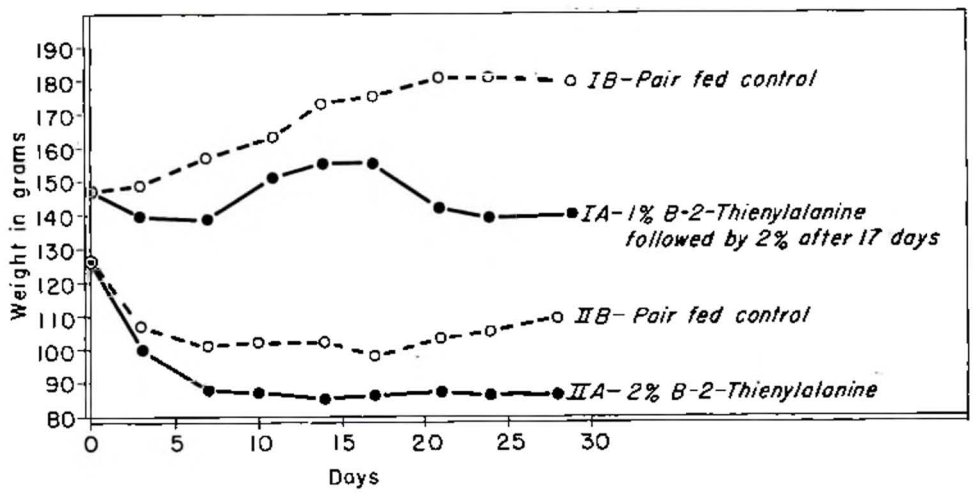

Fig. 1 Weight changes in rats receiving a β-2-thienylalanine supplement.

of the experiment and in the animals fed a 2% supplement for the entire period.

The myocardial fibers were slightly narrowed in the experimental animals when compared with the controls. The nuclei were plumper. Small amounts of sudanophilic material were noted in the myocardium in both the experimental animals and their pair-fed controls, but the amount was somewhat greater in the former.

The mucosa of the esophagus of the experimental animals was slightly atrophic. In the stomach, the cells of the pyloric glands were less vacuolated and contained less mucin (fig. 2A). The cytoplasmic basophilia was present diffusely throughout the entire cell. The nuclei were enlarged, rounded and vesicular. Occasionally, prominent nucleoli were present. In the controls, the cytoplasm was clear and vacuolated and the basophilia was perinuclear and basal. The nuclei were less rounded and the chromatin was dense and homogeneous (fig. 2B). Similar changes were noted in Brunner's glands of the duodenum of the animals in the experimental group (fig. 3). In addition, the glands were distorted and shrunken.

In contrast with the normal findings in the control group, the pancreatic acini were shrunken and the cells contained pyknotic nuclei and vacuolated cytoplasm. There was occasional loss of individual acinar cells. The slight decrease in basophilia was similar to that in the pair-fed controls (fig. 4).

In the periportal areas of the liver many of the nuclei were vesicular and contained large prominent nucleoli. No changes were seen in the parotid or sublingual glands. In the submaxillary glands, there was decrease in the size of the acini. The nuclei were rounded, increased in size and tended to be located centrally. The cytoplasm contained vacuoles (fig. 5).

Kidneys were enlarged. The changes were confined to the entire inner cortical zone and represented damage to the proximal convoluted tubules (fig. 6). There was swelling of the tubular epithelial cells so that the lumens were decreased in diameter. The cytoplasm was pale and the nuclei were vesicular. Occasionally, cells with pyknotic nuclei and eosinophilic cytoplasm were noted. These cells were detached from the basement membrane and some were free in the lumens. A few mitotic figures were seen in one animal indicating regeneration. The cytoplasm of the convoluted tubules contained fat which was graded from 2 to 4 plus with an average of 3 plus. One control animal contained a small amount of fat (1 plus). Otherwise, all kidneys were normal.

The seminiferous tubules were slightly decreased in size and contained fewer mature spermatozoa. There was no arrest in spermatogenesis until the sper-

matid stage was reached (fig. 7). The ductus epididymis was decreased in diameter and contained very few spermatozoa, but degenerated immature forms were present. The epithelium was thickened due to an increase in cytoplasm which was coarsely vacuolated (figs. 8 and 9).

The epithelium of the prostate glands was low and atrophic (fig. 10). In the controls, the secretion was homogeneous and filled the entire lumen; whereas in the experimental group, the contents were granular and foamy and decreased in amount. Similar atrophic changes were seen in the epithelium of the seminal vesicles.

The spleens in the experimental group were smaller than those in the control rats. There was a decrease in the size of Malpighian corpuscles and a marked decrease in the mature lymphocytes (fig. 11). The reticular cells were prominent and in many situations appeared to fill the sinusoids.

There was an overall decrease in the number of lymphocytes in both the medulla and cortex of the thymus. The epithelial reticulum was less apparent in the medulla.

The cytoplasm of the acidophilic cells of the pituitary was decreased and stained less intensively.

No striking changes were observed in the thyroid gland. Occasionally, the follicles were smaller and the epithelium lower in the experimental than in the control animals.

The adrenal size was not altered. In the controls a distinct narrow area was observed between the zona glomerulosa and the zona fasciculata which did not stain for fat. In the experimental animals the fat-free area was indistinct. Apart from this, there was no alteration in the fat distribution and content as seen histologically.

The epiphyseal plates of the tibias were thinner. No apparent difference was noted in the zone of resting cartilage. The zones of proliferating, maturing and calcifying cartilage were markedly narrowed. The bony trabeculae were short, blunt and decreased in number (fig. 12).

The only changes noted in the skin were hyperkeratosis of the epidermis and atrophy of the hair follicles (fig. 13).

DISCUSSION

The weight changes observed with the use of 1 and 2% thienylalanine are similar to those reported by Ferger and du Vigneaud ('49), inasmuch as a 1% supplement caused an initial loss of weight for the first week, followed by a weight gain. This weight gain was halted when the amount of thienylalanine in the diet was increased to 2%. When 2% thienylalanine was given from the beginning of the experiment, the animals lost weight during the first week and their weights remained at a low but constant level (fig. 1).

Some effects of β-2-thienylalanine on tissue were described by Jacquez et al. ('53). These workers were interested in the effects of this substance on sarcoma (T-241) and hence did not give detailed descriptions of tissue changes in all organs. They described the lymphocytes in the spleen, however, as being decreased in number and an arrest of spermatogenic activity in three mice examined. These observations were similar to those found by us. In addition, we noted changes in the heart, esophagus, stomach, duodenum, pancreas, liver, submaxillary salivary glands, kidney, epididymis, prostate, seminal vesicles, thymus, pituitary, thyroid, adrenals, bone and skin.

Hruban and Wissler ('60), using β-3-thienylalanine, another phenylalanine antagonist, reported changes in the pancreas, liver, spleen and thymus which resembled in some respects the changes described by us. We did not note the hepatic necrosis as reported by them, however.

A comparison of the effect on the tissues of phenylalanine deficiency with the changes elicited by its analogue β-2-thienylalanine shows that changes which are described above are similar to those described in animals with phenylalanine deficiency in the testis, bones, thymus, pituitary, thyroid, seminal vesicles and epididymis (Maun et al., '45; Schwartz et al., '51).

Lesions which were observed following the administration of β-2-thienylalanine

that have not been described in phenylalanine deficiency were noted in the upper gastrointestinal tract, skin, spleen, renal tubules, pancreas, and liver. The adrenal changes described by Maun et al. ('45) and Schwartz et al. ('51) with phenylalanine deficiency were not observed in our animals, however; neither did Jacquez et al. ('53) note microscopic changes in the adrenals in mice fed thienylalanine.

None of the lesions described could be considered specific or unique for β-2-thienylalanine inasmuch as some of them were similar to those noted with ethionine, another amino acid antimetabolite. For instance, the changes in bone (Klavins et al., '59), submaxillary glands, (Kinney et al., '60), prostate and seminal vesicles (Kaufman et al., '56), skin,[4] and gastrointestinal tract[5] were similar to those seen with ethionine. The pattern of the distribution and extent of some of the lesions were different, however. Changes in the pancreas were very slight and there was no associated cellular infiltrate in contrast with the marked damage with ethionine (Kinney et al., '60) and the testicular and renal changes were less marked with β-2-thienylalanine. There were no changes, however, in the epididymis with ethionine (Kaufman et al., '56), but there was striking epithelial thickening with β-2-thienylalanine and furthermore, ethionine produces marked liver damage (Klavins et al., '55) but only slight changes were noticed with β-2-thienylalanine.

SUMMARY

β-2-Thienylalanine, a phenylalanine antagonist, was fed to rats receiving a purified synthetic diet in amounts of 1 and 2 % for 4 weeks. A marked loss of weight was noted in all the animals so fed.

The pyloric glands of the stomach and Brunner's glands of the duodenum were altered. The pancreatic acini were shrunken and the cytoplasm was vacuolated. The acini of the submaxillary glands were atrophied. Kidneys were enlarged and the proximal convoluted tubules were damaged. There was spermatogenic arrest at the spermatid level. The ductus epididymis was narrowed and the epithelial lining was thickened. Prostatic epithelium was atrophic and the glands contained a decreased amount of secretion. There was a decrease in the mature lymphocytes of the spleen and of the thymus. The skin was hyperkeratotic and the hair follicles were atrophic. Thinning of the epiphyseal plate was observed, with a decrease in growth of bone. Less marked changes were noted in the heart, pituitary gland, thyroid, adrenals, and the liver.

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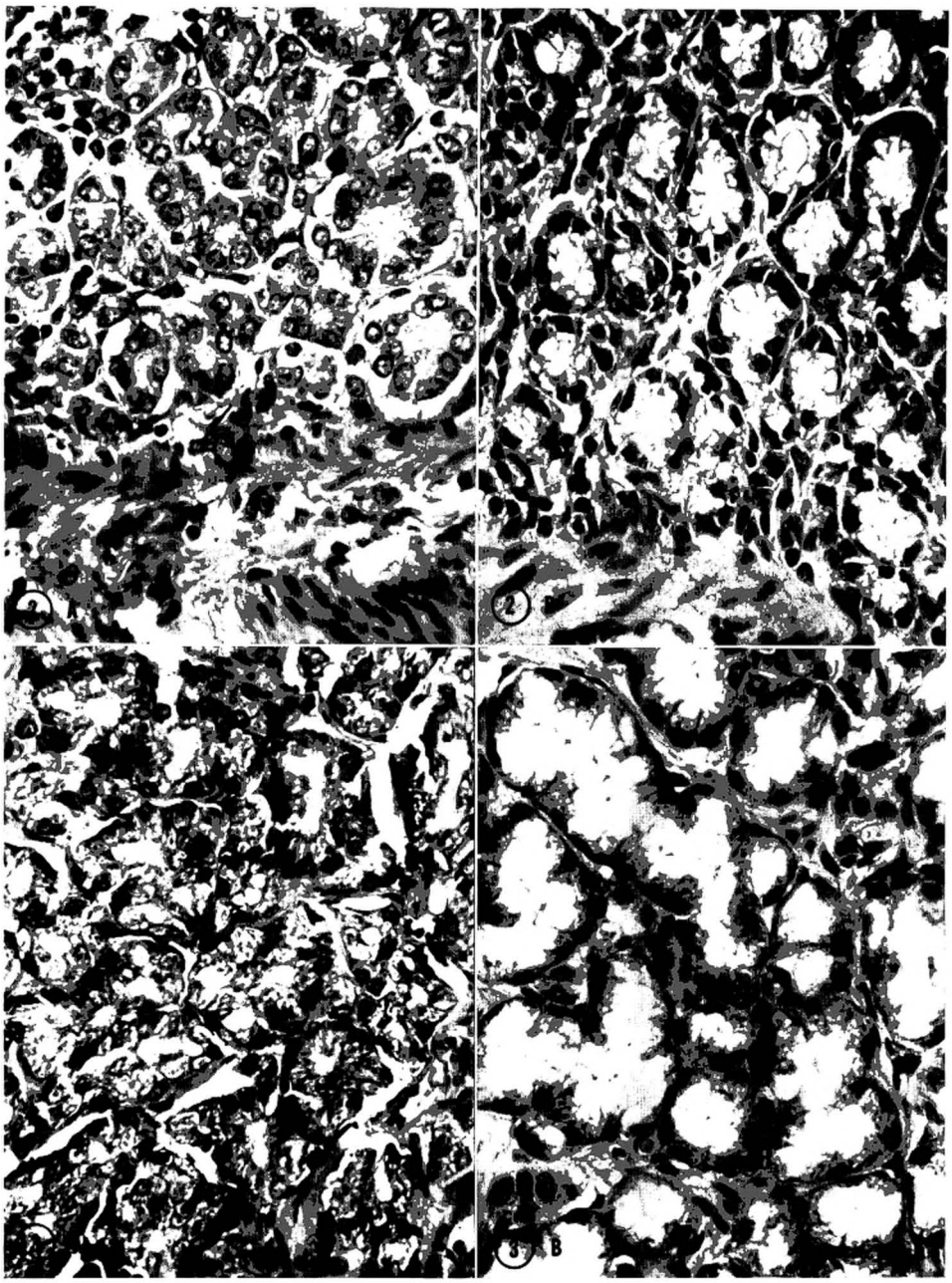
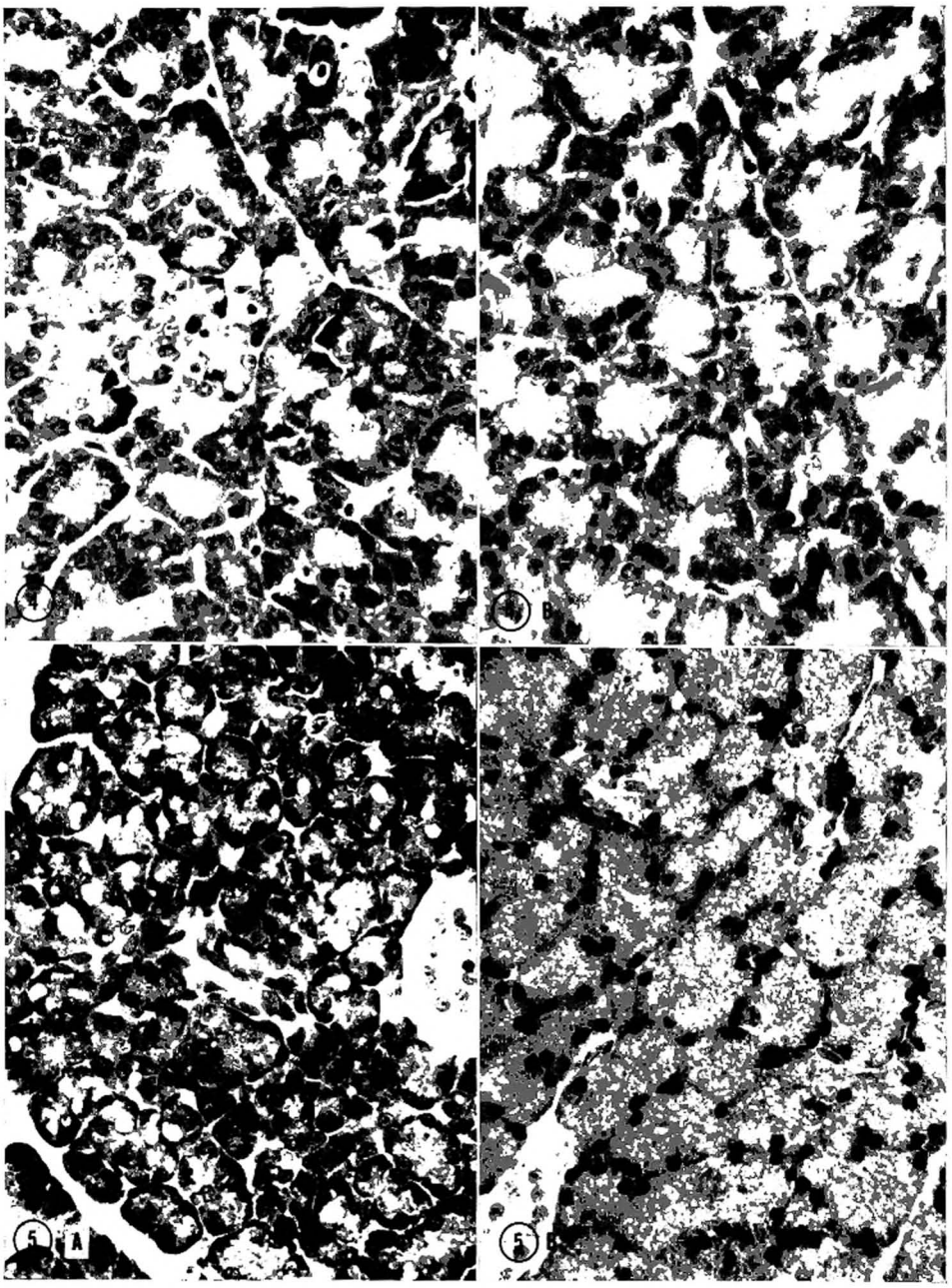


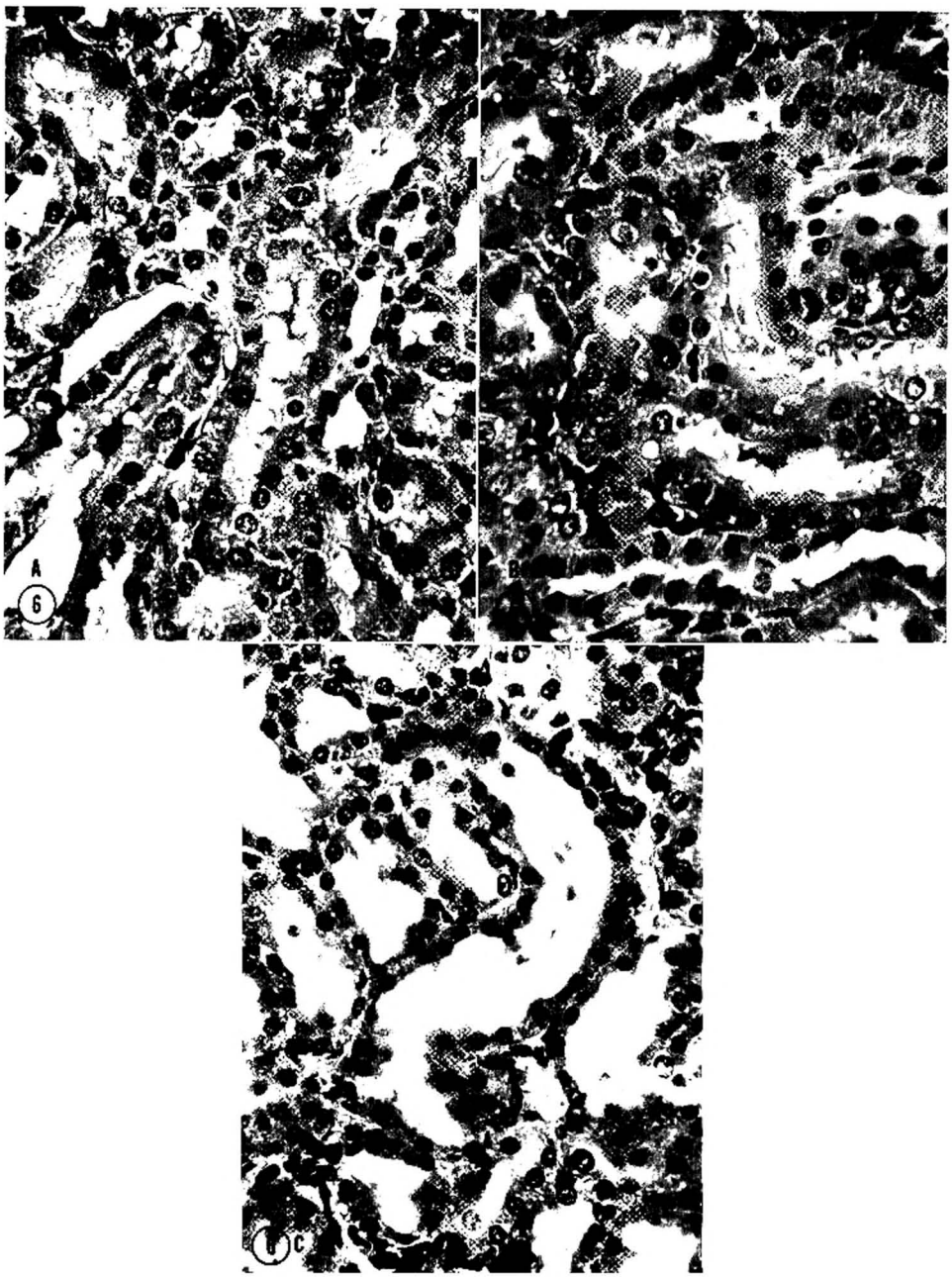
2 Sections of pyloric glands of stomach. H. and E. × 320. A, experimental animal. Note vesicular, rounded and enlarged nuclei and decrease in cytoplasmic vacuolization. Compare with B, pair-fed control.

3 Sections of Brunner's glands of duodenum, H. and E. × 320. A, experimental animal. The glands are distorted and shrunken. The histologic changes are similar to those seen in the pyloric glands in figure 2A. Compare with B, pair-fed control.

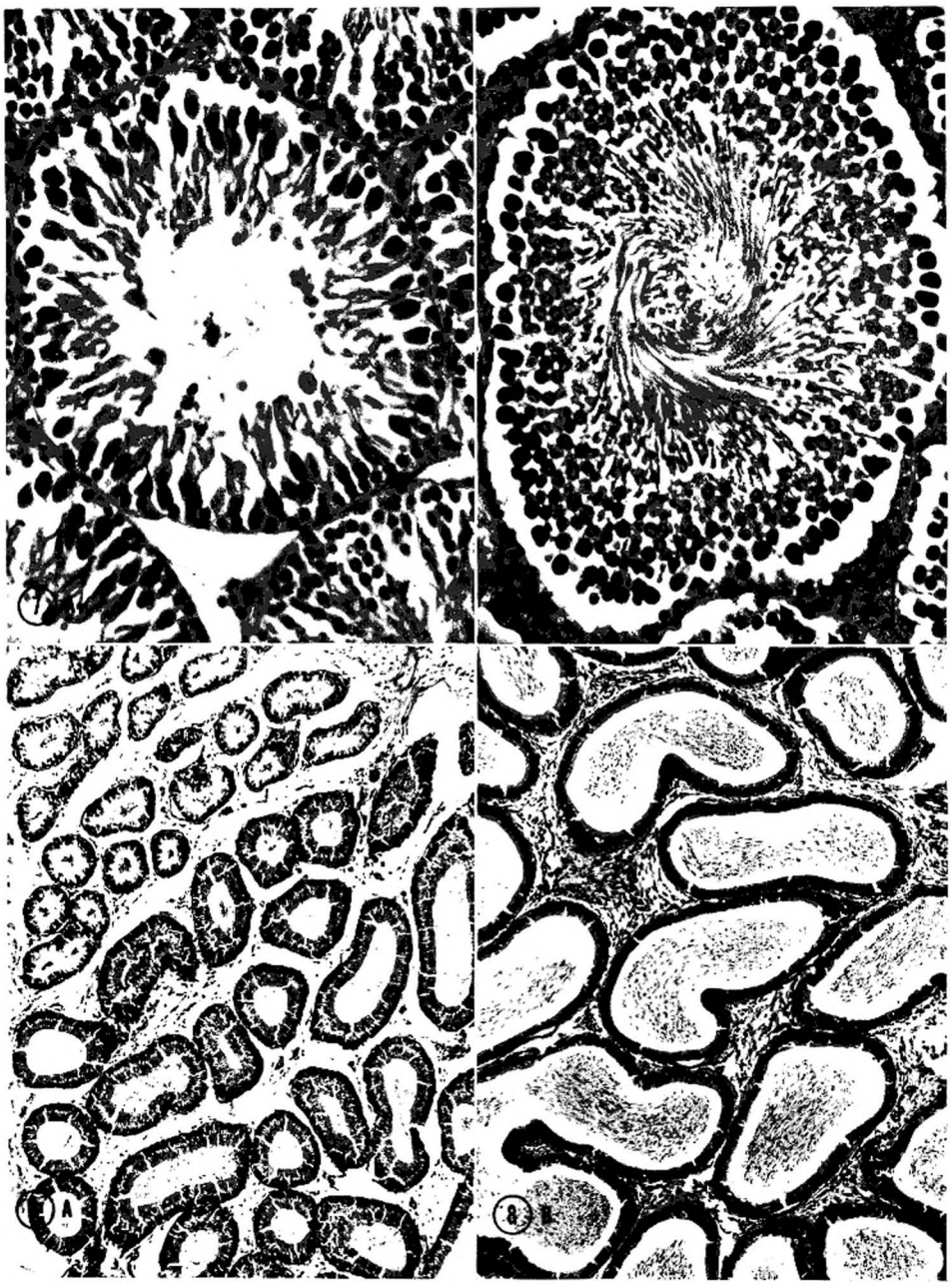


4 Sections of pancreas, H. and E. × 320. A, experimental animal. The acini are shrunken with loss of cells and pyknosis of some nuclei. Compare with B, pair-fed control.

5 Section of submaxillary salivary gland, H. and E. × 320. A, experimental animal. Note decrease in size of acini lined by cells with large rounded nuclei, vacuolated cytoplasm. Compare with B, pair-fed control.

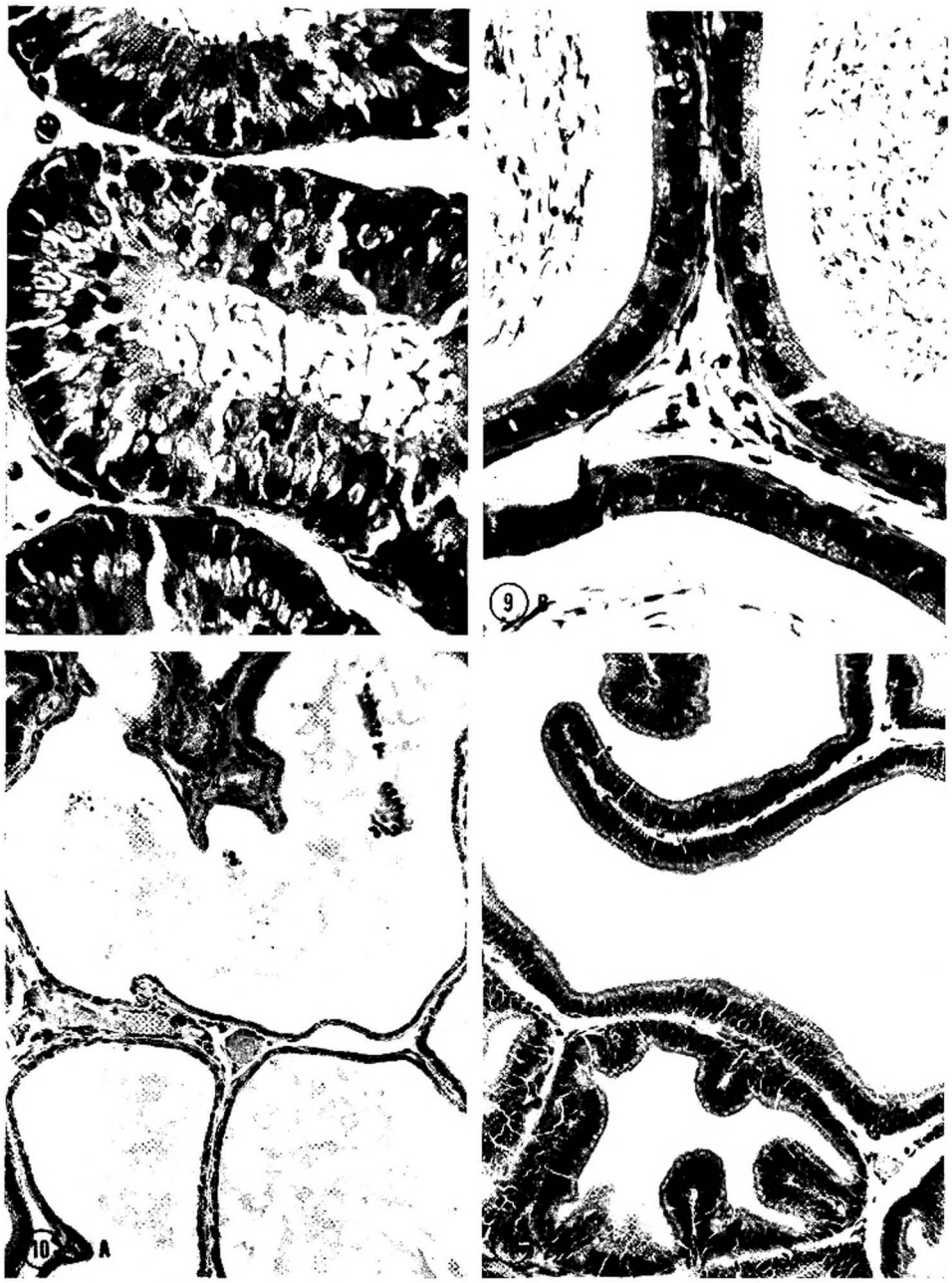


6 Sections of inner cortical zone of kidney, H. and E. × 320. A, and B, experimental animals. Note swelling of epithelial cells and vesicular as well as pyknotic nuclei. In the center of A, a mitotic figure is present. C, pair-fed control for comparison with A and B.



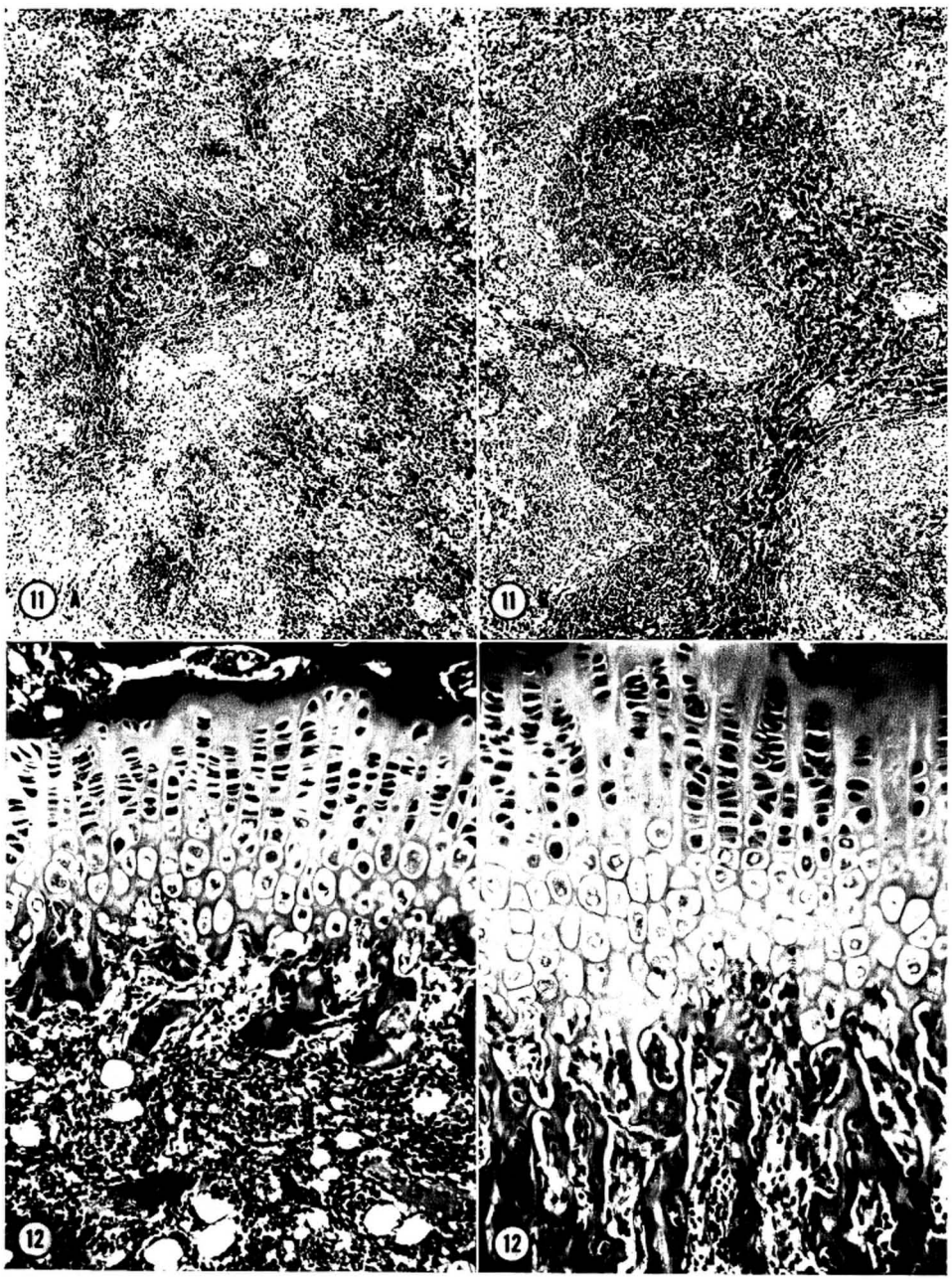
7 Sections of testis, H. and E. × 220. A, experimental animal. Note decrease in diameter of seminiferous tubules with spermatogenic arrest at spermatid level. Compare with B which shows a section of a pair-fed control with numerous mature spermatozoa present.

8 Sections of epididymis, H. and E. × 57. A, experimental animal. The epididymis contains very few spermatozoa, the duct is narrowed and the epithelium is thickened. B, pair-fed control for comparison (see fig. 9).



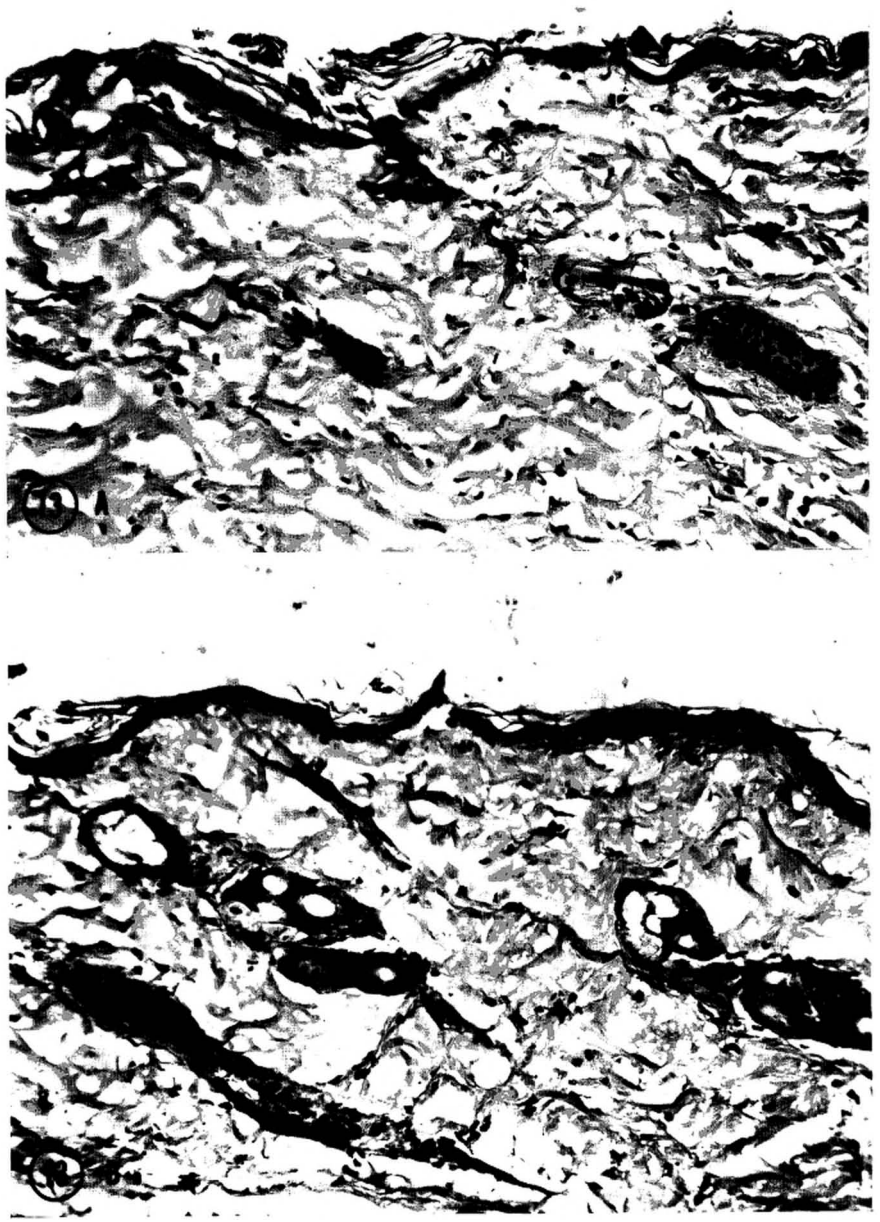
9 Sections of epididymis, H. and E. × 320. A, experimental animal (see fig. 8). B, pair-fed for comparison.

10 Sections of prostatic gland, H. and E. × 140. A, experimental animal. The glandular epithelium is low. The gland contains granular, foamy material. Compare with B, from a pair-fed control where the epithelium is tall, intact and the gland contains homogeneous secretions which fill the entire lumen.



11 Sections of spleen, H. and E. × 85. A, experimental animal. Note decrease in size of Malpighian corpuscles and number of mature lymphocytes. Compare with B, from a pair-fed control.

12 Sections through the epiphyseal plate of tibias, H. and E. × 140. A, note thinning of the entire plate and the blunted and shortened bony trabeculae which are decreased in number. B, section from pair-fed control for comparison.



13 Section of skin, H. and E. × 195. A, experimental animal. Note hyperkeratosis and atrophy of hair follicles. B, section from pair-fed control for comparison.

Effect of Dietary Gallic Acid and Pyrogallol on Choline Requirement of Rats

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We have reported numerous examples in rats of *in vivo* O-methylation of compounds containing a catechol nucleus and a carboxyl side chain including 3,4-dihydroxyphenylacetic acid (Booth et al., '56), caffeic (3,4-dihydroxycinnamic) acid (Booth et al., '57), 3,4-dihydroxyphenylalanine (DeEds et al., '57), and gallic (3,4,5-trihydroxybenzoic) acid (Booth et al., '59). Soon after our first publication, it was shown by Armstrong et al. ('57) that in man the major route of inactivation of epinephrine also is via O-methylation. The enzyme system involved is catechol O-methyl transferase. Evidence that O-methylation of epinephrine is inhibited by pyrogallol both *in vivo* in mice and *in vitro* has been reported (Axelrod and Laroche, '59). In our *in vivo* metabolism studies with rats and rabbits the major urinary metabolite of gallic acid was identified as 4-O-methyl gallic acid but there was very little evidence of O-methylation when pyrogallol was administered (Booth et al., '59).

The source of methyl groups for the O-methylation of gallic acid undoubtedly involves principally choline and methionine. The present study with rats was designed to determine whether a choline deficiency (fatty liver) could be produced by adding gallic acid or pyrogallol to a diet containing marginal amounts of choline and methionine.

METHODS

Weanling albino rats from our colony were divided into similar groups of 5 according to weight. The animals were housed in individual wire-bottom cages and fed the experimental diets ad libitum. Weekly records of body weights and food consumption were maintained.

For experiment 1, male rats were fed a low-choline diet that contained the following ingredients: (in per cent) yellow corn meal, 30; cornstarch, 43; crude casein, 10; corn oil, 10; cod liver oil, 3; alfalfa meal, 2; bone ash, 1.5; and sodium chloride, 0.5.

Because of the rather poor body weight gains obtained in the first trial, a second experiment was conducted using a modified basal diet which contained the following ingredients: (in per cent) yellow corn meal, 40; cornstarch, 32; crude casein, 10; corn oil, 10; alfalfa meal, 2; salts (USP 14), 4; and vitamin mixture in sucrose, 2. The vitamin mixture contained the following nutrients: (in mg per kg diet) thiamine·HCl, 10; riboflavin, 10; Ca pantothenate, 15; pyridoxine·HCl, 10; niacin, 30; folic acid, 5; and 2-methyl-1,4-naphthoquinone, 10. Vitamin A (25,000 I.U. per kg) and vitamin D_3 (3000 I.U. per kg) were also included in the vitamin mixture. In this trial weanling female rats were used, the duration of the experiment was increased from 19 to 27 days, and the effects of pyrogallol and methionine on liver fat were investigated.

When the experiments were terminated the rats were anesthetized, decapitated, bled, and the entire liver removed, blotted and weighed. Liver fat was determined by diethyl ether extraction of each liver, which had been first oven-dried (103°C) and ground.

RESULTS

For experiment 1, the effects of feeding gallic acid to weanling male rats fed a diet

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TABLE 1

Effect of gallic acid and choline on weight gain and liver fat of weanling male rats after 19 days

Diet treatment	Food intake	Weight gain[1]	Dry liver fat[1]
	gm/rat/day	*gm*	*%*
Low-choline control	7.2	29.2 ± 3.33	13.81 ± 0.71
Low-choline control + 1% gallic acid	7.5	23.8 ± 1.78	43.44 ± 2.72
Low-choline control + 0.28% choline chloride	7.2	32.8 ± 3.12	9.06 ± 1.23
Low-choline control + 1% gallic acid and 0.28% choline chloride	7.5	29.6 ± 2.98	10.90 ± 0.78

[1] Mean ± standard error.

TABLE 2

Effect of gallic acid, pyrogallol, choline and methionine on growth and liver fat of weanling female rats after 27 days

Diet treatment	Food intake	Weight gain[1]	Dry liver fat[1]
	gm/rat/day	*gm*	*%*
Low-choline, low-methionine, control	8.2	74.0 ± 1.48	19.91 ± 1.21
Control + 0.3% choline HCl + 1% DL-methionine	7.9	72.6 ± 2.73	12.93 ± 1.05
Control + 1% gallic acid	7.0	56.8 ± 3.31	37.96 ± 2.33
Control + 1% pyrogallol	5.9	36.6 ± 2.23	23.36 ± 3.08
Control + 1% gallic acid + 0.3% choline HCl	7.6	67.2 ± 3.12	11.17 ± 0.31
Control + 1% gallic acid + 1% DL-methionine	7.7	69.6 ± 0.92	14.45 ± 0.20

[1] Mean ± standard error.

low in choline and methionine are shown in table 1. A statistically significant increase in liver fat ($P < 0.01$) for the group receiving 1% of gallic acid for 19 days was observed. Prevention of fatty livers was clearly evident for the group receiving 1% of gallic acid and 0.28% of dietary choline.

In the second experiment (table 2), the addition of 1% of gallic acid to the diet again caused a significant increase in liver fat ($P < 0.01$), and the response was prevented by the addition of either choline or methionine to the diet. The feeding of a diet containing 1% of pyrogallol did not significantly increase the liver fat, whereas growth was significantly decreased ($P < 0.01$). Food intake and weight gain were directly related for all groups. In both experiments the increased amount of liver fat of the control groups indicates that the choline-methionine content of the basal diets was slightly inadequate.

DISCUSSION

Development of fatty livers in rats fed gallic acid was prevented by increasing the choline or methionine intake. Undoubtedly the methyl groups of choline and methionine are utilized in the O-methylation of gallic acid. These observations are in complete agreement with those of our metabolic fate studies in which it was shown that the major urinary metabolite of gallic acid is 4-O-methyl gallic acid. Very little O-methylation of pyrogallol was observed, however. Thus our *in vivo* studies indicate that the *in vitro* methylation of pyrogallol reported by Archer et al. ('60) does not take place in the intact animal. Since there is little evidence of methylated pyrogallol metabolites in the intact animal, it follows that inhibition of O-methylation of epinephrine by pyrogallol *in vivo* probably involves a mechanism of action other than as a methyl group acceptor. The absence of a carboxyl group in pyrogallol could be an important factor.

Many years ago it was established that N-methylation of specific compounds in the animal body is dependent on choline and methionine as a source of methyl groups. Thus Stetten and Grail ('42) were

able to produce fatty livers by feeding guanidoacetic acid to rats in a diet containing marginal amounts of methionine and choline. Guanidoacetic acid is methylated to form creatine. Likewise, Handler and Dann ('42), reported that the N-methylation of niacin was responsible for the production of fatty livers in rats which could be prevented by dietary choline.

The use of gallic acid as an experimental tool for the assay of dietary methionine-choline content and for the production of a choline deficiency in growing rats fed ordinary diets should be considered. Whether a toxic effect (fatty liver) would or would not result from the use of gallates as food additives would depend on the amount of gallate ingested and the adequacy of the diet with respect to choline and methionine. Furthermore, estimations of dietary labile methyl group requirements of animals and man should allow for the amount of choline or methionine required for the detoxification of all ingested catecholic acids, such as chlorogenic and caffeic, which are present in coffee and most fruits and vegetables.

SUMMARY

Rats fed a low-methionine, low-choline diet containing 1% of gallic acid developed fatty livers. Added choline or methionine prevented the increased liver fat. Pyrogallol, in contrast, did not cause fatty livers. These results are in agreement with the metabolism studies in that gallic acid, but not pyrogallol, is O-methylated in the animal body.

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Effect of Dried Egg Yolk, Oils and Fat on Chick Growth

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The initial work of Denton et al.[1] suggesting that egg yolk contained an unidentified growth promotant for chicks stimulated considerable research on this material. Arscott ('56) confirmed these findings and stated that egg yolk contains, along with the fish factor, at least one additional growth factor. On the other hand, Hopper et al. ('56) postulated that egg yolk owed its major growth-promoting ability to the fish factor contained therein. In corroboration of previous work cited above, however, Arscott et al. ('57) reported the existence of at least two separate unidentified growth factors in egg yolk. Menge et al. ('57) presented evidence that the factor in dried egg yolk is fat soluble. The growth stimulant was shown to be present in the nonphospholipid and nonsaponifiable portions of yolk lipid and to be organic since activity was destroyed by ashing. The factor could not be identified with oleic acid, linoleic acid, cholesterol, or soybean lecithin.

These observations were later confirmed and enlarged by Arscott et al. ('59) who also found that palmitic and stearic acids, sodium oleate, orotic acid, and adenosine were ineffective. More recently, Wiese et al. ('60) reported their results on the fractionation of egg yolk which also indicated the presence of an unidentified growth promotant. Scott et al. ('58) presented evidence to show that chick growth responses were identical for both corn oil and egg oil when fat calories were substituted for carbohydrate calories on an isocaloric basis and in isonitrogenous diets. These workers concluded that substitution of fat calories for carbohydrate calories is sufficient to stimulate chick growth. Greene et al. ('60), from the same laboratory, reported that the major portion of the growth stimulation obtained from fluid egg yolk can be attributed to a better balance of known nutrients rather than to an unidentified growth factor(s). Because of these reports it was considered advisable to reappraise our findings and subject the so-called egg yolk factor to a more critical examination using isocaloric-isonitrogenous, high-energy diets at two protein levels. These diets were fed both ad libitum and on an equalized nutrient intake plan.

STUDIES WITH HIGH-PROTEIN, HIGH-ENERGY DIETS

Experimental procedure. Purebred White Rock male (sexed) chicks were used in experiment 1, and crossbred males (New Hampshire male × Barred Plymouth Rock female) in all of the other studies. Each dietary treatment in experiment 1 was triplicated and fed to 120 chicks. In experiments 2, 3, 5, 6, and 7 duplicate groups of 10 chicks were fed each dietary supplement. In experiment 4, 4 groups of 10 chicks received each dietary treatment. In each case the chicks were selected at random after hatching, being wing-banded, placed in their respective groups and weighed collectively. The length of the experimental period was 4 weeks in all trials with the exception of experiments 1 and 2 in which 8-week growth data were obtained. Electrically heated batteries were used in all trials except experiment 1 in which floor pens containing sawdust litter were used.

The chicks had free access to feed in experiments 1, 2, and 3. Experiments 4, 5, 6, and 7 were designed to equalize nutritive intake. In experiment 4, protein and/or energy sources in the basal diet, A,

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[1] Denton, C. A., R. J. Lillie and J. R. Sizemore 1954 Effect of egg yolk, fat and fish solubles on growth of chicks. Federation Proc., *13*: 455 (abstract).

(table 1) were replaced by lard, egg yolk, corn oil, or egg oil on an isocaloric-isonitrogenous basis. This resulted in "short" diets in which 91.7 parts of the egg yolk diet and 87.7 parts of the lard and oil diets equaled 100 parts of the basal. The diets were fed at 91.7 and 87.7% of the quantity of the basal diet consumed during the previous 24 hours. In experiments 5, 6, and 7, the egg oil in the basal diet, C, (table 1), was replaced by equal quantities of fat or other oils. Since these substitutions did not change protein or energy levels the resulting diets were fed in quantities equal to those of the basal diet consumed during the previous 24-hour period.

The diets used in the high-protein, high-energy studies (table 1) were calculated to be isocaloric and isonitrogenous with the exception of those used in experiments 1 and 2. In these experiments the diets were isonitrogenous. Diets A, B, and C were used in experiments 1 and 2. Diets A, B, D, and E were used in experiments 3 and 4. Diet C containing 17.5% of egg oil was used as the control diet in experiments 5, 6, and 7.

The dried egg yolk used in these studies was obtained from a commercial source.[2] The yolk oil was prepared in this laboratory by exhaustive extraction of dried yolk using equal parts of ethanol and petroleum ether (b.p. 60 to 71°C). On the average, 60% of the original dried yolk was extracted as oil. The residue was designated as yolk protein.

Fraps' productive energy values as tabulated by Titus ('55) were used throughout with the following exceptions. Since there

[2] Furnished by Henningsen, Inc., Central Laboratories, Springfield, Missouri.

TABLE 1

Composition of basal and experimental diets (high-protein, high-energy)

Ingredient	Basal A	Experimental B	Basal C	Experimental D	Experimental E
Soybean oil meal, 50%	40.0	34.66	40.0	40.0	32.08
Casein	7.0	6.066	7.0	7.0	5.62
Gelatin	5.0	4.333	5.0	5.0	4.01
Glucose monohydrate	40.2375	26.8785	22.7375	17.4375	43.5275
Oil or fat[1]	—	—	17.5	10.5	—
Dried egg yolk	—	12.0	—	—	—
Yolk protein[2]	—	- -	—	—	7.0
DL-Methionine	0.5	0.5	0.5	0.5	0.5
Choline supplement[3]	0.7	0.7	0.7	0.7	0.7
DPPD[4]	0.0125	0.0125	0.0125	0.0125	0.0125
Antioxidant[5]	0.05	0.05	0.05	0.05	0.05
Vitamin mix[6]	0.5	0.5	0.5	0.5	0.5
Mineral mix[7]	6.0	6.0	6.0	6.0	6.0
Diet, %	100.0	91.7	100.0	87.7	100.0
Protein, % (calc.)	31.71	34.65	31.71	36.16	31.71
Productive energy/pound	974.0	1,065.0	1,259.0	1,112.0	974.0
Calorie:protein	30.7:1	30.7:1	39.7:1	30.8:1	30.7:1

[1] One of the following oils or fat used: corn oil (Mazola); stabilized animal fat (lard containing 0.05% antioxidant described in footnote 5 below); or egg oil extracted from dried egg yolk using equal parts of EtOH and petroleum ether (b.p. 60 to 71°C) and containing 0.05% antioxidant (described in footnote 5 below).

[2] Residue of dried yolk extraction described in footnote 1 above was substituted.

[3] Contains 113,500 mg of choline/pound.

[4] Diphenyl-*p*-phenylenediamine.

[5] Twenty per cent of butylated-hydroxyanisole and 20% of citric acid in propylene glycol solvent.

[6] Vitamin mix to provide (mg/kg of diet): thiamine·HCl, 50; niacin, 100; riboflavin, 16; D-Ca pantothenate, 20; folacin, 4; 2-methyl-1, 4-naphthoquinone, 5; pyriodoxine·HCl, 8; biotin, 0.3; vitamin B_{12} (0.1%), 20; stabilized vitamin A (10,000 USP units/gm), 1,100; stabilized vitamin D_3 (15,000 ICU/gm), 59; stabilized vitamin E (44 ICU/gm), 700; plus chlortetracycline, 20; and glucose monohydrate, 290 (to 0.5% of diet).

[7] Mineral mix (in per cent): steamed bonemeal, 3.5; ground limestone, 1; iodized salt (96 parts NaCl, 4 parts $MnSO_4 \cdot H_2O$), 0.5; $MgCO_3$, 0.18; $FeC_6H_5O_7 \cdot 5H_2O$, 0.04; $ZnCO_3$, 0.004; H_3BO_3, 0.001; $CuSO_4 \cdot 5H_2O$, 0.002; KI, 0.004; $Na_2MoO_4 \cdot 2H_2O$, 0.0009; $CoSO_4 \cdot 7H_2O$, 0.0002; $Na_2SeO_4 \cdot 10H_2O$, 0.0004; glucose monohydrate 0.7675 (to 6% of diet).

were no values for dried yolk or yolk oil, we assigned a value of 1950, 1030, and 2900 Cal. of productive energy per pound of dried yolk, yolk protein (residue), and egg oil, respectively.[3] The other oils and fat used in these studies were corn oil,[4] soybean oil,[5] and lard.[6] These materials together with egg oil were assigned a value of 2900 Cal. of productive energy per pound and were substituted accordingly in the experimental diets. Iodine values for the oils and fat were determined in this laboratory by the Wijs' method as described in AOAC ('55). The results of each experiment were subjected to statistical treatment for the analysis of variance and Duncan's new multiple range test as described by Li ('57).

Results. The substitution of dried yolk, lard, or corn oil in experiments 1 and 2 (table 2) elicited a growth response significant over the basal at the 1% level. The results obtained in experiment 3 (table 3) show a significant (1% level) growth response to corn oil, egg oil, and dried yolk supplementation when compared with the basal and the yolk protein groups. Apparently the growth-promoting activity contained in dried yolk was extracted with the fat solvents since the chicks receiving the yolk protein (residue) grew at the same rate as the basal group.

Dietary substitutions in experiment 1, 2, and 3 were made in terms of their caloric values. It follows therefore that whenever a high-energy supplement was substituted at the expense of a lower energy ingredient the final total would not add to 100 parts. Thus, the 12% yolk diet contained 91.7 parts, and the 10.5% lard and 10.5% corn oil diet, 87.7 parts (table 1). This made it possible to maintain a constant ratio of energy to protein and all other nutrients in the experimental diets, and to eliminate any possibility that added cellulose might interfere with feed consumption or have an effect on the intestinal flora. Although these "short" diets contained the same ratio of protein to energy and nutrients as the control diets, they contained more nutrients per unit. Calculations based on feed consumption records showed that the total nutrient intake of chicks receiving the "short" diets was greater than those fed the basal diet. Therefore, to study the specific effect of dried yolk, or other energy-rich substances on chick growth, it was necessary to equalize nutrient intake. Paired-group feeding was used in experiments 4, 5, 6, and 7. In experiment 4, (table 3) paired-group feeding was compared with ad libitum feeding (experiment 3). Supplements of

[3] Protein and fat were assigned a productive energy value of 1030 and 2900, respectively. The value for dried yolk was obtained through the use of the formula: % protein × 1030 plus % fat × 2900 (dried yolk, 35% protein and 54.8% fat).

[4] Corn oil (Mazola, Corn Products Refining Company, New York) iodine value, 121.0, was supplied by E. F. Drew and Company, Inc., New York.

[5] Refined-deodorized soyabean oil (edible), iodine value 128.7.

[6] Stabilized animal fat, iodine value, 65.9.

TABLE 2

Effect of dried yolk, corn oil, and lard on chick growth (ad libitum feeding)

	Experiment 1, ad libitum feeding			
Supplement	None	12% dried yolk	17.5% lard	17.5% corn oil
Average weight, gm	1567	1728	1749	1808[1]

White Rock males (8 weeks), 120 chicks/treatment

	Experiment 2, ad libitum feeding			
Supplement	None	17.5% lard	12% dried yolk	17.5% corn oil
Average weight, gm	1154	1271	1362	1387

N.H. × B.P.R. males (8 weeks), 20 chicks/treatment

[1] No significant difference at the 1% level between means underscored by same line.

TABLE 3

Effect of ad libitum feeding vs. equalized nutrient intake on chick growth responses to dried yolk, egg oil, corn oil, and yolk protein

Supplement	Experiment 3, ad libitum feeding None	7% yolk protein	10.5% corn oil	10.5% egg oil	12% dried yolk
Average weight, gm	383	387[1]	420	429	443

N.H. × B.P.R. males (4 weeks), 20 chicks/treatment

Supplement	Experiment 4, equalized nutrient intake None	10.5% lard	10.5% corn oil	12% dried yolk	10.5% egg oil
Average weight, gm	349	371	379	381	388

N.H. × B.P.R. males (4 weeks) 40 chicks/treatment

[1] No significant difference at the 1% level between means underscored by same line.

dried yolk, egg oil, corn oil, and lard promoted a growth response significant over that of the basal groups (1%) in both feeding systems.

The average weight of the chicks in experiment 4 (paired-group feeding), was somewhat less than that observed in experiment 3 (ad libitum feeding). The chicks assigned to the regular feeding plan had access to feed up to the time they were weighed, whereas experimental procedure necessitated the removal of feed from chicks assigned to the equalized intake plan approximately 18 hours before weighing. Observations in this laboratory show an approximate 12% differential in body weight resulting from this procedure. This would account for the weight difference noted in experiments 3 and 4 (table 3).

Experiments 5, 6, and 7 were conducted to study the effect of substituting soybean oil, corn oil, or lard, for egg oil. These groups were all fed according to the equalized intake plan using as the control the feed consumption of the group fed the 17.5% of egg oil. The results of these experiments (table 4) show no significant effect on growth through the interchange or substitution of any of the oils and fat tested.

STUDIES WITH 22% PROTEIN HIGH-ENERGY DIETS

Experimental procedure. Chicks used in the following studies were crossbred males (New Hampshire male × Barred Plymouth Rock female). In experiments 8, 9, 10, and 12 duplicate groups of 10 chicks were fed each dietary supplement. Experiments 11 and 13 consisted of 5 quadruplicated treatments making a total of 4 groups of 10 chicks receiving each dietary treatment. The chicks were selected at random after hatching, being wing-banded, placed in their respective groups and weighed collectively. The length of the experimental period was 4 weeks in all trials. Electrically heated batteries were used. Feed was available ad libitum in experiments 8, 9, and 10, and provided on an equalized nutrient intake basis in experiments 11, 12, and 13. In the latter experiments, the basal and experimental groups received a percentage of the amount of feed consumed by the control groups (two groups of 10 chicks each) that started the basal diet 24 hours previous to the actual start of the experiment. Thus, on the first day of the experiment, the basal group was given an amount of feed equivalent to that consumed by the control groups started the day before. The dried yolk diet was fed at 91.7%, and the oil and fat diets at 87.7%.

The composition of the basal and experimental diets is presented in table 5. All diets were calculated to be isocaloric and isonitrogenous. The diets used in experiments 8, 9, and 10 (diets F, G, and H) were similar to those used by Arscott et al. ('57) with the exception that all supplements were substituted on an iso-

caloric-isonitrogenous basis. The composition of the diets used in experiments 11, 12, and 13 (diets I, J, and K) are also shown in table 5. These diets all contained 0.5% of corn oil as a source of essential fatty acids. Corn oil, lard, egg oil, and the coconut oils[7] were all assigned equivalent productive energy values and were substituted in their respective diets accordingly. The results of each experiment were subjected to statistical treatment for the analysis of variance and Duncan's new multiple range test as described by Li ('57).

Results. In experiment 8, 9, and 10 (table 6), the isocaloric-isonitrogenous substitution of dried yolk, corn oil, or lard gave significant growth responses (1% level) when fed ad libitum. The results of experiments 11, 12, and 13 (table 7) also showed that growth was improved significantly (at the 1% level) when either dried yolk, corn oil, lard, egg oil, a combination of lard and egg oil, soybean oil, or coconut oil (iodine value, 9.2) was substituted isocalorically and isonitrogenously and fed on an equalized nutrient intake basis. The coconut oil used in experiment 12 was just as effective as the other oils. In experiment 13, however, this coconut oil did not promote as rapid growth as soybean or egg oil, although the growth response was significantly more rapid than that of the basal. In the same experiment a sample of highly saturated coconut oil (iodine value, 0.26) did not stimulate a significant increase in growth over that obtained with the basal diet.

DISCUSSION

The isocaloric-isonitrogenous substitution in either a 22 or a 32% protein, low-fat ration with dried yolk, yolk oil, lard, and corn oil resulted in significant growth responses. The use of an equalized intake feeding plan has also shown that egg oil, corn oil, lard, or soybean oil supplementation to the diet of the growing chick results in a highly significant increase in growth. These responses are not attributable to variations in intake of nutrients or to the essential fatty acids since the latter were included in the basal diet. Scott et al. ('58) have presented data to show that the isocaloric substitution of egg yolk oil or corn oil for carbohydrate calories in an isonitrogenous diet results in a similar

[7] Coconut oil, iodine value, 9.2, edible coconut oil. Coconut oil, iodine value, 0.26, was furnished by the Procter and Gamble Company, Cincinnati.

TABLE 4

Effect on chick growth by replacing egg oil with other oils (equalized nutrient intake)[1]

Treatment	Av. weight, 4 weeks
	gm
Experiment 5	
17.5% Egg oil	412
13.125% Egg oil + 4.375% lard	425
8.75% Egg oil + 8.75% lard	427
4.375% Egg oil + 13.125% lard	415
17.5% Lard	412
Experiment 6	
17.5% Egg oil	416
13.125% Egg oil + 4.375% soybean oil	412
8.75% Egg oil + 8.75% soybean oil	422
4.375% Egg oil + 13.125% soybean oil	426
17.5% Soybean oil	434
Experiment 7	
17.5% Egg oil	390
13.125% Egg oil + 4.375% corn oil	391
8.75% Egg oil + 8.75% corn oil	406
4.375% Egg oil + 13.125% corn oil	393
17.5% Corn oil	393

[1] N.H. × B.P.R. males, 20 chicks/treatment.

TABLE 5

Composition of basal and experimental diets (22% protein-high energy)

Ingredients	Basal F	Experimental G	Experimental H	Basal I	Experimental J	Experimental K
Soybean oil meal, 44%	49.0	39.45	49.0	—	—	—
Soybean oil meal, 50%	—	—	—	28.5	22.607	28.5
Zein	0.4	0.4	0.4	—	—	—
Casein	—	—	—	5.0	4.037	5.0
Gelatin	—	—	—	3.5	2.826	3.5
Sucrose	44.6875	32.6875	27.2975	—	—	—
Glucose monohydrate	—	—	—	54.8875	42.1175	32.5875
Corn oil	—	—	—	0.5	0.5	0.5
Oil or fat[1]	—	—	8.0	—	—	10.5
Dried egg yolk	—	12.0	—	—	12.0	—
DL-Methionine	0.2	0.2	0.2	0.35	0.35	0.35
Choline supplement[2]	0.7	0.7	0.7	0.70	0.70	0.70
DPPD[3]	0.0125	0.0125	0.0125	0.0125	0.0125	0.0125
Antioxidant[4]	—	—	—	0.05	0.05	0.05
Vitamin mix[5]	0.5	0.5	0.5	0.5	0.5	0.5
Mineral mix	4.5[6]	4.5[6]	4.5[6]	6.0[7]	6.0[7]	6.0[7]
Diet,%	100.0	90.45	90.61	100.0	91.7	87.7
Protein, % (calc.)	22.1	24.43	24.3	22.42	24.45	25.6
Productive energy/pound	962.0	1,064.0	1,062.0	1,050.0	1,143.0	1,198.0
Calorie:protein	43.5:1	43.6:1	43.5:1	46.8:1	46.8:1	46.8:1

[1] Corn oil (Mazola); stabilized animal fat (lard) containing antioxidant as described in table 1, footnote 5; egg oil as described in table 1, footnote 1; coconut oil (iodine value, 9.2); coconut oil (iodine value, 0.26).

[2] Contains 113,500 mg choline/pound.

[3] Diphenyl-*p*- phenylenediamine.

[4] Described in table 1, footnote 5.

[5] Described in table 1, footnote 6.

[6] Mineral mix (in per cent): $CaCO_3$, 0.3; $Ca_3(PO_4)_2$, 2.4; K_2HPO_4, 0.9; NaCl, 0.5; $MgCO_3$, 0.18; $FeC_6H_5O_7 \cdot 5H_2O$, 0.11; $MnSO_4 \cdot H_2O$, 0.03; $CuSO_4 \cdot 5H_2O$, 0.002; KI, 0.004; $ZnCO_3$, 0.004; H_3BO_3, 0.001; $CoSO_4 \cdot 7H_2O$, 0.0002; $Na_2MoO_4 \cdot 2H_2O$, 0.0009; $Na_2SeO_4 \cdot 10H_2O$, 0.0004; glucose monohydrate, 0.0675 (to 4.5% of diet).

[7] Described in table 1, footnote 7.

TABLE 6

Effect of dried yolk, corn oil, and lard on chick growth (ad libitum feeding)

		Experiment 8		
Supplement	none	8% corn oil	8% lard	12% dried yolk
Average weight, gm	362	409	417	434[1]
		Experiment 9		
Supplement	none	8% corn oil	12% dried yolk	8% lard
Average weight, gm	366	412	429	434
		Experiment 10		
Supplement	none	8% lard	8% corn oil	12% dried yolk
Average weight, gm	361	420	429	459

N.H. × B.P.R. males (4 weeks) 20 chicks/group

[1] No significant difference at the 1% level between means underscored by same line.

TABLE 7

Effect of dried yolk, egg oil, lard, and coconut oil on chick growth (equalized nutrient intake)

		Experiment 11			
Supplement	none	10.5% lard	5.25% lard 5.25% egg oil	12% dried yolk	10.5% egg oil
Average weight, gm	353	390	395	405	418[1]

N.H. × B.P.R. males (4 weeks), 40 chicks/treatment

		Experiment 12			
Supplement	none	10.5% coconut oil[2]	10.5% lard	10.5% egg oil	10.5% corn oil
Average weight, gm	336	371	389	392	394

N.H. × B.P.R. males (4 weeks), 20 chicks/treatment

		Experiment 13			
Supplement	none	10.5% coconut oil[3]	10.5% coconut oil[2]	10.5% soybean oil	10.5% egg oil
Average weight, gm	345	367	381	409	420

N.H. × B.P.R. males (4 weeks), 40 chicks/treatment

[1] No significant difference at the 1% level between means underscored by same line.
[2] Iodine value, 9.2.
[3] Iodine value, 0.26

chick growth pattern. The results presented in this paper confirm these findings and show that lard and soybean oil can also be used as a substitute for carbohydrate calories in the promotion of chick growth.

The growth responses obtained by supplementing the basal diet with either the fat or the oils may have been due to the lower specific dynamic action of fat. Forbes and Swift ('44) reported that dietary fat significantly improved the utilization of energy. Forbes et al. ('46), and French et al. ('48) showed that an increase in the fat level of the diet of rats receiving an equalized intake caused an

improvement in the digestibility and retention of protein as well as the growth rate.

The chick growth responses obtained by the addition of oil or fat to the diet may have been due to a growth stimulant present in these materials. Rand et al. ('58) have presented evidence to show that improvement in growth and protein retention obtained by corn oil supplementation may be due to unidentified factors. Dam et al. ('59) have also suggested that improved growth was the result of an unknown substance in vegetable fats, or of unrecognized characteristics of the known components of the fats. The results of the present study are in agreement with those reported by these investigators. Although fats and oils have heretofore been considered mainly as a source of calories, it is possible that these materials may provide additional benefits over and above their caloric value.

That animal fat (lard) was equally as effective in growth promotion as the vegetable oils is not in agreement with the observations of Dam et al. ('59), who reported the growth responses from vegetable oils to be significantly greater than those observed with lard. Also it was apparent that the degree of saturation of the oils and fat used in this study had no effect on growth with the exception of the almost completely saturated coconut oil. Both corn and soybean oil have a relatively high iodine value (corn, 121 and soybean oil, 129), whereas lard and egg oil have much lower iodine values (lard, 66 and egg oil, 73). Nevertheless, no differences in growth were observed when corn oil, soybean oil, or lard was substituted for all or part of egg oil in the diet of chicks receiving an equalized nutrient intake. When the almost completely saturated coconut oil was used, however, no growth stimulation was observed. These data are in accordance with the results of Dam et al. ('59) who found that the growth-promoting property of vegetable oil was reduced noticeably during hydrogenation. The positive results of these investigators with coconut oil led them to postulate that the growth stimulation they observed was not due to fatty acids because of the widely differing composition of the unsaturated vegetable oils and coconut oil. No description of the coconut oil is given in their report, but according to our results, coconut oil with an iodine value of 9.2 promoted a growth response significant over that of the basal at the 1% level. A specially prepared coconut oil (iodine value, 0.26) did not stimulate chick growth. These data are indicative of the involvement of unsaturated fatty acids; or it may well be that an unidentified growth stimulant present in the coconut oil and possibly the other oils and fat are labile to the hydrogenation process. These results are in agreement with those reported by Hopkins et al. ('60).

SUMMARY

A highly significant increase in chick growth rate was obtained by the isocaloric-isonitrogenous substitution of dried yolk, yolk oil, corn oil, or soybean oil in either a 32 or a 22% protein, high-energy diet. The substitution of fat calories for carbohydrate calories on an isocaloric basis in isonitrogenous diets resulted in significantly improved weight gains. This was true for equalized nutrient intake as well as for ad libitum feeding. Highly significant differences in growth were noted even though the Calorie/protein ratios were constant. If the growth response to yolk is due to a growth promotant, then these results indicate that corn oil, soybean oil, lard, and coconut oil also contain a common growth stimulant, and that this can be extracted from dried yolk with fat solvents, hence giving the response with egg oil. These results also show that animal fat was just as effective as vegetable oil in growth stimulation. The relatively more saturated lard, coconut oil, and egg oil were just as effective as the less saturated corn and soybean oil. Although this difference in degree of saturation had no effect on growth, lack of growth stimulation from hydrogenated coconut oil suggests that unsaturated fatty acids are involved, or that the growth promotant present in coconut oil is inactivated by hydrogenation.

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Growth Promoting Properties of 6-Ethyl-7-methyl-9-(1′-D-ribityl)isoalloxazine and 6-Methyl-7-ethyl-9-(1′-D-ribityl)isoalloxazine[1,2]

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Riboflavin is 6,7-dimethyl-9-(1′-D-ribityl)-isoalloxazine. It was the first member of the vitamin B-complex to be synthesized and characterized. (Karrer et al., '35b; Kuhn et al., '35). For a short time immediately preceding and following its synthesis, several compounds possessing structural similarity to the vitamin had been synthesized. It was not, however, until the need for a D-ribityl chain in position 9 was discovered, that material possessing any appreciable degree of vitamin-like activity was found. Three flavins which were synthesized shortly after the structure of riboflavin had become known were reported to have vitamin-like activity in the riboflavin-deficient rat; they were 6-methyl-9-(1′-D-ribityl)isoalloxazine (Karrer and Strong, '35; Kuhn et al., '37) 7-methyl-9-(1′-D-ribityl)isoalloxazine (Karrer et al., '35a) and 6-ethyl-7-methyl-9-(1′-D-ribityl)isoalloxazine (Karrer and Quibell, '36). When any one of these materials was administered as the sole flavin in the diet of a riboflavin-deficient rat, it caused a growth response.[3] In terms of our present knowledge we can state that these growth studies were very limited and were not especially critical. This is not said disparagingly. At the time the work was done it was accepted, if only by implication, that a growth response in a riboflavin-deficient rat following the administration of a flavin was adequate evidence that it could replace riboflavin for biochemical purposes. We now know that a growth response is inadequate evidence for a conclusion that the material is able to replace riboflavin in the nutrition of this animal.

A later observation (Snell and Strong, '39) that these three homologs of riboflavin were also the only three of several tested to support the growth of *Lactobacillus casei*, lent support to the reasonableness of the belief of their activity in the rat. It appeared that a flavin which could serve as the sole flavin in the nutrition of *L. casei* was also able to perform this function in the rat.

A general interest in chemotherapy and the mechanism of action of metabolic antagonists has prompted us to synthesize, and study the biological responses of several analogs, homologs and isomers of riboflavin. One of these, 6,7-diethyl-9-(1′-D-ribityl)isoalloxazine was equivalent to riboflavin for the flavin requirement of *L. casei* over most of the range of limiting concentrations (Lambooy, '51). On the basis of what was known about flavins at that time, we expected that it would have considerable activity as a riboflavin substitute in the nutrition of the rat as well. It was found, however, that while the administration of small quantities were ineffective, intermediate quantities caused a prompt and dramatic growth response, improvement in the animal's appearance and unexpectedly, its sudden death. When still larger quantities were given the homo-

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[1] This investigation was supported in part by grants from the United States Public Health Service. The most recent, CY-2940, is from the Cancer Institute.
[2] Some of the results of this work appeared in a brief preliminary report (Lambooy, '58b).
[3] For more detailed discussion of the activity of these materials and several pertinent references see Lambooy and Aposhian ('52).

log was clearly an inhibitor (Lambooy and Aposhian, '52). An even more striking growth response in the riboflavin-deficient rat was observed when it was given any quantity of the riboflavin analog 6-chloro-7-methyl-9-(1'-D-ribityl)isoalloxazine (Haley and Lambooy, '60). This material is the best substitute for riboflavin in the growth of the rat under these circumstances; it is also the most lethal riboflavin antagonist in this animal to have been described. The mechanism by which such growth is accomplished is in the final stages of study and has been discussed before (Lambooy and Aposhian, '52; Lambooy, '55; Haley and Lambooy, '60).

Diethyl riboflavin stimulates the growth of the riboflavin-deficient rat but it cannot be considered capable of complete utilization in the place of riboflavin in this animal. In view of this discovery one might be justified in harboring a certain curiosity about the flavin 6-ethyl-7-methyl-9-(1'-D-ribityl)isoalloxazine. The only information which we had about its activity in the riboflavin-deficient rat was a brief statement (Karrer and Quibell, '36) that at 10 μg per day it was very active. Is it active like riboflavin or is it active like diethyl riboflavin? We were interested to seek an answer to this question.

The procedure used by Karrer and Quibell in the original synthesis of 6-ethyl-7-methyl-flavin had been suspected of leading to small amounts of contaminating isomeric flavins by Tishler and associates ('47), and was actually found to do so by Haley and Lambooy ('54) and by Lambooy ('58a).[4] Since comparative studies of biological activity require that the materials be of highest purity, an alternative procedure was used to accomplish the unequivocal synthesis of this material of unquestionable purity. 6-Methyl-7-ethyl-9-(1'-D-ribityl)isoalloxazine, an isomeric form of the flavin discussed above should be of interest because it bears a similar relationship to riboflavin and diethyl riboflavin. These two flavins, 6-ethyl-7-methyl-9-(1'-D-ribityl)isoalloxazine and 6-methyl-7-ethyl-9-(1'-D-ribityl)isoalloxazine were synthesized for biological evaluation (Lambooy, '58a).

6-Ethyl-7-methyl- and 6-methyl-7-ethyl-9-(1'-D-ribityl)isoalloxazine have been found to serve independently as the sole source of flavin in the nutrition of *L. casei* with potencies equivalent to riboflavin throughout limiting concentrations.

There can be no doubt concerning the ability of these materials to stimulate the growth of the riboflavin-deficient rat. All the evidence we have at this time indicates that the ability of these materials to support the growth of this animal is due to their serving as replacements for riboflavin. We are, however, unwilling to commit ourselves concerning the completeness of the interchangeability of these materials with riboflavin because, while they appear to satisfy the flavin requirement for the growth of the rat, they do not meet this need for the reproductive function (Lambooy, '58b).

METHODS

Acid production by L. casei. The procedure employed was essentially that used for the preparation of a standard curve for the microbiological assay of riboflavin (Association of Vitamin Chemists, '51). Standard solutions of the three flavins were prepared to contain 2.66×10^{-8} moles per ml in water. These solutions were diluted immediately before use to concentrations of 2.66×10^{-10}, 5.32×10^{-10} and 26.6×10^{-10} moles per ml to constitute the "working standards." Four sets of three series of tubes were prepared; a series consisting of tubes containing graded increments of one of the

[4] Shortly after the appearance of this paper Dr. P. Karrer requested a sample of our 6-ethyl-7-methyl-9-(1'D-ribityl)isoalloxazine to compare with his. He promptly informed us that our two samples were identical as determined by infrared analysis and by mixed decomposition point. While we consider these methods to be of insufficient sensitivity to have revealed the presence of from 5 to 6% of isomeric impurity, we believe that Dr. Karrer's sample was free of a contaminant. We assumed that his original sample was recrystallized from water several times in preparation for micro combustion. We recrystallized our preparation made by Karrer's procedure, and which contained an isomeric contaminant, from water several times also. This led to the removal of the contaminant. This flavin has been incorrectly named in a paper by W. Forter and P. Karrer, Helv. chim. acta, 36: 1530 (1953).

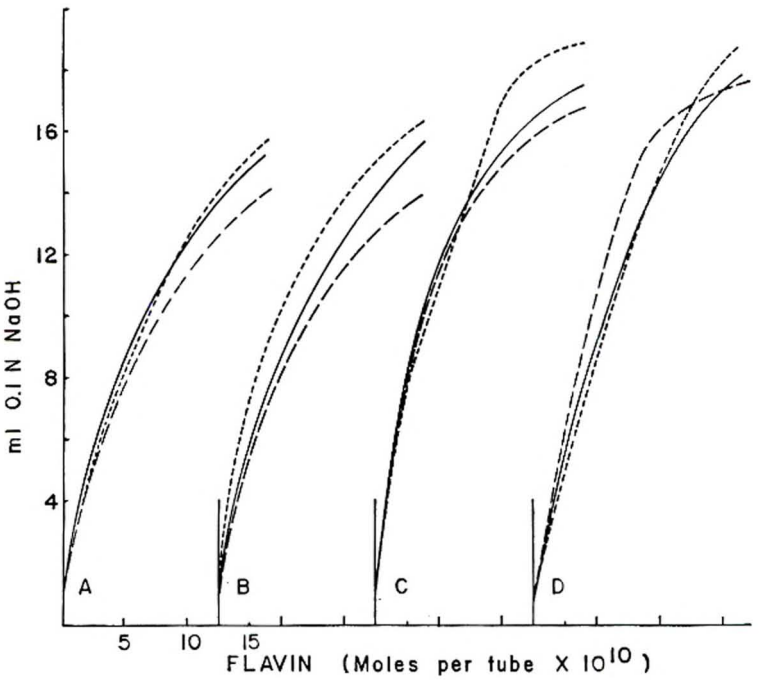

Fig. 1 Acid production by *L. casei* (American Type Culture Collection, no. 7469) in the presence of the flavins. Riboflavin —; 6-Et-7-Me-flavin - - -; 6-Me-7-Et-flavin – –. A, the three flavins inoculated by "stock" *L. casei*. B, the three flavins inoculated by *L. casei* which had been subcultured 6 times in riboflavin at the same time and under the same conditions as in C and D. C, the three flavins inoculated by *L. casei* subcultured 6 times in 6-Et-7-Me-flavin. D, the three flavins inoculated by *L. casei* subcultured 6 times in 6-Me-7-Et-flavin.

flavins from zero to 15.96×10^{-10} moles per tube. One such series for each flavin constituted a set. One set was inoculated with cells which had been freshly subcultured from an agar stock stab (fig. 1A). A second set was inoculated with cells which had been subcultured into media containing riboflavin,[5] every third day for 6 passes (fig. 1B). The third and 4th sets were inoculated with cells which had been subcultured into media containing 6-ethyl-7-methyl-flavin (fig. 1C) and 6-methyl-7-ethyl-flavin (fig. 1D), respectively, for 6 passes also. The acid produced was measured by titration with 0.1 N sodium hydroxide after 120 hours of incubation at 37°C.

Higher concentrations of the flavins were also used (to 106.4×10^{-10} moles per tube), but the data are not shown. The acid production at flavin concentrations above those plotted reached a plateau a little above that produced by 15.96×10^{-10} moles per tube (0.600 μg per tube for riboflavin and 0.624 μg per tube for the homologs).

Rat growth. Weanling male Wistar rats were used as described before (Lambooy and Aposhian, '60). When an animal had become deficient in riboflavin as evidenced by its failure to gain weight during a period of 14 days, it was placed in one of the 16 groups of animals used for this assay. The group assignments were made in a random manner, except that every 6th animal was placed in the deficient control group. The deficient control group consisted of 8 animals; all other groups consisted of three or 4 animals. The diets of the animals in the experimental groups were supplemented with 2, 4, 5, 10 or 20 μg of riboflavin or 5, 10, 20,

[5] The subculturing was done into solutions containing 7.98×10^{-10} moles of flavin per 10 ml of medium.

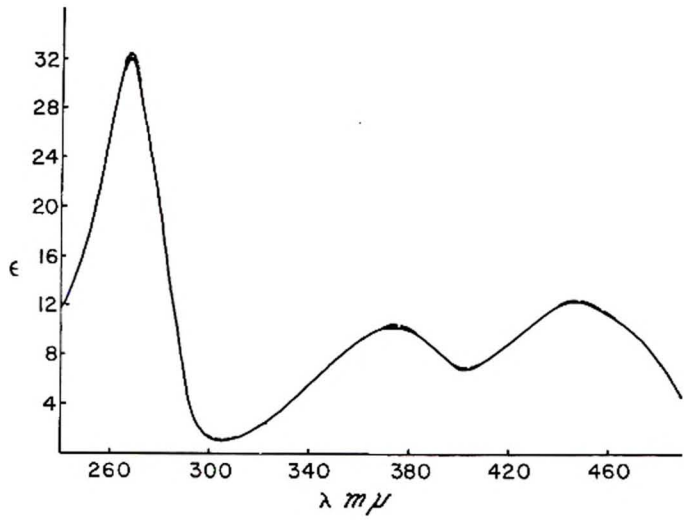

Fig. 2 Ultra-violet absorption spectra of the three flavins. Riboflavin —; 6-Et-7-Me-flavin · · ·; 6-Me-7-Et-flavin – –. The concentration was 1.33×10^{-5} moles per liter in water. Optical density determination made on a Beckman Spectrophotometer, model DU.

40 or 500 μgEq[6] of either the 6-methyl-7-ethyl-flavin or the 6-ethyl-7-methyl-flavin. The supplements were given in 0.5 ml of water solution by stomach tube immediately before the animals were fed on each of the 28 days of the test period. The deficient group received only 0.5 ml of water. During the test period the food was withdrawn for a period of 6 hours each day.

The riboflavin used throughout the study was United States Pharmacopeia Riboflavin Reference Standard ('55). The ultraviolet absorption spectra (fig. 2) indicate that the two homologs are as pure as the riboflavin standard. The ultraviolet absorption spectra are also given to emphasize the similiarity in one of the physical properties among these compounds. Several other physical and chemical properties show comparable similarities.

Flavin equivalence for food utilization. The results of the rat growth study indicated that the 6-ethyl-7-methyl-flavin had 47% of the activity of riboflavin while the 6-methyl-7-ethyl-flavin had 36% of the activity of riboflavin. These results were based on growth resulting from the daily administration of quantities of the flavins to animals which had essentially unlimited access to food. If the three flavins were actually equivalent to riboflavin in the ratio of 100 to 47 and 100 to 36 for the 6-ethyl-7-methyl-flavin and 6-methyl-7-ethyl-flavin, respectively, they should, when given in equivalent amounts, provide for equivalent utilization of food. A test of this sort requires that the quantities administered be within a limiting range and, since on a former occasion we had found 5 μg of riboflavin per gm of food to be sufficient for good growth but not sufficient for optimal growth (Lambooy and Aposhian, '60), this was chosen as the standard.

With consideration of no other factors than a standard level of 5 μg per gm of food for riboflavin and based on the appropriate ratios of activity alone, identical diets were mixed except that they contained the above quantity of riboflavin, 10.6 μg per gm[7] of food of the 6-ethyl-7-methyl-flavin or 13.9 μg per gm of food for the 6-methyl-7-ethyl-flavin. On one occasion the diets containing riboflavin or the 6-ethyl-7-methyl-flavin were fed to 15 pairs of weanling male Wistar rats for

[6] The symbol μgEq means microgram equivalent. This is a corrected value based on the differences in the molecular weights of the flavins. This ratio is equal to 1.04 and thus 10 μg of riboflavin is equivalent to 10.4 μg of one of the homologs.

[7] The ratio $100/47 \times 5 = 10.6$ and $100/36 \times 5 = 13.9$.

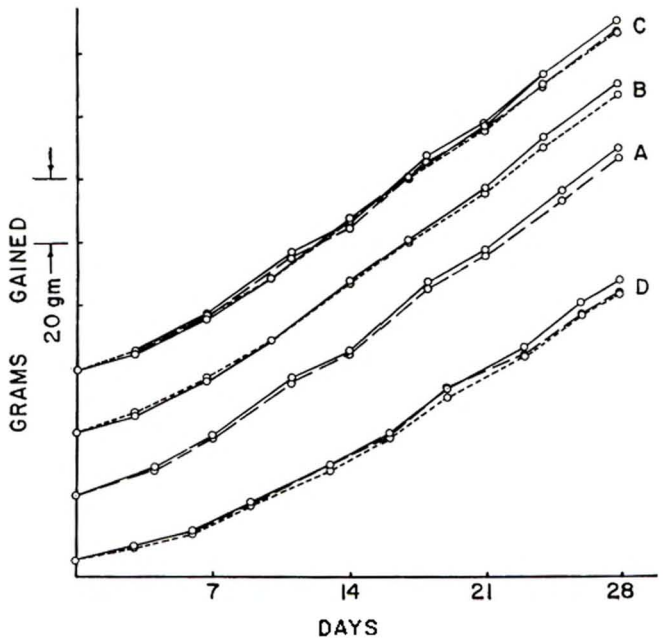

Fig. 3 The equivalence of the three flavins for food utilization. A, riboflavin (5 μg/gm) — vs. 6-methyl-7-ethyl-flavin (13.9 μg/gm) – –. B, riboflavin (5 μg/gm) vs. 6-ethyl-7-methyl-flavin (10.6 μg/gm) - - -. C, A and B superimposed. Studies A and B were done at times separated by several months. D, the three flavins given in the above concentrations to three groups of animals by triplicate feeding. Flavin supplements mixed in the diets in the quantities given above.

28 days by the paired-feeding technique. When the quantity of food consumed by a pair was the same and all that given the previous day had been consumed, the quantity offered was increased for both animals by 0.5 gm. Some months later the study was repeated in as nearly an identical manner as possible using instead, diets containing riboflavin or the 6-methyl-7-ethyl-flavin. The results (fig. 3, A, B, C) were of the type which prompt one to seek additional confirmation. The entire study was repeated with the following changes: an "uninformed" investigator[8] was given the problem, 15 trios of animals were used and feeding done by the "triplicate" technique. When the quantity of food consumed by a trio was the same and all that given the previous day had been consumed, the quantity offered was increased for all animals by 0.2 gm.

RESULTS

Acid production by L. casei. In figure 1 it is shown that the three flavins stimulate the growth of *L. casei* almost equally well (fig. 1A). We did not observe a greater response as a result of frequent, closely spaced subculturing, perhaps because the stock culture was passed regularly once each week (fig. 1A). Considerable stimulation of the organism did result from subculturing in either of the homologs. The appearance of more rapid and extensive growth in the 6-ethyl-7-methyl-flavin as a result of the subculturing being carried out in its presence is true for only a short range of concentrations (fig. 1C). The range of concentrations in which the growth of *L. casei* seems to be superior in the 6-methyl-7-ethyl-flavin following subculturing in its presence is even shorter and decreases when the concentration is 15.96×10^{-10} moles per tube (fig. 1D). In neither case do we consider it significant and, indeed, in other cases the apparent improvement

[8] This study was undertaken by Willard Christiansen as a Medical Student Summer Research Project.

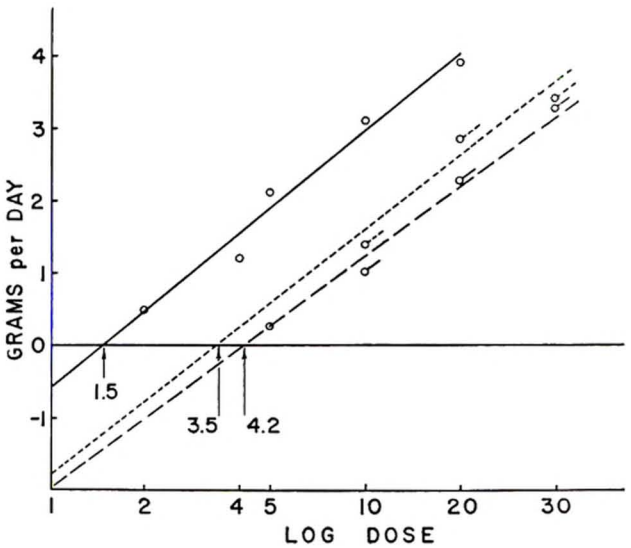

Fig. 4 Rat growth on the flavins; grams per day vs. log dose. Riboflavin, $Y = (3.55 \pm 0.32)$ Log X $-(0.60 \pm 0.27)$, —; 6-Et-7-Me-flavin, $Y = 3.38$ Log X -1.82, - - -; 6-Me-7-Et-flavin, $Y = 3.19$ Log X -1.97, – –. Grams per day indicate the final weight gain of the animal divided by 28 days. 6-Et-7-Me-flavin activity $= 1.5/3.5 \times 100 = 43\%$. 6-Me-7-Et-flavin activity $= 1.5/4.2 \times 100 = 36\%$. Flavin supplements given by stomach tube. Regression equation of the form $Y = a + bX$;

$$a = \frac{\Sigma X^2 \Sigma Y - \Sigma X \Sigma (XY)}{N\Sigma X^2 - (\Sigma X)^2}; \qquad b = \frac{N\Sigma(XY) - (\Sigma X)(\Sigma Y)}{N\Sigma X^2 - (\Sigma X)^2}$$

shown in part C and D was sometimes more and sometimes less than that shown. By the time the concentrations reached 21.28×10^{-10} moles per tube the organisms grown in riboflavin gained ascendency regardless of their immediate previous history. From approximately this latter point until a concentration of 106.4×10^{-10} moles per tube is reached the activity of each of the two homologs is about 90% of that observed for riboflavin.

When the studies of acid production of *L. casei* were first begun we found that each time the inoculum was prepared from an agar stab in the routine manner, the cells placed in the medium containing the 6-ethyl-7-methyl-flavin showed a remarkable decrease in response between the concentrations of 15.96 and 26.6×10^{-10} moles per tube. The acid production at the latter concentration was approximately 60 to 70% of that observed at the former. This lower response was maintained to a concentration of 106.4×10^{-10} moles per tube. Subculturing the organism once into a medium containing 6-ethyl-7-methyl-flavin produced organisms which gave a normal response. Two years later when we decided to renew a study of this phenomenon we found that our new culture of *L. casei* responded as shown in figure 1, and in no case was a decrease in response observed.[9]

Rat growth. Growth data were compared in 5 ways. In figure 4 the average weight gains per group are plotted against the logarithm of the daily dose of flavin. This analysis indicates that the 6-ethyl-7-methyl-flavin and the 6-methyl-7-ethyl-flavin have 43 and 36%, respectively, of the activity of riboflavin.

In figure 5 is shown an evaluation based on the average growth of a group expressed as arbitrary units of area "under" the entire growth curve plotted against the logarithm of the daily dose. The plotted line represents the best fit of the groups for which the diet was supplemented with various quantities of riboflavin. The response of any group receiving the various quantities of one of the

[9] This observation was part of a Second Year Medical Student Project undertaken by A. Allen Levitan.

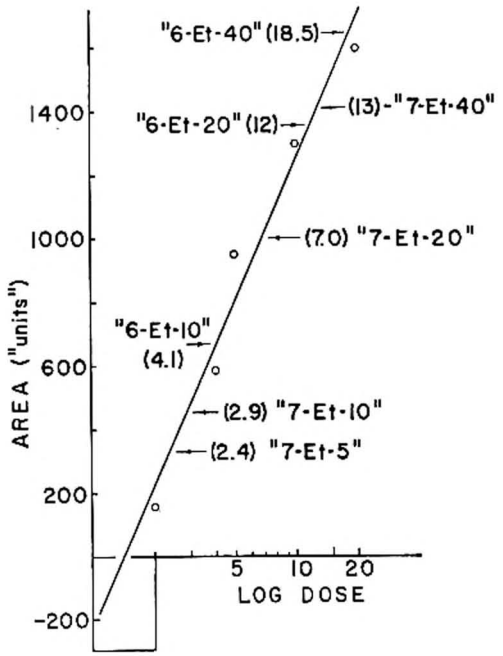

Fig. 5 Growth of rats on the flavins; area vs. log dose. Equation for the riboflavin growth response, $Y = (1462 \pm 149) \log X - (224 \pm 126)$. The notation "6-Et-10"(4.1) → is located at the point corresponding to the area under the curve for the growth for the animals on this quantity of the flavin. The (4.1) indicates that this point is equivalent to 4.1 μg of riboflavin. The activity for the 6-Et-7-Me-flavin at this test point, 4.1/10 × 100 = 41%. The values for the 6-Et-10, -20 and -40 are 41, 60, 46% for an average value of 49%. Similarly for 7-Et-5, -10, -20 and -40 the values are 48, 29, 35 and 33% for an average value of 36%. Flavin supplements given by stomach tube.

homologs can be found in terms of riboflavin directly from the graph. This analysis indicates that the 6-ethyl-7-methyl-flavin and the 6-methyl-7-ethyl-flavin have 49 and 36%, respectively, of the activity of riboflavin.

The other three methods are given in brief form to support the above analyses. In one case the average growth curve for each group was plotted so as to be enclosed by auxiliary curves determined by the standard deviations for each point. One might view such a plot as a growth pattern. A growth pattern for any one of the groups receiving a homolog was placed between the growth patterns of the two nearest standard riboflavin groups. An estimate was then made as to the general growth of the experimental group in terms of riboflavin. By these means the 6-ethyl-7-methyl-flavin and the 6-methyl-7-ethyl-flavin have 45 and 35%, respectively, of the activity of riboflavin. In another case the activity for any experimental group, in terms of area under the growth curve, was determined by interpolation between the nearest two riboflavin group values. This gave values for the 6-ethyl-7-methyl-flavin and 6-methyl-7-ethyl-flavin of 52 and 38%, respectively, of the activity of riboflavin. The last method involved the comparison of the increase in growth (in terms of area) between two adjacent quantities of one of the homologs with the increase in growth between the two quantities for riboflavin producing the most nearly identical increase in the same growth range. This gave values for the 6-ethyl-7-methyl-flavin and 6-methyl-7-ethyl-flavin of 46 and 35%, respectively, of the activity of riboflavin.

The average values based on all these methods of evaluation are 47% for the 6-ethyl-7-methyl-flavin and 36% for the 6-methyl-7-ethyl-flavin. At one time or another, all of these procedures have been used to evaluate growth data. We can find no reason to choose among them; the most subjective method (the third) and the most objective method (the last) give almost identical results and both are very close to the average values.

Flavin equivalence for food utilization. The results are reported in graphic form in figure 3. Growth curves A show the response of pair-fed animals to the same diet supplemented with 5 μg of riboflavin per gm or 13.9 μg of 6-methyl-7-ethyl-flavin per gm. The average grams gained by the riboflavin group was 110 ± 3[10] gm with a food utilization value of 0.431 ± 0.009 gm of weight gained per gm of food consumed. The average grams gained by the 6-methyl-7-ethyl-flavin group was 107 ± 2 gm with a food utilization value of 0.420 ± 0.009 gm of weight gained per gm of food consumed. Growth curves B show the response of pair-fed animals to the same diet supplemented with 5 μg of riboflavin per gm or 10.6 μg of 6-ethyl-7-methyl-flavin per gm. The average grams

[10] The ± values are standard errors.

gained by the riboflavin group was 110 ± 3 gm with a food utilization value of 0.437 ± 0.005 gm of weight gained per gm of food consumed. The average grams gained by the 6-ethyl-7-methyl-flavin group was 106 ± 2 gm with a food utilization value of 0.420 ± 0.003 gm of weight gained per gm of food consumed.

We are able to use this information to discover the quantity of each of the homologs which should have been used to produce the same growth as the pair fed riboflavin controls.

$$\frac{\frac{\mu g\ 7\text{-Et/gm food}}{\mu g\ Rb\ /gm\ food}}{\frac{gm\ gain\ 7\text{-Et diet}}{gm\ gain\ Rb\ diet}} = \frac{X}{1.00}$$

$$\frac{\frac{13.9}{5}}{\frac{107}{110}} = \frac{X}{1.00}\ ;\ \frac{2.78}{0.972} = \frac{X}{1.00}\ ;\ X = 2.86.$$

The correct quantity of 6-methyl-7-ethyl-flavin should have been 5 × 2.86 or 14.3 μg. Similarly, for the 6-ethyl-7-methyl-flavin:

$$\frac{\frac{10.6}{5}}{\frac{106}{110}} = \frac{X}{1.00}\ ;\ \frac{2.12}{0.964} = \frac{X}{1.00}\ ;\ X = 2.20.$$

The correct quantity of 6-ethyl-7-methyl-flavin should have been 11.0 μg.

These quantities, 14.3 and 11.0 μg, are in excellent agreement with the theoretically equivalent amounts, based on the differences in molecular weights,[11] of 14.5 μg (13.9 × 1.04) and 11.0 μg (10.6 × 1.04) for the 6-methyl-7-ethyl-flavin and the 6-ethyl-7-methyl-flavin, respectively.

The values for the food utilization can be used in this way to arrive at the same answers since the pair-fed animals consumed the same amount of food.

The differences in the final weights of the animals were small, the largest being 4 gm in the case of the 6-ethyl-7-methyl-flavin vs. riboflavin. A difference of 4 gm over a 28-day growth period may seem insignificant; however, the paired-feeding technique used in this way is sufficiently sensitive to reveal that a real difference exists between these groups ($P = 0.009$).

The results plotted in curves D show that the average grams gained by the riboflavin group, 6-methyl-7-ethyl-flavin group and the 6-ethyl-7-methyl-flavin group were 87 ± 2, 84 ± 1 and 83 ± 2 gm, respectively. The values for the food utilization given in the same order were 0.385 ± 0.011, 0.372 ± 0.008 and 0.366 ± 0.011 gm gained per gm of food consumed. When the quantities of the homologs which would have produced equivalence is calculated from these data, they were found to be 14.4 and 11.1 μg for the 6-methyl-7-ethyl-flavin and the 6-ethyl-7-methyl-flavin when based on either the weights gained or the grams gained per gram of food consumed.

The next question which might be said to follow from the above was whether the quantities of one of the flavins available to an animal would influence his utilization of food. In this study 6 pairs of animals were pair-fed the same diet except that one animal of each pair received 1.1 μg of the 6-ethyl-7-methyl-flavin per gm of food and the other received 9.9 μg per gm of food.[12] At the end of the experimental period of 23 days the group receiving the larger quantity of flavin showed an average of 9 ± 1.0 gm greater increase in weight ($P > 0.000001$). The food utilization for the group receiving the larger amount of flavin was 0.315 ± 0.020 gm gained per gm of food consumed while that for the group receiving the smaller amount of flavin was 0.237 ± 0.022 ($P > 0.000001$).

DISCUSSION

In the simplest possible terms we can summarize the current state of our knowledge of the biochemical function of riboflavin by stating that it constitutes part of the coenzyme or prosthetic group of a number of flavoprotein enzymes. While riboflavin may exist in some other forms under certain circumstances (Huennekens et al., '52), only two forms, flavin mononucleotide (FMN) or riboflavin phosphate (RP) and flavin adenine dinucleotide (FAD) are known to serve as coenzymes. Free riboflavin which has no known coenzyme function, is generally found in

[11] See footnote 6.

[12] This study was part of a Second Year Medical Student Project undertaken by Janet C. Register and E. Roger Newman.

small amounts in the presence of these materials.

These two coenzymes appear in all living cells under natural conditions and it seems unlikely that *any form* of life can exist if they are absent. It is possible, however, and there is no evidence to exclude the possibility, that in certain living cells, only one of these may be a functional oxidative coenzyme. The other may serve as a precursor of the active form or an inactive or stored form of the functional unit. While the two forms are invariably present in all cells studied for this information, we have insufficient evidence to indicate that they are both indispensable to the metabolism of all cells.

If some material other than riboflavin can be shown to be utilized by such riboflavin-requiring micro-organisms or mammals in the place of the vitamin, it follows that the substance must serve as the structural basis for the coenzyme units F'MN and F'AD.[13] The adequacy of such a substance for the metabolism and multiplication of micro-organisms such as we have shown in this case for *L. casei*, for example, through several subculturing steps, can be viewed in no other way. Lyxoflavin (Huennekens et al., '57), diethyl riboflavin and 6-chloro-7-methyl-flavin (Scala and Lambooy, '58) have been shown to constitute the structural basis of the coenzymes utilized by this organism. In a similar way we have found that the 6-ethyl-7-methyl-flavin also maintains its structural identity in the formation of FMN-like and FAD-like coenzymes when it is the sole flavin available to *L. casei*.[14] This is also, obviously, the case with respect to the few other flavins which are able to support metabolism and multiplication of such micro-organisms even though the existence of the nucleotides has not been demonstrated.

In the case of mammals, however, the situation is quite different. Normal weanling animals are made riboflavin-deficient by withholding riboflavin from their diet. When they become riboflavin-deficient their tissues still contain a considerable amount of riboflavin. The fact that these tissues do not show a higher degree of depletion might, conceivably, be related to the well known *in vitro* observation that in general, the flavoproteins are not readily or extensively dissociated. There is, then, a considerable pool of riboflavin which is obviously inadequate for the animals' needs. This same quantity of riboflavin might appear to have become adequate if it could be redistributed; some of it going to critical apoenzymes requirements of which must be extremely low and highly specific. The quantity of the vitamin present in the tissues of a young, riboflavin-deficient animal may be of the order of 2.5 to 2.8 μg per gm. A 30-gm weanling animal may weigh 45 gm by the time it is deficient and, with an average body content of 2.8 μg per gm, the animal might have in his tissues approximately 126 μg of riboflavin. When animals in this condition are fed diets containing either the 6-ethyl-7-methyl-flavin or the 6-methyl-7-ethyl-flavin the result is what appears to be normal growth into adulthood and normal survival.

This is not the same sequence of events that we observed when the deficient animals were given comparable amounts of diethyl riboflavin or 6-chloro-7-methyl-flavin. In these latter two cases the growth is not sustained because the animals die. We have suggested that small quantities of diethyl riboflavin and 6-chloro-7-methyl-flavin stimulate growth of the riboflavin-deficient rat by actually functioning as the coenzyme units of some of the flavoproteins.

We further postulated that some other apo-(flavo)-enzymes may not function with these abnormal coenzymes. They must, however, become functional by some means when these materials (e.g., diethyl riboflavin) are given or the animal would not grow nor would he recover from the signs of his apparent state of riboflavin deficiency. We feel that a portion of the 126 μg of riboflavin retained in the tissues of a riboflavin-deficient rat is liberated by "displacement" by these flavins (diethyl riboflavin and 6-chloro-7-methyl-flavin). The displaced riboflavin is now free to be used by those apo-(flavo)-enzymes which can not function with the abnormal coen-

[13] F-prime is used to indicate that at least the flavin portion must be different.

[14] This study was part of a Second Year Medical Student Project undertaken by Arnold Werner.

zymes. The form in which the riboflavin is released is immaterial; the enzymes required for its interconversion into appropriate forms are present. Perhaps these latter enzymes are the ones which enter into an ultimate inhibitory response to these "displacing" flavins.

This is apparently not the case with respect to 6-ethyl-7-methyl-flavin and 6-methyl-7-ethyl-flavin. If these two flavins function by displacement as described above the utilization of the reclaimed riboflavin must be remarkably efficient. If the growth and survival of these animals is due to the utilization of riboflavin by one or more very critical enzymes, we must admit that the small total amount of riboflavin available (from displacement, from intestinal bacterial synthesis, from the very small quantity in the food) is not excluded from these sites by the overwhelming quantity of the flavin which is being given. When either of these flavins is fed in quantities of 500 μg per day for 28 days, the growth is better than when the quantity is 40 μg per day. When mixed in the diet, 41.6 μg per gm of food is harmless up to 10 months. We have seen no signs that either of these materials is inhibitory. Our current interpretation of the evidence eliminates "displacement" as the mechanism of action of these two homologs.

The evidence presented supports the conclusion that the 6-ethyl-7-methyl-flavin and the 6-methyl-7-ethyl-flavin are fully able to replace riboflavin for the growth and survival requirements of the rat. This would imply that these requirements are satisfied by either of these flavins as F'MN or F'AD. If this is true we are at a loss to account for the failure of rats given these flavins to reproduce (Lambooy, '58b), unless we postulate the existance of either a unique coenzyme or apoenzyme required for reproduction which is not present or critical during the manifestations of growth and survival.

We are unwilling at this time to postulate the existance of an enzyme which is critical during reproduction but not critical during growth and survival. We are forced to conclude, therefore, that 6-ethyl-7-methyl-flavin and 6-methyl-7-ethyl-flavin are not able to completely replace riboflavin in the nutrition of the rat.

It seems unlikely that a compound can resemble a vitamin in physical, chemical and biological properties more closely than these two flavins resemble riboflavin, and yet they are unable to replace riboflavin in the nutrition of a mammal. One might raise the question whether a compound will ever be designed and synthesized which will fully replace a vitamin in its biochemical functions in a mammal. If such a compound is produced it seems likely that it will ultimately be found to already exist in some form of life and that it is serving this purpose in some naturally occurring system.

SUMMARY

The flavins 6-ethyl-7-methyl- and 6-methyl-7-ethyl-9-(1'-D-ribityl)isoalloxazine have been found to be indistinguishable from riboflavin in the nutrition of *Lactobacillus casei* when either is the only flavin available to this micro-organism throughout limiting concentration ranges.

These two flavins are also able to satisfy the requirement for a flavin in the growth and survival of the rat. The 6-ethyl-7-methyl-flavin and the 6-methyl-7-ethyl-flavin have 47 and 36%, respectively, of the activity of riboflavin for growth of this animal.

These two flavins can not be considered as completely adequate replacements for riboflavin in the nutrition of the rat, however, since they are unable to satisfy the need for a flavin in the over-all function of reproduction in this animal.

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Influence of Feeding Various Levels of Velvet Beans to Chicks and Laying Hens[1]

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Velvet beans (*Stizolobium* species) are grown as a soil-improving crop in the southern states and often used as a livestock feed. Tempelton et al. ('17) reported that velvet beans were relished by steers, but were unpalatable to dairy cows. These workers found that two-and-one-half pounds of velvet beans ground in the pod were nutritionally equal to one pound of good cottonseed meal when included in dairy cattle feed.

Satisfactory results have been reported with the use of velvet beans for fattening hogs. Tempelton et al. ('17) found that feed for fattening pigs could contain 50% of velvet beans, but results were not as good as those obtained when other sources of protein were used. Scott ('17) reported that satisfactory gains were obtained with pigs when the feed contained shelled corn, velvet beans in the pod, and Japanese cane. Later results (Tempelton, '20) indicated that for best performance the feed for pigs should not contain more than one part of velvet beans for each 4 parts of corn.

Sure and Read ('21) observed that raw velvet beans were injurious to young rats when constituting 40% of the total ration. They also reported that autoclaving the seed for one hour at 15 pounds pressure destroyed most of the toxic effect, making it possible to include 60% of autoclaved beans in the ration. Read ('23) confirmed the observation that autoclaving of the beans destroyed the toxic factor.

Salmon ('22) reported that velvet beans were toxic to pigeons. He found that on the second day of feeding, birds showed ruffled feathers and appeared sleepy. Mortality commenced on the 4th day, and survivors were in poor condition, having lost about 80 gm.

No data were found in the literature as to the effect of feeding velvet beans to chickens. Since the beans are grown in many southern states, it is possible that they might be unintentionally included in feeds; therefore, experiments were initiated to determine the effect of graded dietary levels fed to chickens.

EXPERIMENTAL PROCEDURE

Chick studies. The velvet beans used in these experiments were purchased from a local seed store, and ground into a coarse meal before incorporation into the experimental diets. The beans had not received any previous treatment.

Composition of the two basal diets used in these experiments is shown in table 1. The soybean meal basal diet contained no velvet beans and was fed to one group of chicks which served as the control in order to establish normal gains. The other basal diet contained 50% of velvet beans. The two basal diets were blended in appropriate amounts to give three other experimental diets containing 12.5, 25, and 37.5% of velvet beans. This resulted in 5 experimental diets.

The two basal diets were formulated to contain comparable levels of protein and productive energy. Since no productive energy value was available for velvet beans, an approximate value of 980 Cal. of productive energy per pound was used. This value was obtained by using "percentage multipliers" according to Titus ('55). All other calculations were based on values according to Titus ('55). In order to furnish an adequate supply of amino acids to support chick growth, 10%

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TABLE 1

Composition of basal diets (chick studies)

Ingredients	Velvet bean basal	Soybean meal basal
	pounds/100 pounds	
Velvet beans (23% protein)	50.0	
Soybean oil meal (50% protein)	—	17.0
Fish meal (60% protein)	10.0	10.0
Yellow corn	32.6	66.9
Stabilized animal fat	2.0	0.7
Dehydrated alfalfa meal (17% protein)	3.0	3.0
Ground limestone	0.8	0.8
Defluorinated phosphate[1]	0.3	0.3
Iodized salt	0.4	0.4
Vitamin premix[2]	0.9	0.9
Protein,[3] %	21.1	21.0
Productive energy, Cal./pound[3]	993.6	994.6

[1] Contains 17% P and 34% Ca.

[2] Supplied/pound of feed: (in milligrams) riboflavin, 2; Ca pantothenate, 9; niacin, 18; choline chloride, 261; oxytetracycline, 10; oleandomycin, 2; ethoxyquin, 57; iron, 9; copper, 0.9; iodine, 5; manganese sulphate, 80; manganous oxide, 35; methionine hydroxy analogue calcium, 318; glycine, 270; and (in micrograms) vitamin B_{12}, 10; zinc, 45; cobalt, 90; and also vitamin A, 2268 I.U. and vitamin D_3, 340 I.C.U.

[3] Calculated, based on values according to Titus ('55), except for velvet beans which were assumed to contain 980 Cal. of productive energy/pound, based on "percentage multipliers."

of fish meal was included in the basal diets. This also insured an adequate level of vitamin K.

Two experiments were conducted with replicated pens of day-old chicks fed each of the 5 diets. Experiments 1 and 2 were identical, except that each replicated pen contained 5 male and 5 female Vantress × White Rock chicks in experiment 1, and 10 Single Comb White Leghorn males in experiment 2. Experiment 1 was terminated after 4 weeks, and experiment 2 at the end of the second week. Chicks were wing-banded and randomly assigned to pens in thermostatically controlled, electrically heated battery brooders with raised wire floors. Experimental diets and tap water were supplied ad libitum.

Chicks were individually weighed at one, two and 4 weeks of age in experiment 1, and at one and two weeks of age in experiment 2. All birds that died were subjected to complete postmortem. Selected tissues for histopathological study were fixed in 10% neutral formalin, embedded in paraffin, cut at 6 μ, and stained with hematoxylin-eosin.

Statements of probability are based on the analysis of variance according to Snedecor ('56) with significant differences between treatment means determined by the method outlined by Duncan ('55).

Hen study. One experiment, involving 40 hens of commercial egg-production type, was conducted to determine the nutritional value of velvet beans for laying hens. The velvet beans used in this experiment were obtained and prepared in the same manner as those used in the chick studies.

The composition of the two basal diets used in this experiment is shown in table 2. The soybean meal basal diet was fed as a control in order to establish normal egg production rate for the experimental period. The other basal diet contained 50% of velvet beans. Because fish meal was not included in the basal diets, supplementary vitamin K was added. Three other diets were prepared by blending appropriate amounts of the two basal diets which resulted in diets containing 12.5, 25, and 37.5% of velvet beans, resulting in 5 experimental diets.

Replicated groups of 4 individually caged hens were fed each of the 5 diets. Hens were assigned to the groups on the basis of past egg production so that average production of all groups was approximately the same. Diets were fed for 25

TABLE 2

Composition of diets (hen study)

Ingredient	Velvet bean basal	Soybean meal basal
	pounds/100 pounds	
Yellow corn	31.8	65.5
Soybean meal (50% protein)	5.2	22.6
Dehydrated alfalfa meal (17% protein)	5.0	5.0
Velvet beans (23% protein)	50.0	—
Stabilized animal fat	1.2	—
Ground limestone	3.9	4.2
Defluorinated phosphate[1]	2.2	2.0
Iodized salt	0.4	0.4
Vitamin premix[2]	0.3	0.3
Protein, %	18.0	18.0
Productive energy, Cal./pound[3]	910.2	910.5

[1] Contains 34% Ca and 17% P.

[2] Supplied/pound of feed: (in milligrams) riboflavin, 2; Ca pantothenate, 4; niacin, 6; choline chloride, 227; menadione sodium bisulfite complex, 0.5; manganese sulphate, 80; also vitamin A, 2000 I. U.; vitamin B_{12}, 6 μg; vitamin D_3, 700 I.C.U.; and vitamin E, 2.5 I.U.

[3] Calculated, based on values according to Titus ('55), except for velvet beans which were assumed to contain 980 Cal. of productive energy/pound, based on "percentage multipliers."

TABLE 3

Body weight and percentage mortality of Vantress × White Rock chicks fed graded levels of velvet beans (exp. 1)

Velvet beans in diet	Body weight (gm)									Mortality
	1-week			2-week			4-weeks[1]			
	M	F	Av.	M	F	Av.	M	F	Av.	
%										%
—	102.4	105.3	103.8	194.5	195.7	195.1	312.3	312.2	312.2	0.0
12.5	78.5	81.7	80.1	155.7	150.5	153.1	249.0	246.3	247.6	0.0
25.0	75.0	81.8	78.4	139.2	139.9	139.5	230.6	224.7	227.6	0.0
37.5	61.6	65.9	63.7	101.9	112.8	107.3	169.4	177.4	173.4	0.0
50.0	47.1	47.2	47.1	76.0	67.6	71.8	123.6	110.0	116.8	25.0

[1] All treatments are significantly different according to Duncan's multiple range test ('55).

days. Daily egg production records were kept for individual hens. Rate of egg production was calculated on a hen-day basis for each 5-day period.

Significant differences in treatment means in rate of egg production for the 25-day experimental period were determined according to the procedure outlined by Duncan ('55).

RESULTS AND DISCUSSION

Chick studies. A depression in growth rate was noted with chicks fed diets containing all 4 levels of velvet beans used in these studies (tables 3 and 4). This depression in growth was detected at the end of the first week of feeding. Statistical analysis of body weights at 4 weeks of age in experiment 1, and at two weeks of age in experiment 2, indicated significant differences between all experimental treatments.

An increase in velvet bean content of the diet resulted in a greater depression of growth. This would indicate a greater content of the growth depressant in the diets containing the higher levels of velvet beans. A portion of this growth depression might be attributed to the poor amino acid content of the velvet beans. Since the basal diets contained 10% of fish meal, however, sufficient amino acids should have been present to support a greater rate of growth. Therefore, it is concluded that the poor results were attributed to an unknown toxic factor in the velvet beans.

TABLE 4

Body weight and percentage of mortality of White Leghorn cockerels fed graded levels of velvet beans (exp. 2)

Velvet beans in diet	Body weight		Mortality
	Week 1	Week 2[1]	
%	gm	gm	%
—	102.0	168.2	0.0
12.5	92.5	157.6	0.0
25.0	77.0	128.3	0.0
37.5	64.0	100.4	5.0
50.0	51.8	69.8	15.0

[1] All treatments are significantly different according to Duncan's multiple range test ('55).

These results agree with the observations of Sure and Read ('21) that velvet beans contain a toxic factor as indicated by rat growth, and with Salmon ('22) who found that these beans were not well tolerated when fed to pigeons.

It is possible that velvet beans could be rendered nontoxic for poultry by heat treating or autoclaving, as has been possible for rats (Sure and Read, '21; Read, '23). Since sufficient quantities of velvet beans are not grown for use in commercial feeds, this problem was not investigated.

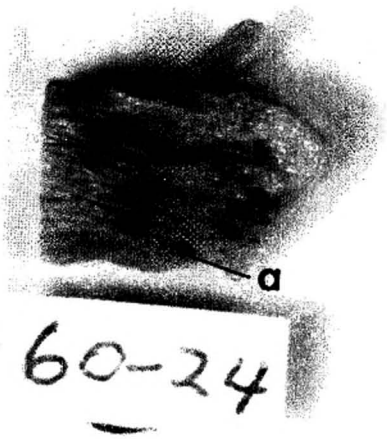

Fig. 1 Esophagus of chick that died following feeding of 50% of velvet bean meal. Note hemorrhages (a) on mucosal surface.

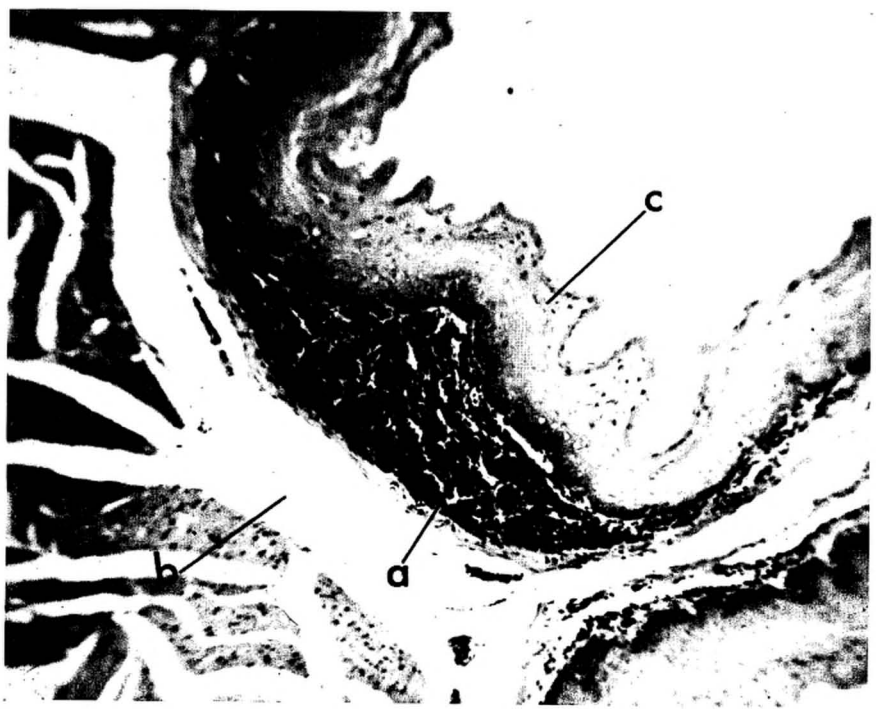

Fig. 2 Crop of chicken, showing hemorrhage (a) and edema (b) in the lamina propria. The surface stratified squamous epithelium is shown (c). H & E stain. × 80.

TABLE 5

Rate of egg production from hens fed various levels of velvet beans in the diet

Velvet beans in diet	Feeding period, days					Average[1]
	0–5	6–10	11–15	16–20	21–25	
%	% production[2]					
—	82.5	70.0	82.5	80.0	77.5	78.5
12.5	70.0	77.5	55.0	62.5	62.5	65.5
25.0	67.5	35.9	21.6	55.0	53.3	46.6
37.5	67.5	35.0	20.0	33.5	27.5	36.5
50.0	62.5	5.0	2.5	12.5	5.0	17.5

[1] All treatments are significantly different according to Duncan's multiple range test ('55).
[2] Calculated on a hen-day basis.

Feeding diets containing 50% of velvet beans caused 25 and 15% mortality in experiment 1 and 2 respectively (tables 3 and 4). Most of this mortality occurred during the first week.

Hemorrhage was the principal lesion found on autopsy. These were seen in the thigh musculature, and on the mucosal surface of the duodenum and esophagus (fig. 1). Histologically, there was edema and hemorrhage in the lamina propria (fig. 2).

Hen study. A reduction in rate of egg production resulted from feeding the levels of velvet beans used in this experiment (table 5). The rate of decrease in egg production was directly proportional to the amount of velvet beans contained in the diet. A gradual restitution in rate of egg production followed continued feeding of velvet beans, and resumption was slowest with hens receiving the diets containing the most beans. This would indicate the presence of a factor in velvet beans which interferes with the normal performance of laying hens, and that over a prolonged feeding period at low levels it may be possible for the hens to become partially adjusted to them. No mortality occurred during this laying study.

SUMMARY

Two chick experiments and one laying hen experiment were conducted to determine the feeding value of velvet beans for poultry. Results from these tests indicated that velvet beans are not a satisfactory feedstuff for use in poultry feeds. Feeding of diets containing velvet beans at levels used in these studies decreased performance of chicks as measured by mortality and reduced body gains, and decreased performance of laying hens as measured by rate of lay.

There appeared to be some factor(s) in velvet beans that interferes with the normal processes of growth and egg production in chickens.

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Proceedings of the Twenty-Fifth Annual Meeting of the American Institute of Nutrition

AMBASSADOR HOTEL, ATLANTIC CITY, NEW JERSEY
APRIL 10–14, 1961

COUNCIL MEETINGS

The American Institute of Nutrition Council met on Saturday evening, April 8, and on Sunday morning and evening, April 9. Formal actions of the Council were reported at the Institute business meetings and are included in the following minutes.

SCIENTIFIC SESSIONS

A full 5 days of scientific sessions on nutrition and related sciences were held as part of the annual meetings of the Federation of American Societies for Experimental Biology. A total of 209 abstracts was submitted to the Institute for inclusion on the scientific program. Of these, 27 were transferred to the programs of other societies and intersociety sessions. Forty-six abstracts were received by transfer from other societies, of which 29 were included in the intersociety sessions on atherosclerosis which were sponsored by the AIN. A nutrition program of 15 half-day sessions was arranged and published in the March issue of *Federation Proceedings*, part 2. In addition, 5 half-day intersociety sessions on atherosclerosis were held. Of special interest were the two half-day symposia on vitamin K and selenium. All abstracts were published in the March 1961 issue of *Federation Proceedings*. The symposia papers will be published in future issues of *Federation Proceedings*.

BUSINESS MEETINGS

Business meetings were held on Monday, April 10, and Thursday, April 13, attended by approximately 240 and 150, respectively. Dr. Floyd S. Daft, president, presided at both meetings.

I. Minutes of 1960

The minutes of the 1960 meeting, as published in *The Journal of Nutrition*, 72: 473, December, 1960, were approved.

II. Election

The Secretary transmitted the sealed ballots to the Teller's Committee, Dr. Bacon Chow, chairman, Dr. D. V. Frost, Dr. J. G. Bieri, Dr. J. H. Williams and Dr. Carl Douglas. At the second business meeting the Committee reported the election results from 419 ballots received, as follows:

Effective July 1, 1961:
President: Paul György
President-Elect: L. C. Norris
Councilor (three-year term): Willard A. Krehl
Effective May 1, 1961:
Editorial Board, JOURNAL OF NUTRITION (4-year term):
George K. Davis
A. E. Axelrod
Joseph T. Anderson
Richard H. Follis, Jr.

The names of individuals with the 10 highest number of votes for suggested members of the Nominating Committee were submitted to the President. (See complete list of officers and committees at the end of these proceedings.)

III. Constitutional Amendments

By over two-thirds of all votes cast, the following amendments to the Constitution and By-Laws were adopted by mail vote (please refer to the September issue of *Federation Proceedings, 19:* 722, 1960, for the former wording):

A. By a vote of 358 for, to 12 against, Article VI has been changed to read as follows:

Article VI. Financial

Section 1. Dues. The dues shall be an annual assessment which shall be determined by majority vote at the annual meetings, upon recommendation of the Council, plus the annual cost of a member subscription to one journal, as follows: A. Division members, when required to subscribe to the official journal of their division, shall not be obligated to subscribe to the official journal of the American Institute of Nutrition. B. All other members shall subscribe to the official journal of the Institute. Dues shall be

paid within two months after the annual meeting. All members have the privilege of subscribing to any official journal of the Institute or its division(s) at members' rates. A member on attaining the age of 65 may elect to be relieved from all the above financial obligations.

B. Article VII has been changed by a vote of 375 for, to 17 against. Dr. Daft reported that the Council suggested that the proposed change in Section 2, which would have permitted the appointment of the Editor-in-Chief by the Publications Committee, be ruled out of order on technical grounds since the old wording did not appear in the ballot. There being no objections from the floor, the President ruled that the Council's action be adopted. This article, as changed, reads as follows:

Article VII. Publications

Section 1. The Council of the American Institute of Nutrition shall designate the official organ(s) of publication of the Institute.

Section 2. Publications Committee. The management of the official publications of the Institute shall be under the supervision of a Publications Committee. This committee shall be appointed by the President of the American Institute of Nutrition with approval of the Council. It shall advise the Council on matters of publication management, designation of the official journal(s) of the Institute and shall negotiate for the approval of the Council agreement with publishers of the official journal(s).

Section 3. The Official Journal(s) of the Institute. A. The editorial responsibility for the official journal(s) of the Institute shall be vested in an Editorial Board of 16 members and the Editor-in-Chief. B. The Editor shall be chosen by the Editorial Board to serve a term of 5 years beginning July 1 of the year in which he is chosen, and shall be eligible for re-election. The Editor shall have the power to appoint an assistant and such an appointee shall be called Associate Editor. C. Four members of the Institute shall be nominated by the Nominating Committee for membership on the Editorial Board each year to serve a term of 4 years and to take office May 1 of the year in which they are elected. D. Retiring members of the Editorial Board shall not be eligible for renomination until one year after their retirement.

C. By a vote of 370 for, to 7 against, Article IX has been changed to read as follows:

Section 4. Members of divisions of the American Institute of Nutrition will be assessed the basic annual assessment of the American Institute of Nutrition, the assessment of the Federation, divisional dues as determined by the individual divisions and subscription to one official publication (of the American Institute of Nutrition or one of its divisions. See Article VI, Section 1). In addition, each member may subscribe at members' rates to any other publication(s) of the American Institute of Nutrition.

(NOTE: The Constitution and By-Laws of the Institute are printed each year in the September issue of *Federation Proceedings*.)

IV. Membership Status

The Secretary reported that as of April 1, 1961, there were 747 members in the Institute, 686 active, 51 retired and 10 honorary members. This is a net increase of 179 members since last year. Two members resigned during the year. The Clinical Division reported a total membership of 69.

Members present at the business meeting stood for a moment of silence in memory and in recognition of the following 6 members of the Institute who had passed away since April 1, 1960:

Norman R. Blatherwick, January 13, 1961
Edward C. Bubl, June 28, 1960
Joseph S. Butts, April 9, 1961
*Albert G. Hogan, January 25, 1961
William H. Peterson, July, 1960
*Barnett Sure, June 3, 1960

* Charter members of the Institute.

Appropriate resolutions which had been received for deceased members were read and approved. Resolutions were read in honor of charter member, Dr. Barnett Sure, and past president and charter member, Dr. Albert G. Hogan, and for Dr. Joseph S. Butts, the notice of whose untimely death arrived at about the time of the business meeting. Resolutions for charter members are included in the minutes as per custom.

RESOLVED, That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, in its Annual Meeting, April 13, 1961, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of its distinguished member, Barnett Sure.

Upon receiving the doctorate at the University of Wisconsin in 1924, Dr. Sure, or "Barney," as he was known from university days, went to the Department of Agricultural Chemistry at the University of Arkansas, where he spent his entire professional career, retiring in 1957. Dr. Sure was a zealous and dedicated investigator, meticulous and thorough in his work. During his early studies upon reproduction in the rat, he obtained evidence for the existence of a new factor, which he called vitamin X. He was thus a co-discoverer with Evans and Burr of the nutritional role of the tocopherols in reproduction. With his usual painstaking approach, he made and published a long series of studies on the requirements of the rat in reproduction and lactation. A similar extended series of studies showed

the interrelationship between certain hormones and vitamins. Protein and amino acid nutrition also fascinated him. Sensing the importance of low-cost protein foods for many people, he instituted a comprehensive evaluation of the cereal grains and other seeds as sources of protein. During these investigations, he made an exhaustive study of the nutritional value of rice—one of the major crops of his adopted state, Arkansas. During the depression he developed a low-cost, high-quality protein food consisting largely of grains and nuts. With the passing of Barnett Sure, the American Institute of Nutrition has lost one of the pioneer nutritionists of America.

RESOLVED, That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, in its Annual Meeting, April 13, 1961, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of its distinguished member, Albert G. Hogan.

The death of Dr. Albert G. Hogan on January 25, 1961, terminated a long and distinguished career in research, teaching, and administration in nutrition and biochemistry. From 1920 to his retirement in 1955 he was Professor of Animal Nutrition at the University of Missouri and, for the last 32 years of that period, he was chairman of the University's Department of Agricultural Chemistry. He made many important research contributions relating to basic and applied animal nutrition. His research and his personal life were a challenge and inspiration to his staff and to the many graduate students who received their training under his guidance. He became Professor Emeritus of Animal Nutrition in 1955 and continued an active interest in nutrition research at the University and as Research Consultant for the National Live Stock and Meat Board. During the month of November, immediately preceding his terminal illness, he traveled extensively in behalf of the Board's program. Dr. Hogan had received numerous awards for his research achievements. Among these were the Morrison Award from the Society of Animal Production, and the Mead Johnson Award, the Borden Award, and the Osborne-Mendel Award from the Institute of Nutrition. He was a charter member of the Institute and served as a member of the Editorial Board of the Journal 1937–42, as Vice President of the Institute in 1940–41 and President in 1941–42. He was elected a Fellow in 1959 and was a member of the Fellows Committee at the time of his death. It is appropriate, therefore, that we the members of the American Institute of Nutrition, in business meeting assembled on this the 13th day of April, 1961, should express our appreciation in the form of this resolution as a tribute to the enduring contributions of Albert G. Hogan.

V. *New Members*

The Council received 75 nominations for active membership, of which 73 were unanimously approved by members at the business meeting. All of these have accepted membership in the Institute. They are as follows:

NEW MEMBERS—1961*

†Arthur F. Abt
Lucile F. Adamson
George H. Arscott
C. Willet Asling
Robert Leon Atkinson
Stanley L. Balloun
Maurice Bender
George Berryman
Orson D. Bird
Robert B. Bradfield
Myrtle L. Brown
Richard E. Brown
William K. Calhoun
Lowell William Charkey
Bessie B. Cook
Fred E. Deatherage
Arnold E. Denton
John P. Feaster
Marina Flores
Henry L. Fuller
Karl E. Gardner
†Ira Gore
Andrew W. Halverson
Kenneth E. Harshbarger
Elmer W. Hartsook
†James Iacono
Ronald R. Johnson
Kenneth W. Keane
Barbara Kennedy
George O. Kohler
Stanislaw Kazimierz Kon
Harriott O. Kunkel
Allen A. Kurnick
John P. Lambooy
Fred Lester Losee
Robert W. Mason
J. K. Matsushima
†William Hyde Meroney
Jack Metcoff
John Edgar Monagle
Kenneth Morgareidge
Malden Charles Nesheim
Paul Medford Newberne
Gordon W. Newell
Lawrence E. Ousterhout
Tilden Wayne Perry
Wilson G. Pond
John Mack Prescott
Rodney LeRoy Preston
Walter J. Pudelkiewicz
Orrea Florence Pye
Miloslav Rechcigl, Jr.
Ulma D. Register
Bobby L. Reid
Ruth Renner
†Milton Emanuel Rubini
Frances A. Schofield
†Joseph Seitchik
Herschel Sidransky
John T. Sime
Herbert Sprince
Thomas W. Sullivan
William C. Supplee
†Stanley A. Tauber
John William Thomas
Stewart G. Tuttle
Erma S. Vanderzant
Alva B. Watts
C. Edith Weir
Oliver H. M. Wilder
Walter S. Wilkinson
Dorothy E. Williams
Daisy Yen Wu

* For institutional affiliations and addresses of new members see the September issue of *Federation Proceedings*.
† Elected to membership in the Clinical Division.

VI. *Treasurer's Report*

A report by the Treasurer, J. B. Allison, from April 9, 1960, to April 1, 1961, was read and approved. The Auditing Committee, Walther H. Ott and Harry W. Titus, submitted a report that the treasurer's accounts were correct and complete. The report was approved.

Dues. The President, Dr. Daft, reported on the recommendation of the Council regarding dues for the coming year. The Council of the Federation of American Societies for Experimental Biology, in anticipation of an additional building at their headquarters located at Beaumont House, passed a resolution recommending an increase in the individual membership assessment to the Federation from $4.00 to $6.00 for the year. The AIN Council recommended that the previous assessment

Balance brought forward			
Cash		$3,220.53	
U. S. Series K Bond		500.00	$ 3,720.53
Receipts			
Institute dues from 688 members at $2.00		$1,376.00	
Federation dues, 687 at $4.00		2,748.00	
Subscriptions to *Journal of Nutrition*, 659 at $8.50	$5,601.50		
2 partial subscriptions	8.30	5,609.80	
Assessments for Fifth International Congress on Nutrition, 684 at $2.00		1,368.00	
Contributions to Fifth International Congress on Nutrition: Individual	$ 298.50		
Hershey Chocolate Corporation	500.00	798.50	
Interest on U. S. Series K Bond		13.80	
Wistar Institute, for editorial office		9,000.00	
Federation, share of 1960 Annual Meeting registration fees		966.00	
Contributions to Symposium on Selenium Toxicity:			
Abbott Laboratories	$ 500.00		
Eli Lilly and Company	500.00		
The Nutrition Foundation, Inc.	250.00	1,250.00	
Bank premiums on foreign checks		1.20	23,131.30
Total receipts and balance brought forward			$26,851.83
Expenditures			
Federation office:			
Dues	$2,804.00		
Mailing of March, 1960	21.41		
Mailing of manuscripts	32.04		
Addressing dues notices	11.88	$2,869.33	
Wistar Institute, subscriptions to *Journal*		5,635.50	
Fifth International Congress on Nutrition		2,166.50	
Secretary's office:			
Expenses	$ 350.00		
Complete set of Murlin *Journals* for permanent record	179.85	529.85	
Treasurer's Office: Secretary		100.00	
Editor's office, Cornell University		9,000.00	
American Society of Biological Chemists, one-half of smoker, 1960		60.70	
Expenses of Council Meeting in Chicago, 1960		11.10	
Reimbursement of contribution to International Congress by foreign member		6.00	
New Zealand Government Tourist Bureau, for plane fare for Dr. W. J. Hartley		1,210.00	
Bank charges		1.38	
Total expenditures			$21,590.36
Balance on hand, April 1, 1961			
Cash		$4,761.47	
U. S. Series K Bond		500.00	
TOTAL BALANCE			$ 5,261.47

of dues for the AIN of $4.00 be reduced to $2.00 (previous assessment included $2.00 support of the Fifth International Congress on Nutrition). Thus, the current assessment to both Federation and the AIN remains at $8.00 per year. For those members receiving *The Journal of Nutrition* (members of the Clinical Division are required to take the *American Journal of Clinical Nutrition* with the option of also subscribing to *The Journal of Nutrition*) the Council recommended increasing membership subscriptions to $10.00 (see report by the Ad Hoc Committee on Jour-

nal Management). A motion for approval of the increased subscription rate for *The Journal of Nutrition* to $10.00 per year was seconded and passed.

VII. Editor's Report

The Editor of *The Journal of Nutrition*, Dr. Richard H. Barnes, submitted his report covering January 1, 1960, to December 31, 1960. It was approved and is summarized below.

Dr. Barnes reported that the total cost of operation of the editorial office from July 1, 1959, to March 15, 1961, was $14,212.27 and projected expenses covering through June 30, 1961, would include a total expenditure of $17,869.38. Estimated additional office expense for the period of July 1, 1961, to June 30, 1962, was $9,689.88. In an agreement negotiated with The Wistar Institute, expenses of the editorial office have been reimbursed by The Wistar Institute at a rate of $9,000 per year. The forthcoming fiscal year they have agreed to reimburse the editor, if necessary, with the increased budgetary cost up to $9,689.88, applying the unused portion of the first two years budgetary support.

Editing and Publication Operations:

	1960	1959
Volumes of *Journal of Nutrition*	70,71,72	67,68,69
Pages published (new format)	1,486	
Papers published (not including 3 biographical sketches)	205	171
Papers rejected	29	34
Pages per paper (new format)	7	
Supplements	—	—
Biographies: Samuel Brody, Alfred Fabian Hess, and Michel Eugene Chevreul		
Average time lapse from receipt of manuscript to publication date (including three months at Wistar)	5.4 months	

VIII. Report of Committees and Representatives

A. *Ad Hoc Committee on The Journal of Nutrition Management:* W. J. Darby, chairman, P. György, O. L. Kline, H. A. Schneider.

As a result of numerous meetings with officials of The Wistar Institute, the following agreements were reached:

1. The Wistar Institute agrees to a one-year extension of the present working relationship between the Wistar Press and the American Institute of Nutrition for the publication and editing of *The Journal of Nutrition* covering the period July 1, 1961, to June 30, 1962.

2. The Wistar Institute agrees to publish all issues and supplements within three months from the time manuscripts are received from the editorial office and accept all manuscripts submitted by the editorial office of the *Journal*, estimating that this may amount to an approximate increase of 15 per cent in current publication space.

3. The Wistar Institute will make payment to the editorial office of the *Journal* at the present rate and schedule of payments, but in addition will underwrite the total cost of the editorial office up to the level estimated by the Editor, Dr. Barnes, namely, $9,689.88, for the period July 1, 1961, to June 30, 1962. The Wistar Institute agrees that any savings accruing out of the first two years' budget provided to the editorial office may be carried into the third year, but that any difference between the scheduled payment of the $9,000 plus the savings will be made up by additional payments up to a maximum sum of $9,689.88.

4. The Wistar Institute agrees to make available to all members of the American Institute of Nutrition who are required to subscribe to *The Journal of Nutrition* and to those members of the American Society of Clinical Nutrition who have the option to subscribe to *The Journal of Nutrition*, the journals at the members' rate of $10.00 per year for the period of September 1, 1961, to August 31, 1962.

5. Within approximately 6 months The Wistar Institute agrees to meet with the Publications Management Committee of the American Institute of Nutrition for negotiations beyond the period of June 30, 1962. Prior to this meeting, the Publications Management Committee and the Council are to receive from The Wistar Institute the accountant's audit of *The Journal of Nutrition's* cost.

This report was unanimously approved by the Council and by the Society membership. A motion was made and seconded

to accept the report by Dr. Darby and to commend the Ad Hoc group for their guidance and efforts in negotiations regarding the official journal of the American Institute of Nutrition. The motion was unanimously approved.

B. *Representatives to the AAAS Council:* Paul L. Day and E. L. Hove.

These representatives reported that current membership of the AAAS is over 62,000 and that the Council of the AAAS is composed of the Association's officers, representatives from the 18 sections of the Association and representatives from 294 affiliated societies. The American Institute of Nutrition has representatives to Section C, Chemistry, and to Section N, Medical Sciences. Of the total AIN membership, 413 members, or 58 per cent, also belonged to the AAAS in 1960.

C. *Representative to the Division of Biology and Agriculture of the National Research Council:* N. R. Ellis.

Dr. Ellis reported the following:

The proceedings of the conference on "Beef for Tomorrow," held in the fall of 1959 under the auspices of the Conference Committee of the Agricultural Research Institute were published during 1960 as NAS-NRC Publication no. 751. The Committee on Agricultural Pests of the Agricultural Board is developing plans for a symposium on factors determining the behavior of plant pathogens in soil. Another committee is considering the promotion of research on aerial photography as a means of assessing damage to crops caused by plant pests. This is an outgrowth of an earlier resumé on "Losses Due to Agricultural Pests."

The Committee on Animal Health has published a review of "Animal Health Research Needs" with emphasis on losses from pests and diseases in livestock production. Publication no. 824 on the "Fluorosis Problem in Livestock Production" appeared during the year. The Committee on Animal Nutrition and its subcommittees were active in continuing reviews of new information and revisions of publications on nutrient requirements. The revision on poultry has been published and a new one on Nutrient Requirements of Laboratory Animals is due this summer.

Dr. LeRoy Voris now devotes full time to the Food and Nutrition Board, his place as Secretary of the Agricultural Board being taken by Mr. R. E. Krauss.

The Food and Nutrition Board prepared a statement in regard to tolerances for pesticide residues in milk. The statement sets forth views on zero tolerances and bases for scientifically justified regulations. The Food Protection Committee was especially active and has issued several reports dealing with toxicity, food additives and chemical contaminants.

Attention should be called to the prominent role that the Division of Biology and Agriculture plays in broad aspects of science on the national as well as the international scene. There is every evidence that this role of interpreter and advisor will increase due to current world conditions.

D. *Public Information Committee:* Philip L. White, chairman, R. W. Engel, S. S. Negus, L. Voris, A. E. Schaefer (ex officio).

Dr. Engel reported in Dr. White's absence that approximately 5,000 copies of the leaflet "Career Opportunities in Nutrition" were distributed during the year in response to individual requests. The Committee received and responded to many hundreds of letters from the public. Most of the letters requested information about foods and nutrition. Considerable Committee time was required to respond to these letters.

The Committee received excellent cooperation from the members submitting papers for presentation at the annual meeting with regard to possible news releases. The necessity for individual cooperation in public information is obvious and the Committee recommended that the membership continue and extend their loyal support in the project to interpret scientific findings in the field of nutrition into newsworthy articles.

E. *U. S. National Committee of the International Union of Nutritional Sciences (IUNS):* Paul György.

Dr. Paul György reported on the activities of the IUNS during the past year. One of the functions of the IUNS was to assist in sponsoring the Fifth International Congress on Nutrition. (See report

by Dr. C. G. King on the Fifth International Congress on Nutrition.) Dr. György reported that as a result of the funds collected to support the Fifth International Congress on Nutrition, there was approximately $25,000 remaining. Upon recommendation of the organizing committee of the Fifth International Congress on Nutrition, with approval of the U. S. National Committee of IUNS and the Council of AIN, the following disposition of the fund is being negotiated. The fund is to be transferred to the National Academy of Sciences—National Research Council, for use by the U. S. National Comittee of the International Union of Nutritional Sciences with the following provisions:

1. That this fund, designed "U.S.-IUNS," be placed in the National Academy of Sciences' "Investment Fund," sharing fully in both capital gains and dividend earnings; that no handling or overhead charges by the Academy be made against this fund.

2. That the U. S. National Committee of the IUNS may utilize this fund to:

a. Support travel grants of United States scientists attending future meetings of the International Congress on Nutrition.

b. Support travel expenses of U. S. nutritionists who, in the opinion of the U. S. National Committee of IUNS, should represent the United States at international working conferences on nutrition. Normally such conferences should be supported by funds from the International Relations Division of the National Academy of Sciences—National Research Council. Thus, in only exceptional cases should it be necessary to draw on the present fund for such uses.

3. Authorization for expenditures of earnings or principal fund requires a majority affirmative vote of the members of the U.S.-IUNS.

4. The U. S. National Committee of the IUNS will furnish the Treasurer of AIN a financial statement on January 1 of each year.

F. *Representatives to Federation Board:* F. S. Daft, chairman, D. W. Woolley, Paul György.

Dr. Daft summarized the activities of the Federation Board and its Advisory Committee. The Board unanimously approved proceeding with the construction of a new building on the Federation property in Bethesda, Maryland. Various means and methods of financing the cost of this new construction were discussed. The Council acted favorably upon the motion to increase the registration fee for the annual meeting of the Federation from $10.00 to $15.00, with the provision that the possibility of maintaining the $10.00 registration fee for graduate students be investigated. Additional support for financing will be received from increased rates for exhibits.

Dr. Daft announced that the next meeting of the Federation would be in Atlantic City, April 16–21, 1962.

G. *Fifth International Congress on Nutrition:* Dr. C. G. King, president.

Through 4 years of continuous planning for the Fifth International Congress on Nutrition, the primary emphasis was to make the scientific program as strong as possible in terms of (1) research papers and (2) opportunities to build around these reports, maximum provision for educational and practical developments to follow. Attainment of these goals required travel assistance for selected scientists, extensive hospitality features for members and guests, exhibits by industry, government agencies and academic institutions, simultaneous translations in Spanish, German, French and English, and publication of abstracts, panel papers and the Proceedings of the Congress (*Federation Proceedings*, March, 1961).

The total attendance reached about 2,300, of whom nearly 600 were from 67 countries other than Canada and the United States.

The program included an opening plenary session addressed by President Eisenhower, and by Maurice Pate, representing the United Nations, Vincent du Vigneaud, the National Academy of Sciences, David Cuthbertson, the International Union of Nutritional Sciences, E. W. McHenry, the Canadian Nutrition Society, and Floyd Daft, American Institute of Nutrition. There followed 7 half-day panel discussions based on invited papers, 5 organized groups of submitted research reports daily, and a final day's symposium on "World

Food Needs and Food Resources" addressed by Secretary Benson, Department of Agriculture; Secretary Flemming, Department of Health, Education and Welfare; Director General Sen of the Food and Agriculture Organization; Director General Candau of the World Health Organization; Director Horwitz of the Pan-American Health Organization, and 6 of the world's leading research scientists. The closing banquet session was addressed by Executive Director Pate of the United Nations Children's Fund; H. J. Heinz, II, honorary chairman of the Finance Committee; and D. P. Cuthbertson, chairman of the International Union of Nutritional Sciences.

Financial commitments for the Congress were necessarily kept within the total of assured funds. Hence as a result of receiving a few late grants that we could not be certain of in advance, and finding that some of the final charges were below budgeted allowances, we managed to meet all costs and had a remaining fund in the range of $25,000 from total receipts of about $332,000.

According to present plans, the next Congress will be held in Edinburgh in 1963, and the following one in Germany, in 1966.

On behalf of the Organizing Committee, it is a pleasure and obligation to again express our thanks for the generous personal and financial support that made the Fifth International Congress on Nutrition a very successful and notable occasion.

IX. Report of the Clinical Division (American Society for Clinical Nutrition)

This report was presented by Dr. Robert Olson, vice-president and president-elect of the Clinical Division.

Officers for the past year have been: President, R. W. Vilter; Vice-President, President-Elect, R. E. Olson; Secretary-Treasurer, R. E. Hodges; Councilors, W. B. Bean, R. S. Goodhart and W. A. Krehl.

The charter members at their meeting May 1, 1960, voted to join the AIN as a Clinical Division. This was finalized by mail ballot to all AIN members in July which carried by overwhelming majority. All charter members, numbering 69, had previously been elected to the American Institute of Nutrition.

The Clinical Division, in addition to participating in the annual meeting of the Federation, officially held its annual meeting at the time of the clinical meetings in Atlantic City on April 29, 1961.

Dr. Olson reported that a contract had been signed between the officers of the American Society for Clinical Nutrition and the publishers of the *American Journal of Clinical Nutrition*. This contract has been approved by the Council of AIN. The Journal is owned by the Yorke Publishing Group, now a subsidiary of the Reuben H. Donnelley Corporation. The AIN Council approved having the Journal become the official organ of the Clinical Division. After July 1, 1961, the Journal will become a monthly issue. The contract specified that the American Society for Clinical Nutrition has the right to elect the editor and editorial board. It also has the right and privilege to pass on all advertising.

The officers of the Clinical Division have worked closely with the officers of AIN and the Council and indeed look forward to the coming year—an active one—for furthering the aspects of clinical nutrition.

X. Report of AIN Council Actions

The Council met on November 5, 1960, in Washington, D. C., and on April 8 and 9, 1961, in Atlantic City. Dr. Daft reported some of the primary concerns of the Council were the negotiations regarding *The Journal of Nutrition* and the establishment of procedures and policies to insure closer coordination and assistance to the Clinical Division of AIN so as to insure its function within the framework of the Institute.

XI. Acknowledgments

The Secretary wishes to gratefully acknowledge the excellent secretarial assistance of Mrs. Sylvia Gladsden and the staff of the Federation Office for many hours of devoted help in connection with Institute activities.

ANNIVERSARY BANQUET AND PRESENTATION OF FELLOWS AND AWARDS

The annual banquet was held on April 12 at the Ambassador Hotel with approximately 325 members and guests in attendance. Dr. Daft, as toastmaster, introduced the special guests and awardees.

Dr. W. D. Salmon, chairman of the Committee on Fellows, presented Certificates of Fellow to the following persons selected in 1961 for their distinguished careers in nutrition:

HELEN T. PARSONS

"For her important research contributions relating to vitamin C, the physiological effects of proteins, availability of the vitamins from yeast, soybeans as a source of protein, and human requirements for folic acid, thiamine and riboflavin."

R. ADAMS DUTCHER

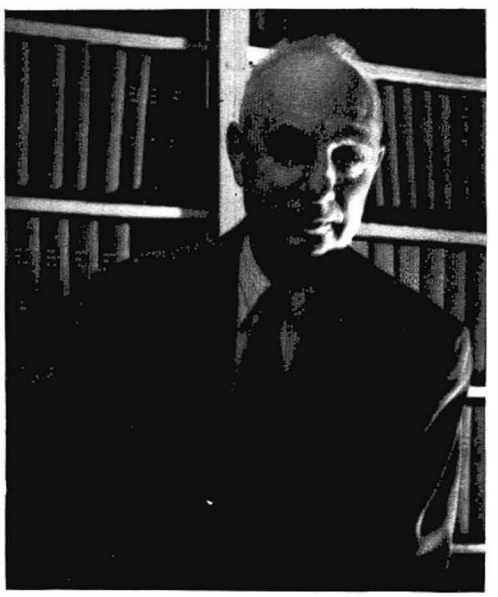
"For his outstanding research contributions in the fields of nutrition, vitamin stability, vitamin function and the deficiency diseases."

ARTHUR H. SMITH

"For his research related to intermediary metabolism, nutrition, the biological value of proteins, mineral metabolism and water balance."

HELEN T. PARSONS

R. ADAMS DUTCHER



ARTHUR H. SMITH

The 1961 Borden Award in Nutrition of $1,000 and a gold medal was presented to Dr. Klaus Schwarz, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, for his highly significant research contributions relating to the discovery of the nutritional role of selenium and its relationship to vitamin E as

the protective factor for dietary liver necrosis and the role of trivalent chromium in glucose metabolism.

The 1961 Osborne-Mendel Award of $1,000 and a scroll was presented to Dr. Max K. Horwitt, Director of the L. B. Mendel Research Laboratory, Elgin State Hospital, Elgin, Illinois, and Associate Professor of Biological Chemistry at the University of Illinois College of Medicine, Chicago, Illinois, in recognition of his contributions in determining thiamine and riboflavin requirements of men and the relationship of niacin to tryptophan and tocopherol to fatty acid metabolism in human nutrition, and in the field of protein malnutrition.

KLAUS SCHWARZ

MAX K. HORWITT

Instead of holding the joint Biochemistry-Nutrition Smoker this year, coffee hours were initiated. As measured by the consumption of coffee during the entire session, the lounge was indeed frequently and numerously visited.

OFFICERS AND COMMITTEES—AMERICAN INSTITUTE OF NUTRITION

July 1, 1961–June 30, 1962

Council

President: Paul György, Philadelphia General Hospital, Philadelphia, Pennsylvania

President-Elect: L. C. Norris, Cornell University, Ithaca, New York

Past-President: F. S. Daft, NIAMD, National Institutes of Health, Bethesda, Maryland

Secretary: A. E. Schaefer, Building 16A, National Institutes of Health, Bethesda, Maryland (1963)

Treasurer: J. B. Allison, Rutgers, the State University, New Brunswick, New Jersey (1962)

Councilors: W. H. Griffith (1962), R. W. Engel (1963), W. A. Krehl (1964)

Committees

Nominating Committee: C. G. King, chairman, G. M. Briggs, Robert Olson, R. W. Engel, J. S. Dinning

Committee on Nomenclature (joint with American Society of Biological Chemists): P. L. White (1962), Robert S. Goodhart (1963)

Committee on Publication Management: W. J. Darby, chairman, O. L. Kline, George Davis, R. W. Vilter, R. W. Engel, Bernard Oser, P. György (ex officio), A. E. Schaefer (ex officio), R. H. Barnes (ex officio)

Nominating Committee for Borden Award: E. E. Snell, chairman (1962), G. M. Briggs (1963), Robert E. Shank (1964)

Nominating Committee for Osborne-Mendel Award: Grace A. Goldsmith, chairman (1962), L. C. Norris (1963), Cosmo G. Mackenzie (1964)

Fellows Committee: E. W. McHenry, chairman (1962), David Hand (1963), W. H. Sebrell, Jr. (1963), Icie Macy Hoobler (1964), H. E. Robinson (1964)

Public Information Committee: P. L. White, chairman (1962), L. Voris (1962), S. S. Negus (1963), G. M. Briggs (1963), A. E. Schaefer (ex officio) (1963)

Committee on Recommended Constitutional Changes (ad hoc): A. E. Harper, chairman, O. L. Kline, Bernard Oser, Robert Hodges, E. L. R. Stokstad, A. E. Schaefer (ex officio)

Auditing Committee: H. W. Titus, chairman (1962), Robert L. Squibb (1962)

Tellers Committee: P. L. Harris, chairman (1962), D. M. Hegsted (1962)

U. S. National Committee—IUNS

R. W. Engel, secretary (1963), Gladys Emerson (1962), J. M. Hundley (1962), A. E. Schaefer (1962), G. M. Briggs (1963), L. J. Teply (1963), F. S. Daft (1964), W. J. Darby (1964), W. H. Sebrell, Jr. (1964)

Also, ex officio (voting) members are Paul György (1962), Grace Goldsmith; and ex officio (non-voting) members, H. B. Steinbach, R. K. Cannan, W. W. Atwood, Jr.

Representatives to other organizations

Federation Board: F. S. Daft (1962), Paul György (1963) and L. C. Norris (1964)

Federation Advisory Committee: F. S. Daft (1963)

National Research Council boards and divisions: N. R. Ellis (1963)

American Association for the Advancement of Science: E. L. Hove (Section C, Chemistry) (1962), Joseph H. Roe (Section N, Medical) (1963)

Food and Agriculture Organization: L. J. Teply (1962)

Officers, American Society for Clinical Nutrition
(a division of the American Institute of Nutrition)

Robert E. Olson, president; William B. Bean, president-elect; R. W. Vilter, past president; R. E. Hodges, University of Iowa School of Medicine, Iowa City, Iowa, secretary-treasurer; councilors, W. A. Krehl, R. S. Goodhart, George V. Mann

Editorial Board
Journal of Nutrition

R. H. Barnes, editor (1964), O. L. Kline (1962), E. S. Nasset (1962), H. Pollack (1962), D. V. Frost (1963), A. E. Harper (1963), O. Mickelsen (1963), G. F. Combs (1964), R. M. Leverton (1964), G. V. Mann (1964), M. O. Schultze (1964), G. K. Davis (1965), A. E. Axelrod (1965), J. T. Anderson (1965), R. H. Follis, Jr. (1965).

Respectfully submitted,
ARNOLD E. SCHAEFER, *Secretary*

INVITATIONS FOR NOMINATIONS FOR 1962 AMERICAN INSTITUTE OF NUTRITION AWARDS AND FELLOWS

Nominations are now being invited for the 1962 A. I. N. awards and Fellowships.

Nominations for the 1962 *Borden Award in Nutrition* must be submitted by October 1, 1961, to Dr. E. E. Snell, Department of Biochemistry, University of California, Berkeley 4, California.

Nominations for the 1962 *Osborne and Mendel Award* are due also by October 1, 1961, and should be sent to Dr. Grace A. Goldsmith, Department of Medicine, Tulane University School of Medicine, New Orleans 12, Louisiana.

The deadline for receipt of nominations for *A. I. N. Fellows* is October 1, 1961. These should be sent to Dr. E. W. McHenry, School of Hygiene, University of Toronto, Toronto, Ontario, Canada.

Full details of the rules for these awards and lists of former recipients are given in the July 1961 issue of the *Journal of Nutrition*.