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NORMAN H. JOLLIFFE

Norman H. Jolliffe
— A Biographical Sketch

(August 18, 1901 — August 1, 1961)

My acquaintance with Norman Jolliffe began at the time of the first Hearings of the Food and Drug Administration on the advisability of a standard for enriched flour. Already, at that time, Jolliffe had become outstanding for his recognition of the prevalence of deficiency diseases in the clinics of our cities. Pellagra and beriberi were then very prevalent especially among alcoholics and derelicts of the streets and it was his testimony very largely that made it evident that these diseases were not confined to remote countries but were practical medical problems in this country.

Of course, among the people who proposed to testify at the Hearings there was a considerable diversity of views. The proponents included millers and bakers, leading nutritionists and many members of the then proposed Food and Nutrition Committee, later to become the Food and Nutrition Board of the National Research Council. There had long been a strong feeling that something should be done to re-enforce our flour and bread, but there was poor agreement as to just what measures should be taken. I recall very distinctly that it was Jolliffe's genial attitude which resulted in a substantial concurrence among the various people who offered testimony without the omission of any of the essential facts. Jolliffe, more than any other person among the participants at the testimony, emphasized the medical need for the remedial measures.

For many years thereafter Jolliffe and I were closely associated in the promotion of wise measures for cereal enrichment. This biography of Dr. Jolliffe will be confined very largely to his contribution in the field of nutrition but he was very active in other related fields. Notably he was an early student of alcoholism, and a biography especially bearing on this aspect of his work appeared in the Quarterly Journal of Studies on Alcohol (vol. 22: 531, ('61)) of which journal he was long an editor. As we look back at the matter, there was much that we did not know at the time about the subject of nutritional deficiencies among the general population. Particularly there was no real idea as to the extent of the human need for nicotinic acid or niacin as it became presently to be known. The original standard was fixed far too low to be effective and the revision came gradually over the following two or three years. Another major problem was the extent of ariboflavinosis, and Jolliffe added very much to our knowledge of the occurence of these symptoms among the deficiently fed people of the country.

Unlike many nutrition and medical people of the period, Jolliffe did not share the suspicion of many that the desire for enrichment on the part of the baking and milling industries was dictated entirely by their desire to profit by it. He gave everyone credit for being honest in his intentions and soon became recognized as one of the most trustworthy contributors to the subject. The American Bakers Association and the American Institute of Baking recognized this large contribution by Jolliffe in awarding him a diploma at a dinner held by them on the twentieth anniversary of enrichment.

My further associations with Norman Jolliffe were broadened very considerably in 1941 when he became a member of the Williams-Waterman Fund Committee and continued until his death to advise with the Committee very frequently about the entire range of nutrition projects with which the Committee was concerned. The following resolution was enacted at the Committee's meeting immediately after Norman's death:

In Memoriam, Norman Jolliffe, 1901–1961

It is with sincere sorrow and a deep sense of loss that we record the passing on August 1, 1961 of our esteemed colleague, Norman Jolliffe, Member of the Williams-Waterman Fund Committee since 1941.

From almost the very beginning of the Fund's operations, Dr. Jolliffe helped shape the policy and direction of this organization's activities, thereby lending his beneficial influence and wise counsel to worldwide work in nutrition over a period of two decades. In addition, he undertook for the Fund the leadership of investigations in Formosa and Cuba where his friendly nature instantly won the hearty collaboration of those peoples whose customs and traditions were largely foreign to his own.

Dr. Jolliffe will be remembered for his many professional contributions toward better health and for the ever-constant qualities of sincerity, integrity, and good judgment which he brought to every endeavor. He will be remembered equally for his inspirational example of unflagging zeal and determination in the face of, what would have been to lesser men, overwhelming physical handicaps. He will be remembered as a great and kindly man, a good friend. It is not possible to assess his contributions, for they will be endless. One can only rejoice that building will continue on the firm bases he has established, and that the lives he has touched will in turn transmit his influence to other lives.

In this time of sorrow, we join in extending to his widow, Lillian Jolliffe, the sincere sympathy of those who were so fortunate as to know him as a friend and to be associated with him in one of the organizations through which his admirable characteristics were made manifest.

THEREFORE, BE IT RESOLVED, that an engrossed copy of this memorial be forwarded to Mrs. Jolliffe.

Norman Jolliffe was born on August 18, 1901, in Knob Fork, West Virginia. He took his B.S. degree at West Virginia University and received the M.D. degree from the New York University College of Medicine in 1926. He interned and was a resident physician at Bellevue Hospital, and in 1932 became Chief of the Medical Service of the Psychiatric Division where he remained until 1946. In 1945 he opened the first nutrition clinic in the New York City Department of Health and in 1949 became Director of the Bureau of Nutrition, Department of Health, City of New York.

He was a Diplomate of the American Boards of Internal Medicine and Preventive Medicine and Public Health; Fellow of the American College of Physicians; Fellow of the New York Academy of Medicine; and a member of numerous scientific societies.

Dr. Jolliffe was a participant in both the 1944 and the 1948 Newfoundland Survey teams, an international group of nutrition experts who assessed the nutritional status of that island's population. In 1951 he visited Brazil on the invitation of public health authorities of Sao Paulo to advise them on nutrition surveys in connection with their proposed enrichment program. In 1952 and 1953 he visited Rome on invitation of the Centro per lo Studio dell'liment azione Infantile, Consiglio Nazionale Delle Richerche, to assist them with nutrition problems.

Of course, we had many consultations with Dr. Jolliffe concerning his participation in the introduction of enriched flour and bread in Newfoundland, and his assistance in preparing reports on this subject was invaluable. As it happens, the evidence of benefit of enrichment of cereals is more clear-cut and unequivocal in Newfoundland than in any other country. In the U. S. the introduction of enrichment accompanied many changes in the economic conditions of the country, especially the development of better transportation through good roads and the migration to the South of many war industries. These features so greatly modified the economic condition of the people as to make difficult the separation of enrichment from other causes of dietary improvement. However, the almost simultaneous disappearance of metabolic and nutritional diseases from both rural and city clinics made the story quite convincing. There was really no reason why improvement of economic conditions had anything to do with the sharp decline in beriberi or pellagra among alcoholics on skid-row in Chicago.

As time went on the Williams Waterman Fund Committee increasingly turned its attention to foreign countries and began its major activities in India and especially in the Philippines. Jolliffe took an extraordinary interest in these studies and when, in 1954, there was an occasion for a nutrition survey of the civilian population of Formosa, I did not hesitate to ask Jolliffe if he could not undertake it. He readily assented and anyone who goes

to Formosa today will find among the nutritionists and physicians of the country a host of very warm friends of Norman and Lillian Jolliffe. A word is in order at this point about Lillian, whom Norman married as a secretary at Bellevue Hospital. She was always keenly interested in his work and active as an assistant in many aspects of it. When it came to organizing the school children of Formosa into groups for inspection, Lillian was almost as valuable as Norman himself in effecting a systematic examination. The Jolliffes continued their journey to Hong Kong, Manila, Bangkok, and some cities in Northern India, and at each place Norman held consultations with the local scientists and gave lectures to large groups. He thus made a great impression throughout Asia concerning the importance of nutritional disease and the means of combating it.

Two years later, when a nutrition survey of Cuba seemed in order, Norman also gladly undertook that and Lillian played much the same role that she had in Formosa. Norman very effectively organized a large group of young doctors to participate in the survey and to learn the diagnosis of deficiency diseases. Both Norman and Lillian are as much loved in Cuba as they are in Formosa.

Norman undertook studies of many medical topics other than those I have mentioned. At the end of World War II, the New York City Department of Health recognized his high standing in nutrition by appointing him Chief of its first Nutrition Clinic and a few years later, Director of its Bureau of Nutrition. In toto, he made more than 150 contributions to the medical literature, among which were two popular books. He was co-editor of the medical textbook, *Clinical Nutrition*, published in 1950 by Paul Hoeber, which has become the standard work in the field. Best known of his writings was *How to Reduce and Stay Reduced.* A second edition of *Clinical Nutrition* was prepared by Norman before his death and has appeared under the same publisher in 1962.

In his later years he concentrated a great deal of his attention on heart disease and conducted the so-called "Anti-Coronary Club" in the New York City Department of Health which collected a vast amount of data on the relationship of fat and cholesterol to the incidence of coronary problems.

The Mayor's annual message to the Council included the following statement:

"A research project of immense potential importance, the study of the relationship of diet to heart disease, is being conducted by the Health Department. Our physicians, by following carefully the results of especially prepared diets, are trying to learn the effect of fat and cholesterol in the diet on hardening of the arteries and heart disease. I believe this work, done quietly and efficiently, is an important and necessary addition to the responsibilities of local government."

Although Norman was always a very convivial and jovial spirit at any gathering, few knew of the very serious handicap from which he had suffered since his student days. He was a severe diabetic and only by the most careful control which he asserted with utmost nonchalance, was he able to keep going and particularly to partake in various lunches and dinners in his honor. Most men would have crumpled under the weight of his disability but Norman's spirit rose above his bodily weakness and urged him to continue without letup his arduous duties as teacher, researcher and administrator. How often I admired the nonchalance with which he dismissed his ever-present cares from his mind.

Robert R. Williams
Summit, New Jersey

Fat Accretion and Growth in the Rat[1]

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ABSTRACT The normal course of fat accretion in male rats from birth to 3 years is described. The relative amount of fat and relative size of fat pads increased steadily throughout life (except for a brief cessation in fat accretion at weaning), in a manner closely related to body weight and independent of age. Several new rat strains with extremes of growth or fat accretion, fed the same diet, were used to define and illustrate courses of fat accretion: very large and very small non-obese strains and a hereditarily obese rat. Rats of different known strains fed a variety of more refined diets show a course of fat growth different from that obtained with stock diets. Several types of obesity are discussed: insulin obesity, nutritional obesity, and hereditary obesity. Hereditary size and growth of the rat were readily (in a few generations) altered up or down, by consistently using the largest or the smallest 25% of the population for breeders. It is probable that the large increase in size of some laboratory rats over the past 40 years has been accomplished not only by improvement in stock diets and colony conditions, but also by selection for increased hereditary size.

This report deals with the body fat of the rat both under conditions that would be called "normal" and conditions that justify classification as obesity. Relatively few data on "normal" fat accretion are available. We will reproduce some of these for comparison with our data.

On the subject of obesity in laboratory animals the following should be considered. In mice Fenton (1, 2) found that some strains become obese when fed high fat diets, while others do not. Mickelsen et al. (3) studied such nutritional obesity in rats and more recently found[2] indications that in this species also a genetic predisposition is a factor. Extreme obesity can be produced in rats, mice, monkeys and dogs by surgical interference with the intactness of the hypothalamus (4). In mice hypothalamic lesions with resulting obesity also follow injection of goldthioglucose (5, 6). Continued injection of protamine insulin in rats and mice causes increased lipogenesis and fat accretion (7). In some species (mouse and man, but not rat) corticosteroids can lead to hyperinsulinism with resultant obesity (8). In every case it appears that obesity is made possible by an increased caloric intake.

Besides the above instances which depend on conditions imposed by the experimenter, there are also obesities related to a cause entirely within the animal. Four mutations in mice are known which lead to obesity. Two of these are due to single recessive genes — the gene *ob*, called obese (9), and the gene *ad*, called adipose, which is at a different locus from *ob* (10). The yellow obese mouse depends on a dominant gene A^y which is an allele of agouti (11). These 3 types of obesities, with genetically different mechanisms, presumably also operate through physiologically different mechanisms. With regard to Bielschowsky's (12) NZO obese mouse the genetic situation has apparently not been worked out.

Recently we described an obese mutation in the rat (13, 14) which is dependent on a single recessive gene *fa*, called fatty. It was found that this obesity is not caused by any hereditary lesion of the hypothalamus.[3]

We are concerned in this report principally with three questions:

(1) Is fat accretion related to age or body weight?

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[1] This investigation was supported in part by research grants C-4241 and A-4085 from the National Institutes of Health.

[2] Personal communication from Dr. Mickelsen.

[3] We are greatly indebted to Dr. James Q. Miller of the Neuropathology Laboratory at the Children's Hospital Medical Center, Boston, for his study of the hypothalamic region in two old and one young fatty. He reported: "The brains were removed, after sacrificing the animals, fixed in formalin and embedded in celloidin. Serial step sections were stained with cresyl violet and hematoxylin and eosin. Fat rat brains and controls were examined and compared to one another. No evidence of degenerative, neoplastic, or infectious disease was found, and there was no anatomical defect identified in the region of the hypothalamus in any of the specimens."

(2) Is there, under conditions which can be considered "normal" for the laboratory rat, a definable controlled path of fat accretion?

(3) Can a reasonable distinction be made between "normal" fat accretion and obesity?

first question is of importance since in most extant studies fat accretion has been related to age. To decide the question it is necessary to have rats that differ largely in weight for a given age. Widdowson and McCance (15), using early (preweaning) food restriction, concluded that fat is related to weight, not age. As appears below, we have used animals of hereditarily different growth rates, thus avoiding any further imposed experimental conditions. The third question deserves consideration because old rats have frequently been considered obese with the implication of a pathological state associated with senility.

METHODS AND MATERIALS

Random-bred rats. Male rats from a random-bred colony of Sherman origin, with body weights from 5 g (newborn) to 500 g (about 6 months old), were used for the carcass analysis data of figures 1, 7 and 8, as well as the data reported in the following paper (16). For older ages these were supplemented by rats of Sprague-Dawley (SD) origin in groups aged 5 months, 2 and 3 years; they came from the colony of Dr. H. S. Simms, a colony which is maintained for studies in aging. These particular old animals had remained in good physical condition and had shown no weight losses.

Rats selectively bred for body size. Two long-time experiments were carried out by breeding heavy to heavy rats and lightweight to lightweight rats over many generations with avoidance of inbreeding. The heaviest (or lightest) 25% of the available rats in each generation were used

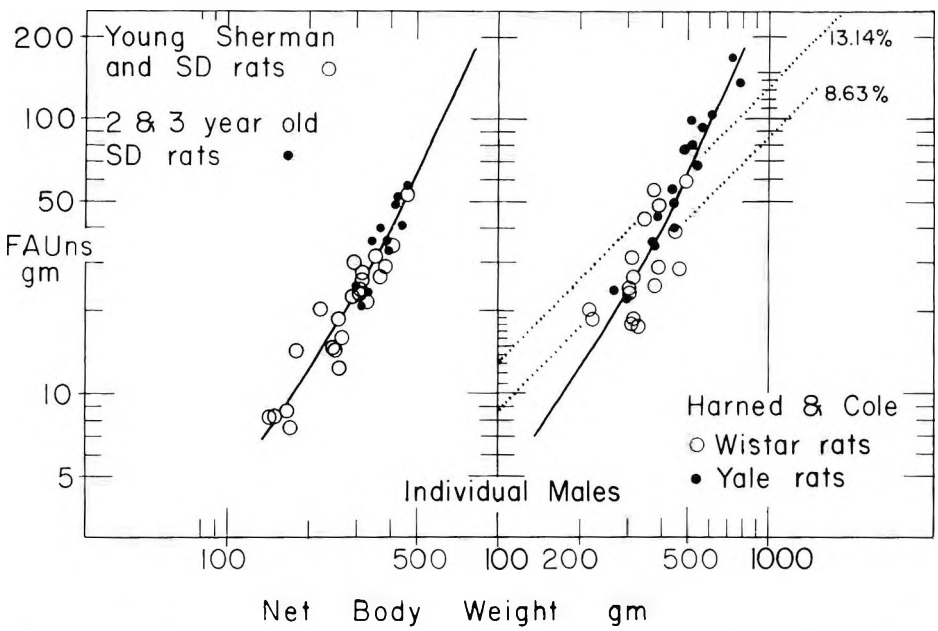


Fig. 1 (left) and 2 (right) Post-weaning fat accretion; log-log plots of carcass analysis data for individual male rats of 4 rat stocks. FAUns indicates fatty acids plus unsaponifiable material. Net weight is gross weight minus intestinal contents. (Fig. 1) The diet was a commercial pelleted stock diet containing whole milk powder, soybean meal, meat and bone scrap, yellow corn, barley, oats, wheat, small quantities of alfalfa, molasses, salt and calcium carbonate. (Fig. 2) The data line is the same as that of figure 1. The 2 dotted lines represent 13.14 or 8.63% of fat; these are the mean percentages as given by the authors (22) for their Yale and Wistar rats. The individual values for FAUns shown in the graph were calculated from the individual percentage values (22). The diet was a commercial pelleted stock diet supplemented with lettuce twice weekly.

for breeders, and changes were very apparent within a few generations. The first of these experiments was carried out with the above-mentioned Sherman rat stock. The earlier results of this experiment have been described in a progress report (17). Three sizes of strains resulted: the small 9B strain, the medium size 13C, and the large 14C. These strains are the subject of figure 3. The second size selection experiment was carried out on a foundation stock made up by crossing 4 known rat stocks: 13C mentioned above; an albino strain of Wistar origin given to us by Dr. Oser of the Food Research Laboratories; Long-Evans hooded rats from the colony of Dr. P. E. Smith at Columbia University; and a black strain from the Merck Institute for Medical Research. The purpose of setting up such a foundation stock was to provide a larger gene pool. The foundation stock and selective breeding scheme have been described together with some experimental data (18, 19). The 2 strains resulting from this experiment are designated 4StS (small) and 4StL (large). Photographs and growth curves for these rats are shown in figures

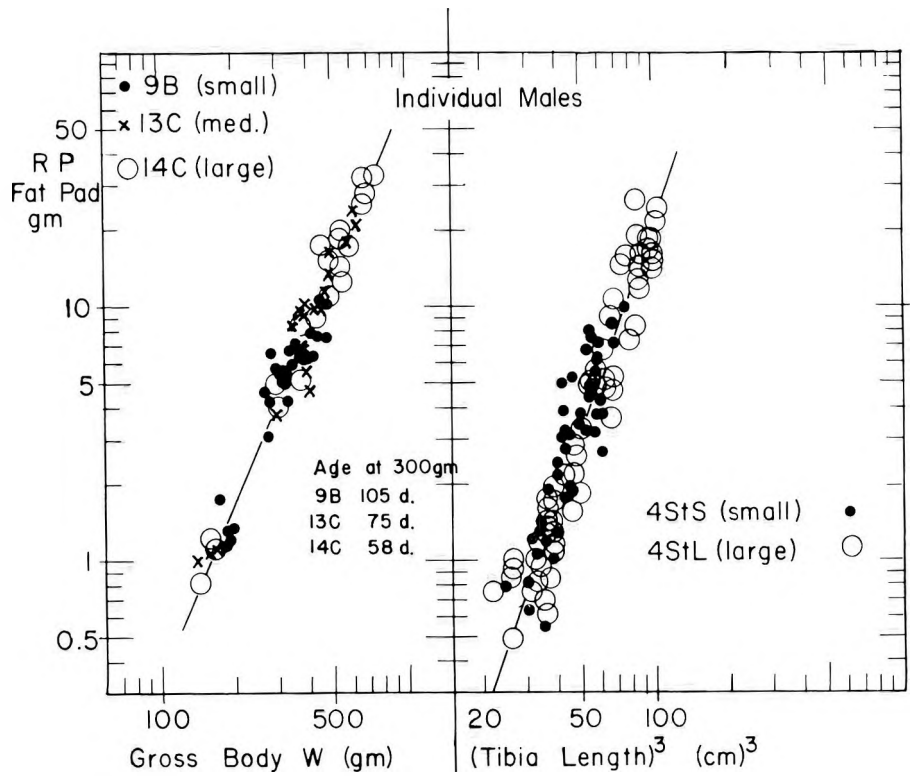


Fig. 3 (left) and 4 (right) Post-weaning growth of retroperitoneal fat pad. (Fig. 3) Log-log plot against gross weight for individual male rats of 3 strains derived by selective breeding for body size from our Sherman stock. These rats were fed an unpelleted stock diet composed of wheat, 25; cottonseed flour (Proflo, Traders Oil Mill Co., Fort Worth, Texas), 20; whole milk powder, 25; lactalbumin, 10; glucose, 14; small amounts of bone ash, yeast, salt, added vitamins and trace elements. (Fig. 4) Log-log plot against the cube of tibia length for individual male rats of 2 strains derived by selective breeding for body size from a cross of 4 known rat stocks. The appearance and growth of these strains are shown in figures 5 and 6. The diet was a commercial pelleted stock diet containing soybean meal, fish meal, skim milk powder, yellow corn, wheat, and small quantities of alfalfa, corn oil, salt and calcium carbonate, supplemented with spinach weekly and a monthly vitamin supplement. Up to 7 weeks of age there was also available ad libitum a lactation diet composed of wheat, 45; skim milk powder, 35; lactalbumin, 10; yeast, 2.5; bone ash, 1.9; salt, 0.5; cottonseed oil, 5; and a vitamin and trace element supplement.

TABLE 1

Fat pad weights[1] in individual rats

	Body wt	Tibia length	Xyphoid	Testicular	Retroperitoneal	Dorsal subcutaneous[2]			Thigh muscle pads[3]			
						R Caud	L Caud	Ceph	1-R	1-L	2-R	2-L
		cm										
13 M *fafa* male	692	3.95	0.89	27.5	54.8	29.6	30.4	65.9	0.42	0.47	1.63	1.79
4StL male	693	4.64	1.20	15.1	14.1	3.4	3.1	2.0	0	0	0	0
Ratio $\frac{fafa}{4StL}$			0.75	1.8	3.9	9.2		33				

[1] All weights are in grams.

[2] The dorsal subcutaneous fat, obtained after skinning the rat, was divided into a cephalad half (neck to lower costal border) and a caudad half, which in turn was divided at the midline into right (R Caud) and left (L Caud) portions. Subcutaneous fat is the adipose tissue (nearly 90% is $CHCl_3$ extractable) which is readily separated, by gentle traction and occasional cutting, from both skin and body wall. There is much fat in the skin itself — some 25% in the inner layers in normal rats.

[3] Two fat pads of the thigh muscle, no. 1 R and L, and no. 2 R and L, were partly imbedded in the muscle, underneath and quite separate from the overlying subcutaneous fat layer. Many such intramuscular fat deposits were noted in fatties, where none was found in normals. The fatties also often have moderately fatty livers.

5 and 6, and data on fat in figure 4 and table 1.

Spontaneous mutation to hereditary obesity. In building up the foundation stock for the size-breeding experiment, the 13C and Merck stock M were crossed to give 13M. This was carried on as a random-bred strain to serve as a control. Within this strain a spontaneous mutation occurred which produced obesity, always recognizable by 5 weeks of age and increasing steadily thereafter. The recessive mutant gene is called *fa* (fatty), and obese rats are designated as 13M strain, *fafa* genotype (13, 14). Figure 5 includes a photograph, figure 10 mean growth curves, and figure 10 and table 1 show data on fat in these rats.

Since the fatty condition is due to a single recessive gene, fatty rats (genotype *fafa*) and normal rats (*FaFa* or *Fafa*) regularly occur in the same litter. There is no intermediate condition: a rat is either a fatty or of normal appearance. This is quite different from the result of selective breeding for large and small size, where the size difference is due to the accumulation of appropriate genes at many loci (thought to be of the order of 10 to 100). It is possible to have a continuous gradation in individual rat size from very large to very small; the cross of a large and a small rat will give intermediate-size rats. Random-bred closed colonies will vary around a stable mean size anywhere from large to small, generally in each case with a coefficient of variation of the order of 10. Very large and very small rat strains have mean sizes so far apart that there is no individual overlap.

Fat determinations. After washing out the intestines, the weighed body was cut into convenient pieces with large shears and ground in a meat grinder[4] with a known weight of Na_2SO_4 (1 to 3 times the

[4] Meat grinders with a 4-pronged knife revolving against a perforated plate will not properly comminute connective tissue. Excellent results were obtained with an "Enterprise" grinder (now apparently no longer available) in which the tapering worm with very oblique ground edges works against the side of the tapering shell, which has many perforations towards the forward end. We have also used a "Universal" food and meat chopper no. 1 (Landers, Frary and Clark, New Britain, Connecticut) which has the cutter outside the perforations. Sieve analysis showed the superiority of the "Enterprise" grinder. Extremely fine comminution has been obtained by running the oven-dried product from a meat grinder through a hammermill.

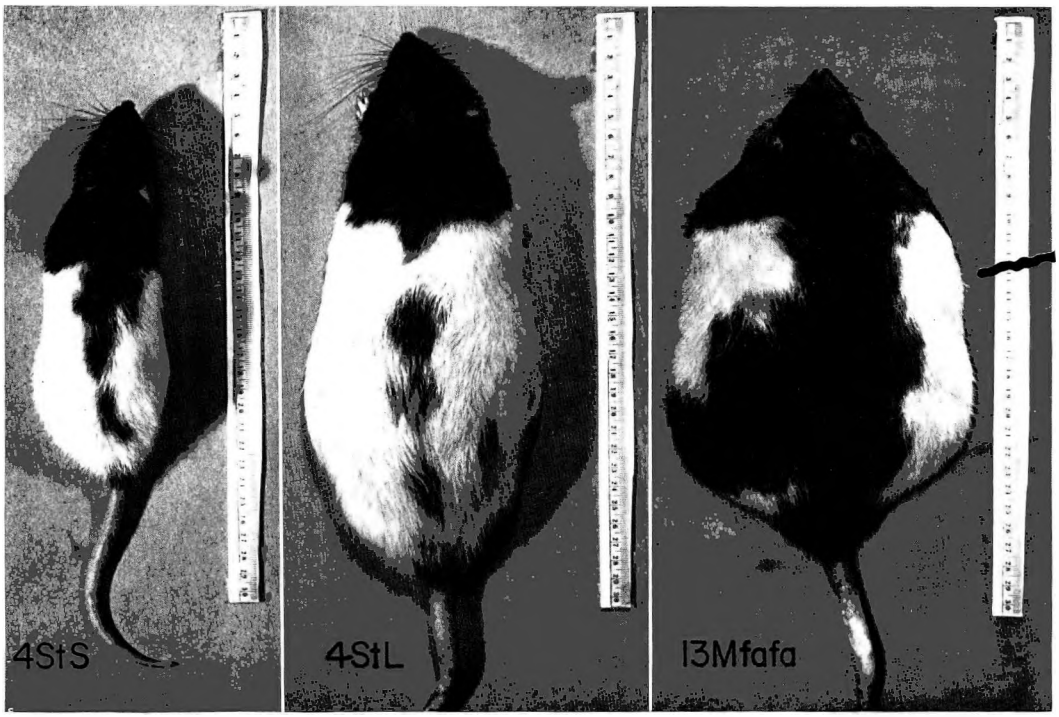


Fig. 5 Adult males of three rat strains — 4StS, 4StL and 13M *fafa*. They were all 45 weeks old (within a few days); weights were in order from left to right 254, 837 and 1025 g. The scale is 30 cm.

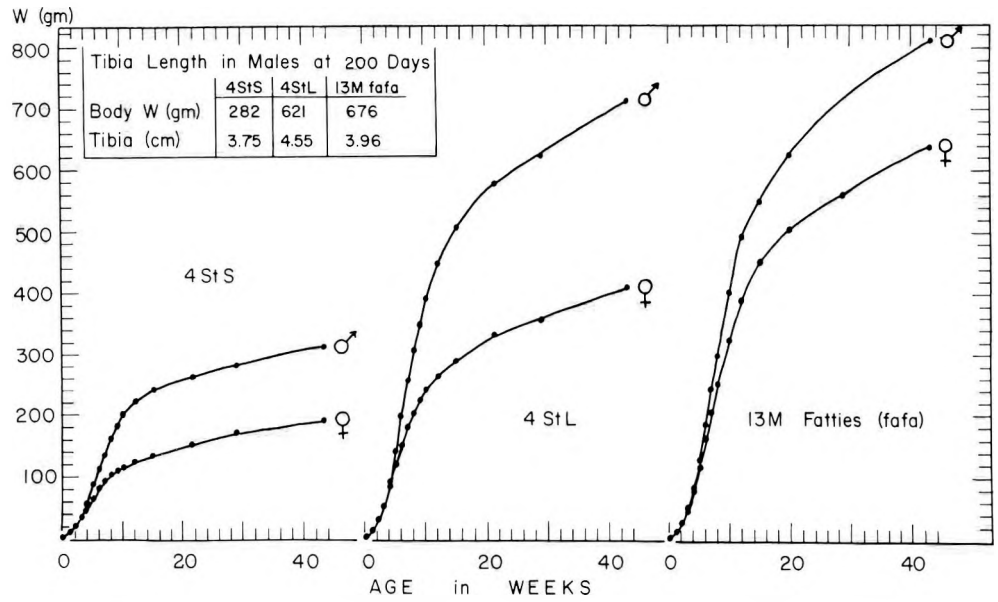


Fig. 6 Average growth curves for males and females of the 3 strains illustrated in figure 5. The 13M *fafa* curves represent a random selection (56 males, 30 females) from all the rats of this type ("fatties") born during one year. The 4StS and 4StL groups shown here represent the fourteenth generation of 2-way selection for body size. The extreme values for individual body weights at 9 weeks of age in the entire generation comprising 33 to 44 rats of each sex and strain were as follows: the largest 4StS male weight 222 g, the smallest 4StL 320; the largest 4StS female weighed 143, the smallest 4StL female 201. Diets were as in the legend to figure 4.

carcass weight). The material was mixed by hand and again run through the grinder. It was left for approximately an hour to allow the mixture to set. The shears and grinder parts were scraped off and the mixture was given a final grinding, resulting in a sufficiently homogenous mass. The mixture was air-dried in a thin layer for several hours, thoroughly mixed, transferred to a bottle with well-fitting closure, and weighed. At this stage the mixture was by no means dry, in the sense of water-free, but was stable under refrigeration, did not support mold growth, and could be sampled by weight. Total water loss to this point could be calculated as the difference between body weight plus Na_2SO_4, and air-dried weight. Aliquots of a few grams each were analyzed in duplicate for the remaining water (oven at 105°) and for total nitrogen and lipid components; sampling for all these constituents was quite satisfactory. Results on water,[5] nitrogen,[5] phosphatides and cholesterol (16) will be reported elsewhere. The samples for lipid analysis (4-g) were heated to boiling in an alcohol ether mixture (3:1), the suspensions were cooled, made to volume, and allowed to settle overnight. Aliquots of the supernatant solution were concentrated, transferred quantitatively to the apparatus of Stetten and Grail (20), and saponified. After acidification, the fatty acids and unsaponifiable matter (FAUns) were removed by continuous extraction with petroleum ether for 2 hours, and finally weighed after evaporating the solvent. All solvent evaporations were carried out in a stream of CO_2. The rat-Na_2SO_4 mixtures could be stored in the refrigerator for as long as 2 weeks before analysis. Data on rats below 15 g represent pooled carcasses of 2 to 5 individuals.

To show that the course of fat accretion is not related to conditions in one laboratory, we present quantitative comparisons of data from several other laboratories. In several such cases fat was determined by direct solvent extraction of a dried carcass or aliquot (without saponification). We have made reasonable reductions in such values in order to make them comparable with FAUns: for petroleum ether we used 15% (see fig. 12); for ethyl ether we used 20% (data of Pickens et al. (21) in fig. 8). In the following paper which deals with phosphatides and cholesterol (16) we suggest a reasonable correction of FAUns to approximate triglyceride values which more closely represent storage fat.

Diet compositions are given in the figure or table legends where they apply.

Fat in relation to body weight or age

As an example of the relation between body weight and carcass fat we have in figure 1 an allometric (i.e., log-log) plot of these two quantities in post-weaning male rats of our Sherman strain and the Simms SD strain, all fed the same commercial pelleted stock diet. Data for young and old rats overlap and all are closely related to body weight.

In a paper of Harned and Cole (22), body weight and percentage of fat (determined as FAUns) are given for rats of Wistar and Yale origin. The Yale rats were heavier for any given age than the Wistars. Since the animals had been bred for several generations in their laboratory under identical conditions including diet, they assumed—and gave their reasons for it—that the Yale rat had in the past been bred for larger body size. In groups of the 2 strains aged approximately 100, 160, 225 and 400 days, Yale rats had at each age a higher average percentage of body fat, and on this basis they designated the Yale rat as obese. For figure 2 we have calculated their data back to weight of FAUns and plotted as in figure 1. The line (slightly curved) drawn through the points is identical with the line of figure 1 fitted to our data. The average values for percentage of fat in the 2 strains were 13.14 and 8.63. In such a graph equal percentages of fat lie of necessity along a line of slope 1 (45°). The 2 dotted lines of this slope are drawn through the mean values for Yales and Wistars and indicate the percentage values at the abscissa for 100 g body weight. The observed much steeper slope corresponds to a constantly increasing percentage of fat, whereas the adherence to the same course by all 4 rat stocks of widely varying age for a given body weight (as shown in figs. 1 and 2) means

[5] Unpublished data.

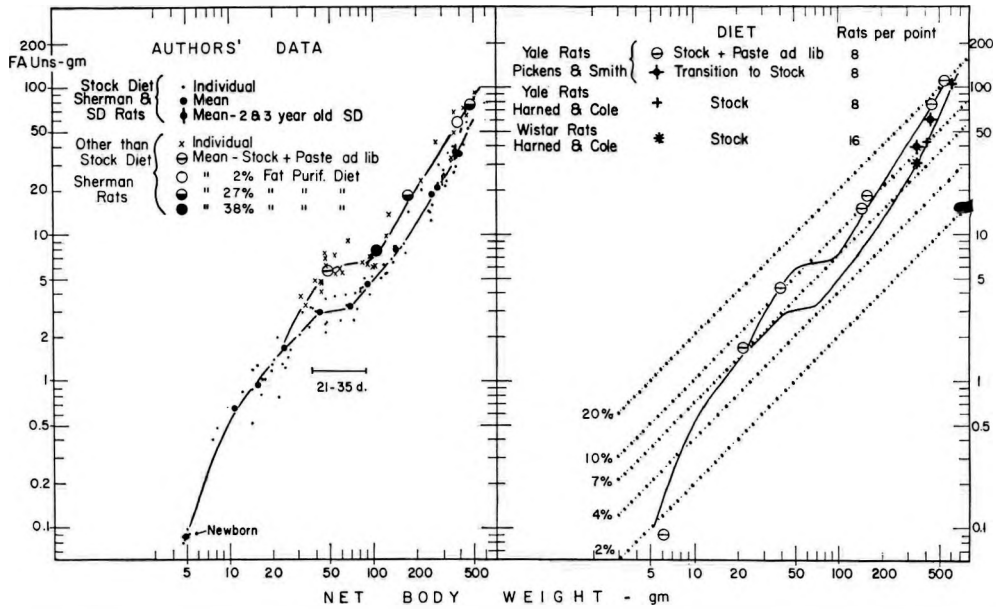


Fig. 7 (left) and 8 (right) Lifetime fat accretion. There are two patterns, corresponding to crude stock diets (lower curve) and more refined diets (upper curve). (Fig. 7) In the lower curve individual rats (or pooled samples for rats under 15 g) are represented by dots. Means for successive small groups are shown as small filled circles; mean ages in days for these groups are 0 (23 rats), 4 (6 rats), 10 (14 rats), 15 (9 rats), 25 (11 rats), all preweaning. In the postweaning period ages and numbers are 36 (6), 39 (7), 54 (9), 162 (8) and 201 (4). For the Simms SD rats ages and numbers are 5 months (13) and 2.5 years (7 two-year and 5 three-year); the mean point for the old rats has a vertical line through it. The commercial pelleted stock diet used was described in the legend to figure 1.

In the upper curve individual rats are represented by small crosses. These have been averaged to give 5 points, one for each of 3 diets and 2 points for the fourth diet. The paste food supplement was composed of whole milk powder, 25; wheat germ, 20; crude casein, 25; hydrogenated vegetable oil, 28; bone ash, 1; and calcium carbonate, 1. There were 14 rats in this group, all unweaned, with an average age of 25 days. The diet with 2% of fat contained crude casein, 27.9; glucose, 61.4; carotene in oil, 0.1; cod liver oil, 0.1; wheat germ oil, 0.2; cottonseed oil, 1.6; salts, 3.2; rice bran extract, 5; yeast extract, 0.5; and added riboflavin. There were 6 rats in this group, with an average age of 163 days. The diet with 27% of fat was obtained by isocaloric substitution of equal parts of cottonseed oil and hydrogenated vegetable oil for glucose in the preceding diet. The 5 rats fed this diet were combined into 2 means, one averaging 48 days, the other 181 days. The diet with 38% of fat contained purified casein, 27.8; dextrin, 28.5; hydrogenated vegetable oil, 31.4; cottonseed oil, 7; salts, 5.3; and complete vitamin supplement. The 7 rats in this group were all 37 days old.

(Fig. 8) Data from other laboratories. The data lines are the same as in figure 7. Positions for several different percentages of fat are indicated by the dotted lines. Harned and Cole (22) fed a commercial pelleted diet. The paste food differed from that in the legend to figure 7 in having 30% of lard instead of 28% of hydrogenated vegetable oil plus 2% of salts. The stock diet of Pickens et al. (21) was an unpelleted mixture of linseed oil meal, skim milk powder, corn, barley, oats, wheat and small amounts of blood meal, bone meal, salt and cod liver oil, plus supplements of lettuce and yeast. Pickens et al. determined fat as ethyl ether extract, without saponification; their values have been adjusted down by 20% in an attempt to make them comparable with FAUns.

that fat is closely related to body weight irrespective of age.

The relation of carcass fat to body size rather than to age has been verified in several other experiments. Figures 3 and 4 deal with the several strains bred for body size, and therefore differing greatly in growth rate, or body weight at a given age; fat is represented by the weight of the excised retroperitoneal fat pad. According to Hausberger (8): "Changes in body fat closely reflect the weight changes of dissectable adipose tissue." The rats of figure 3 are the 3 strains of different growth rate which resulted from our first size-selection experiment (see Methods and

Materials section). As an illustration of the determining role of body weight rather than age, we note that at body weights of about 300 g all 3 strains are represented in figure 3; at this weight the mean ages for the 3 strains were 105, 75 and 58 days. Over the body weight range of 150 to 600 g the points fall on a single line of high slope irrespective of age.

Later we will deal with obese rats whose fat content was so large that it became a very appreciable part of body weight. To avoid any "spurious correlation" in using body weight as abscissa we substitute in figure 4 a skeletal measure of body size, namely the cube of the tibia length. These data were obtained from the rats of our second size-selection experiment, the 4StS and 4StL strains. Here again the fat pad data fall on a straight line of slope similar to that of figure 3. One-year-old 4StS rats and 9-week-old 4StL rats of the same skeletal size appear together, with the same fat, in the graph of figure 4. In figures 5 and 6 the two left-hand parts show in photographs and growth curves the obvious differences between 4StS and 4StL rats.

Lifetime fat accretion

In figure 7 the data on carcass fat (FAUns) and (net) body weight of figure 1 are repeated, together with additional earlier points. Both colonies represented (Sherman and Simms SD) had been fed the same colony diet for generations. The break in the curve at about 10 g body weight may stand for some unknown change in developmental pattern. Alternatively the events surrounding birth may deplete the fat. The near horizontal section around weaning is also unexplained, but is quite widely observed and will be taken up later. From about 25 g body weight (about 15 days) the graph shows 2 diverging courses. The lower one represents animals that remained on the crude colony diet. In the upper curve are data for rats fed more concentrated or refined diets, the composition of which is given in the figure legend.

The upper curve of figure 7 has been drawn through 5 mean points; the rats represented in these means are shown individually by small crosses. The first mean (large circle with horizontal bar) represents 14 rats aged 19 to 28 days and not yet weaned. They received from the age of 2 weeks a paste food supplement ad libitum, with stock diet pellets also available. The next mean (large filled circle) represents 7 rats that had received stock diet and paste food ad libitum to the age of 25 days and were then weaned to a casein dextrin diet containing 38% of fat (7% of cottonseed oil, 31% of hydrogenated vegetable oil); they were killed at 37 days of age. The remaining rats received no paste food supplement preweaning. The open circle represents 6 rats with an average age of 163 days fed since weaning a casein glucose diet with only 2% of fat (cottonseed oil). The 2 half-filled circles represent rats fed a diet with 27% of fat (one-half cottonseed oil, one-half hydrogenated vegetable oil) isocalorically substituted for glucose in the preceding diet; average ages for the 2 points were 48 (2 rats) and 181 (3 rats).

The effect of preweaning supplements of the paste food was rather transitory; such rats transferred to stock diet at 28 days of age were on the stock diet line by 40 days of age. Such rapid adjustment to a new body fat level either up or down has been illustrated by Peckham et al. (23).

All 4 of the experimental diets led to significantly fatter rats than stock diet; the significance ratio t was over 4 for each diet. For this calculation we used the curve drawn through the mean points for stock diet animals and measured the deviations of points for individual rats in the vertical direction.

In figure 8 we have repeated the lines of figure 7, and plotted some data from the laboratory of A. H. Smith (21) on his Yale rats. Each point represents the mean of 8 rats. The large circle with horizontal bar signifies a diet of paste food ad libitum in addition to stock diet. The first 2 such points (newborn and 14 days old) agree well with our preweaning curve. The later points are in excellent agreement with our upper curve for a variety of refined diets; these consist of a group at 21 days, 2 groups at 42 days (one of which got somewhat less than an ad libitum intake

of paste), and groups at 110 and 230 days. They also had groups aged 110 and 230 days which had received paste food ad libitum to 21 days, one-half of the ad libitum intake to 42 days, and thereafter none, with stock diet fed ad libitum throughout. These 2 groups, represented by filled circles with horizontal and vertical bars, are approaching the stock line and are well below the upper experimental line. This upper course of fat accretion is also related to size and independent of age. The rats of Pickens et al. are the large Yale strain; they are much younger at any given weight than our Sherman strain. Yet both strains fed this type of diet lie on the same plotted course. Lying on the stock diet line in figure 8 are mean values for the Harned and Cole rats on stock diet (the same data shown individually in figure 2).

The data of Widdowson and McCance (15) give further confirmation of controlled fat accretion. These investigators were interested in body composition with varying growth rate which they produced by limiting some litters to 3 pups, while increasing others to 15 to 20 pups per dam. Figure 9 (data obtained as age of fat from their figure 11 and recalculated to absolute weight of fat) shows that except for the near horizontal parts around weaning, both the fast-growing and slow-growing rats show the steep slope for fat accretion. Also, rats weighing 200 g or more have body fat content values that fall on the same line — i.e. body weight determines fat content. As soon as growth-inhibited rats had undergone enough realimentation, the fat content was determined by body weight independently of age. As far as fat is

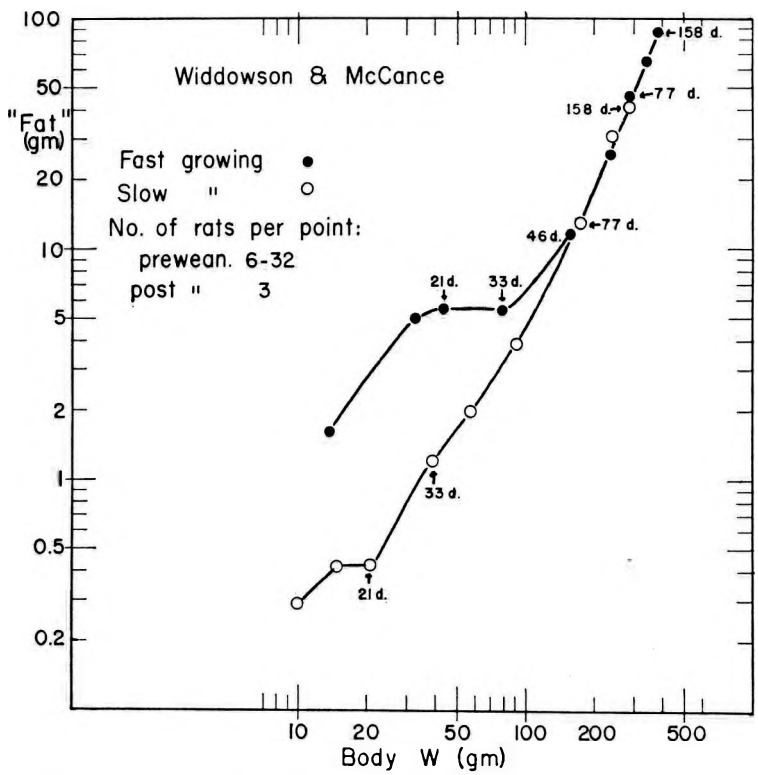


Fig. 9 Effect of very large early difference in food supply on the pattern of fat accretion (15). The lower curve corresponds to a litter size of 15 to 18, the upper curve to a litter size of 3. Fat was determined as petroleum ether extract, without saponification, and is plotted as such without adjustment.

concerned, experimental differences disappear, although there are still large differences in body weight for a given age. Their data do not show the same absolute values as presented in figure 7, presumably because of differences in experimental conditions. The curves clearly show periods when body fat does not increase even though body growth is continuing. This phenomenon, which is quite similar to the pause in fat accretion around weaning shown in figure 7, is also shown by data of Mayer and Vitale (24); groups of rats with mean body weights of 38 g at 21 days and 48 g at 24 days had 2.07 and 2.02 g of fat, respectively. Indications of the same thing are reported in a number of other papers.

Data so far shown demonstrate several points: (1) at all times except for a short period around weaning, the rate of fat accretion as shown by the steep slope of the lines is much greater than the rate of body growth (approximately twice); (2) rats as old as 3 years adhere to the same controlled path of fat accretion as rats 5 months old; (3) certain differences in the diet, independent of its fat content which varied between 2 and 38%, alter the course of fat accretion in the post-weaning period in a controlled manner.

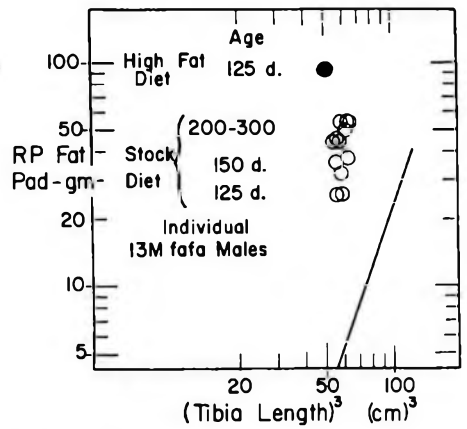
Obesity

Hereditary obesity. The 13M *fafa* rat presents an obviously obese appearance (figure 5) and is even heavier as an adult than the skeletally very large 4StL strain, although it is skeletally not much larger than the very small 4StS strain (compare mean tibia lengths of figure 6). The fatty (genotype *fafa*) is also skeletally smaller than its normal 13M sibs (genotypes *FaFa* or *Fafa*) which are, judged by tibia length and body weight, about midway in size between 4StS and 4StL. One variety of obese mouse (genotype *obob*) also shows a smaller frame than its normal sibs (25). Figure 10 shows the state of adipose tissue in the hereditarily obese rat. Part of the line of figure 4, for the retroperitoneal fat pad in relation to the cube of tibia length, is repeated; the points represent individual *fafa* rats. All but one of these fatties were raised with the stock diet.[6] Due to hyperphagia (14) these rats deviate from the "normal" pattern and the longer this process goes on — the older the rats — the more obese they become. However, the fattest of all is a single *fafa* rat which was raised with a high-fat diet; obviously this was more efficient for fattening than the stock diet. The obese hyperglycemic mouse (*obob*) is said to do poorly with a high fat diet (26).

In a fat animal various fat depots may not all be enlarged to the same degree. It is well known for man that there are various characteristic patterns of excess fat distribution, some definitely associated with particular causes of obesity (e.g. Cushing's syndrome). The fatty is characterized by emphasis on subcutaneous fat, especially about the neck, chest and upper back. Table 1 shows weights of various fat depots in a representative fatty, compared with a 4StL (a large non-obese rat) of the same body weight. From the ratio of fat pad weights in the 2 rats it appears that the retroperitoneal fat pad used in figure 10 to measure the obesity of fatties is of an intermediate degree of enlargement. Sibs of fatties which are normal in appearance but which carry one fatty gene (genotype *Fafa*) became somewhat obese when fed a high fat diet (14). Such rats displayed a different distribution of excess fat, with primary emphasis on retroperitoneal and mesenteric fat depots. In the obese mouse (*obob*) fat accumulation in the hind quarters is characteristic (25).

Insulin obesity. This is well delineated by data of Hausberger and Hausberger (7). Their experiment was performed with rats started at a body weight of 240 g; all experiments lasted 14 days and each group contained 4 to 6 rats. In figure 11 the groups representing initial and final untreated control rats (open circles) fit our line for stock diet animals. The rats treated with protamine insulin were but slightly heavier than the 14 day controls but had a much higher fat content. Cortisone alone did not increase fat but rather diminished it (unlike the situation with mice and men), but rats receiving cortisone and the standard insulin dose (lower asterisk) were obese, and when the in-

[6] Soy protein used was ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.



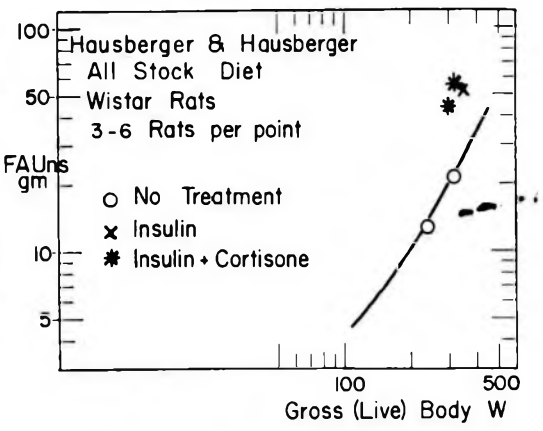


Fig. 10 (left) The relation of weight of retroperitoneal fat pad to tibia length in hereditary obesity. The line was taken from figure 4. Each plotted point represents an individual male fatty. All but one of these were raised with the stock diet regimen described in the legend to figure 4. One fatty was raised with a high fat diet containing cottonseed oil, 24.5; hydrogenated vegetable oil, 15.5; cod liver oil, 0.2; carotene in oil, 0.2; casein, 26; soy protein (see footnote 6 in text), 20; glucose, 6; salts, 6; cellulose, 1.6; and complete vitamin supplement.

Fig. 11 (right) Carcass fat in insulin obesity (7). The diet was a commercial pelleted diet. The line is our line of figure 1 (lower curve of fig. 7).

sulin dosage was further increased (upper asterisk), the obesity was as great as on the lower dose of insulin alone.

Nutritional obesity. The demonstration by Mickelsen et al. (3) that immensely obese rats can be produced by feeding diets with as much as 60% of fat has opened up the study of nutritional obesity in the rat. Peckham et al. (23) set up 2 groups of weanling rats, one fed a pelleted stock diet and the other Mickelsen's 60% fat diet. After 31 weeks the diet of one-half of each group was switched to the other diet; at 64 weeks a switchback was made. Both in body weight and body fat an adjustment to the new diet was apparent in a few weeks. To present the Peckham data in the form of a relation of fat to body weight, the fat-inflated body weight has to be considered. In figure 12 we show lines for the same rats as in figure 7, but based on fat-free net weight rather than net weight. Their 4 points for stock diet (open circles) fit our stock line quite well. Their points for rats fed the high fat diet (crosses) rise further and further above the line as the animals become older. We have also added to figure 12 some data from Mickelsen and Anderson (27). The 2 stock diet groups (filled circles) agree well enough with our line. The single rat fed a high fat diet (asterisk), with a fat-free carcass weight of 440 g, had petroleum ether-soluble fat weighing 770 g, which we have corrected down to 655 for comparison with our FAUns data. This marked obesity shows to what extent fat accumulation can progress above "normal" fat accretion.

DISCUSSION

It appears that under conditions of good health and good food, with no extraneous procedures, fat accretion in the laboratory rat is a controlled process characterized by a relative rate of fat increase about twice that of body weight increase. This is independent of age and, within limits, independent of fat content of the diet. Data from several laboratories for body weights above 150 g are in good agreement. The path of fat accretion at smaller body weights is not as well established and presents several unsolved problems.

We are concerned with 2 processes: increase in body size and increase in fat. The most objective presentation results from using weight of fat as such (not percentage), or weight of a fat pad, plotted against a measure of body size. Body size can be measured by live weight, net weight, fat-free weight, or a skeletal meas-

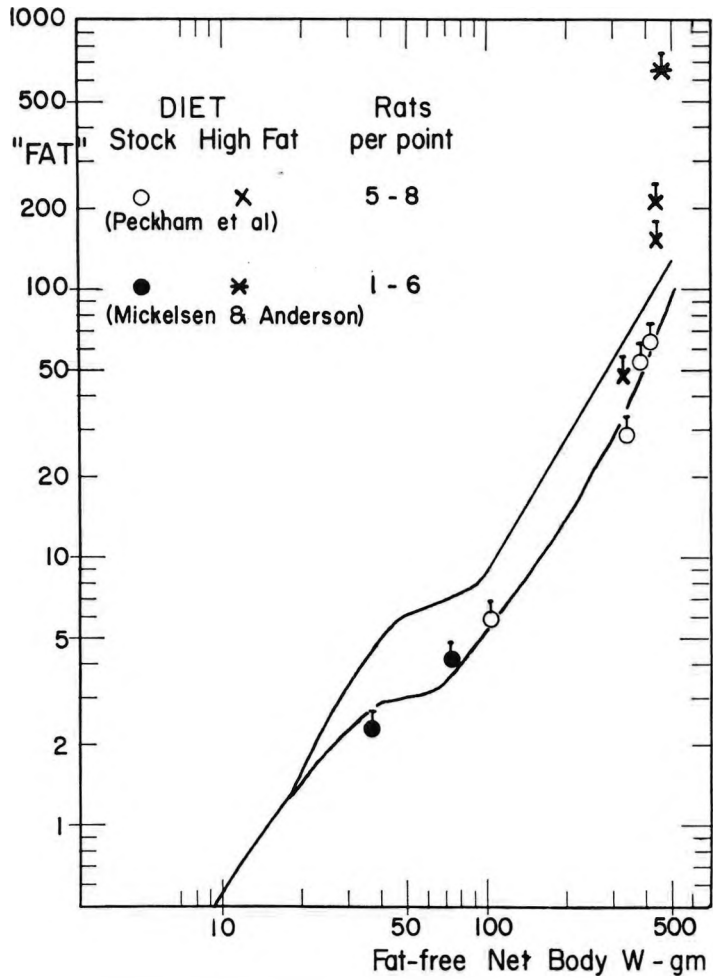


Fig. 12 The relation of carcass fat to fat-free net weight in nutritional obesity (23, 27). The same 60% fat diet and analytical procedures were used by both groups of investigators. Fat was determined as petroleum ether extract, without saponification; the values have been adjusted down by 15% to make them more comparable with FAUns values. The original unadjusted values are also indicated (upper end of the vertical line through the symbol). The abscissa is fat-free net weight. The lines have been fitted to our data; they differ only slightly from the lines fitted to plots against net weight.

urement; if the latter is a length measurement it should be cubed. To visualize the relations most clearly, allometric (i.e. log-log) plotting is used. This type of plotting is based on the fact that growth is multiplicative, not additive. It results in a very uniform dispersion of individual points around the mean course over the entire life; the steadily increasing spread of individual points around the mean course when one uses observed values as such, rather than their logs, is a familiar feature of ordinary time-growth curves, and is illustrated for the bone-to-body weight relation by figure 2 of Zucker and Zucker (28). The reason that the various measures of body size are admissible is that they are all related to each other allometrically. The slope of the line will differ slightly according to which measure of body size is used.

In carcass analysis percentage composition has its place for many types of presentation. However, when we are interested

in how much of each component is produced, the use of actual weights is recommended. Percentage values change without change in the actual amounts of the component under consideration, whenever any other component changes appreciably. Therefore expressing composition as percentage can be seriously misleading. Thus Widdowson and McCance (15), discussing a plot of percentage of fat, state: "All the animals gained fat during suckling and lost it afterwards." This hides the fact that fat content did not decrease, but merely remained constant, whereas other body components increased (see our figure 9 in comparison with their figure 12).

The effect on fat accretion produced by crude and more refined diets adds another example to the list of such unexplained differences that have been observed. Just as Ganther and Bauman (29) recently observed something in a crude stock diet that radically affects the metabolic paths involved in selenium excretion, so we may postulate an unknown factor which influences fatty acid metabolism.[7] Alternately, it may be that more rapid absorption of soluble carbohydrate from refined diets stimulates insulin production which in turn leads to more fat accretion. Also the greater bulk of stock diets may spread out food intake and lessen any peaks of carbohydrate absorption. Measurements of food intake and fecal weight in adult rats have resulted in a figure of 31 g of fecal solids /100 g of food solids (31%) for the stock diet used in figure 7, as against 6% for the purified casein-sugar diet containing 2% of fat, and 10% for the purified diet containing 27% of fat.

Although we are concerned here only with laboratory rats, it may be asked whether the fat accretion described may apply only to animals confined in cages, not in their natural habitat. It may however be noted that many animals in the wild state store fat in the summer to have its energy available during the winter. For such a species the ability to store considerable amounts of fat may be a condition for survival.

Let us again consider the difference in body weight for a given age as seen in the data of Harned and Cole (22) and in our rat strains which were selectively bred for body size. Breeding practices involving the mating of large to large and small to small will, in succeeding generations, produce animals of distinctly different body sizes for a given age, i.e., it will very quickly change the growth rate. This has been amply established for mice (discussion in Falconer (30)) and for rats (figure 6, and Zucker (19)). Since the inception of nutrition work with rats, there has been a steady increase in average body size of colony rats. This has been illustrated by Mendel and Hubbell (31), who describe the arduous task of improving stock diets. In the handling of a colony it is an almost common practice that one selects the biggest and healthiest looking animals for breeders; thus it is almost inevitable that selection for large hereditary size will occur. In the Mendel and Hubbell paper it is stated that at an earlier time, definite but unsuccessful attempts were made to select for increased hereditary size. However, this was a period when the rats were fed a distinctly inferior diet. Apparently at that time the inferior diet was growth limiting so that the genetic potential for growth could not assert itself. With the improvement in rations since then, the combined effect of hereditary size and good nutrition is quite apparent.

Falconer (32) notes that in mice, guinea pigs and rabbits there is an association between body size and litter size. With respect to rats, we have observed that in large-size and small-size strains (4StL and 4StS) the first litters averaged 13.8 and 6.5 young per litter, with maximal numbers reaching 22 and 10, respectively. The repeated observation in independent experiments of such an association suggests that it is genetically based. Thus the expectation that the frequent practice of breeding from females with numerous offspring per litter will also gradually increase body size suggests another path by which body size has increased through the years.

[7] Since submission of this manuscript, the paper by Di Giorgio, J., R. A. Bonanno and D. M. Hegsted (J. Nutrition, 78: 384, 1962) has appeared. They find that lipogenesis is less with stock diet than with purified diet when epididymal fat tissue is observed *in vitro*, and suggest the presence of an unknown food factor in stock diet.

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LITERATURE CITED

1. Fenton, P. F. 1956 Growth and fat deposition in the mouse. A definition of obesity. Am. J. Physiol., *184:* 52.
2. Fenton, P. F., and M. T. Dowling 1953 Studies on obesity. I. Nutritional obesity in mice. J. Nutrition, *49:* 319.
3. Mickelsen, O., S. Takahashi and C. Craig 1955 Experimental obesity. I. Production of obesity in rats by feeding high-fat diets. Ibid., *57:* 541.
4. Brobeck, J. R. 1946 Mechanism of the development of obesity in animals with hypothalamic lesions. Physiol. Rev., *26:* 541.
5. Brecher, G., and S. H. Waxler 1949 Obesity in albino mice due to single injections of goldthioglucose. Proc. Soc. Exp. Biol. Med., *70:* 498.
6. Marshall, N. B., R. J. Barnett and J. Mayer 1955 Hypothalamic lesions in goldthioglucose injected mice. Ibid., *90:* 240.
7. Hausberger, F. X., and B. C. Hausberger 1958 Effect of insulin and cortisone on weight gain, protein and fat content of rats. Am. J. Physiol., *193:* 455.
8. Hausberger, F. X. 1958 Action of insulin and cortisone on adipose tissue. Diabetes, *7:* 211.
9. Ingalls, A. M., M. M. Dickie and G. D. Snell 1950 Obese, a new mutation in the house mouse. J. Hered., *41:* 317.
10. Falconer, D. S., and J. H. Isaacson 1959 Adipose, a new inherited obesity of the mouse. Ibid., *50:* 290.
11. Grueneberg, H. 1952 The Genetics of the Mouse, ed. 2. Martinus Nijhoff, The Hague, p. 40.
12. Bielschowsky, M., and E. Bielschowsky 1956 New Zealand strain of obese mice; their response to stilbestrol and to insulin. Austral. J. Exp. Biol. Med. Sci., *34:* 181.
13. Zucker, L. M., and T. F. Zucker 1961 Fatty, a new mutation in the rat. J. Hered., *52:* 275.
14. Zucker, T. F., and L. M. Zucker 1962 Hereditary obesity in the rat associated with high serum fat and cholesterol. Proc. Soc. Exp. Biol. Med., *110:* 165.
15. Widdowson, E. M., and R. A. McCance 1960 Some effects of accelerating growth. I. General somatic development. Proc. Roy. Soc. (Ser. B), *152:* 188.
16. Zucker, T. F., and L. M. Zucker 1963 Phosphatides and cholesterol in the rat body: effects of growth, diet and age. J. Nutrition, *80:* 20.
17. Zucker, T. F. 1953 Problems in Breeding for Quality, in Rat Quality, a Consideration of Heredity, Diet and Disease, a symposium. National Vitamin Foundation, Inc., N. Y., p. 48.
18. ——— 1957 Pantothenate deficiency in rats. Proc. Animal Care Panel, *7:* 193.
19. Zucker, L. M. 1960 Two-way selection for body size in rats, with observations on simultaneous changes in coat color pattern and hood size. Genetics, *45:* 467.
20. Stetten, D. W., Jr., and G. F. Grail 1942 Microextraction and microtitration of fatty acids. Ind. Eng. Chem., Anal. Ed., *15:* 300.
21. Pickens, M., W. E. Anderson and A. H. Smith 1940 The composition of gains made by rats on diets promoting different rates of gain. J. Nutrition, *20:* 351.
22. Harned, B. K., and V. V. Cole 1939 Evidence of hyperfunction of the anterior pituitary in a strain of rats. Endocrinol., *25:* 689.
23. Peckham, S. C., C. Entenman and H. W. Carroll 1962 The influence of a hypercaloric diet on gross body and adipose tissue composition in the rat. J. Nutrition, *77:* 187.
24. Mayer, J., and J. J. Vitale 1957 Thermochemical efficiency of growth in rats. Am. J. Physiol., *189:* 39.
25. Vlahakis, G., and W. E. Heston 1959 Relationship between recessive obesity and induced pulmonary tumors in mice. J. Hered., *50:* 90.
26. Mayer, J. 1960 The obese hyperglycemic syndrome of mice as an example of "metabolic" obesity (see table 2). Am. J. Clin. Nutrition, *8:* 712.
27. Mickelsen, O., and A. A. Anderson 1959 A method for preparing intact animals for carcass analysis. J. Lab. Clin. Med., *53:* 282.
28. Zucker, T. F., and L. M. Zucker 1946 Bone growth in the rat as related to age and body weight. Am. J. Physiol., *146:* 585.
29. Ganther, H. E., and C. A. Bauman 1962 Selenium metabolism. I. Effects of diet, arsenic and cadmium. J. Nutrition, *77:* 210.
30. Falconer, D. S. 1953 Selection for large and small size in mice. J. Genetics, *51:* 470.
31. Mendel, L. B., and R. B. Hubbell 1935 The relation of the rate of growth to the diet. III. A comparison of stock rations used in the breeding colony of the Connecticut Agricultural Experiment Station. J. Nutrition, *10:* 557.
32. Falconer, D. S. 1960 The genetics of litter size in mice (see especially discussion by Wright and Falconer, p. 166). J. Cell. Comp. Physiol., *56:* 153.

Phosphatides and Cholesterol in the Rat Body: Effects of Growth, Diet and Age[1]

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ABSTRACT Data are presented on the content of phosphatides, free and total cholesterol, and neutral fat in 152 male rats from birth to 3 years of age. The ratio of phosphatides to total cholesterol remained remarkably constant at near 4 (mol ratio of 2) for the first year of life with a variety of diets. Two- and three-year-old rats showed a highly significant decrease in this ratio, corresponding to an increase in total cholesterol of about 10%, with no change in phosphatides. The percentage of free cholesterol decreased steadily throughout life from 90 to 72, diet and senescence having no effect thereon. The pattern of accretion of neutral fat with stock diet was in many respects very different from the common pattern observed for both phosphatides and cholesterol. The substitution of more concentrated, refined or purified diets of varying fat content (2% to approximately 40%) for the crude stock diet led to no change in phosphatides or cholesterol. These same rats showed large increases in neutral fat.

In the preceding paper (1) we discussed rat carcass fat as estimated either by extracting fatty acids plus unsaponifiable material (FAUns) or by direct solvent extraction of the dried carcass. Storage or reserve fat is considered to be essentially in the form of triglycerides (the element variable). Other lipids, essentially phosphatides and cholesterol, represent the cellular components (the element constant). The present report deals with phosphatides and cholesterol in the carcasses of some of the same rats used in the preceding paper. These data have their own significance and also afford means of calculating good approximations to the actual triglyceride content.

METHODS AND MATERIALS

Lipid determinations. The grinding and sampling procedures and estimation of FAUns have been described (1). From the same alcohol ether extract used to determine FAUns, other aliquots were taken for the determination of lipid P (2) and total and free cholesterol (3). Neutral fat was estimated by correcting FAUns values for cholesterol, for fatty acids esterified with cholesterol, and for 2 fatty acids (or one fatty acid and one fatty alcohol or aldehyde of similar weight) for each P. The calculation is shown in the figure legend.

Rats. Males of our Sherman stock from birth to 321 days were included; also 3 groups of Simms rats of Sprague-Dawley origin (Simms SD), aged 5 months, 2 and 3 years.

Diets. Most of the Sherman rats and all of the Simms SD rats were raised with a single commercial pelleted stock diet. In addition, 24 of our Sherman rats were fed a variety of more refined diets that led to somewhat fatter carcasses. These diets, although varying in fat content from 2 to approximately 40%, appeared to be equivalent in their effect on carcass fat. They are characterized in the table legend; for details of composition see Zucker and Zucker (1).

RESULTS

In figure 1 the top graph shows mean values for the calculated approximation to triglycerides, for rats fed crude stock diets up to 3 years of age. As before, it is an allometric (log-log) plot of the lipid data against net body weight. An interesting difference between this and the FAUns plot of the preceding paper is the elimination of curvilinearity in the postweaning data. This places the data for neutral fat more in line with the expected rectilinearity of such log-log plots, as illustrated by the fat pad data of the preceding paper and the phosphatide and cholesterol data of the present report.

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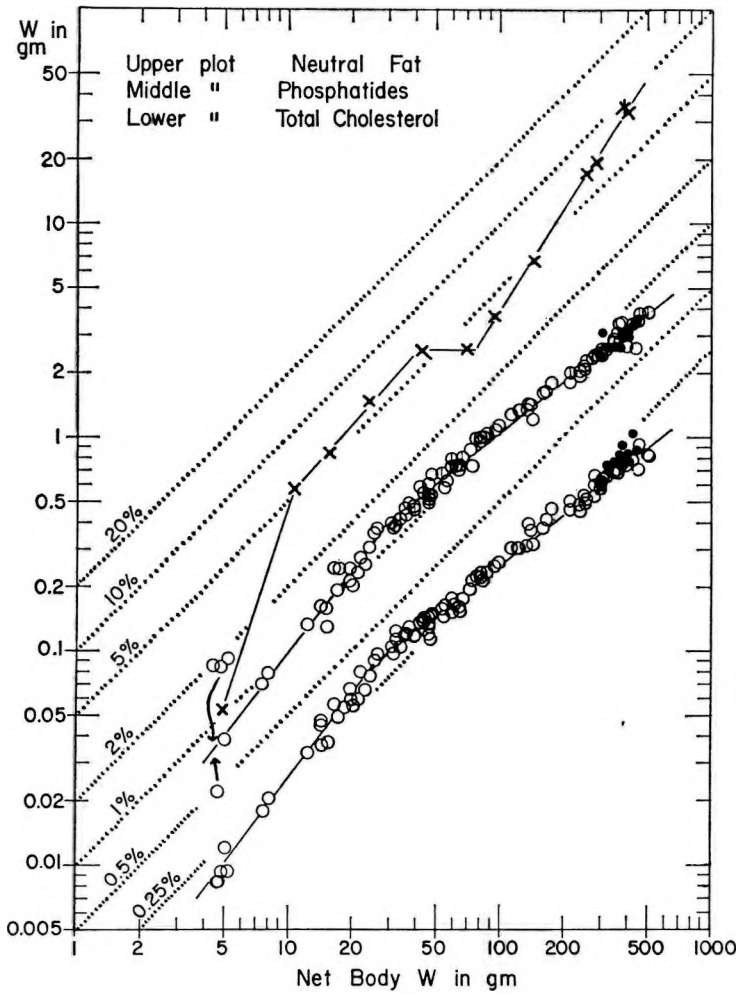

Fig. 1 Relative growth of storage fat (neutral fat or triglycerides), phosphatides and total cholesterol in male rats.

Phosphatides were calculated as 25.1 × lipid P. Neutral fat was calculated as follows: FAUns − 0.7 × phosphatides − free cholesterol − 1.7 × esterified cholesterol = fatty acids (FA) of neutral fat; this was multiplied by 1.05 to get triglycerides (neutral fat). The FAUns (fatty acids plus unsaponifiable) results have been presented elsewhere (1).

In the upper plot (neutral fat), mean values are shown, and for stock diet rats only; the values for the low-bulk diets, being far above the line, are omitted. The symbol for the 2- and 3-year-old rats has a vertical line through it. In the middle and lower plots individual data are shown, including both stock and low-bulk diets. The old (2 and 3 years) rats are symbolized by filled circles, all others by open circles. Diet made no difference in these two plots.

The dotted lines represent various constant percentage values. The data lines in the two lower plots follow the equations $\log y = b_1 \log \times + \log a_1$ up to 30 g body weight, and the same form of equation with parameters b_2 and a_2 thereafter. The values of the parameters are:

	a_1	b_1	a_2	b_2
Phosphatides	0.0052	1.27	0.026	0.80
Total cholesterol	0.0013	1.27	0.0063	0.80

The ratios of the intercepts are 4 and 4.1 for the 2 periods; slopes (b) are the same in each period. While percentages, i.e. y/x, are constantly changing during growth, the straight lines mean that ratios of the form y over x raised to a power are constant; thus in the first period, for both lipids, $y/x^{1.27}$ remains constant, and in the second period $y/x^{0.80}$ is a constant.

In the middle plot are the data for weight of phosphatides obtained on the same individual rats for which FAUns was reported in the preceding paper. Similarly the lowest plot reports total cholesterol values. The remarkable parallelism of phosphatides and cholesterol is evident. The ratio of phosphatides to cholesterol is about 4 throughout (mol ratio of 2). In figure 1 dotted lines of slope 1 are drawn in. These lines describe the path along which constant percentage values lie. For instance, the line for 2% passes through 0.02 at one g body weight, and 20 at 1000 g. In both the phosphatide and cholesterol plots the slope before the break is 1.27 and after this it is 0.80. This means that up to 30 g body weight the percentage values increase, and after that point they decrease. At the weight of 30 g which is at about 17 days of age, the rats began to partake of diet other than mother's milk. This also coincides approximately with the time when myelination of the nerve fibers is complete.

The wide fluctuation in phosphatides of the newborn was not observed elsewhere among phosphatide values or in the cholesterol or fat data.

During growth the pattern of phosphatides and cholesterol is entirely different from that of neutral fat. There is no reflection in the upper plot of the break shown at about 30 g (body weight) by both phosphatides and total cholesterol. On the other hand there is a sharp break between birth and 5 days corresponding to a very high rate of fat accretion right after birth; there is a cessation of neutral fat accretion around weaning (21 to 35 days of age) and a rather steep course of fat accretion from there on. During growth none of these features is reflected in the pattern of phosphatides and cholesterol.

In the previous paper it was shown that rats of various ages fed various experimental diets of low bulk are consistently fatter than rats fed stock diet. In figure 1, values for the rats fed these experimental diets have been omitted from the plot of neutral fat. They would lie well above the line. However, they are included individually in the plots of phosphatides and total cholesterol. Because of crowding it was not feasible to designate these points by separate symbols, but they were not in any way distinguishable in distribution around the lines from the points representing stock diet.

Other information can be derived from the data of table 1. 1. The proportion of cholesterol that is unesterified (% free) decreases steadily during growth. The overall trend is highly significant statistically, although neighboring small groups do not always show a significant difference. 2. The ratio of phosphatides to total cholesterol is fairly constant throughout the first year, with an overall value of around 4 (mol ratio of 2). This has already been noted in connection with the parallelism of the lines in figure 1, but the means and standard errors in the table make a more precise analysis possible. From birth to about 17 days of age, when the rats are ingesting mother's milk only, and while myelination is being completed, the ratio is consistently slightly lower than later. The difference between the 2 means shown in the table, 3.84 and 4.17, is statistically significant. Thus this ratio is not precisely constant during growth; nevertheless even the very close approach to constancy is rather remarkable.

In old age (cf. lines 14 and 15) there is a striking and highly significant lowering of the ratio, from 4.2 to 3.7. This is referable to an increase in cholesterol content, with no particular deviation in phosphatides (see fig. 1, where the old rats are distinguished by a separate symbol). Deuel (4, p. 759) has cited evidence of an increase in brain cholesterol in human old age. We have observed[2] another deviation in the carcass analysis of these old rats; there is a very significant increase in the proportion of water to nitrogen, a change also indicated by Shock (5). Neutral fat, on the other hand, appears to be about the same as in the younger rats of the same size.

On lines 11, 12 and 13 of table 1 are data for the 3 groups of rats fed low-bulk diets. In each case there is approximately twice as much neutral fat as in stock diet carcasses of comparable lean mass (net weight minus adipose tissue). This difference can be seen by comparing the neutral fat value on line 11 (5.37 g) with that

[2] Unpublished data.

TABLE 1

Carcass lipids in male rats

Line	N[1]	Mean age	Body weight				Phosphatides	Cholesterol		Phosphatides
			Gross	Net	Lean[2] mass	Neutral[3] fat		Total	% free	Total cholesterol
		days	*g*	*g*	*g*	*g*	*g*	*g*		
					Stock diet, Sherman rats					
1	5(23)	0	5.2	4.95	4.87	(0.053)[4]	(0.038)[4]	0.0094	90.2 ±1.0[5]	(4.05)[4]
2	4(11)	5.2	11.9	10.7	9.8	0.57	0.109	0.0284	85.3 ±0.8	3.84
3	9(14)	9.7	17.3	15.9	14.6	0.84	0.177	0.0461	86.1 ±1.3	3.84
4	9	15	26.0	24.1	21.9	1.47	0.286	0.0745	83.7 ±1.2	3.83
						Mean and standard error (mother's milk only)				3.84 ±0.056[5]
5	11	25.3	48.3	42.7	38.9	2.54	0.514	0.128	79.3 ±0.6	4.03
6	6	36	81.1	69.6	65.7	2.61	0.79	0.181	80.1 ±1.3	4.35
7	7	39.3	105.6	92.0	86.4	3.73	1.05	0.243	80.8 ±0.9	4.34
8	10	53.3	166.0	143.7	133.7	6.66	1.43	0.353	78.7 ±1.3	4.05
9	8	162	269.6	254.7	228.7	17.3	2.15	0.509	72.6 ±1.3	4.22
10	4	201	427.6	400.0	349.0	34.0	3.24	0.773	72.1 ±1.7	4.20
						Mean and standard error (mixed feed)				4.17 ±0.048
					Low-bulk diets,[6] Sherman rats					
11	14	24.5	53.0	48.6	40.5	5.37	0.571	0.139	79.3 ±0.7	4.12 ±0.058
12	4	150	422.4	406.7	311.1	63.8	3.00	0.672	74.1 ±0.4	4.47 ±0.103
13	6	163	401.8	382.4	294.9	58.4	3.11	0.697	74.1 ±0.4	4.45 ±0.103
					Stock diet, Simms SD rats					
14	13	162.5	298.9	281.0	251.7	19.5	2.34	0.557	72.2 ±1.1	4.20 ±0.045
15	12	888	394.0	371.7	318.7	35.3	2.88	0.779	71.3 ±1.4	3.70 ±0.058

[1] N is the number of analytical samples; where this differs from the number of rats, the latter is given in parentheses.

[2] Lean mass is net weight minus adipose tissue. Adipose tissue was estimated as 1.5 times the weight of neutral fat; this allows approximately for the water and nonfat solids of adipose tissue (unpublished data; see also Brozek (7)).

[3] For the calculation of neutral fat, see the figure legend.

[4] Phosphatides in the newborn were very variable (see fig. 1); values enclosed in parentheses are uncertain for this reason. Possibly the extremely high storage of phosphatides in the liver just prior to birth as described in Everett (6, p. 232) has some bearing on our observations.

[5] SE of mean.

[6] Low-bulk diets; this is a purely descriptive term, and we do not wish to infer that low bulk is the reason that these diets produce fatter carcasses. The diet for line 11 was a paste food consisting of approximately equal parts of whole milk powder, wheat germ, casein, and hydrogenated vegetable oil. The diet for line 13 was a purified casein-glucose diet with only 2% of fat; the diet for line 12 contained 27% of fat isocalorically substituted for glucose in the preceding diet. For details of composition see Zucker and Zucker (1).

of similarly sized rats fed stock diet, on line 5 (2.54 g), and by comparing the values on lines 12 and 13 (averaging about 60 g) with any reasonable interpolation between the values for stock diet animals on lines 9 and 10 (possibly about 30 g). This large difference in fatness has practically no effect on phosphatides and cholesterol (table 1, fig. 1).

DISCUSSION

The division of lipids into element constant and element variable was based originally on the intactness of the former during starvation. While this concept may be in need of some amendment (see (4, p. 597 ff); (8, p. 104)), we see in the foregoing further evidence for the differential behavior of the groups of lipids under conditions other than starvation. During growth the patterns of accretion of the triglycerides on the one hand and of phosphatides and cholesterol on the other differ markedly. Differences also exist in the response to certain dietary effects and old age.

The close parallelism of phosphatides and cholesterol appears to indicate functional interaction of these components.

As we have shown by the use of rats of widely differing body weights at a given age, composition expressed as either absolute weight or percentage is a function of body weight, not of age (1). It has been quite customary to express results of carcass analysis in percentage values, usually related to age. Unless body weights are also reported, the actual weights of components, which are often essential information, are lost to the record. This is unfortunately true of the paper of Williams et al. (9). However, this paper and others by the same authors make a very important plea for more discerning methods of lipid analysis. The fullest significance of this has been impressed on us only in the course of studying our own results. It is reassuring that recently simpler and more reliable methods have appeared, which can be used for the direct determination of lipid entities, especially triglycerides.

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LITERATURE CITED

1. Zucker, T. F., and L. M. Zucker 1963 Fat accretion and growth in the rat. J. Nutrition, *80:* 6.
2. Sperry, W. M. 1942 Electrophotometric microdetermination of phosphorus in lipide extracts. Ind. Eng. Chem., Anal. Ed., *14:* 88.
3. ——— 1938 A micromethod for the determination of total and free cholesterol. Am. J. Clin. Path., Tech. Suppl., *8:* 91.
4. Deuel, H. J., Jr. 1955 The Lipids, vol. 2. Interscience Publishers, Inc., New York.
5. Shock, N. W. 1956 Hormones and the Aging Process. Academic Press, Inc., New York, p. 95.
6. Everett, M. R. 1946 Medical Biochemistry, ed. 2, revised. Paul B. Hoeber, Inc., New York.
7. Brozek, J. 1961 Body composition. Science, *134:* 920.
8. Rossiter, R. J., and K. P. Strickland 1960 The metabolism and function of phosphatides. In Lipide Metabolism, ed., K. Bloch. John Wiley and Sons, New York.
9. Williams, H. H., H. Galbraith, M. Kaucher and I. G. Macy 1945 The influence of age and diet on the lipid composition of the rat. J. Biol. Chem., *161:* 463.

Nutritional Studies with the Guinea Pig

VIII. EFFECT OF DIFFERENT PROTEINS, WITH AND WITHOUT AMINO ACID SUPPLEMENTS, ON GROWTH

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ABSTRACT Both casein and purified soy protein produce good growth rates in young guinea pigs fed purified diets, especially if they are used in the ration at levels of 35 to 40%. To obtain a rapid rate of growth with protein levels of 30%, the casein ration must be supplemented with 0.3% arginine and the soy protein with 0.5% DL-methionine. Poor growth occurred when these proteins were used at levels of 20%. Marked improvement occurred with the addition of 1.0% of arginine to a 20% casein diet and of 1.0% methionine to a 20% soy protein diet. No further improvement occurred when methionine and tryptophan were added to the arginine-supplemented casein diet and this was also true of arginine and tryptophan added to the methionine-supplemented soy diet. Diets containing both casein and soy protein, each at a 10% level, produced better growth than when either protein was used alone at a 20% level. Supplementation of the ration containing both proteins with methionine improved the growth rate. The use of a mixture of soy protein and gelatin, each at a 10% level, produced poor growth. Addition of a mixture of the essential amino acids, except arginine and tryptophan yielded a considerable improvement in growth and with the further addition of tryptophan, a fairly good rate of growth was achieved. Substitution of an isonitrogenous amount of alfalfa for 20% of the casein of the purified diet resulted in a growth rate equal to that obtained with the commercial ration.

Previous results reported from this laboratory with guinea pigs reared with a semipurified diet containing 30% casein showed a growth rate which equalled that observed with a commercial pelleted diet (1). Male guinea pigs (Hartley strain), 2 to 5 days old with an average weight of 104 g, attained an average weight after 6 weeks of 350 to 365 g when fed either the purified or commercial pelleted diet containing approximately 18% protein. A few years later, however, commercial pelleted diets containing approximately 25% protein were formulated to suit the special needs of the guinea pig. In tests made in our laboratory with these latter diets and without any supplements, an average weight was obtained after 6 weeks of 469 ± 16 g. The new diets contained alfalfa and, judging by the brighter green color of the pellets, it was of higher quality with more leafy material than that formerly used. By rapid curing of the alfalfa and selection of plants in an active stage of growth, an improvement might occur, not only from gains in quality and quantity of proteins as such, but also from the presence of other nitrogenous substances such as peptides and free amino acids (2–4). A change in the amount and quality of the alfalfa may have produced an improvement in the nutrient balance of the ration and may have overcome a partial arginine deficiency. Alfalfa is a good source of this amino acid which the guinea pig requires in large amounts (5–7). The possibility that the alfalfa contributed special carbohydrates of nutritive value is also recognized.

The present study was planned to evaluate in the guinea pig the growth-promoting effect of different proteins fed at different levels, in different combinations and with a variety of amino acid supplements; also, to attempt to obtain clues as to the factors responsible for the better growth with the commercial than with the purified diets.

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METHODS

The same methods were used as described in previous publications (1, 8), namely the use of male guinea pigs (Hartley strain) 3 to 5 days of age, ranging in weight from 95 to 115 g and with an average weight of approximately 103 g. Variations in the protein level of the diet were made by altering the amount of starch[2] in diet no. 13 (1).

To study the protein requirement and growth-promoting value of amino acid supplements, diets were prepared with vitamin-free casein,[3] purified soybean protein[4] and gelatin. The levels of the proteins and the various combinations with or without amino acid supplements, are indicated in the tables.

RESULTS AND DISCUSSION

Amino acid supplements. The rate of growth with 30% casein, although only 70% of that obtained with a commercial stock diet (as is shown later) is considered to be very good for a purified diet. With a 20% level of casein, growth was much poorer (table 1). Growth was increased with the addition of 0.3% arginine to a diet containing 30% casein. The increase with a 1.0% arginine supplement was slight, if any. No increase occurred with the addition of methionine or tryptophan. Addition of the latter amino acids, together with arginine at a 1.0% level, yielded the same result as the 0.3% arginine supplement. The addition of 1.0% arginine to a 20% casein diet resulted in a marked increase in growth. No improvement in

[2] To minimize the danger of changes in flavor such as would occur if sugar were the only variable.

[3] The vitamin-free casein was obtained from Nutritional Biochemicals Corporation, Cleveland, or from General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] ADM C-1 Purified Soybean Assay Protein, Archer-Daniels-Midland, Minneapolis.

TABLE 1

Effect of amino acid supplements on the growth of guinea pigs fed purified rations containing 30 or 20% casein

Casein	Amino acid supplements, %	No. experiments	No. animals	Avg wt after 6 weeks
%				g
30	None	8	45	348 ± 31[1]
30	0.3 L-Arginine·HCl	2	10	411 ± 29
30	1.0 L-Arginine·HCl	3	19	363 ± 50
30	0.5 DL-Methionine	2	9	369 ± 36
30	1.0 L-Arginine + 0.5 DL-methionine	2	10	369 ± 45
30	0.25 DL-Tryptophan	2	10	370 ± 51
30	0.5 DL-Methionine + 0.25 DL-tryptophan	1	5	374 ± 28
30	1.0 L-Arginine + 0.5 DL-methionine + 0.25 DL-tryptophan	3	15	400 ± 34
20	None	5	27	245 ± 52
20	1.0 L-Arginine·HCl	3	13	354 ± 36
20	0.5 DL-Methionine	2	10	241 ± 48
20	0.1 L-Tryptophan	1	5	267 ± 28
20	1.0 L-Arginine + 0.5 DL-methionine	3	16	352 ± 40
20	1.0 L-Arginine + 0.1 L-tryptophan	3	15	355 ± 36
20	1.0 L-Arginine + 0.5 DL-methionine + 0.1 L-tryptophan	3	14	374 ± 22
20	1.0 L-Arginine + 0.5 DL-methionine + 1.5 glycine	1	5	350 ± 35

[1] SD.

growth occurred with the addition of methionine or tryptophan. The addition of methionine or tryptophan, together with arginine, did not produce an increase in growth over that secured with the 20% casein diet supplemented with 1.0% arginine. There appeared to be a slight increase in growth when all three amino acids were added to the diet simultaneously. None of the 20% casein diets supplemented with amino acids produced as good growth as the 30% casein diet supplemented with arginine, methionine and tryptophan. This suggests that the 20% casein diet was deficient in one or more of the other amino acids. The substitution of glycine for tryptophan in the amino acid mixture added to the 20% casein diet appeared to reduce growth slightly.

Good growth was secured with the 30% soy protein ration (table 2) but growth was poor when the protein was used at a 20% level in agreement with previous results (9). Addition of 0.5% DL-methionine to a diet containing 30% soy protein stimulated growth but increasing the methionine level to 1.0% did not produce significantly better growth than that of the basal group. The addition of tryptophan to the basal diet or to the one containing 0.5% methionine produced no better growth than did the diet without it. The addition of arginine to the mixture gave no increase in growth.

Adding 0.5% DL-methionine to a 20% soy protein diet resulted in a marked increase in growth. A further increase occurred with the addition of 1.0% methio-

TABLE 2

Effect of amino acid supplements on the growth of guinea pigs fed purified rations containing 30 or 20% soy protein

Protein	Amino acid supplements, %	No. experiments	No. animals	Avg wt after 6 weeks
%				g
30	None	8	54	353 ± 34[1]
30	0.5 DL-Methionine	4	24	394 ± 37
30	1.0 DL-Methionine	2	16	367 ± 40
30	0.25 DL-Tryptophan	1	5	349 ± 30
30	0.5 DL-Methionine + 0.25 DL-tryptophan	2	10	397 ± 21
30	0.5 DL-Methionine + 0.25 DL-tryptophan + 0.5 L-arginine	2	10	396 ± 35
20	None	2	10	262 ± 16
20	0.5 DL-Methionine	5	26	344 ± 34
20	1.0 DL-Methionine	2	10	368 ± 23
20	2.0 DL-Methionine	1	5	341 ± 30
20	1.0 DL-Methionine + 0.25 DL-tryptophan	1	5	331 ± 35
20	0.5 DL-Methionine + 0.2 L-arginine·HCl	2	10	346 ± 39
20	0.5 DL-Methionine + 0.2 L-tyrosine	2	10	341 ± 30
20	0.5 DL-Methionine + 0.2 DL-tryptophan	2	10	345 ± 38
20	0.5 DL-Methionine + 0.2 DL-tryptophan + 0.2 L-arginine	1	5	346 ± 14

[1] SD.

nine, but a 2.0% level appeared to be too high. No improvement was noted with the addition of tryptophan, arginine or tyrosine to a 20% soy protein diet supplemented with 0.5% DL-methionine. The addition of arginine, methionine and tryptophan simultaneously gave the same result as the addition of methionine alone.

Protein mixtures. With 10% each of casein and soy protein, a complementary effect on growth was observed (table 3). Growth was further stimulated with the addition of 0.25% of DL-methionine but no benefit resulted from the addition of 0.1% of L-tryptophan. Addition of the methionine and tryptophan with 0.3% of L-arginine·HCl appeared to have a greater stimulatory effect than the addition of methionine alone.

The addition of 10% gelatin to a 10% soy protein ration produced poorer growth than was obtained with a 20% level of soy protein, probably a consequence of an amino acid imbalance involving an excessively high arginine supply and an inadequacy of tryptophan and methionine. Addition of the amino acid mixture, previously described (see Methods section) resulted in a definite improvement in growth. With the further addition of 0.1% L-tryptophan there was an additional improvement both in growth and in protection of the eyes from cataracts (8).

The apparently high protein need of the guinea pig is chiefly a consequence of a high arginine requirement (5–7). For this reason it is not unexpected that a small supplement of arginine (0.3%) added to a 30% casein ration produces an improvement in growth and a much greater improvement when added (1.0%) to a 20% casein ration. In diets prepared with soy protein, methionine is the chief limiting amino acid (table 4). In agreement with previous results (10) growth was poor when guinea pigs were fed a 20% soy protein ration. These observations with soy protein are to be expected since legumes are recognized as deficient in methionine (11, 12).

In agreement with results reported by Heinicke et al. (6) growth with a 35% casein diet was found to be greater in another study (387 ± 39, Reid (10)) than that with a 30% diet (354 ± 34). Growth as good as that secured with the 35%

TABLE 3

Supplementary effect of protein mixtures with and without amino acid supplements

Diet no.	Protein and amino acid supplements, %	No. experiments	No. animals	Avg wt after 6 weeks
				g
A	20 Casein	5	27	245 ± 52[1]
B	20 Purified soy protein	2	10	262 ± 16
C	10 Casein + 10 soy protein	4	20	300 ± 35
D	Diet C + 0.25 DL-methionine	3	15	327 ± 27
E	Diet C + 0.1 L-tryptophan	1	5	298 ± 27
F	Diet C + 0.25 DL-methionine + 0.1 L-tryptophan	1	5	330 ± 48
G	Diet C + 0.25 DL-methionine + 0.1 L-tryptophan + 0.3 L-arginine·HCl	2	10	349 ± 33
H	10 Soy protein + 10 gelatin	3	14	234 ± 23
I	Diet H + amino acid mixture[2]	4	20	286 ± 34
J	Diet I + 0.1 L-tryptophan	3	15	339 ± 21

[1] SD.

[2] Mixture of essential amino acids (except for tryptophan and arginine) to bring their level in the diet up to that in a 20% soy protein diet plus additional methionine. The mixture of amino acids, added as g/kg of diet consisted of: L-cystine, 0.5; L-glutamic acid, 5.0; L-histidine·HCl, 1.5; L-leucine, 4.0; DL-isoleucine, 10.0; L-lysine·HCl, 2.5; DL-methionine, 5.0; DL-phenylalanine, 3.0; DL-threonine, 5.0; L-tyrosine, 3.0; and DL-valine, 7.0.

TABLE 4

Amino acids in diets

	Commercial[1]	Purified, 30% by weight	
		Casein[2]	Soybean[3]
	%	%	%
Arginine	1.49	1.26	2.49
Cystine	0.36	0.12	0.18
Glycine	1.06	0.63	1.23
Histidine	0.57	0.96	0.78
Isoleucine	1.35	2.25	1.95
Leucine	1.86	3.00	2.25
Lysine	1.32	2.55	2.04
Methionine	0.44	1.05	0.30
Phenylalanine	1.17	1.89	1.50
Threonine	0.92	1.35	1.17
Tryptophan	0.31	0.39	0.30
Valine	1.25	2.31	1.65

[1] Purina Guinea Pig Chow, (Ralston Purina Company, St. Louis); composition given on page 34 of Purina Laboratory Manual, 1962.
[2] Values from Block and Bolling (20) based on a content of 16% N.
[3] Values from ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis, also based on a N content of 16%.

TABLE 5

Composition of commercial and purified diets

	Commercial[1]	Purified[2]
	%	%
Protein	24.62[3]	30.0[4]
Fat	4.20	7.3
Fiber or cellulose	10.40	15.0
Ash	8.50	7.5
Nitrogen-free extract	40.88	38.1
Total accounted for	88.60	97.9[5]

[1] Purina Guinea Pig Chow; see footnote 1, table 4.
[2] Calculated from diet composition, not by proximate analysis.
[3] Based on nitrogen content of 16.0%.
[4] Diet contains 30% vitamin-free casein of which 90% is protein. Therefore, the level of protein in the diet is 27.0%.
[5] 2.1% of the diet consisted of vitamins and acetate from 2.5% of added potassium acetate.

casein diet was noted in the present study with diets containing 30% casein supplemented with 0.3% L-arginine. With the soy protein diet a weight of 404 ± 38 g after 6 weeks was obtained (10) by increasing the protein level to 40%, whereas in the present study the same result was achieved with a 30% protein diet supplemented with 0.5% DL-methionine (394 ± 37). When a diet containing a mixture of 10% casein and 10% soy protein was fed, much better growth was obtained than with 20% of either of the proteins alone. Although casein contains from 2.6 to 3.5% of methionine, the total amount in the mixture (0.36 to 0.45% in the diet) was inadequate. An addition of methionine produced an increase in growth.

The 10% gelatin, 10% soy protein mixture was undoubtedly deficient in most of the essential amino acids except arginine, and for this reason, produced poorer growth than was obtained with a 20% level of soy protein. By supplying the essential amino acids in sufficient amount to this protein mixture, fairly good growth was obtained.

Availability of amino acids in proteins for guinea pigs. The availability of amino acids in a protein may be an important factor in influencing the growth rate. This was true of the tryptophan in casein present in a diet lacking niacin (9). With the soy protein, however, the utilization of tryptophan was good. This previous work by one of the authors showed that if the diet contains ample niacin, the tryptophan requirement was about 0.2% of the diet. This is present in a diet containing 20% of soy protein. In the present experiments, no response was obtained with tryptophan added to a 20% soy protein diet.

The results with a diet containing a mixture of 10% casein and 10% soy protein, suggest that not all of the methionine in this ration is utilized. The soy protein and casein provide 0.45% methionine, whereas the commercial ration[5] (table 4) which produces good growth contains 0.44% methionine. Despite the similarity in methionine levels, the purified ration produced even better growth when extra methionine was added to it.

There is evidence that part of the arginine in casein cannot be fully utilized by the guinea pig. Heinicke et al. (7) reported that a diet containing 35% casein provided 1.22% of arginine, whereas the commercial pellets[6] then in use were found by microbiological assay to contain 0.94%. They considered it doubtful that more than 70% of the arginine in casein is available for the young guinea pig. Since in the present studies the most rapid growth with the casein diets occurred with a 30% level (1.26% arginine) supplemented with 0.3% arginine, it appears that the arginine requirement with this protein is approximately 1.56% of the diet.

[5] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.
[6] Rockland Guinea Pig Pellets. A. E. Staley, Peoria, Illinois.

Growth with purified diets compared with that with diets composed chiefly of natural materials. Animals reared with the commercial diet especially adapted to the needs of the guinea pig, showed an average gain of 126 g over that obtained with the purified diet prepared with 30% protein. Supplementation of the purified diets with suitable amino acids reduced the difference by 50%. The major constituents of the 2 types of diets are shown in table 5 and the percentages of amino acids in table 4. In the commercial diet, 11.4% of its content is unaccounted for.

The purified diet has a higher content of protein, fat and cellulose. The mineral constituents of the 2 diets are not shown in table 5. The chief differences are the higher iron and lower iodine levels in the commercial ration. The zinc content was also higher than in the earlier studies with purified diets. The substitution of the N-salt mixture (13) for the salts used in the earlier studies, increased the zinc content of the purified diet to a slightly higher level than that in the commercial ration. This change in zinc content was not associated with any change in the growth rate. Compared with the commercial ration, the purified diet has a higher concentration of the B-vitamins and ascorbic acid but a considerably lower level of tocopherol and vitamin A plus carotene.

Changes in the purified diet to make its composition more nearly equal to that of the commercial one, produced no further increase in growth rate (table 6). These changes included a reduction of the fat to 5% and cellophane to 10%, a substitution of the N-salt mixture for the previous mixture (14), a doubling of the iron content and a tripling of the levels of vitamins A and E.

It appears that the greater growth of the guinea pigs fed the commercial ration was probably related to its amino acids or carbohydrates, or to both. Possibly the amino acid composition of the improved commercial diets more closely approaches the guinea pig's requirements than do the purified diets. The chief differences in the amino acid content of the commercial and purified rations involve arginine, methionine and tryptophan (table 4). The purified diet with 30% casein is lower in arginine and cystine but considerably higher in methionine than the commercial ration. The purified diet with 30% soy protein has a higher concentration of arginine but a lower level of methionine and cystine than the commercial ration. The present work shows that amino acid supplementation of the purified diets to levels comparable to those in the commercial ration, increased the growth rate but it still did not equal that produced by the commer-

TABLE 6

Comparison of the growth rate with the commercial and purified diets, the latter with various alterations and additions

	No. experiments	No. animals	Avg wt after 6 weeks
			g
Commercial diet[1]	2	13	474 ± 24[2]
Purified diet (30% casein)	8	45	348 ± 31
Purified diet + 0.3% arginine	2	10	411 ± 28
Purified diet (30% soy protein)	8	54	353 ± 34
Purified diet (30% soy protein + 0.5% methionine)	4	24	394 ± 37
Modification of 30% casein diet[3]	1	5	356 ± 15
Modification of 30% soy protein diet[3]	1	5	340 ± 18
30% Casein diet	1	5	347 ± 25
27%[5] Casein diet + 13.7% alfalfa[4]	1	5	370 ± 36
24%[6] Casein diet + 27.4% alfalfa	1	5	469 ± 16
21%[7] Casein diet + 41.1% alfalfa	1	5	439 ± 59

[1] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.

[2] SD.

[3] Modifications of purified diet to make it more like the commercial diet. The changes were: change of the salt mixture to the N-salt mixture (13), reduction of the fat to 5%, of the cellophane to 10%, doubling the iron, and tripling the amounts of vitamins A and E.

[4] Rapidly dried alfalfa harvested in budding stage. The alfalfa was supplied by the Bureau of Plant Industry, U.S.D.A., Department of Agronomy, University of Maryland, and by the Field Crops Laboratory, U.S.D.A., Albany, California.

[5,6,7] 10, 20, and 30% casein substitution levels, respectively, with isonitrogenous amounts of alfalfa.

cial ration. There is a possibility that further refinement in establishing the amino acid requirement of the guinea pig may bring the growth rate produced by the purified diet up to that of the commercial ration.

To determine whether alfalfa had any effect on the growth of guinea pigs fed the purified diet, the casein level of the rations was reduced progressively by 3% increments and an isonitrogenous amount of alfalfa was added. Increases in growth were observed at the 10, 20 and 30% substitution levels,[7] with the greatest effect at the 20% level (table 6). After 6 weeks the average weight of the animals fed the 20% substitution level of casein with alfalfa was 469 ± 16 g, a weight equal to that obtained with the commercial pelleted diet. The gain over that secured with the basal diet containing 30% casein amounted to 35%. It thus appears that the increased growth obtained with the improved commercial diet may have been largely due to changes in quality and possibly also in quantity of the alfalfa component of the diet.

Ershoff (15) found no significant increase in body weight gains as a result of adding 15% of oven-dried alfalfa to diet 13 (1). In his tests the alfalfa was added to the 30% casein diet, not as a substitution for an equivalent amount of nitrogen in the casein. Briggs,[8] however, obtained a 17% increase in weight as a result of adding 10% alfalfa leaf-meal to the basal ration.

Comparison of the protein and amino acid requirements of the guinea pig as compared with those of the rat. With casein as the dietary protein, the minimal requirement of the rat for maximal growth ranges from 16.3% (16) to 25 to 30% (17), whereas for the guinea pig it is about 35% (6, 10). The sequence in which the amino acids in casein become limiting for growth of the guinea pig is arginine, tryptophan and methionine. With the rat, however, the sulfur-containing amino acids are most limiting, threonine is next, then tryptophan, isoleucine, leucine, histidine, valine and phenylalanine all are equally limiting. Lysine is slightly less so than the preceding 6, and finally arginine is the least limiting (18). Table 7 summarizes the requirements of the rat and the guinea pig for arginine, tryptophan and methionine.

TABLE 7

Comparison of the requirement for arginine, tryptophan and methionine by the rat and guinea pig

Amino acids	Requirement	
	Rat[1]	Guinea pig
	% of diet	*% of diet*
Arginine	0.2	0.8[2]
Tryptophan	0.2	0.2[3]
Methionine	0.4	0.48[4]

[1] Harper (18).
[2] Approximate requirement if fully utilized.
[3] Reid (9).
[4] Unpublished data obtained by the author with diet containing 0.12% cystine.

Suitability of dietary proteins for the guinea pig. Purified casein[9] and soybean protein are both good proteins for the young guinea pig if supplied at an adequate level, namely 35 to 40%. Because of its high arginine content the soybean protein is somewhat better adapted to the guinea pig's needs and some of its amino acids such as tryptophan are more readily available. Its low methionine content, however, explains why a dietary intake of 35 to 40% is needed to insure a rapid rate of growth. Casein at a 30% level, supplemented with 0.3% arginine produces an excellent rate of growth. For long-continued studies with purified diets, present evidence is insufficient to justify a statement as to which of these 2 proteins better serves the nutritional needs of the guinea pig.

The differences herein observed in comparative value of casein and soy protein for the guinea pig are considerably less

[7] The casein levels were 27, 24, and 21%, respectively.
[8] Briggs, G. M. 1961 An unidentified growth factor(s) from plant sources for guinea pigs — an improved assay. Federation Proc., 20: 454 (abstract).
[9] Difficulties were encountered in a few experiments with diets containing casein (not included in this report). Poor growth occurred, mortality was high, and little difference in growth was seen between experimental groups which should have shown wide variations. All of the animals produced soft feces. Dr. Robert Habermann, the pathologist who examined the animals, was unable to identify an infective organism. He invariably found large amounts of white material in the cecum and large intestine, evidently undigested casein. At the time this condition was occurring, similar groups of animals were fed soy protein diets. They all grew well from the start. This suggested strongly that the unsatisfactory results were due to a certain lot of casein which may have been a result of some nutritional damage that occurred in the preparation.

striking than those found by Hove and Herndon (19) in the rabbit. They obtained daily growth rates of 15 g with a 20% casein diet, 30 g with an equivalent level of defatted soybean meal and 30 g with a commercial ration. Supplements of arginine, glycine and tryptophan improved growth with the casein diet and accounted for part of the soybean meal effect. The type of carbohydrate in the diet had a minor effect on rabbit growth.

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LITERATURE CITED

1. Reid, M. E., and G. M. Briggs 1953 Development of a semi-synthetic diet for young guinea pigs. J. Nutrition, *51:* 341.
2. Osborne, T. B., A. J. Wakeman and C. S. Leavenworth 1922 The water-soluble constituents of the alfalfa plant. J. Biol. Chem., *53:* 411.
3. Clarke, M. F. 1938 The nitrogen distribution in alfalfa hay cut at different stages of growth. Canad. J. Res., *16C:* 339.
4. Randle, S. B., H. A. Sober and G. O. Kohler 1940 The distribution of the grass juice factor in plant and animal materials. J. Nutrition, *20:* 459.
5. Woolley, D. W., and H. Sprince 1945 The nature of some new dietary factors required by guinea pigs. J. Biol. Chem., *157:* 447.
6. Heinicke, H. R., A. E. Harper and C. A. Elvehjem 1955 Protein and amino acid requirements of the guinea pig. I. Effect of carbohydrate, protein level and amino acid supplementation. J. Nutrition, *57:* 483.
7. ——— 1956 Protein and amino acid requirements of the guinea pig. II. Effect of age, potassium, magnesium and type of protein. Ibid., *58:* 269.
8. Reid, M. E., and L. Von Sallmann 1960 Nutritional studies with the guinea pig. VI. Tryptophan with ample dietary niacin. Ibid., *70:* 329.
9. Reid, M. E. 1961 Nutritional studies with the guinea pig. VII. Niacin. Ibid., *75:* 279.
10. ——— 1963 Nutritional studies with the guinea pig. IX. Effect of dietary protein level on body weight and organ weights in young guinea pigs. Ibid., *80:* 33.
11. Haag, J. R. 1931 The physiological effect of rations restricted principally or solely to the alfalfa plant. II. Cystine as a limiting factor in the nutritive value of alfalfa proteins. Ibid., *4:* 363.
12. Johanson, R. 1948 The cystine plus cysteine and methionine contents of the seed proteins and sulphur distribution of seeds of Vicia Spp (Vetches) with notes on their cyanogenesis. Australian J. Exp. Biol. Med. Sci., *26:* 259.
13. Fox, M. R. Spivey and G. M. Briggs 1960 Salt mixtures for purified-type diets. III. An improved salt mixture for chicks. J. Nutrition, *72:* 243.
14. Briggs, G. M., M. R. Spivey, J. C. Keresztesy and M. Silverman 1952 Activity of citrovorum factor for the chick. Proc. Soc. Exp. Biol. Med., *81:* 113.
15. Ershoff, B. H. 1957 Beneficial effects of alfalfa and other succulent plants on the growth of immature guinea pigs fed a mineralized dried milk ration. J. Nutrition, *62:* 295.
16. Goettsch, M. 1960 Comparative protein requirement of the rat and mouse for growth, reproduction and lactation using casein diets. Ibid., *70:* 307.
17. McCoy, R. H. 1949 Dietary requirements of the rat. In The Rat in Laboratory Investigation, ed. 2, eds., E. J. Farris and J. Q. Griffith. Lippincott Company, Philadelphia, p. 86.
18. Harper, A. E. 1959 Sequence in which the amino acids of casein become limiting for growth of the rat. J. Nutrition, *67:* 109.
19. Hove, E. L., and J. F. Herndon 1957 Growth of rabbits on purified diets. J. Nutrition, *63:* 483.
20. Block, R. J., and D. Bolling 1951 The amino acid composition of proteins and foods. Charles C Thomas, Springfield, Illinois.
21. McCoy, R. H. 1949 Dietary requirements of the rat. page 86 In "The Rat in Laboratory Investigation." Edited by E. J. Farris and J. Q. Griffith, 2nd Ed., Lippincott Co.

Nutritional Studies with the Guinea Pig

IX. EFFECT OF DIETARY PROTEIN LEVEL ON BODY WEIGHT AND ORGAN WEIGHTS IN YOUNG GUINEA PIGS

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ABSTRACT Male guinea pigs were supplied with purified diets containing either vitamin-free casein or purified soybean protein at 2 to 5 days of age and were maintained with the diets for 6 weeks. The protein levels ranged from 20 to 70%. Protein level of the diet influenced growth of the young guinea pig most markedly during the first 2 to 4 weeks. The minimal protein level for maximal growth was 35% with both casein and soy protein. The maximal dietary protein level compatible with good growth was 60% for casein and 70% or possibly more for the soy protein.

The dietary protein level affected notably the rate of growth of the liver and testes. The relative weights (as a percentage of body weight) of the adrenals, pancreas and heart, were not influenced by the dietary protein level. The level of soy protein had a slight effect on the relative weights of the kidneys and spleen, whereas casein had little, if any, effect. The relative weights of both liver and kidneys were greater in the animals fed soy protein than in those fed casein.

Many types of laboratory animals thrive well with diets high in protein if adequately supplied with minerals, vitamins, and essential fatty acids. It is difficult to compare the effects on growth of wide variations in dietary protein levels because few studies have been reported in which the dietary variations involved only the protein and carbohydrate components. There is a lack of reliable information concerning the relationship between the dietary protein level and the organ-to-body weight ratios. Although a number of investigators recorded the weights of the internal organs of their animals, very few of these studies were related to changes in dietary protein levels. Huxley (1) noted that for different animals the growth of the organs was proportional to growth of the body. Webster and Liljegren (2) determined the relative change in weight of the liver, kidneys, lungs, heart and spleen with increasing body weight in mongrel guinea pigs, randomly mated in a closed colony. Eaton (3) reported that the liver and lungs of the guinea pig varied with the body weight, whereas the heart, spleen, kidneys, adrenals and testes in the adult were practically the same weight, regardless of body weight. Walter and Addis (4) observed that in the rat, organ weights may fluctuate within wide limits. Dietary changes, introduced under otherwise constant conditions gave results which indicated that the weight of the liver and kidneys but not the heart varied directly with the protein content of the diet. Hamilton (5) reported similar findings. Bong et al. (6) observed that exclusive ingestion of proteins and peptones by dogs produce an increase in weight of the liver, pancreas and kidneys. Addis et al. (7) observed a decreased weight of the liver and an increased weight of the kidneys in rats maintained with a diet containing 70% casein. No pathological changes were observed in the kidneys.

Osborne et al. (8, 9) reported that there is an almost invariable increase in size of the kidneys of the rat when the protein content of the diet exceeds 50%. The hypertrophic condition appeared when the protein content of the diet was approximately 40%. The kidney response occurred with a variety of proteins. The period required for renal enlargement was unexpectedly brief and retrogression occurred when the protein level was lowered. No reports have been found concerning the relation of the dietary protein level to the

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weight of the spleen, testes, adrenals, pancreas and heart.

An investigation has been under way in this laboratory to determine the effect on growth of the young guinea pig of 1) different proteins; 2) protein mixtures; and 3) amino acid supplements with dietary protein levels of 20 and 30%.[1] Since the laboratory and dietary conditions, except for variations in protein and carbohydrates, were so constant it appeared desirable to study a wider range of dietary protein levels and to determine the effect of protein level on growth of the body and on some of the internal organs.

METHODS

Male guinea pigs of the Hartley strain with a weight range of 95 to 115 g were supplied with the experimental diets at 3 to 5 days of age. From 5 to 15 animals were used in each group. The basal diet (no. 13) and the procedures for the care of the animals were described previously (10). The ration consisted of the following: (in %) protein, 20 to 70; vitamin-free casein[2] or purified soybean protein;[3] corn oil, 7.3; sucrose, 10.3; cellulose, 15.0;[4] cornstarch, 20; glucose, 7.8;[5] potassium acetate, 2.5; magnesium oxide, 0.5; salts, 6 (Briggs et al. (10)); and 0.2 each of choline chloride, ascorbic acid and inositol, and liberal amounts of the known vitamins.[6] Variations in the amount of protein were made by altering the quantity of starch, sucrose and glucose monohydrate.[7] Table 1 shows the variations in carbohydrates made in the formulation of diets containing 20 to 70% protein. The animals were maintained with the diets for a period of 6 weeks, at the end of which time they were killed and the organ weights determined.

RESULTS

Maximal gains in weight for the 6-week period were secured when guinea pigs were fed a ration containing 35% of casein or soybean protein[8] (table 2). At protein levels up to 60%, growth was about the same for the 2 proteins. No additional increase occurred when the casein level was increased to 60% or the soy protein to 70%. Diets containing 70% casein reduced survival and growth. A total of 30 animals were fed the latter ration — of these, 8 died early and 7 grew slowly but succumbed before the end of the test. The apparent unpalatability of the 70% casein diet was attributed in part to its lack of sugar. The addition of a noncaloric sweetening solution[9] appeared to improve its acceptability somewhat but it was unnecessary to use the sweetener with the 70% soy diet. Some differences in growth depression were noted with different lots of casein but all showed the effect to a considerable extent. No fat was observed around the internal organs of the animals

[1] The results of this study are reported in paper no. VIII of this series (11).

[2] The nitrogen content of the casein, determined by Kjeldahl analysis ranged in different lots from 13.26 to 14.00%.

[3] ADM-CIO made by Archer-Daniels-Midland Company, Cincinnati, Ohio. The nitrogen content by Kjeldahl analysis was 12.98%.

[4] Cellulose in the form of Cellophane Spangles, obtained from the Rayon Processing Company of Pawtucket, Rhode Island.

[5] The glucose was used in preparing the mixes of the B-vitamins.

[6] The vitamin E content of the diet (no. 13) was increased in the present study. The amount used consisted of 2.5 mg DL-α-tocopheryl acetate + 2.5 mg DL-α-tocopherol/100 g of diet. This change not only increased the amount of vitamin E but also increased the protection of the vitamin A in the diet.

[7] Cerelose, Corn Products Company, Argo, Illinois.

[8] The differences in weight between the 30 and 35% levels of the 2 proteins are not significant. However, when the values for the 30% protein levels obtained in another experiment by Reid and Mickelsen (11) are used (45 animals, 348 ± 31 g with casein; 54 at 353 ± 34 with soy protein), the differences become significant.

[9] Sucaryl, Abbott Laboratories, North Chicago, Illinois.

TABLE 1

Percentage amounts of protein and carbohydrates in diets

Protein	20	25	30	35	40	50	60	70
Starch	30.0	25.0	20.0	20.0	15.0	5.0	0.0	0.0
Sucrose	10.3	10.3	10.3	5.3	5.3	5.3	0.3	0.0
Glucose monohydrate[1]	7.8[2]	7.8	7.8	7.8	7.8	7.8	7.8	0.0
Cellophane[3]	15.0	15.0	15.0	15.0	15.0	15.0	15.0	10.0[4]

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Cerelose used in preparing the B-vitamin mix.
[3] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.
[4] B-vitamin mix prepared in 3.1% Solka Floc (The Brown Company, Berlin, New Hampshire) instead of Cerelose and the Cellophane Spangles was reduced accordingly.

TABLE 2

Effect of type and level of protein on early growth

Protein	Amount	No. of animals	Average weights			Gain in 6 weeks
			2-weeks	4-weeks	6-weeks	
	%		g	g	g	g
Casein	20	12	128 ± 13[1]	174 ± 27	245 ± 32	136 ± 28
	25	14	136 ± 20	207 ± 30	281 ± 33	176 ± 31
	30	11	156 ± 15	261 ± 27	354 ± 34	247 ± 42
	35	10	164 ± 10	270 ± 27	387 ± 39	281 ± 39
	40	10	167 ± 15	279 ± 23	388 ± 42	282 ± 51
	50	10	168 ± 20	278 ± 26	382 ± 34	277 ± 23
	60	5	194 ± 4	298 ± 14	379 ± 26	269 ± 25
	70	15[2]	171 ± 23	233 ± 32	277 ± 48	169 ± 53
Purified soybean protein	20	14	125 ± 11	188 ± 14	235 ± 30	130 ± 23
	25	9	127 ± 8	197 ± 8	258 ± 23	153 ± 24
	30	15	158 ± 16	257 ± 24	355 ± 35	250 ± 35
	35	9	166 ± 10	283 ± 16	386 ± 32	280 ± 30
	40	9	172 ± 16	297 ± 22	404 ± 38	297 ± 39
	50	5	185 ± 12	300 ± 15	384 ± 25	280 ± 27
	60	5	194 ± 14	297 ± 12	386 ± 17	280 ± 16
	70	9	183 ± 28	292 ± 20	398 ± 29	293 ± 27

[1] SD.
[2] The number of animals that survived to 6 weeks. A total of 30 animals were started with the diet.

fed the 70% casein diet but there was an abundance of it in the 70% soy protein group. Animals fed a 20% level of either casein or soy protein grew very slowly during the first 2 weeks and thereafter growth gradually increased. The retarded growth was associated with a low rate of food intake, which, because of the difficulty in making accurate measurements, was not determined directly. Food consumption was estimated from the length of time a kilogram of diet lasted for a definite number of animals. As the protein levels were increased to 50%, the rate of food consumption and growth both increased during the first 2 to 4 weeks. The average rate of gain in weight per day throughout the 6-week period was 6.7 g for the 35% casein diet and 7.0 for the 40% soy protein diet. Increases of 8.0 g/day occurred in some of both the casein and soy protein groups during the second to fourth weeks.

Effect of protein level on organ weights. In table 3 are shown organ weights expressed as percentage of body weight. The results indicate that the liver and testes were more affected by the level of dietary protein than were those of the other organs.[10] In the animals fed casein, liver weights of the group fed 35, 40 or 50% protein were significantly heavier ($P < 0.05$) than the livers of animals fed 20 or 25% protein.[11] In the soy protein group, the liver weights at 20 and 25% levels were not different, but all higher levels were significantly greater than those of the 2 lower protein groups. The 35% soy protein produced heavier livers than did 30% ($P < 0.01$) but higher levels showed no appreciable increase (the high value for the 50% protein may be a result of too few animals).

The greatest effect of the dietary protein level on organ-to-body weight ratios was noted in the testes, particularly in the animals fed the soy protein. The differences in the relative weights at successively higher levels of soy protein up to 35% were all significant ($P < 0.05$). With both proteins the testes weights reached a plateau at levels higher than 35%. The

[10] In the late phases of this study it became evident that the level of dietary protein influenced the weight of the cecum and large intestine in relation to body weight. At the lower protein levels there was a tendency for a greater accumulation of contents in the cecum and large intestine. In a later study, comparison of these changes at high and low protein levels were measured and will be subsequently reported. Even with this allowance for differences in weight of cecum and large intestine at different protein levels, a clear-cut difference persists in the percentage weight of the liver and testes.

[11] In calculating the averages for the standard deviation values (Sigma) of all organs of animals fed the casein diets the first 7 groups only were included. The eighth group, with the 70% casein level, was omitted because it obviously belonged in a class by itself due to its toxic effect.

TABLE 3

Effect of variations in amount and type of dietary protein on organ weight as percentage of body weight of young guinea pigs fed diets for 6 weeks

Protein	Amount	No. of animals	Liver	Kidneys	Spleen	Testes	Adrenals	Pancreas	Heart
	%								
Casein	20	12	5.64 ± 0.43[1]	1.03 ± 0.04	0.137 ± 0.02	0.137 ± 0.023	0.062 ± 0.013	0.355 ± 0.04	0.324 ± 0.023
	25	9	5.46 ± 0.66	0.942 ± 0.109	0.123 ± 0.015	0.145 ± 0.039	0.0480 ± 0.004	0.303 ± 0.046	0.280 ± 0.019
	30	11	5.26 ± 0.39	0.869 ± 0.052	0.135 ± 0.017	0.166 ± 0.023	0.0407 ± 0.005	0.325 ± 0.057	0.297 ± 0.014
	35	10	6.19 ± 0.70	0.950 ± 0.101	0.172 ± 0.040	0.191 ± 0.025	0.041 ± 0.005	0.306 ± 0.051	0.302 ± 0.024
	40	10	6.06 ± 0.87	1.006 ± 0.126	0.187 ± 0.024	0.181 ± 0.031	0.0403 ± 0.008	0.311 ± 0.037	0.298 ± 0.014
	50	10	6.34 ± 0.43	1.107 ± 0.105	0.151 ± 0.023	0.199 ± 0.037	0.0514 ± 0.007	0.347 ± 0.038	0.321 ± 0.026
	60	5	5.40 ± 0.40	1.104 ± 0.12	0.155 ± 0.015	0.181 ± 0.040	0.0545 ± 0.009	0.334 ± 0.044	0.310 ± 0.024
	70	15	5.37 ± 0.74	1.20 ± 0.09	0.135 ± 0.030	0.126 ± 0.067	0.0700 ± 0.006	0.417 ± 0.058	0.300 ± 0.027
Purified soybean protein	20	14	4.84 ± 0.61	0.960 ± 0.10	0.110 ± 0.049	0.120 ± 0.040	0.0530 ± 0.01	0.350 ± 0.03	0.297 ± 0.03
	25	9	5.21 ± 0.20	0.957 ± 0.055	0.123 ± 0.019	0.154 ± 0.032	0.0531 ± 0.0045	0.294 ± 0.046	0.274 ± 0.032
	30	21	5.74 ± 0.72	0.971 ± 0.098	0.132 ± 0.023	0.183 ± 0.029	0.0399 ± 0.0069	0.301 ± 0.037	0.292 ± 0.018
	35	9	6.81 ± 0.78	1.120 ± 0.12	0.138 ± 0.024	0.217 ± 0.025	0.0480 ± 0.007	0.285 ± 0.034	0.315 ± 0.019
	40	9	6.65 ± 0.49	1.121 ± 0.080	0.156 ± 0.033	0.215 ± 0.040	0.0404 ± 0.0052	0.307 ± 0.030	0.306 ± 0.023
	50	5	7.41 ± 0.77	1.187 ± 0.121	0.168 ± 0.054	0.215 ± 0.051	0.0499 ± 0.0089	0.337 ± 0.023	0.291 ± 0.015
	60	5	6.36 ± 0.85	1.31 ± 0.20	0.133 ± 0.020	0.212 ± 0.055	0.0511 ± 0.0072	0.383 ± 0.027	0.321 ± 0.022
	70	9	6.43 ± 0.70	1.29 ± 0.11	0.149 ± 0.029	0.241 ± 0.038	0.0475 ± 0.005	0.349 ± 0.07	0.307 ± 0.02

[1] SD.

much lower value with 70% casein was a result of the toxicity previously mentioned.

The kidneys of animals fed the soy protein were not different at 20 and 25% levels but all higher amounts increased the weight of the kidneys over the two lower groups ($P < 0.05$). Differences in casein levels produced no significant changes in kidney weights. The weights of the spleen in relation to body weight appeared to be increased slightly by increases in the level of protein; the effect was greater with casein than with soy protein. The relative weights of the adrenals were not influenced by the level of protein in the diet. This was true also of the pancreas. Because of the diffuse nature of the pancreas in the guinea pig, an accurate measurement of its weight is difficult, if not impossible, to make. There was no effect of the dietary protein level on the weight of the heart in relation to body weight.

DISCUSSION

The young guinea pig, unlike the rat, shows no depression in growth when the dietary protein level reaches 60% with casein and up to 70% with soy protein. However, the slowing in the growth rate which occurs after a few weeks at levels of 50% and higher, suggests a limitation to the protein tolerance levels. This was definitely so with casein at a 70% level. The lack of any accumulation of fat around the internal organs of the animals fed the 70% casein diet suggests a deficiency in the conversion of protein to fat. J. N. Williams, Jr.[12] observed that maximal growth of rats was not achieved with casein levels higher than 50%. The minimal level of dietary protein for maximal growth in the guinea pig is approximately 35% with both casein and the soy protein. The minimal level is determined by the peculiar need of the guinea pig for certain amino acids. Its high arginine requirement sets it apart from the rat. By increasing the protein level of the diet a deficiency of an essential amino acid can be overcome. Arginine is the critical amino acid in casein, and methionine is critical in soy protein. It has been shown (11) that maximal growth can be achieved with a 30% level of casein or soy protein if a small amount of the deficient amino acid is supplied. Other studies have shown that the guinea pig does not have a requirement for protein as such since it grows well with a diet containing a well-balanced mixture of amino acids.[13] The stimulating effect of high protein diets on the early growth of the guinea pig, followed later by a slowing in the rate, agrees with observations on rats by Osborne et al. (9). They noted that rats fed high protein diets grew rapidly up to a body weight of 200 to 250 g. After that the body weight increased at a slower rate.

The guinea pig shows an increase in liver weight with increase in dietary protein, particularly with the soy protein. The maximal size of the liver occurred with a 35% level of casein and with a 50% level of the soy protein.

In the guinea pig, in contrast to the rat (7, 8) changes in the dietary casein level, produced little or no change in weight of the kidneys but with the soy protein there appeared to be a slight enlargement at very high levels. On the basis of present knowledge, comparisons between the rat and guinea pig as to the effect of protein level on kidney size, are not justified since the guinea pig studies deal only with the early stages of growth whereas most of the work with rats has been with older animals.

The only other organ in the guinea pig to show a marked response to level of protein was the testis. Growth in weight of this organ appears to be directly related to the protein level of the diet. This was true both with casein and soy protein. The results indicate that even though body weight gains of young guinea pigs are better at high dietary protein levels than normal, the testes increased in weight at a greater rate. Since no histological studies were made, no conclusions can be drawn as to a possible relation of the increased size to the rate of maturation of the tissues. Jackson (12), however, found that by holding the body weight of very young rats constant by underfeeding (milk), there was a marked increase in the weight of the testes accompanied by a normal differentiation of the tissues. It is conceivable that this feeding procedure may have pro-

[12] Personal communication.
[13] Unpublished data by the author.

duced a result somewhat similar to that of an increase in the dietary protein level. Another illustration of the growth of the testes during periods of starvation is that which occurs in the salmon during their migration back to their breeding grounds.

Evidence from the present studies with guinea pigs, together with the results reported by other workers with rats and salmon, strongly suggest the importance of a high dietary nitrogen level for growth of the testes.

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LITERATURE CITED

1. Huxley, J. S. 1924 Constant differential growth-ratios and their significance. Nature, *114:* 895.
2. Webster, S. H., and E. J. Liljegren 1949 Organ: body weight ratios for certain organs of laboratory animals. III. Guinea pig. Am. J. Anat., *85:* 199.
3. Eaton, O. N. 1938 Weights and measurements of the parts and organs of mature inbred and crossbred guinea pigs. Am. J. Anat., *63:* 273.
4. Walter, F., T. Addis 1939 Organ work and organ weight. J. Exp. Med., *69:* 467.
5. Hamilton, T. S. 1939 The growth, activity, and composition of rats fed diets balanced and unbalanced with respect to protein. J. Nutrition, *17:* 565.
6. Bong, E., L. Hilgenberg and P. Junkersdorf 1933 Animal experiments on the effect of unphysiological diets on the composition of organs and metabolic processes. III. Experiments with the exclusive administration of protein and protein-decomposition products. Arch. Exp. Path. Pharmakol., *171:* 16.
7. Addis, T., E. M. MacKay and L. L. MacKay 1926 The effect on the kidney of the long continued administration of diets containing an excess of certain food elements. J. Biol. Chem., *71:* 139.
8. Osborne, T. B., L. B. Mendel, E. A. Park and D. Darrow 1923 Kidney hypertrophy produced by diets unusually rich in protein. Proc. Soc. Exp. Biol. Med., *20:* 452.
9. Osborne, T. B., L. B. Mendel, E. A. Park and M. C. Winternitz 1927 Physiological effects of diets unusually rich in protein or inorganic salts. J. Biol. Chem., *71:* 317.
10. Reid, M. E., and G. M. Briggs 1953 Development of a semi-synthetic diet for young guinea pigs. J. Nutrition, *51:* 341.
11. Reid, M. E., and O. Mickelsen 1963 Nutritional studies with the guinea pig. VIII. Effect of different proteins, with and without amino acid supplements, on growth. Ibid., *80:* 25.
12. Jackson, C. M. 1932 Structural changes when growth is suppressed by undernourishment in the albino rat. Am. J. Anat., *51:* 347.

Effect of Chromium, Cadmium and Other Trace Metals on the Growth and Survival of Mice[1]

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ABSTRACT Seven hundred weanling white mice were fed a diet of rye, corn oil and dried skim milk containing no detectable cadmium and only small amounts of chromium, titanium, lead and nickel. They were housed in quarters from which most contaminating metals were excluded. Groups of 100 or more animals were given 5 ppm of these metals in drinking water as soluble salts. Trivalent chromium increased the growth of both sexes and lessened the mortality of male animals up to 17 months of age. Tetravalent titanium increased growth, but did not significantly affect mortality. Divalent lead, nickel and cadmium had no marked effect on growth, mature weights nor mortalities up to 18 months of age, but after that, cadmium and lead significantly increased death rates in males when tissue levels were less than or equal to those of adult human beings. Mice "cadmium-free" by microanalytical methods (< 0.02 μg/g, wet tissue) grew normally and did not differ in any obvious way from those given the metal, except for prolonged survival of males. Chromium appears to act as an accessory trace metal for growth and survival while cadmium is not essential in these terms, having an innate "toxicity" similar to lead.

Several "abnormal" trace metals have been found to accumulate with age in the tissues of man (1, 2), these include cadmium (3), lead (4), titanium (5) and tin. Six considered "essential" as having important metabolic functions (manganese, iron, cobalt, copper, zinc, molybdenum), do not accumulate, despite relatively large exposures in food and water. Three other transitional metals also do not increase in concentration with age, except in lung, but their biological significances are not known; vanadium, chromium (6) and nickel (7).

The purpose of this communication, the first of several on the subject, is to report the effects on growth and survival of 5 "abnormal" trace metals given to weanling mice fed a diet adequate for growth and reproduction. The metals were present in the diet in low concentrations or were absent, whereas the "essential" ones were provided in adequate amounts. "Abnormal" metals were given in concentrations designed to reproduce in so far as possible the tissue concentrations found in man (2). Environmental contamination was kept at a minimum. One metal, trivalent chromium, appeared to show biological activity on both these parameters, while lead and cadmium affected only survival.

METHODS

About 700 weanling white mice of the Charles River CD strain were housed in acrylic plastic cages with stainless steel covers (8). Six mice occupied each cage. Kiln-dried wood chips were used for bedding. Water bottles were of polyethylene with rubber stoppers and stainless steel or Pyrex drinking tubes (depending on the metal given). Stoppers were protected from nibbling by mice.

The animal quarters, constructed of wood, were situated at the top of a remote hill 500 m high at the end of a mile-long (1.609 km) dirt road, in order to avoid airborne contaminants, especially those from motor vehicle exhausts (4). Material used in the building was analyzed for the trace metals studied, and contamination prevented by triple coats of plastic varnish.[3] Cage racks were of white pine;

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[2] Requests for reprints should be sent to 75 Linden Street, Brattleboro, Vermont.

[3] Fabulon, Pierce and Stevens Chemical Corporation, Buffalo, New York.

all nail heads were covered with plastic wood. A minimum of varnished metal objects was allowed in the quarters and no galvanized metal was used inside or outside. Quarters were heated by butane gas vented externally and were dehumidified. Environmental temperatures were kept between 21 and 28° C. Air inlets, near a forest 100 feet (30 m) from the road, consisted of two electrostatic precipitators continuously operated; negligible quantities of dust accumulated in them yearly. Maximum precautions were taken against introducing metallic contaminants from the outside, and shoes were removed when entering the animal room. All procedures were performed in a separate room.

The diet consisted of whole ground seed rye,[4] 60%; powdered skim milk, 30%; corn oil, 9%; and sodium chloride, 1%. The rye was ground into flour at a local mill. To each kilogram was added: 100 mg ferrous sulfate; 5,000 IU vitamin A; 1,000 IU vtamin D; 10 mg Ca pantothenate; 50 mg niacin; and 1.0 mg pyridoxine hydrochloride. These amounts, plus others present in rye and milk, were similar to the vitamin contents of standard commercial diets. Concentrations of "abnormal" trace metals are shown in table 1. Food was prepared twice a week. Diet was given ad libitum.

Soft water was supplied from a forest spring through 210 feet (65 m) of polyethylene pipe, passed through a bulk water softener, a bulk demineralizer[5] and a laboratory demineralizer.[6] It contained no detectable metal and showed a resistance of more than one million ohms. To it was added in ppm metal: Zinc acetate, 50; cupric acetate, 5; manganous citrate, 10; cobaltous chloride, one; and sodium molybdate, one; these amounts being comparable to those found in commercial diets when converted to a mean water intake of 4 g/day.[7] This solution was known as "basic water." To it was added one of the following at 5 ppm metal: Chromic acetate, potassium titanium oxalate, nickelous acetate, cadmium acetate, or lead acetate. The final solutions were clear and colorless.

Water bottles were weighed to the nearest gram and tabulated continuously to measure the total intakes of metals. The mice were weaned 21 to 23 days after birth and immediately presented with the appropriate water solution, which remained identical for their lifetimes. They were weighed in groups of 6 at weekly intervals for 2 months and thereafter every month: they were handled as little as possible and no therapeutic measures were used. Cages, washed in demineralized water, were not interchanged from one group to another. Unnecessary noise and traffic in the quarters was restricted. Pilot studies with metal-containing and deionized water revealed no aversion to the basic water. During the period of the experiment no epidemics occurred in the animal quarters.

Data were analyzed statistically to determine the significance of differences between two means, using Students' *t* test, and for abnormal distribution, using chi square. Tissues of mice dying of "natural" causes were analyzed by microanalytical chemical methods previously reported (3–7).

TABLE 1

Concentrations of "abnormal" trace metals in mouse diets and bedding

Metal	Commercial diets	Special diet[1]	Bedding (softwood chips)
	μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*
Chromium	ND[2]– 2.3[3]	0.10	0.11
Titanium	ND –11.7[3]	0.03	2.31
Nickel	0.4 – 3.0[3]	0.40	0.32
Cadmium	0.1 – 0.2	ND	0.05
Lead	0.4 – 1.9[3]	0.19	1.83

[1] The intake in food per day per mouse was approximately 4 times the amounts shown.
[2] ND indicates not detected (< 0.005 μg/g).
[3] Obtained by Keenan, see footnote 7 in text.

[4] Untreated seed rye, one lot for a year's supply, was obtained from Eastern States Farmers Exchange and stored in a tightly closed space provided with dehumidification. No gross spoilage occurred.

[5] Culligan Water Institute, Northbrook, Illinois.

[6] Barnstead Still and Sterilizer Company, Boston.

[7] According to Keenan, commercial diets contain the following "essential" metals by spectrographic or chemical methods (ppm): Zinc, 58.8 to 66.7; copper, 7.1 to 11.7; manganese, 6.3 to 20.7; cobalt, < 0.4 to < 08; molybdenum, < 0.5 to 5; iron, 130 to 600. A diet similar to ours devised by Schneider, using whole wheat, showed: Zinc, 50.1; copper, 3.4; manganese, 6.0; molybdenum, not detected; and iron, < 0.5 ppm. (Keenan, R. G., U. S. Public Health Service 1958 Occupational Health Field Headquarters, Cincinnati, personal communication). Therefore, the intake of trace metals on our regimen was somewhat high for zinc but was well within the ranges of other metals found in commercial diets considered adequate for nutrition and without mineral imbalances.

RESULTS

Growth and mature weights. The mean weights of the mice given the several metals are shown in tables 2 and 3. No significant differences from the controls appeared in those receiving lead, cadmium and nickel. Males and females given chromium and titanium, however, gained weight more rapidly and when mature were heavier than their controls. Although significant differences were not evident at 2 months of age, the growth curves diverged at 80 to 100 days of age (figs. 1–5). These two metals increased mean body weight of males about 14% at 6 months and 22% at 12 months of age; they likewise affected female mice approximately 9 to 16% at both intervals.

Mortalities. Under the conditions of the experiment, all female mice were healthier than males in terms of survival. Tables 4 and 5 illustrate survival at various periods. No significant differences in mortality occurred among the female groups. Males given chromium had a lower mortality at one year of age than did the controls ($P < 0.001$), which continued to 16 months ($P < 0.02$) and 17 months ($P < 0.05$) but not later. Therefore, chromium appeared to affect male mortality in the first half of life.

Cadmium and lead, on the other hand, caused increased mortalities appearing in the latter half of life. The administration of cadmium was associated with significant differences at 21 months ($P < 0.001$) and 24 months ($P < 0.01$) of age, as compared with their controls, 98.0 and 81.1%, respectively, having died. Similarly more males given lead were dead at 21 months ($P < 0.05$). These increased mortalities were not apparent at 16 or 18 months. Neither titanium nor nickel obviously affected mortality at any interval up to 18 months of age.

TABLE 2

Mean weights of male mice given various trace metals (5 ppm in drinking water)

Metal	No. of animals	Body weight at indicated ages		
		60 days	180 days	360 days
		g	*g*	*g*
Control	62	35.6 ± 0.38[1]	45.2 ± 1.09[1]	45.1 ± 1.61[1]
Chromium	54	36.3 ± 0.89	51.6 ± 1.19	55.4 ± 1.15
P[2]			< 0.005	< 0.001
Titanium	54	36.7 ± 1.84	52.4 ± 1.40	54.8 ± 1.36
P			< 0.005	< 0.001
Nickel	50	34.9 ± 1.27	45.6 ± 2.05	45.8 ± 2.35
Cadmium	65	35.4 ± 0.62	46.0 ± 1.57	51.5 ± 1.77
Lead	52	33.5 ± 1.56	46.7 ± 1.97	48.8 ± 2.18

[1] Mean weight ± SE of mean.
[2] Probability of difference being due to chance by Student's *t* test. Only significant *P* values are shown.

TABLE 3

Mean weights of female mice given various trace metals (5 ppm in drinking water)

Metal	No. of animals	Body weight at indicated ages		
		60 days	180 days	360 days
		g	*g*	*g*
Control	88	29.4 ± 0.38[1]	41.0 ± 0.73[1]	46.4 ± 1.20[1]
Chromium	54	30.7 ± 0.62	45.0 ± 1.32	50.7 ± 1.64
P[2]			< 0.025	~ 0.03
Titanium	53	31.3 ± 1.08	46.2 ± 1.19	53.9 ± 1.86
P			< 0.005	< 0.005
Nickel	54	29.8 ± 1.45	39.7 ± 2.05	43.7 ± 1.63
Cadmium	60	28.6 ± 0.46	43.1 ± 1.00	49.5 ± 1.12
Lead	52	27.9 ± 1.22	43.2 ± 2.52	48.6 ± 2.46

[1] Mean weight ± SE of mean.
[2] P indicates probability of difference being due to chance by Student's *t* test. Only significant P values are shown.

Significant differences between groups are shown in table 5. Males given chromium outlived those fed cadmium, lead and nickel. Females given lead outlived those receiving cadmium.

When the half-lives of the male groups (time when 50% were dead) were compared, the various metals appeared in the following order of lethal effects: Nickel > lead > cadmium > titanium = control >> chromium. The first 3 metals decreased the half-lives of their groups by 1 to 2 months from that of the controls while chromium increased it by 100 days (19.6%).

Nutritional adequacy of diet. Growth curves provided by the manufacturer[8] of a commercial diet showed that male mice 60 days old weighed about 27 g and females 24 g. Although the strain of mice fed this diet may have differed from ours, our control animals at 6 weeks of age were about equal in weight to those fed the commercial diet at 8 weeks, and were about 15 and 28% heavier at 2 months. None of the metals depressed growth or mature weights. The rate of growth of animals given cadmium, lead and nickel was almost identical to that of controls. In this sense the diet appeared adequate. There was, however, a somewhat excessive mortality confined to males (tables 4 and 5), 20% of the controls having died at 9 months and 31% at 12 months of

[8] Rockland Mouse Diet, Rockland Farms, New City, New York.

TABLE 4

Mortality of mice given various trace metals

Metal given	No. animals at 2 months of age	% Surviving, months				Half-life
		6	12	18	21	
						days
Male						
Controls	61	96.7	68.8	47.3	35.9	510
Chromium	54	100.0	92.6	61.1	38.8	609
P[1]			<0.001			
Titanium	54	98.1	85.2	42.1	27.8	513
P			<0.05			
Nickel	50	100.0	66.0	38.0	30.0	449
Cadmium	65	83.1	60.0	32.2	8.0	476
P		<0.02			<0.001	
Lead	52	96.2	67.3	28.8	19.2	463
P	—				<0.05	
Total	336					
Female						
Controls	88	97.7	93.2	72.7	53.4	662
Chromium	54	98.1	98.1	72.2	48.2	639
Titanium	53	96.2	88.7	78.6	49.1	624
Nickel	54	100.0	92.4	71.7	57.4	673
Cadmium	60	98.3	90.0	66.7	50.0	636
Lead	52	100.0	92.3	78.8	69.2	704
Total	361					

[1] P indicates probability of the differences from controls being due to chance by χ^2 analysis.
[2] Series incomplete.

TABLE 5

Significant differences between groups by χ^2 analysis, P

	Months	Cadmium	Lead	Nickel
Male				
Chromium	12	<0.0005	<0.001	<0.001
	18	<0.005	<0.001	<0.0005
	21	<0.0005	~0.025	
Female				
Cadmium	21		<0.05	

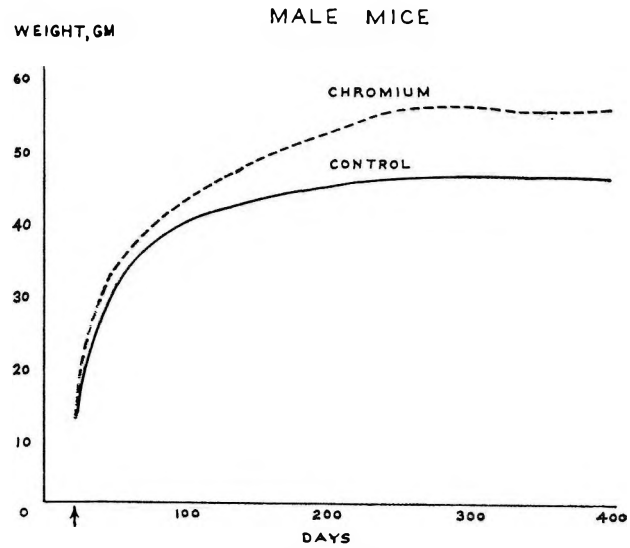

Figure 1

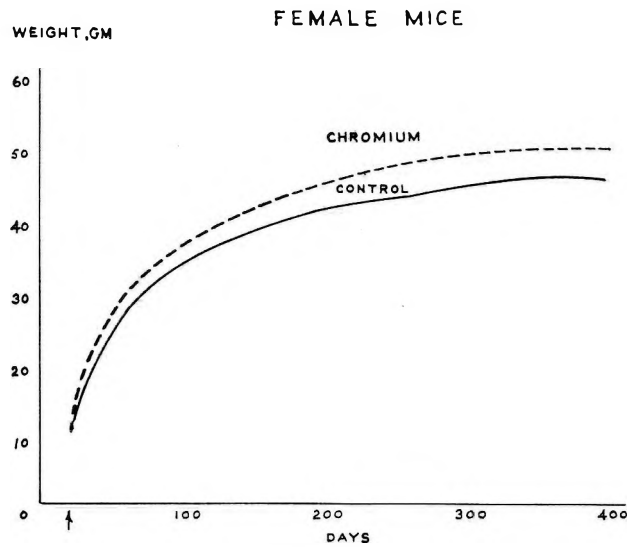

Figure 2

Figs. 1 and 2. Curves of growth of white mice given trivalent chromium at 5 ppm in drinking water from the time of weaning (shown by arrow). The continuous line indicates the mean weights of the controls, the interrupted line those of the treated animals. The abscissa shows days of age. Differences are statistically significant at 180 and 360 days of age (see tables 2 and 3).

age. This early mortality was shared by the groups given cadmium, nickel and lead, but not by those fed chromium and titanium. The survival of all female groups for the duration of the experiment was reasonable and in them the regimen showed no innate toxicity.

Absorption of trace metals. Analyses of several organs for each of the trace metals given to mice revealed that concentrations were increased, whereas less was usually found in tissues of the other 5 groups serving as internal controls. Detailed results showing rates of accumula-

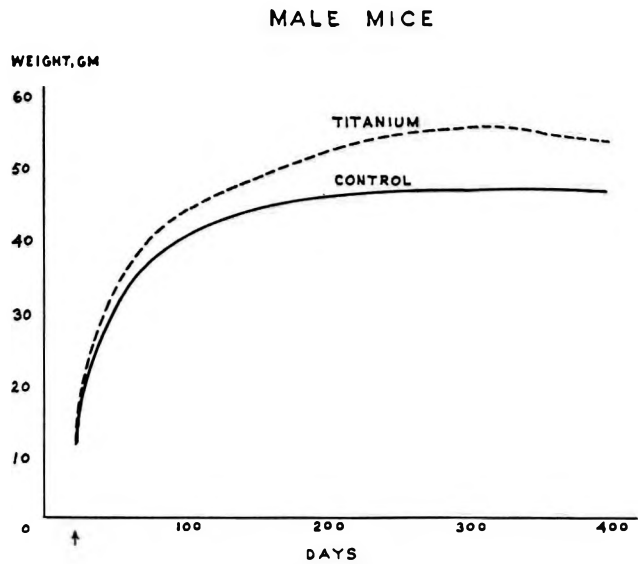

Figure 3

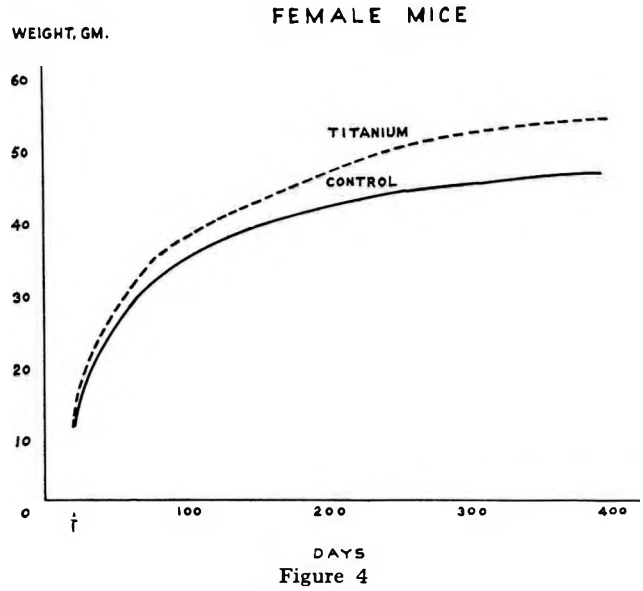

Figure 4

Figs. 3 and 4 Curves of growth of white mice given tetravalent titanium at 5 ppm in drinking water. Notations same as in figure 1. Differences are statistically significant at 180 and 360 days of age (see tables 2 and 3).

tion will be reported in a subsequent communication, but a brief summary is indicated in table 6. No cadmium (< 0.02 μg/g, wet weight) was detected in more than 50 kidneys and livers from the control and the other 4 groups not given this metal. These animals can be considered virtually "cadmium-free." Renal and hepatic cadmium were regularly found in mice given it, but not in the high concentrations usually seen in adult American human beings (1). Little chromium appeared in tissues from 5 groups; while concentrations exceeded those of adult

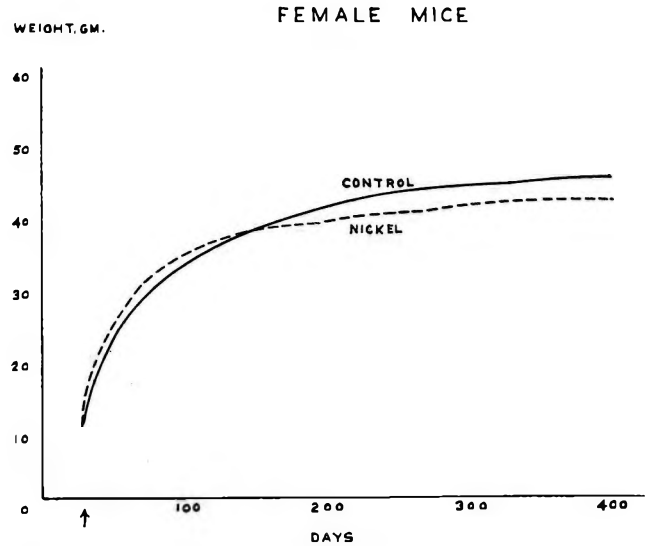

Fig. 5 Curves of growth of white mice given divalent nickel at 5 ppm in drinking water from the time of weaning. Notations are same as in figure 1. Differences were not significant.

TABLE 6

Abnormal trace metals in tissues of mice and man[1]

Group	Organ[2]	Mice		Adult man[3]	
		Not given metal	Given metal	Mean	Range
		μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*
Chromium	liver	0.0 –0.1	0.87– 1.28	0.02	<0.001– 0.3
Titanium	lung	2.5 –4.0	7.7 –18.1	2.8	0.1 –11.0
Cadmium	kidney	0.0	2.9 – 6.14	32.0	11.0 –66.0
Lead	liver	0.05–0.71	0.67– 3.3	2.0	0.5 – 3.2
Nickel	kidney	0.08–0.59	0.9 – 3.7	0.09	<0.05 – 0.9

[1] Data based on analyses of animals dying at 360 to 480 days of age.
[2] Organ of high concentration.
[3] From Tipton (1).

man many fold in animals receiving it. Nickel was considerably increased in mice given it, whereas their controls showed lesser amounts.

We were unable to prevent accumulation of lead and titanium by animals receiving little of these metals in the diet, probably because of the large amounts present in bedding. Control animals had pulmonary concentrations of titanium in the range of those of adult human beings and accumulated several times as much when given the metal orally. Mice given lead, on the other hand, showed hepatic and renal levels comparable to those of adult man, with lesser amounts in the controls.

DISCUSSION

The purpose of these experiments was to duplicate in mice the tissue concentrations of metals found in man and to observe any obvious effects. We were partly successful. Thus, "cadmium-free" mice were grown and compared with mice having renal concentrations up to one-fourth of that of man. Mice with relatively low levels of hepatic lead were compared with animals having concentrations approximately similar to the human. On the other hand, control mice with concentrations of chromium, titanium and nickel comparable to those of adult human beings were paired with animals accumulating larger quantities.

Cadmium-fed mice grew normally, with almost identical growth curves to those "cadmium-free." The striking difference between the 2 male groups was the late mortality in that given the metal. This difference was significant at 21 and 24 months. Females did not show this trend. The data indicate that cadmium is not essential for the growth of mice; on the contrary, it shortened the life span of males at renal and hepatic concentrations considerably less than those found in adult human beings. Therefore, cadmium can be considered a metal with innate toxicity in terms of the survival of male mice.

The effects of lead were similar, but less strikingly so with male mice, whereas females tolerated it better than any other metal. A late mortality appeared when hepatic concentrations were in the human range. These sex differences in results are unexplained.

The action of chromium on growth and survival differed considerably. Growth rates were enhanced in both sexes. Furthermore mortality up to 17 months of age was partly inhibited in male animals, who also outlived their counterparts given cadmium, lead and nickel. Therefore, chromium appeared to act as an accessory trace metal in terms of growth and of male survival.

It is unlikely that the enhancement of growth by chromium was the result of an action on intestinal bacteria such as occurs with certain antibiotics. The daily dose was too small, about 20 μg (or < 1 μg/g body weight). There is no evidence that chromium accumulates in the lumen of the large intestine. Trivalent chromium has a low order of toxicity for most living things (6), much less than some of the metals given in water (copper, cadmium, lead).[9] Furthermore, a similar effect with titanium was probably not caused by a favorable action on intestinal flora, for titanium has little or no toxicity to living organisms (5). The animals, however, had access to their feces, and this explanation, although improbable, must be considered.

Titanium at high tissue concentrations did not appear to affect mortality of males, the half-lives of this group and of the controls being similar. Evidence for its action as an accessory trace metal does not include effect on survival. The mechanism of action is unknown. Nickel did not manifest toxicity at any specific interval, although the half-life of the male group was shorter than any other.

More than 200 analyses of various foods were made to obtain a diet cadmium-free and low in the other metals used in the experiment. Our diet represents the best compromise, other than the use of synthetic foods, some of which are also contaminated by metals. The cost is low. Softwood chips were used for bedding despite the lead and titanium present. A number of other materials were analyzed and tested, but none was free from cadmium, lead and chromium and had absorptive qualities. Stainless steel wire cage floors were considered, but were found to donate traces of nickel and chromium to slightly acid solutions. Obviously, the environmental exposures of the regimen were poor for studying titanium and only fair for lead, facts which did not become apparent until tissues were analyzed. On the other hand they were reasonably good for chromium and nickel and excellent for cadmium.

The work of Schwarz and Mertz (10) has indicated that trivalent chromium may be an accessory essential trace metal for rats, preventing the low glucose tolerance induced by deficient diets and, with insulin, promoting *in vitro* the synthesis of glucose from acetate (11). The observations here reported tend to support their idea of the essentiality of chromium for mice, not especially for "normal" growth and development, but possibly for general health or resistance to disease. Parallel effects on these parameters for male rats will be reported subsequently (12). The effect of chromium, cadmium and lead on survival of male mice make these trace metals pertinent to study with other mammals, especially at tissue levels similar to those of man.

The following conclusions may be drawn from these experiments on mice: 1) Cadmium is not "essential" in terms of growth and survival, cadmium-free mice being successfully raised. On the

[9] In fact, chromium ions stimulated the growth of azobacteria (9).

contrary, small doses sufficient to provide renal accumulations about one-fourth of those of adult American human beings caused significantly increased mortality in males. Cadmium, therefore, exhibits an innate toxicity in terms of survival; 2) titanium acts as an accessory growth factor on mature weights when tissue concentrations are high, but does not affect survival; 3) nickel in these doses affects neither mortality nor growth; 4) lead, at hepatic concentrations equal to those of man, is associated with significantly increased male mortality and exhibits an innate toxicity similar to cadmium, although not affecting growth; 5) chromium acts as an accessory growth factor and inhibits male mortality during the first half of life. Thus, chromium resembles an "essential" trace metal in these terms.

LITERATURE CITED

1. Tipton, I. H. 1960 The distribution of trace metals in the human body. In Metal-Binding in Medicine, ed., M. J. Seven. J. B. Lippincott Company, Philadelphia.
2. Tipton, I. H., and M. J. Cook 1963 Trace elements in human tissue II. Adult subjects from the United States. Health Physics (in press).
3. Schroeder, H. A., and J. J. Balassa 1961 Abnormal trace metals in man: Cadmium. J. Chron. Dis., *14:* 236.
4. ——— 1961 Abnormal trace metals in man: Lead. Ibid., *14:* 408.
5. Schroeder, H. A., J. J. Balassa and I. H. Tipton 1963 Abnormal trace metals in man: Titanium. Ibid., *16:* 55.
6. ——— 1962 Abnormal trace metals in man: Chromium. Ibid., *15:* 941.
7. ——— 1962 Abnormal trace metals in man: Nickel. Ibid., *15:* 51.
8. Schroeder, H. A., and W. H. Vinton, Jr. 1962 Hypertension induced in rats by small doses of cadmium. Am. J. Physiol., *202:* 515.
9. Perlman, D. 1945 Some effects of metallic ions on the metabolism of Aerobacter aerogenes. J. Bact., *49:* 167.
10. Schwartz, K., and W. Mertz 1959 Chromium (III) and the glucose tolerance factor. Arch. Biochem. Biophys., *85:* 292.
11. Mertz, W., E. E. Roginski and K. Schwarz 1961 Effect of trivalent chromium complexes on glucose uptake of fat tissue. J. Biol. Chem., *236:* 318.
12. Schroeder, H. A., W. H. Vinton, Jr. and J. J. Balassa 1953 Effects of chromium, cadmium and lead on the growth and survival of rats. J. Nutrition, *80:* 48.

Effects of Chromium, Cadmium and Lead on the Growth and Survival of Rats[1]

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ABSTRACT Eight groups of more than 50 hooded rats were each given (at 5 ppm) cadmium, trivalent chromium or lead in drinking water while fed a diet of rye flour, dried skim milk and corn oil containing no detectable cadmium and only small amounts of chromium and lead. Extensive precautions were taken to avoid environmental metallic contamination. The diet was nutritionally adequate for survival, all but 7 control animals surviving 21 months. Mortality of rats receiving chromium was equally low. Although those given lead and cadmium grew normally, survival was considerably reduced when their tissue concentrations of metal were approximately similar to or less than those of adult human beings. No metallic toxicity appeared. Growth of males was stimulated by chromium and mature animals were larger than the controls. Cadmium was not detected in the livers and kidneys of animals not given it, although they grew normally and survived. Cadmium is not an "essential" trace metal for rats in terms of growth and survival; it shortens their lives at less than human renal concentrations. Lead at human tissue concentrations has a similar innate toxicity in terms of survival. If chromium has a metabolic function, these experiments confine it to male rats.

In a preceding study on white mice (1), we reported that: 1) Trivalent chromium given in drinking water at 5 ppm increased rates of growth and mature weights; 2) chromium appeared to increase survival in males up to 510 days; 3) cadmium and lead at the same concentrations did not interfere with growth; 4) "cadmium-free" mice grew normally, whereas males given cadmium showed an excessive mortality; 5) lead appeared to increase death rates in older males. The present parallel study on rats is concerned with the effects of these trace metals on growth and survival up to 21 months of age. It shows that rats behave similarly to mice in the presence of these metals.

METHODS

Weanling Long-Evans (hooded) rats of the Rockland strain were housed in plastic cages of 4 each in special "metal-free" quarters, as described previously (1). Most of them were raised in the quarters. All 428 animals were fed a diet of rye flour, dried skim milk and corn oil, with added vitamins and iron; it contained no detectable cadmium, 0.1 μg/g chromium and 0.19 μg/g lead. "Essential" trace metals were added to metal-free water exactly as in the parallel study on mice, with 5 ppm chromium, cadmium or lead given in the water to groups of more than 50 males and 50 females from the time of weaning (28 days of age). Two groups receiving none of these metals served as controls. Microanalytical chemical methods were used to control metal intake in food and water at regular intervals and to assess accumulations in tissues of animals dying, as reported (2–4). The conditions of the experiments on rats exactly duplicated the study of mice.

RESULTS

Effects on growth. Table 1 shows the mean weights of the 8 groups at three intervals. Males given chromium grew more rapidly and weighed more when mature than did the controls (fig. 1). Males given lead and cadmium were sig-

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TABLE 1

Weight of rats given chromium, cadmium and lead, 5 ppm in drinking water, at various ages

Metal	No. of animals[1]	Body weights at indicated ages		
		60 days	180 days	360 days
		g	*g*	*g*
Male				
Controls	52	143.5 ± 4.47[2]	329.3 ± 6.28	388.3 ± 8.23
Chromium	54	168.0 ± 3.50	358.6 ± 6.86	440.7 ± 6.48
P[3]		< 0.001	< 0.005	< 0.001
Cadmium	52	165.2 ± 3.73	340.5 ± 5.97	393.1 ± 6.13
P		< 0.005		
Lead	50	157.5 ± 3.15	330.1 ± 4.12	375.2 ± 10.57
P		< 0.01		
Female				
Controls	52	119.2 ± 3.87	219.8 ± 2.93	238.5 ± 3.94
Chromium	54	131.0 ± 3.76	229.4 ± 5.39	256.8 ± 5.92
P		< 0.025		~ 0.01
Cadmium	61	140.1 ± 2.87	233.8 ± 3.14	254.0 ± 4.56
P		< 0.001	< 0.001	~ 0.01
Lead	53	136.3 ± 3.75	225.1 ± 2.89	252.0 ± 7.35
P		< 0.005		

[1] Number started on metal at 28 days of age.
[2] Mean ± SE.
[3] *P* = probability of the difference from the controls being due to chance, by Student's *t* test. Only the significant differences are shown.

nificantly heavier at 60 days, but not later. Females given all 3 metals weighed more at 60 days; those receiving cadmium and chromium were larger than their controls at one year of age (fig. 2), whereas those receiving lead were not. No suppressive effects on growth were apparent in the 3 metal groups. No obvious obesity was observed, except for one female receiving lead, apparently a chance deviation.

There were no significant differences in weight among the 3 metal-fed groups of either sex at 60 days of age, nor among the females at 6 and 12 months. Males fed chromium, however, were heavier at 6 and 12 months than those receiving lead ($P < 0.001$) and cadmium ($P < 0.05$ and < 0.001, respectively), whereas the lead and cadmium groups were alike in this respect.

Mean weights of male rats differed at 21 months of age. The controls, at 401.5 g, were significantly lighter than the chromium group, at 466.1 g ($P < 0.005$). The survivors of the cadmium group, weighing 447.7 g, also differed ($P < 0.01$), whereas those of the lead group, at 435.8 g, did not. Likewise chromium-fed females were heavier than their controls (285.2 and 258.0 g $P < 0.025$) at 21 months of age, whereas groups fed lead (274.5 g) and cadmium (275.5 g) were statistically similar.

The rates of growth at 4 intervals up to 6 months of age are shown in table 2. Both control groups grew more slowly during the second month of life and more rapidly during the third and fourth months than did the others. Rats fed chromium grew somewhat faster after 60 days of age than did those receiving lead and cadmium. Males given chromium gained 10% more weight than their controls, whereas females gained only 7 g more at 6 months of age.

Effects on survival. There were excessive early mortalities in both sexes receiving cadmium and lead, 8 to 11 animals in each group dying before 3 months of age for a total of 37. No such early deaths occurred in the control or chromium groups. Table 3 showing the proportion surviving at various intervals, is concerned with animals alive and apparently healthy at 3 months of age. Deaths of males fed cadmium and females given lead increased steadily, whereas males fed lead and females given cadmium did not begin to die until they were 18 months of age or older. At 22 months cadmium-fed females were showing a relatively high mortality compared with their controls

($P < 0.005$). Significant differences among groups are indicated in table 4. Animals fed chromium outlived those given cadmium and lead. Males given cadmium died more rapidly than did females. No overt signs of metal toxicity were apparent in any of the groups.

Cadmium-free vs. cadmium-fed animals. No cadmium was detected in the organs of the control animals, nor in kidneys

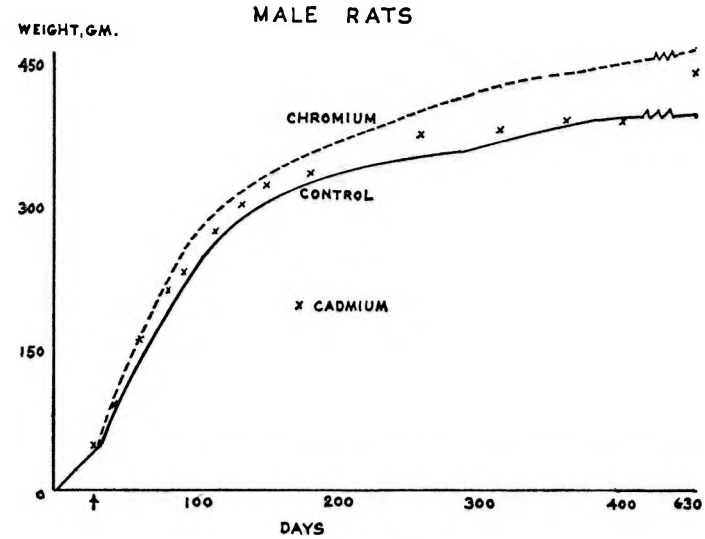

Figure 1

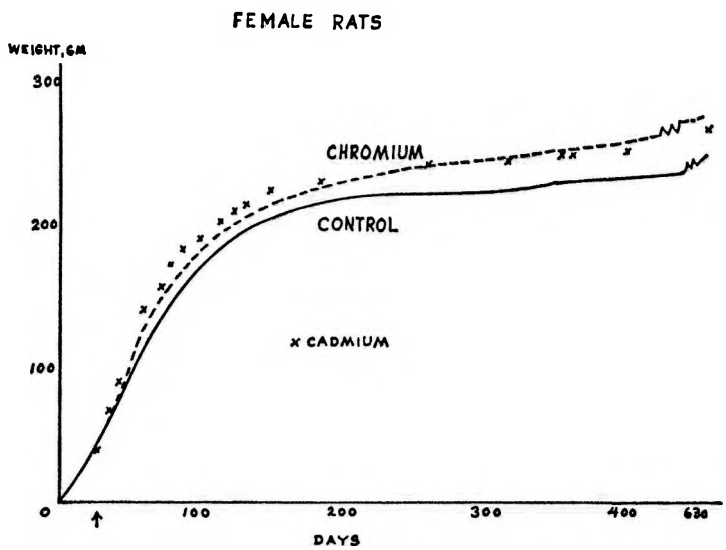

Figure 2

Figs. 1–2 Growth curves of rats taking chromium and cadmium (5 ppm) in drinking water. Control animals were "cadmium-free" by microanalytical chemical methods and their tissues contained only small amounts of chromium. Mean differences in weight of rats given chromium were statistically significant at 2, 6 and 12 months. Older males taking cadmium did not differ from their cadmium-free controls, but females were heavier. From 52 to 61 animals were in each group.

TABLE 2

Growth rates of rats given trace metals[1]

Metal	Total weight gain, 28–180 days of age	Body weight gain during indicated age periods			
		30–60 days	60–116 days	116–180 days	60–180 days
	g	*g/day*	*g/day*	*g/day*	*g/day*
Males					
Control	280	2.92	2.30	0.91	1.55
Chromium	307	3.62	2.27	1.00	1.59
Lead	283	3.47	2.02	0.94	1.44
Cadmium	287	3.47	2.05	1.00	1.46
Females					
Control	172	2.23	1.34	0.41	0.84
Chromium	179	2.55	1.28	0.46	0.81
Lead	179	2.81	1.16	0.40	0.74
Cadmium	186	2.90	1.23	0.40	0.77

[1] Metals were begun at 28 days of age, when rats were weaned.

TABLE 3

Effect of chromium, cadmium and lead on survival of rats

Metal	No. alive at 3 months of age	% surviving, months				
		6	12	18	21	23
Males						
Control	52	100	100	98.1	90.4	90.4
Chromium	54	100	96.3	91.1	91.1	87.0
Cadmium	38	76.3	71.1	60.5	47.4	21.1
P[1]		<0.0005	<0.0005	<0.0005	<0.005	<0.0005
Lead	41	100	97.5	97.5	63.4	43.4
P					<0.005	<0.0005
Total	185					
Females						
Control	52	100	100	96.1	96.1	86.5
Chromium	53	100	100	100	100	96.2
Cadmium	53	100	100	92.5	84.6	54.7
P					<0.02	<0.0005
Lead	44	90.9	81.8	72.7	68.2	50.0
P		<0.05	<0.005	<0.005	<0.0005	<0.0005
Total	202					

[1] *P* = probability of difference from control being due to chance, by χ^2 analysis. Only the significant differences are shown.

TABLE 4

Significant differences in survival among groups of rats at 21 months of age[1] *(P)*

Metal	Chromium	Cadmium	Lead
Males			
Controls	NS[2]	<0.0005	<0.001
Chromium	—	<0.0005	<0.001
Cadmium	—	—	NS
Females			
Controls	NS	<0.02	<0.001
Chromium	—	<0.005	<0.0005
Cadmium	—	—	<0.05

[1] By χ^2 analysis.
[2] NS indicates not significant.

and livers of those taking other metals. Cadmium has been constantly observed in mature breeders bought from the supplier; they had been taking food containing traces (0.1 to 0.2 μg/g) and had been housed in galvanized iron cages, a source of contamination. Five groups of animals not ingesting cadmium grew normally, whereas those given it also grew well. The one distinct difference, shared by the lead-ingesting group, was the considerably higher mortality of those fed cadmium than the controls or those given chromium.

Nutritional adequacy of diet. In terms of survival the diet was adequate for rats not ingesting cadmium or lead. In terms of growth during the first months after weaning, however, both control groups weighed less than their counterparts taking a metal (table 2). Under the conditions of the experiment, all animals were treated alike, except for the metal given. This difference is unexplained. The mean weights at weaning time (28 days) of the various groups were similar, the greatest variation being 7 g (14%) in males and 4 g (8%) in females. In terms of growth after the second month, the diet appeared nutritionally adequate for this breed of rats.

Absorption and accumulation of metal. Only 7 control animals were available for analysis, as the mortality rate was low. All dead animals, however, were analyzed, each group serving as an internal control. The rates of accumulation of metals will be reported in detail subsequently, but results on kidneys are briefly summarized in table 5. No cadmium was detected in the tissues of controls or those given lead and chromium, whereas concentrations up to 8.55 μg/g, wet weight, were noted in the kidneys of rats given the metal. Lead levels in livers of animals receiving only the small dietary quantities (0.1 μg/g) were generally less than 0.5 μg/g, exceeding 1.5 μg in most of those given it. Chromium accumulated in lung and spleen in quantities more than 1.0 μg/g; little was found when it was not given. Human levels for comparison are also shown in the table.

DISCUSSION

The results of this study on rats largely support the conclusions drawn from a similar study on mice (1). Trivalent chromium given in drinking water at 5 ppm appears to have increased the rates of growth and mature weights of male animals as compared with their controls. Unlike the mice, however, cadmium and lead in the same concentrations also affected early growth. Furthermore, few controls or chromium-fed animals had died at 630 days, whereas mortalities of the cadmium- and lead-fed males were similar to or somewhat greater than others have reported with diets fed ad libitum (6, 7). Therefore trivalent chromium is not "toxic" at these concentrations in terms of survival and appears to have had a favorable effect on growth, especially of male rats.

A comparison of cadmium-fed with cadmium-free animals shows the following differences: 1) More rapid early growth in those taking the metal, with heavier mature weights in females but not in males; 2) striking increase in mortality, especially of males at all ages; 3) renal concentrations of cadmium less than those found in adult man. We conclude from this experiment that cadmium is not an essential trace metal for rats; on the contrary, it shortens their lives.

A similar comparison on lead-fed and "low-lead" animals shows these differences: 1) More rapid early growth which does not persist; 2) Strikingly increased mortalities in both sexes, but occurring at different periods of their lives; 3) Renal and hepatic accumulations of lead similar to those in adult human beings. We conclude from this experiment that lead is not an essential trace metal for rats but that concentrations in the range of the human shorten their lives.

TABLE 5

Cadmium, lead and chromium in kidneys of rats

Metal	Rats[1]		Man	
	Not given metal	Given metal	Mean[2]	Range
	μg/g wet wt		*μg/g wet wt*	
Cadmium	0.0	1.2–8.6	32.0	11.0 –66.0
Lead	0.3–0.8	0.7–1.5	1.24	0.28 – 2.53
Chromium	0.0	2.4–2.9	0.03	<0.001– 0.4

[1] Data from analyses of animals at 12 to 18 months of age.
[2] From Tipton (5).

Some overlapping of lead concentrations in the lead and control groups is disturbing (table 5). The obvious source was the bedding (1), although the food contained 0.1 μg/g. Enough lead was probably absorbed from food and bedding to account for this accumulation, for there was no other discoverable source of contamination and air-borne lead was avoided.

This study was designed to reproduce in rats the tissue concentrations of several "abnormal" trace metals found in man. So far these goals have been reasonably well attained. The high mortality caused by cadmium and lead at or below human tissue concentrations is unexpected. Most of the female, but few of the male rats given cadmium exhibited systolic hypertension (8) a condition which probably contributed to female mortality. Little or no hypertension, however, was observed in the lead or chromium groups. Both lead and cadmium in small doses appear to have an innate "toxicity" towards survival of rats of this strain, while not inhibiting growth.

That trivalent chromium may be an accessory "essential" trace metal fulfilling a biological function was suggested by Schwarz and Mertz (9) and Mertz et al. (10). Our parallel study on mice lent support to this idea, in that chormium increased growth and mature weights considerably and appeared to inhibit male mortality up to 17 months of age. In rats, the data are less clear. Survival experiments are incomplete; both control and chromium groups have had exceptionally low mortalities to date.[3] Because cadmium also affected growth and mature weights in females, a specific action of chromium was not shown by these experiments.[4] If chromium has a biological function, the present data on rats suggest that it is largely confined to males, with a slight increase in the rate of growth of females after the fourth month of life.

The question naturally arises as to whether the diet contained some unspecified "toxicity" or mineral imbalance for rats. In the previous study on mice (1) we have concluded that in terms of growth and survival it did not. The lower rates of early growth of the control animals, real before 60 days of age but disappearing after that time, suggest on the surface that some imbalance in the regimen might have been responsible. If so, the "toxicity" or imbalance was a peculiar one. It did not affect all groups of animals. It was correctable by cadmium, lead and chromium. Furthermore, it was favorable for survival in the control (or affected) animals and in the chromium groups. As all groups of animals received the same diet, were under the same regimen, were weaned at the same time after birth, and were born within 3 months of each other (December to February), we cannot conclude that the diet or regimen contained some innate toxicity other than that inherent in the metal given. The cause of the slow early growth of the control animals, however, is unknown (table 2).

Decker et al. (14) gave cadmium in drinking water to Sprague-Dawley rats at a number of concentrations; they noted no adverse effects up to 10 ppm of the metal for a year. Their diet of corn meal, wheat, whole milk and alfalfa probably contained cadmium, according to our analyses (2), and could have as much as 24 μg/g. No comparisons of mortalities are available. Mackenzie et al. (15), from the same laboratory, gave trivalent and hexavalent chromium at several concentrations to rats; they observed no toxic effects and noted no evidence that chromium had any influence on respiratory infections. Byerrum (16), from the same group, reported on survival of rats given these metals; at 12 months 57% of those on 5 ppm cadmium were alive; 71% of those on potassium chromate were alive, but only 38% of the controls survived, the last figure incomparable to ours. The absorption, rates of accumulation and causes of death of our animals will be reported subsequently when all have died "natural" deaths.

[3] Survival curves obtained by McCay and quoted by Comfort (7) show a 60% mortality of male and 50% mortality of female rats at 600 days. Berg stated that 48% of his males survived 800 days, while Verzar's colony had a 50% survival rate at 705 days, under varying conditions (11).

[4] We do not believe that the effect of chromium on growth is due to an antibiotic-like action. Chromium has a low order of toxicity to living things, even stimulating growth of Azobacteria (12) and reversing metavanadate inhibition of *Mycobacterium tuberculosis* (13). The dose was too small for such a direct action on internal flora, although stimulation of growth of favorable strains is a possibility.

From these experiments we may conclude, therefore, that cadmium in small doses exerts a toxic effect on hooded rats in terms of survival, when renal concentrations approach one-third of those in adult American human beings. Furthermore, as no requirement for cadmium could be demonstrated, it is not an essential trace metal for these rats. Similarly, lead at human hepatic concentrations exhibits an innate toxicity on survival but not on growth. Trivalent chromium, on the other hand, increased growth of male rats and may possibly act as an essential micronutrient. Rats "cadmium-free" with low tissue lead and chromium levels by microanalytical methods and those given chromium have very low mortalities under the conditions we have set.

LITERATURE CITED

1. Schroeder, H. A., W. H. Vinton, Jr. and J. J. Balassa 1963 Effect of chromium, cadmium and other trace metals on the growth and survival of mice. J. Nutrition, 80: 39.
2. Schroeder, H. A., and J. J. Balassa 1961 Abnormal trace metals in man: Cadmium. J. Chron. Dis., *14:* 236.
3. Schroeder, H. A., and J. J. Balassa 1961 Abnormal trace metals in man: Lead. J. Chron. Dis., *14:* 408.
4. Schroeder, H. A., J. J. Balassa and I. H. Tipton 1962 Abnormal trace metals in man: Chromium. J. Chron. Dis., *15:* 941.
5. Tipton, I. H. 1960 The distribution of trace metals in the human body. In, Metal-Binding in Medicine, ed., M. J. Seven. J. B. Lippincott Company, Philadelphia, p. 27.
6. Simms, H., B. N. Berg and D. F. Davies 1959 Onset of disease and the longevity of rat and man. Ciba Foundation Colloquia on Ageing, *5:* 72. Little, Brown and Company, Boston.
7. Comfort, A. 1961 The Lifespan of Animals. Scientific American, 205: 108.
8. Schroeder, H. A., and W. H. Vinton, Jr. 1962 Hypertension induced in rats by small doses of cadmium. Am. J. Physiol., 202: 515.
9. Schwarz, K., and W. Mertz 1959 Chromium (III) and the glucose tolerance factor. Arch. Biochem. Biophys., 85: 292.
10. Mertz, R., E. E. Roginski and K. Schwarz 1961 Effect of trivalent chromium complexes on glucose uptake by epididymal fat tissue of rats. J. Biol. Chem., 236: 318.
11. 1959 (Discussion by B. N. Berg and F. Verzar). Ciba Foundation Colloquia on Ageing, 5: 82 and 83. Little, Brown and Company, Boston.
12. Perlman, D. 1945 Some effects of metallic ions on the metabolism of *Aerobacter aerogenes*. J. Bact., 49: 167.
13. Costello, R. L., and L. W. Hedgecock 1959 The effect of metavanadate ion on the growth in vitro of *Mycobacterium tuberculosis*. Ibid., 77: 794.
14. Decker, L. E., R. U. Byerrum, C. F. Decker, C. A. Hoppert and R. F. Langham 1958 Chronic toxicity studies. I. Cadmium administered in drinking water to rats. A. M. A. Arch. Indust. H., *18:* 228.
15. Mackenzie, R. D., R. U. Byerrum, C. F. Decker, C. A. Hoppert and R. F. Langham 1958 Chronic toxicity studies. II. Hexavalent and trivalent chromium administered in drinking water to rats. Ibid., *18:* 232.
16. Byerrum, R. U. 1961 Some studies on the chronic toxicity of cadmium and hexavalent chromium in drinking water. Engineering Bulletin, Purdue Univ., Series no. 106: March.

Quantitative Studies on Tryptophan Metabolism in the Pyridoxine-deficient Rat[1]

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ABSTRACT Female rats weighing about 40 g were fed a pyridoxine-deficient diet. Control animals received the same diet supplemented with 2.5 mg of pyridoxine hydrochloride/kg of diet. After 2 or 3 weeks the animals were given 40 mg of L-tryptophan by the intraperitoneal route. Urine collected for 24 hours before and after the supplementation with tryptophan was analyzed for several tryptophan metabolites. The pyridoxine-deficient rats excreted abnormally large quantities of kynurenine, 3-hydroxykynurenine, acetylkynurenine and xanthurenic acid as compared with the control rats. The deficient animals and the controls excreted similar quantities of indoxyl sulfate, anthranilic acid glucuronide, o-aminohippuric acid and kynurenic acid. Although precursors of both xanthurenic and kynurenic acids were increased in the urine of the pyridoxine-deficient animals after a loading dose of tryptophan, kynurenic acid excretion was no greater than that of the control animals. These results are consistent with the view that the enzyme system concerned with the transamination of kynurenine to form kynurenic acid is distinct from the enzyme system which transaminates hydroxykynurenine to form xanthurenic acid. It is also possible that there is an alternate pathway for xanthurenic acid synthesis which remains active in pyridoxine deficiency.

Abnormal tryptophan metabolism similar to that noted in a pyridoxine deficiency state has been observed in a number of clinical conditions (1). Wachstein and Lobel (2) demonstrated that normal pregnant women exhibited increased urinary excretion of tryptophan metabolites, and these authors commented on the similarity of paper chromatograms of urine from pregnant women and urine from pyridoxine-deficient rats. Brown et al. (3) confirmed and extended the above studies in pregnant women, using quantitative assays of urinary metabolites. They found that after a loading dose of tryptophan, normal pregnant women excreted elevated urinary levels of kynurenine, N-α-acetylkynurenine, 3- hydroxykynurenine, xanthurenic acid and N-methyl-2-pyridone-5-carboxamide (pyridone). Following administration of pyridoxine hydrochloride to these women, the metabolism returned to a more normal pattern, but the excretion of kynurenine, pyridone, and hydroxykynurenine remained somewhat elevated.

It was previously shown (4) that patients receiving pyridoxine antagonists had a pattern of tryptophan metabolism that in some respects resembled the pattern observed in pregnancy (3). The administration of pyridoxine to these patients, however, was associated with a return of the metabolism to normal. Since pyridoxine failed to completely restore the metabolism of pregnant patients to normal, it was suggested (3) that in addition to a functional pyridoxine deficiency, altered hormonal control of enzyme activities was probably a factor in the abnormal tryptophan metabolism in pregnancy.

The above speculation receives support from studies by Mason and Gullekson (5) concerning the effects of estrogen conjugates on the reactivation of resolved kynurenine transaminase. Furthermore, Porter and associates (6) showed that adrenalectomized rats excreted extremely low levels of kynurenic acid following a loading dose of tryptophan even though xanthurenic acid excretion was normal.

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Administration of cortisone to these rats preceding the tryptophan load resulted in the excretion of normal quantities of both kynurenic and xanthurenic acids.

Consequently, it seemed important to study in more detail the effects of pyridoxine deficiency per se on tryptophan metabolism in the rat. Dalgliesh (7) studied tryptophan metabolism in pyridoxine-deficient rats, but the assay procedures were qualitative and semiquantitative in nature. Furthermore, Boone et al. (8) measured xanthurenic acid excretion and Porter et al. (9) determined kynurenine, kynurenic acid and xanthurenic acid in the urine of pyridoxine-deficient rats. A more complete quantitative study, however, was not available. Since the clinical studies conducted in these laboratories entail the measurement of 9 or more urinary metabolites of tryptophan, it was decided to measure these same metabolites in the urine of pyridoxine-deficient rats, using the same quantitative procedures as in the clinical studies.

MATERIALS AND METHODS

Animals and diet. Female albino rats weighing 40 to 45 g each were purchased from the Holtzman Company of Madison, Wisconsin. The rats were housed in steel-wire pens in groups of 5 and were fed ad libitum.

A purified diet was prepared which contained no added pyridoxine (table 1). This pyridoxine-deficient diet was converted to a control diet by the addition of 2.5 mg of pyridoxine hydrochloride/kg of diet.

TABLE 1

Composition of basal diet

	%
Casein, vitamin-test[1]	20
Glucose	70
Corn oil	5
Salts[2]	4.2
Cystine	0.2
Choline	0.1
Vitamin mixture[3]	0.5

[1] Vitamin-Test Casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2] Similar to the mixture used by Phillips and Hart (21), providing in g/kg diet: $CaCO_3$, 13; $CaHPO_4 \cdot 2H_2O$, 3; K_2HPO_4, 13; NaCl, 7; Fe citrate$\cdot 6H_2O$, 1; $MnSO_4 \cdot 2H_2O$, 0.2; KI, 0.5; $MgSO_4 \cdot 7H_2O$, 4; $ZnCl_2$, 0.012; and $CuSO_4 \cdot 5H_2O$, 0.012.

[3] Supplies in mg/kg diet: inositol, 100; α-carotene, 0.8; calciferol, 0.0005; Ca pantothenate, 20; nicotinamide, 10; biotin, 0.1; 2-methylnaphthoquinone, 8; α-tocopheryl acetate, 100; thiamine·HCl, 2; folic acid, 0.2; and riboflavin, 3.

Urine collections. Five rats were fed the pyridoxine-deficient diet and 5 rats were maintained with the control diet for 14 days. The animals were then placed in stainless steel metabolism cages in groups of 5 and a basal 24-hour pooled urine sample was obtained for each group. Each rat was then given an intraperitoneal injection of 40 mg of L-tryptophan in aqueous solution, and a second 24-hour pooled urine sample was obtained from each group. The rats then continued to be fed their respective diets for one additional week, after which time they were returned to the metabolism cages and the urine collections were carried out as outlined above prior to and after a second intraperitoneal injection of 40 mg of L-tryptophan.

Analytical methods. Indoxyl sulfate,[5] anthranilic acid glucuronide, o-aminohippuric acid, acetylkynurenine, anthranilic acid and kynurenine were determined by the previously reported methods of Brown and Price (10). Hydroxykynurenine was determined using the procedure of Brown (11), and analyses for kynurenic acid and xanthurenic acid were performed according to the procedure of Satoh and Price (12).

N-Methyl-2-pyridone-5-carboxamide was not determined since an earlier study by Brown and Price (10) had shown it to be a trace metabolite in rats.

RESULTS

The urinary excretion levels of the various metabolites of tryptophan (table 2) represent data obtained from two separate experiments, in each of which urine collections were made after feeding the respective diets for 2 and 3 weeks. Since there was no apparent difference in the metabolic pattern at 2 weeks as compared with that at 3 weeks, these observations were combined. Thus, the values in table 2 represent the averages of these 4 separate determinations.

The pyridoxine-deficient rats excreted abnormally large quantities of kynurenine, hydroxykynurenine, and xanthurenic acid and moderately elevated levels of acetyl-

[5] Previously referred to as "fraction A."

TABLE 2

Summary of the average values of urinary tryptophan metabolites from 4 experiments

Metabolite	Control		Pyridoxine-deficient	
	Basal	Post-tryptophan	Basal	Post-tryptophan
	μmoles metabolite/rat/day		*μmoles metabolite/rat/day*	
Indoxyl sulfate	3.3 ± 1.3[1]	3.1 ± 1.2	3.3 ± 1.7	4.2 ± 2.7
Anthranilic acid glucuronide	0	0.2 ± 0.1	0	0.3 ± 0.1
o-Aminohippuric acid	0.2 ± 0.1	1.1 ± 0.3	0.2 ± 0.1	1.5 ± 1.2
Acetylkynurenine and anthranilic acid	0.2 ± 0.1	2.3 ± 0.5	0.1 ± 0	4.0 ± 0.9
Kynurenine	0.1 ± 0	1.5 ± 0.7	0.2 ± 0	25.4 ± 9.8
Hydroxykynurenine	0.4 ± 0.3	2.3 ± 2.4	0.2 ± 0	21.3 ± 8.1
Kynurenic acid	0.2 ± 0.2	21.5 ± 9.5	0	21.1 ± 12.3
Xanthurenic acid	0.3 ± 0.3	7.8 ± 0.5	0.3 ± 0.3	38.8 ± 10.4

[1] SD.

kynurenine as compared with the control rats. Kynurenic acid excretion by the pyridoxine-deficient rats was of the same magnitude as that of the control rats.

During the course of the experiments, the rats receiving the control diet had an average weight gain of 59 g/rat/3-week period. The rats fed the pyridoxine-deficient diet had an average weight gain of 25 g/rat/3-week period.

DISCUSSION

The results of these studies corroborate the earlier observations by Dalgliesh (7) and by Wachstein and Lobel (2) of abnormal urinary levels of tryptophan metabolities in pyridoxine-deficient rats and demonstrate that the excretory pattern of tryptophan metabolites in these animals is qualitatively and quantitatively similar to that in the urine of normal pregnant women not receiving supplemental pyridoxine (3).

The magnitude of the increase in kynurenic acid excretion by the pyridoxine-deficient rats following tryptophan supplementation was essentially identical to that of the control animals (table 2). It was previously noted that kynurenic acid excretion by pregnant human females given tryptophan supplements was not significantly different from that of nonpregnant females, whereas xanthurenic acid excretion was elevated. This prompted the speculation that kynurenine transaminase and hydroxykynurenine transaminase may be distinct enzymes which respond differently to the hormonal stimuli of pregnancy. The studies of Porter et al. (6) demonstrate clearly that cortisone had markedly different effects on the *in vivo* synthesis of kynurenic acid and xanthurenic acid in the rat. It appears, therefore, that both hormonal factors and pyridoxine deficiency may have different effects on the biosynthesis of these two acids. Snell (13) pointed out that various pyridoxine-requiring enzymes exhibit a differential sensitivity to pyridoxine deficiency. It may be, therefore, that hydroxykynurenine transaminase is less sensitive to pyridoxine deficiency than is kynurenine transaminase. Thus, in the presence of an accumulation of comparable quantities of kynurenine and hydroxykynurenine, only xanthurenic acid excretion increased, which suggests that hydroxykynurenine transaminase retained high capacity to utilize substrate whereas kynurenine transaminase had suffered from the deficiency of coenzyme.

These results tend to support the suggestion by Brown et al. (3) that the transamination of kynurenine and hydroxykynurenine may involve 2 distinct enzymes with dissimilar characteristics. Another possibility is that an additional pathway for xanthurenic acid synthesis exists which does not utilize pyridoxine-linked coenzymes. Knox (14) has suggested that

oxidative deamination may play a role in this metabolic scheme, and Charconnet-Harding et al. (15) have demonstrated that xanthurenic acid excretion was increased in riboflavin-deficient rats. Henderson and co-workers (16) showed that riboflavin-deficient rats excreted elevated levels of xanthurenic acid following the administration of L-tryptophan but not following the administration of kynurenine or 3-hydroxykynurenine. The latter data support the theory that some alternate pathway for xanthurenic acid synthesis exists.

Ogasawara et al. (17) explained the increased formation of xanthurenic acid in pyridoxine-deficient rats on the basis of a decreased conversion of kynurenine to anthranilic acid and to a lesser extent, a decrease in its conversion to kynurenic acid. This would result in an accumulation of 3-hydroxykynurenine, which is not readily metabolized to 3-hydroxyanthranilic acid, but rather is converted to xanthurenic acid by mitochondrial kynurenine transaminase, which is not impaired extensively by pyridoxine deficiency. However, the results in table 2 show that the precursor of kynurenic acid (kynurenine) accumulated in pyridoxine-deficient animals to levels comparable to the levels of the immediate precursor of xanthurenic acid (3-hydroxykynurenine). Thus, the mechanism suggested by Ogasawara et al. (17) does not account for the failure to observe an increase in the conversion of tryptophan to kynurenic acid in pyridoxine deficiency unless one assumes either that kynurenine transaminase suffers more as a result of the deficiency than hydroxykynurenine transaminase or that there is an alternate pathway to xanthurenic acid which may not involve pyridoxine.

The prior demonstration that administration of pyridoxine to pregnant women was associated with an improvement in tryptophan metabolism was suggestive evidence that pyridoxine deficiency may exist in pregnancy. Furthermore, Willis et al. (18) have reported the successful use of pyridoxine in the therapy of hyperemesis gravidarum, an extremely severe form of nausea and vomiting observed occasionally in pregnancy. In the past 2 decades obstetricians have reported success in the use of pyridoxine in the treatment of nausea of pregnancy (19, 20).

The data obtained in the present studies indicate that pyridoxine deficiency per se may play a role in the abnormal manner in which tryptophan is metabolized by pregnant women. Whether or not there is a correlation between these biochemical abnormalities and the clinical observations of Willis et al. (18) and others is still open to speculation.

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LITERATURE CITED

1. Price, J. M. 1958 Disorders of tryptophan metabolism. Univ. Mich. Med. Bull., *24:* 461.
2. Wachstein, M., and S. Lobel 1954 Abnormal tryptophan metabolites in human pregnancy and their relation to deranged vitamin B_6 metabolism. Proc. Soc. Exp. Biol. Med., *86:* 624.
3. Brown, R. R., M. J. Thornton and J. M. Price 1961 The effect of vitamin supplementation on the urinary excretion of tryptophan metabolites by pregnant women. J. Clin. Invest., *40:* 617.
4. Price, J. M., R. R. Brown and F. C. Larson 1957 Quantitative studies on human urinary metabolites of tryptophan as affected by isoniazid and deoxypyridoxine. Ibid., *36:* 1600.
5. Mason, M., and E. H. Gullekson 1960 Estrogen-enzyme interactions: Inhibition and protection of kynurenine transaminase by the sulfate esters of diethylstilbesterol, estradiol, and estrone. J. Biol. Chem., *235:* 1312.
6. Porter, C. C., H. C. Stoerk and R. H. Silber 1951 The effect of cortisone upon tryptophan metabolism in the rat. Ibid., *193:* 193.
7. Dalgliesh, C. E. 1952 The relation between pyridoxin and tryptophan metabolism, studied in the rat. Biochem. J., *52:* 3.
8. Boone, I. U., D. F. Turney and W. H. Langham 1955 Isoniazid in vitamin B_6-deficient rats. J. Lab. Clin. Med., *46:* 549.
9. Porter, C. C., I. Clark and R. H. Silber 1948 The effect of B vitamin deficiencies on tryptophan metabolism in the rat. Arch. Biochem., *18:* 339.
10. Brown, R. R., and J. M. Price 1956 Quantitative studies on metabolites of tryptophan in the urine of the dog, cat, rat, and man. J. Biol. Chem., *219:* 985.
11. Brown, R. R. 1957 The isolation and determination of urinary hydroxykynurenine. Ibid., *227:* 649.
12. Satoh, K., and J. M. Price 1958 Fluorometric determination of kynurenic acid and

xanthurenic acid in human urine. Ibid., *230:* 781.
13. Snell, E. E. 1953 Summary of known metabolic functions of nicotinic acid, riboflavin and vitamin B_6. Physiol. Rev., *33:* 509.
14. Knox, W. E. 1959 In Symposium on Tryptophan Metabolism. American Chemical Society, Division of Medicinal Chemistry, p. 28.
15. Charconnet-Harding, F., C. E. Dalgliesh and A. Neuberger 1953 The relation between riboflavin and tryptophan metabolism, studied in the rat. Biochem. J., *53:* 513.
16. Henderson, L. M., R. E. Koski and F. D'Angeli 1955 The role of riboflavin and vitamin B_6 in tryptophan metabolism. J. Biol. Chem., *215:* 369.
17. Ogasawara, N., Y. Hagino and Y. Kotake 1962 Kynurenine-transaminase, kynureninase and the increase of xanthurenic acid excretion. J. Biochem., *52:* 162.
18. Willis, R. S., W. W. Winn, A. T. Morris, A. A. Newsom and W. E. Massey 1942 Clinical observations in treatment of nausea and vomiting in pregnancy with vitamins B_1 and B_6. Am. J. Obstet. Gynecol., *44:* 265.
19. Weinstein, B. B., Z. Wohl, G. J. Mitchell and G. F. Sustendal 1944 Oral administration of pyridoxine hydrochloride in the treatment of nausea and vomiting of pregnancy. Ibid., *47:* 389.
20. Dorsey, C. W. 1949 The use of pyridoxine and suprarenal cortex combined in the treatment of the nausea and vomiting of pregnancy. Ibid., *58:* 1073.
21. Phillips, P. H., and E. B. Hart 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

Unidentified Chick Growth Factor in Fish Solubles[1]

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ABSTRACT The unidentified chick growth factor (UGF) of menhaden fish solubles, as measured by stimulation of the growth of chicks fed a purified diet, was found to be organic in nature and partly dialyzable. About one-half the activity was extractable into water or 50% ethanol, and about one-third into 80% ethanol. The water-soluble UGF activity was retained on a strong acid, but not on a weak acid, cation exchanger, and behaved, therefore, as a weak base. About one-half the activity was held on a strong base anion exchanger and could be eluted with sodium hydroxide. The factor was stable under strongly acidic conditions but was partly destroyed by alkali.

Since the identification of vitamin B_{12}, a number of natural products have been reported to stimulate growth of chicks. The literature on this subject has been adequately reviewed by Bird et al. (1). The work reported in this paper was an attempt to determine some of the properties of the unidentified growth factor (UGF) present in fish solubles.

EXPERIMENTAL

Chick assay. The method used to determine the presence of the unidentified growth factor in fish solubles and the various fractions of fish solubles was a 4-week chick assay. New Hampshire × Single Comb White Leghorn chicks of both sexes were used in all experiments with 10 chicks/group and 2 or 3 groups/treatment. The chicks were maintained in electrically heated starting batteries with the treatment groups being distributed randomly throughout the battery. The chicks were individually wing - banded when one-day old and an initial group weight and individual weekly weights were obtained. At 4 weeks of age the sex of the chicks was determined and recorded. The mean 4-week weights were obtained by averaging the sexes within each group and averaging the groups within each treatment.

The composition of the basal diet, which is a modification of the diet developed by Barnett and Bird (2) is shown in table 1. For the first week of the assay 5 g of chicken feces were added/kg of diet, but for the remainder of the experiment feces were not added. Feces were added to the diets in an attempt to establish a more uniform intestinal microflora between groups in each experiment and between experiments. For this purpose feces from basal groups in experiments

TABLE 1

Composition of the basal assay diet

	g
Sucrose	605.0
Isolated soybean protein (C-1)[1]	250.0
Soybean oil	44.0
Dried whey	20.0
Mineral mix[2]	60.0
Methionine hydroxy analogue	7.5
Glycine	3.0
Vitamin mix[3]	5.0
Choline chloride (70%)	2.9
Vitamin A source (5000 IU/g)	2.5
Vitamin E source (10 mg/g)	1.0
Inositol	1.0
Vitamin D_3 (1500 ICU/g)	0.8
Total	1002.7

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland, Cincinnati, Ohio.

[2] Sixty grams of mineral mix contains the following in grams: $CaCO_3$, 22.8; KH_2PO_4, 15.48; $CaHPO_4 \cdot 2H_2O$, 7.14; NaCl, 7.992; $MgSO_4 \cdot 7H_2O$, 4.896; ferric citrate $\cdot 6H_2O$, 1.32; $MnSO_4$, 0.240; KI, 0.0384; $CuSO_4 \cdot 5H_2O$, 0.0144; $ZnCl_2$, 0.0600; $Na_2MoO_4 \cdot H_2O$, 0.0192.

[3] Five grams of vitamin mix contains the following in milligrams: niacin, 50.0; Ca pantothenate, 20.0; vitamin B_{12} (0.1% in mannitol), 20.0; thiamine·HCl, 10.0; riboflavin, 6.0; pyridoxine·HCl, 4.0; folic acid, 4.0; menadione, 0.5; biotin, 0.2; *p*-aminobenzoic acid, 100.0; sucrose to a total of 5000.

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which gave a good growth response were collected and stored in a −10°C freezer.

The sodium chloride levels of the diet were adjusted, where necessary, for sodium present in fractions of fish solubles to be assayed, e.g., fractions obtained by ion exchange. Fish solubles and fractions of fish solubles were added to the basal diet at the expense of an equal weight of sucrose. In all assays conducted, positive (4% fish solubles) and negative (basal) control treatments were included.

Menhaden fish solubles from the same barrel were used in all fractionation procedures and chick assays. This sample of menhaden fish solubles was processed on July 8, 1959 and received at the University of Wisconsin on August 18, 1959. It was found to have a dry matter content of 51.4% and a pH of 4.5. The sample was maintained in a 4°C cooler throughout the experiments.

Fractions of fish solubles were concentrated for assay to a syrup by removing solvents *in vacuo* at 40°C and 10 mm pressure. Acidic fractions were concentrated until crystallization occurred, then neutralized with 6 N NaOH and then further concentrated to a syrup. Basic fractions were neutralized with 1 N HCl prior to concentration *in vacuo*.

Ashing. The fish solubles were ashed under both acid and alkaline conditions. For acid ashing, the fish solubles were first dried at the natural pH of 4.5 in a stream of warm air. The dried cake was then heated with six 250-watt heat lamps and a hot plate at approximately 300°C until the material was carbonized. The carbonized material was ashed in a muffle furnace at 600°C for 6 hours, cooled to room temperature, and ground to a fine powder with a mortar and pestle. The percentage of ash found in 2 separate experiments was 7.9 and 8.9. Alkaline ashing was carried out in the same way except that the fish solubles were first adjusted to pH 9.8 with 6 N NaOH.

The water-soluble and insoluble fractions of fish solubles were also ashed. The water-insoluble fraction was obtained as described below and ashed as above without adjusting the pH. The water-soluble fraction, obtained as described below, was concentrated by means of vacuum distillation to remove most of the water. The pH of the fraction was adjusted to 9.13 with 6 N NaOH, and the material was then ashed as described above.

Water extraction. The water - soluble fraction of fish solubles was used in a number of experimental procedures. It was obtained by diluting the fish solubles 10:1 with distilled water, stirring for 10 to 12 hours at 10°C, and then centrifuging in a Sharples centrifuge to remove the insoluble fraction (fraction 1B). The water-soluble fraction will be called fraction 1A. About two-thirds of the solids of the original fish solubles went into this fraction.

Alcohol extraction. The solubility of the UGF of fish solubles in 50 and 80% ethanol was determined by adding 95% ethanol to give the desired concentration, stirring one hour (experiment 2) or 15 hours (experiment 5) at room temperature and filtering. The final dilution of fish solubles was 3:1 with 50 or 80% ethanol.

The ethanol solubility of the UGF in fractions 1A and 1B was also measured. Fraction 1B was mixed with 3 parts of 80% ethanol and the mixture was homogenized in a Waring Blendor, allowed to stand for 24 hours at room temperature, and then filtered. The residue was extracted 3 additional times in the same manner and the extracts combined and assayed.

Fraction 1A from 500 g of fish solubles (experiment 2) was concentrated *in vacuo* to 205 ml, stirred for 0.5 hour at room temperature with 1750 ml of 95% ethanol and the mixture filtered through no. 1 Whatman paper. In experiment 20, fraction 1A from 1200 g of fish solubles was concentrated *in vacuo* to 1000 ml, stirred 15 minutes at room temperature with 6 liters of 95% ethanol, and filtered. The precipitate was redissolved in 350 ml of distilled water, stirred 15 minutes with 4300 ml of 95% ethanol, filtered and the filtrates combined for assay. The precipitate was dissolved in distilled water and assayed. Allowing for water in the preparations extracted, the ethanol concentrations used were estimated to be in the range of 85 to 90%.

Dialysis. A 900-g portion of fish solubles was placed in a 30.5-cm length of

6.4-cm diameter dialyzing casing and dialyzed against 4 liters of distilled water at 4°C. The water was changed daily for 14 days. The dialyzates were combined and both fractions were assayed.

Ion exchange. A series of ion exchange experiments on fraction 1A were conducted with Amberlites[2] IR-120, IRA-401 and IRC-50. These are strong acid cation, strong base anion, and weak acid cation exchangers, respectively. The resin beds in the columns used had the following dimensions: IR-120, 9 × 90 cm in experiments 15 and 16, and 15.5 × 90 cm in other experiments; IRA-401, 15.5 × 100 cm; IRC-50, 9 × 90 cm. Flow rates of 80 ml/minute were used with all procedures and columns. Fraction 1A was processed at pH 4.5 except in experiment 23 where the pH was adjusted to 2.0 with 1 N HCl.

Columns were prepared by backwashing the resin with distilled water, allowing the resin to settle for 24 hours and again washing with distilled water. The IR-120 columns were converted to the H- form with 1 N HCl of twice the column exchange capacity and washed with 20 liters of distilled water to remove the excess hydrochloric acid. The IRA-401 columns were converted to the OH- form with 1 N NaOH of twice the column exchange capacity and washed with 20 liters of distilled water. The IRC-50 columns were converted to the H- form with 15 liters of 1 N HCl, washed with distilled water, then with 16 liters of 0.5 M sodium acetate, and then with 10 liters distilled water.

In experiment 15, 60 liters of fraction 1A were passed through the IR-120 column and the effluent passed through the IRA-401 column. The final effluent, which had a pH of 7, was assayed. In experiment 16, 24 liters of fraction 1A were passed through the IR-120 column, one-half the effluent was passed through the IRA-401 column and both effluents assayed for UGF activity.

In experiment 17, 70 liters of fraction 1A were processed on IR-120 and one-half the effluent passed through the IRA-401 column. The IR-120 column was eluted with 5 retention volumes of 1 N HCl and the IRA-401 column eluted with 4 retention volumes of 1 N NaOH. The effluents and eluates from both exchangers were assayed.

In experiment 21, 24 liters of fraction 1A were passed through the IRC-50 column, the column was eluted with 15 liters of 1 N HCl and both fractions were assayed. In experiment 22, 20 liters of fraction 1A were processed on IRC-50, the column was eluted with 16 liters of 1 N acetic acid and both fractions were assayed.

In experiment 23, 48 liters of fraction 1A at pH 2.0 were passed through the IR-120 column, the resin was eluted with 36 liters of 1 N HCl and both fractions were assayed. Forty liters of fraction 1A were processed on the IRA-401 column (experiment 18), the column eluted with 4 retention volumes of 1 N NaOH and the 2 fractions were assayed.

Adsorption studies. Fraction 1A was concentrated to one-fourth its original volume and portions equivalent to 1200 g of fish solubles, adjusted with concentrated hydrochloric acid or 6 N NaOH to pH 2.0, 4.5 or 8.5, respectively, were stirred for 45 minutes with 40-g portions of Nuchar[3] C-190-N at room temperature. One hundred-gram portions of Celite[4] were added and the mixtures filtered through 50-gram Celite pads. The filtrates and carbon-Celite mixtures were assayed for UGF activity.

Ten liters of fraction 1A were passed through a Florosil[5] column (5 × 50 cm) at a flow rate of 5 ml/minute and the effluent was assayed. The column was prepared by making a slurry of 400 g of Florosil in water and packing it under 20 mm of water pressure. The column was washed with 3 liters of 1 N acetic acid and with 5 liters of distilled water before fraction 1A was passed through it.

Stability. In experiment 6, 1000 g of fraction 1B were mixed with 30 g of pancreatin and 10 liters of distilled water. The pH was adjusted to 7.45 with sodium carbonate and the mixture placed in a 37°C incubator for one week. The pH was readjusted to 7.45 periodically. Chlo-

[2] Rohm and Haas Company, Washington Square, Philadelphia 5, Pennsylvania.
[3] West Virginia Pulp and Paper Company, New York 17, New York.
[4] Diatomaceous silica filter aid, Johns Manville Company, Milwaukee, Wisconsin.
[5] Floridin Company, Tallahassee, Florida.

roform and toluene were added to the mixture to inhibit bacterial growth. The pancreatin digest was separated into its water-soluble and insoluble fractions by filtration through Celite and both fractions were assayed.

Another portion of fraction 1B (600 g) was heated with 6 liters of 6 N HCl for 5 hours in an autoclave at 121°C. The mixture was filtered through Celite and concentrated to approximately 500 ml before neutralization with 6 N NaOH. Both the soluble and insoluble fractions were assayed.

In experiment 19, 9 liters of fraction 1A were adjusted to pH 1.3 with 1 N HCl. The mixture was concentrated *in vacuo* at 40°C and 20 mm pressure over a 10-hour period to approximately 1500 ml and then neutralized to pH 4.0 with 6 N NaOH.

A second 9-liter portion of fraction 1A was adjusted to pH 11.7 with NaOH. This mixture was allowed to stand at room temperature for 44 hours, neutralized to pH 6.6 with concentrated hydrochloric acid and the water removed *in vacuo* at 40°C and 20 mm pressure in 5 hours.

RESULTS AND DISCUSSION

A positive response to 4% fish solubles was obtained in 25 of 26 assays conducted (table 2). The negative response in experiment 4 is attributed to insufficient replication. The response in the other 25 assays, although variable, clearly indicates the UGF activity of the fish solubles.

Of 10 assays in which fish solubles gave a response of 120% of the basal or greater, 7 occurred in the winter months and one each in the other seasons of the year (table 2). However, assays with responses of less than 110% occurred irrespective of the season. No definite pattern was apparent when the results of all assays in various seasons were compared, although there appeared to be some tendency for the responses to be lower during the spring.

TABLE 2

Comparison of the UGF activity of zero, and 4% fish solubles and the water-soluble fraction of fish solubles

Exp. no.	Date[1]	Mean 4-week gain				
		Basal	4% Fish solubles		Water-soluble fraction[2]	
		g	*g*	*% of basal*	*g*	*% of basal*
1	8/21/59	225	239	106		
2	9/11/59	174	207	119	212	122
3	10/23/59	224	238	106	253	113
4	12/11/59	260	233	90	250	96
5	1/29/60	199	244	123		
6	2/26/60	195	254	130	254	130
7	4/ 1/60	269	275	102		
8	4/13/60	197	207	106	224	113
9	7/ 1/60	158	198	125		
10	7/ 8/60	166	198	119	207	125
11	9/30/60	193	231	120		
12	11/25/60	194	236	122	228	118
13	12/23/60	148	180	122	194	131
14	6/23/61	218	258	118		
15	6/30/61	196	223	114		
16	9/22/61	247	268	109	273	111
17	11/ 3/61	182	233	128	234	129
18	12/ 1/61	165	241	146	229	139
19	12/22/61	215	268	124	272	126
20	2/ 9/62	258	288	111	275	107
21	4/20/62	237	282	120	272	114
22	5/25/62	220	237	108	256	116
23	7/13/62	243	257	106	266	106
24	8/10/62	174	206	118	201	116
25	9/14/62	217	258	119	237	109
26	9/21/62	208	245	118	232	112

[1] Date at which the assay was started.
[2] Fed at levels equivalent to: 4% fish solubles in experiments 2, 3, and 4; 6% fish solubles in experiments 13 and 19; 8% fish solubles in all other experiments.

A low percentage response to the fish solubles supplement was usually the result of high gains with the basal diet rather than to low gains with the supplemented diet.

Ashing. The mean 4-week gain of chicks fed the acid and alkaline ashes of fish solubles, fraction 1A and fraction 1B are shown in table 3. The acid ash at levels equivalent to 4 and 8% fish solubles did not give a response in experiment 8. However, in experiment 9 a response was obtained at the 4 and 8% equivalence level and a much smaller response at the 16% equivalence level. The 16% level contributed 12.8 g of ash/kg of diet. This would not be expected to produce a toxic effect. Although the acid ash gave a response in experiment 9, it did not equal the 4% fish solubles control at either the 4 or 8% equivalence levels and at the 16% level was far below the response of 4% fish solubles.

The assay of the alkaline ash of fish solubles and of fraction 1A showed no response. Similarly, the ash of fraction 1B gave no growth response over the basal. Previous work at this laboratory by Barnett[6] failed to show UGF activity in the ash of fish solubles. Barnett obtained gains in 2 experiments of 205 and 201 g for the basal, 271 and 225 g for 5% fish solubles, and 164 and 140 g for the alkaline ash of 5% fish solubles. An acid ash of 5% fish solubles produced growth well below that of the basal group, but the result was equivocal since in this experiment the group receiving 5% fish solubles grew no better than the basal group.

From these results, despite some apparent response in experiment 9, it appears that the UGF in fish solubles is not inorganic in nature. This supports the results of Menge et al. (3) but differs with those of Mason et al. (4) who reported some response from the ash.

Water solubility. The mean 4-week weight gains produced by fractions 1A and 1B in 3 experiments are shown in table 4. From these results, approximately one-half of the UGF in fish solubles is soluble in water. Additional extraction of the insoluble fraction at pH 2.0, 4.5, and 8.5 did not solubilize additional UGF. Fraction 1A in 18 of 19 assays in which it was included gave a positive response which averaged 17% increase in growth for the 19 assays (table 2). The one assay that gave no response also failed to respond to 4% fish solubles. The data of Menge et al. (3) show a similar equal division between the water soluble and insoluble fractions.

Ethanol solubility. The UGF activity of the ethanol extracts is shown in table 5.

[6] Barnett, B. D. 1956 Dietary factors affecting growth of chicks and turkey poults. Ph.D. Dissertation, University of Wisconsin, Madison.

TABLE 3

Assay of ash of fish solubles and fractions

Exp. no.	Supplement	Level[1]	Mean 4-week gain	
		%	g	% of basal
8	None		197	100
	Fish solubles(1)[2]	4	207	106
	Acid ash of 1	4	190	96
	Acid ash of 1	8	167	85
9	None		158	100
	Fish solubles(1)	4	198	125
	Acid ash of 1	4	182	115
	Acid ash of 1	8	186	118
	Acid ash of 1	16	169	107
12	None		194	100
	Fish solubles (1)	4	236	122
	Alkaline ash of 1	4	196	101
	Alkaline ash of fraction 1A	8	200	103
	Acid ash of fraction 1B	8	185	95

[1] Figures indicate percentages of fish solubles to which supplements are equivalent.
[2] The following abbreviations are used in this table: 1, fish solubles; 1A, water-soluble fraction of fish solubles; 1B, water-insoluble fraction of fish solubles.

TABLE 4

Assay of water-soluble and insoluble fractions of fish solubles

Exp. no.	Mean 4-week gains			
	Basal	4% Fish solubles	Water-soluble fraction	Water-insoluble fraction
		g	*g*	*g*
2	174	207	212[1]	242[1]
6	195	254	254[2]	219[2]
8	197	207	224[2]	244[2]

[1] Fed at levels equivalent to 4% fish solubles.
[2] Fed at levels equivalent to 8% fish solubles.

TABLE 5

Assay of ethanol extracts of fish solubles

Exp. no.	Supplement	Level[1]	Mean 4-week gain	
		%	*g*	*% of basal*
2	None		174	100
	Fish solubles (1)[2]	4	207	119
	Fraction 1A	4	212	122
	50% ethanol-soluble fraction of 1	4	216	124
	50% ethanol-insoluble fraction of 1	4	248	142
	85–90% ethanol-soluble fraction of fraction 1A	4	199	114
	85–90% ethanol-insoluble fraction of fraction 1A	4	202	116
5	None		199	100
	Fish solubles (1)	4	244	123
	80% ethanol-soluble fraction of 1	8	218	110
	80% ethanol-insoluble fraction of 1	8	241	121
	50% ethanol-soluble fraction of 1	8	241	121
	50% ethanol-insoluble fraction of 1	8	244	123
11	None		193	100
	Fish solubles (1)	4	231	120
	80% ethanol-soluble fraction of 1B	18	218	113
12	None		194	100
	Fish solubles (1)	4	236	122
	Fraction 1B	8	225	116
	80% ethanol-soluble fraction of fraction 1B	16	229	118
	80% ethanol-insolnble fraction of fraction 1B	8	234	121
20	None		258	100
	Fish solubles (1)	4	288	112
	Fraction 1A	8	275	106
	85–90% ethanol-soluble fraction of fraction 1A	8	271	105
	85–90% ethanol-insoluble fraction of fraction 1A	8	278	107

[1] Figures indicate percentages of fish solubles to which supplements are equivalent.
[2] See footnote 2, table 3, for abbreviations.

Experiments 2 and 5 appear to indicate that the activity of the original fish solubles divides about equally between the 50% ethanol soluble and insoluble fractions, whereas about one-third went into 80% ethanol. The activity of the water-soluble portion (fraction 1A) again divided about equally on treatment with 85 to 90% ethanol (experiments 2 and 20), and about one-third of the activity of the water-insoluble fraction (1B) went into 80% ethanol (experiment 12). Ethanol thus appears to be a somewhat poorer solvent than water for UGF and to offer little value for purification purposes. Menge et al. (3) reported the UGF to be soluble in 50, 60 and 80% ethanol. The work reported here supports these observations although a smaller proportion of the UGF was found soluble at the 50 and 80% concentrations.

Ion exchange. The mean 4-week growth responses to the fractions obtained by ion exchange are shown in table 6. The nega-

TABLE 6

Assay of fractions from ion exchange experiments using IR-120, IRA-401, and IRC-50

Exp. no.	Supplement	Level[1]	Mean 4-week gain	
		%	*g*	*% of basal*
15	None		196	100
	Fish solubles	4	233	114
	Effluent from passage through both IR-120 and IRA-401	8	168	86
16	None		247	100
	Fish solubles (1)[2]	4	268	109
	Fraction 1A	8	273	111
	Effluent from passage through IR-120	8	258	104
	Effluent from passage through both IR-120 and IRA-401	8	236	96
17	None		182	100
	Fish solubles (1)	4	233	128
	Fraction 1A	8	234	129
	Effluent from passage through IR-120	8	179	98
	Effluent from passage through both IR-120 and IRA-401	8	171	94
	Eluate from IR-120	8	198	109
	Eluate from IRA-401	8	172	94
23[3]	None		243	100
	Fish solubles (1)	4	257	106
	Fraction1A	8	266	109
	Effluent from passage through IR-120	8	231	95
	Eluate from IR-120	8	259	107
21	None		237	100
	Fish solubles (1)	4	282	120
	Fraction 1A	8	272	114
	Effluent from passage through IRC-50	8	259	109
	Eluate from IRC-50	8	244	103
22	None		220	100
	Fish solubles (1)	4	237	108
	Fraction 1A	8	256	116
	Effluent from passage through IRC-50	8	238	108
	Eluate from IRC-50	8	215	98
18	None		165	100
	Fish solubles (1)	4	241	146
	Fraction 1A	8	229	139
	Effluent from passage through IRA-401	8	203	123
	Eluate from IRA-401	8	199	117

[1] Figures indicate percentages of fish solubles to which supplements are equivalent.
[2] See footnote 2, table 3, for abbreviations.
[3] In this experiment the water-soluble fraction of 1 was adjusted to pH 2.0 before exchange on the IR-120 column.

tive response observed from the fraction obtained after passage through both the strong acid cation (IR-120) and strong base anion (IRA-401) exchangers indicates that all the UGF present in fraction 1A was ionizable. The same results were obtained in experiment 16. Also, the effluent of the cation exchanger appeared to give only a slight response, which indicates that most of the UGF was held on the IR-120 column. The results of experiment 17 support these conclusions and in addition show that some of the activity can be eluted from the IR-120 column. However, a large part of the activity was retained on the column since the response to the eluate was only one-third of that to fraction 1A.

The results of the response to the fractions in experiment 23 where the pH of fraction 1A was reduced to 2.0 before passage through IR-120 appear to show that

all of the UGF was held on the column and then eluted. However, the actual response is no greater than in experiment 17, whereas the response to fish solubles and fraction 1A was much less. Under these circumstances, it is difficult to say whether all of the UGF was eluted.

In both experiments 21 and 22 most of the activity appeared not to be held on the weak cationic exchanger (IRC-50). These results, along with those of the IR-120 experiments appear to indicate that UGF is a weak base.

About one-half the activity of fraction 1A was held on the IRA-401 resin (experiment 18, table 6). Most of the activity held by the column was subsequently removed in the eluate. These results might be interpreted to indicate that about one-half of the UGF in fraction 1A has anionic properties.

Dialysis. The chick assay of the dialysis fractions gave only a relatively small response for the positive control (102%) and for the dialyzable (104%) and nondialyzable (105%) fractions. These responses were insufficient to determine the proportions of UGF activity in the 2 fractions but indicated that both had activity. The dialyzable fraction contained 65% of the total solids of the fish solubles. The presence of UGF in this fraction suggests that at least a part of the water-soluble activity is due to relatively low molecular weight compound(s). These results correspond to those obtained by Menge et al. (3) using similar experimental procedures. Mason et al. (4) reported the dialysis residue completly inactive after continuous dialysis against tap water, but dialysis was incomplete after a 7-day dialysis against a daily change of distilled water.

Stability. Table 7 shows the results of acid hydrolysis and pancreatin digestion of fraction 1B and acid and base treatment of fraction 1A. It appears that at least 50% of the UGF in fraction 1B was still insoluble after pancreatin digestion. Slightly more of the UGF in fraction 1B appeared to have been solubilized by acid hydrolysis. Neither of these procedures appeared to cause any loss of UGF activity.

The acid treatment of fraction 1A also did not cause any marked UGF destruction, whereas treatment with base caused an appreciable loss of activity. Mason et al. (4) also reported the UGF to be stable under strong acidic hydrolysis, whereas Menge et al. (3) found it stable at pH 2.0 and above. Menge et al. (3) also observed some UGF destruction under basic conditions whereas Mason et al. (4) reported it stable to basic hydrolysis.

Adsorption. In the 2 experiments conducted with carbon there was no adsorption at either pH 2.0 or 8.5. At pH 4.5, equivocal results were obtained. It is most probable that no adsorption of the UGF occurred at any of the pH levels used.

TABLE 7

Stability studies

Exp. no.	Supplement	Level[1]	Mean 4-week gain	
		%	g	% of basal
6	None		195	100
	Fish solubles (1)[2]	4	254	130
	Fraction 1A	8	254	130
	Fraction 1B	8	219	112
	Water-soluble pancreatin digest of fraction 1B	8	232	119
	Water-insoluble pancreatin digest of fraction 1B	4	234	120
	Water-soluble acid hydrozylate of fraction 1B	8	226	116
	Water-insoluble acid hydrolyzate of fraction 1B	8	217	111
19	None		215	100
	Fish solubles (1)	4	268	124
	Fraction 1A	6	272	126
	Fraction 1A, HCl-treated	6	263	122
	Fraction 1A, NaOH-treated	6	248	115

[1] Figures indicate percentages of fish solubles to which supplements are equivalent.
[2] See footnote 2, table 3, for abbreviations.

This supports the results of Menge et al.[7] who found no adsorption on Norit[8] at pH 3.0.

The effluent from the Florosil column gave a response of 112% of the basal, whereas fraction 1A gave a response of 109%. This would indicate that no adsorption occurred on the Florosil column.

Concentration of active material. In most of the fractionation procedures carried out in this study the UGF activity failed to go predominantly into either fraction, but in general tended to divide in about the same ratio as the total solids. Little purification of the active material was, therefore, achieved. The most active preparation obtained was the 80% ethanol extract of fraction 1B (table 5, experiments 11 and 12) of which 3.4 and 3.8 g/kg of diet gave responses of 113 and 118% of the basal, respectively.

LITERATURE CITED

1. Bird, H. R., F. H. Steinke and F. M. Strong 1962 An unidentified growth factor for chicks in fish meal and solubles. Proceedings 12th World's Poultry Congress, Sydney, Australia, p. 272.
2. Barnett, B. D., and H. R. Bird 1956 Standardization of assay for unidentified growth factor. Poultry Sci., 35: 705.
3. Menge, H., C. A. Denton, J. R. Sizemore, R. J. Lillie and H. R. Bird 1953 Unidentified factors in poultry nutrition. 2. Properties and preliminary fractionation of a growth factor in condensed fish solubles. Poultry Sci., 32: 863.
4. Mason, M. E., J. Sacks and E. L. Stephenson 1961 Isolation and nature of an unidentified growth factor(s) in condensed fish solubles. J. Nutrition, 75: 253.

[7] Menge, H., C. H. Denton, R. J. Lillie and H. R. Bird 1952 Some properties of an unidentified chick growth factor in condensed fish solubles. Poultry Sci., 31: 927 (abstract).

[8] American Norite Company, Jacksonville, Florida.

Utilization of Methionine by the Adult Rat

II. ABSORPTION AND TISSUE UPTAKE OF L- AND DL-METHIONINE

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ABSTRACT The absorption of L- and DL-methionine-$C^{11}H_3$ from the alimentary tract and the distribution of radiocarbon from these sources and DL-methionine-2-C^{14} in blood components and 18 tissues of rats were investigated. Small, but statistically significant differences in the absorption and tissue uptake of L- and DL-methionine were observed in these experiments. The absorption of L-methionine is calculated to be 1.1 times that of its D- isomer.

After 30 minutes, the plasma and plasma proteins of rats fed L-methionine-methyl-C^{14} contained greater activity than the corresponding components of rats fed the DL-form of this compound. More radiocarbon was observed in plasma, protein-free plasma, and plasma protein of rats fed DL-methionine-2-C^{14} than in those components of rats fed the methyl-labeled DL-compound.

The variation in tissue response of individual animals similarly treated to uniform doses of L- and DL-methionine-methyl-C^{14} and DL-methionine-2-C^{14} has been measured. During a 30-minute interval, the greatest amount of variability in uptake of L-methionine occurred in pancreas, liver, bone marrow and thyroid.

Significantly greater concentrations of radiocarbon were present in brain, lungs, liver, and heart of rats fed L-methionine-methyl-C^{14} than in those tissues of rats fed the DL- form of this amino acid. The concentration of the methyl carbon of methionine in liver was significantly greater than that of the *alpha*-carbon.

Recent investigations have suggested that the absorption of the D- and L- isomers of amino acids may differ. Gibson and Wiseman (1) observed that the rate of disappearance of the L- isomers of several amino acids was greater than that of the corresponding D- enantiomorphs when solutions of DL- amino acids were placed in isolated loops of rat small intestine. In these experiments, the absorption of the L- isomer of methionine was 1.6 times greater than that of the D- amino acid. Delhumeau and co-workers (2) have established the pattern of absorption *in situ* of individual amino acids from mixtures of 18 L- amino acids introduced into sacs of rat intestine.

Taylor and co-workers[1] have studied the intestinal absorption of methionine in chickens with Thiry-Vella fistulas. In their studies, L-methionine was absorbed more rapidly than its D- isomer.

In human small intestine, also, the naturally occurring L- isomer of amino acids disappears more rapidly than the D-form (3). Orten[2] has established the pattern of absorption from an equimolar solution of 18 amino acids in man. The rate of absorption of DL-methionine from human intestine during a 30-minute period has been measured by Cummings (4).

One phase of our studies of the metabolism of methionine has involved an investigation of the absorption of this amino acid in the intact rat. Both L- and DL-methionine-methyl-C^{14} were used in these experiments. In addition, we investigated the uptake of L- and DL-methionine by 18 tissues in these rats. Similar experiments were conducted with DL-methionine-2-C^{14} to permit comparison of tissue uptakes of the *alpha*- and methyl carbons of the amino acid.

EXPERIMENTAL

Male, adult rats of Wistar Strain, 6 months $\pm$ 2 weeks of age, were secured

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[1] Taylor, M. W., H. J. Newman and C. M. Paine 1959 Intestinal absorption of methionine and histidine by the chicken. Federation Proc., *18:* 548 (abstract).

[2] Orten, A. U. 1961 Absorption of amino acids and sugars from a Thiry loop in man. Federation Proc., *20:* 2 (abstract).

from the Nutrition Laboratory of Iowa State University of Science and Technology. L-Methionine-$C^{14}H_3$, DL-methionine-2-C^{14} and DL-methionine-$C^{14}H_3$ were obtained commerically.[3] The specific activities of these compounds were 3.8, 0.7, and 4.5 mc/mmole, respectively. Paper chromatograms of the amino acids developed in methyl-ethyl ketone indicated that the radioactivity was present as methionine.

Following a 10-hour fast, 15 animals were fed by stomach tube 0.5 ml of an aqueous solution containing 18 mg of the amino acid. The stomach tube was rinsed with 0.5 ml of distilled water. So that the same concentration of radiocarbon, 9.2 million count/min would be administered to all groups, nonradioactive L- or DL-methionine was added to an appropriate amount of methionine-$C^{14}H_3$ to provide a total of 18 mg.

The rats were placed individually in a plastic cage with wire mesh floor and were not restrained. They were killed 30 minutes after the feeding of the radioactive material. After light ether anesthesia, the heart was exposed and the animal was exsanguinated by cardiac puncture. The tissues, including the gastrointestinal tract, were removed quickly.

The esophagus was tied with silk thread close to the stomach; the stomach was separated from the gastrointestinal tract. The small intestine was cut into 3 segments of approximately 35 cm each. The contents of the stomach, 3 segments of small intestine, the cecum, and large intestine were expressed individually into calibrated tubes. The inner walls of the tissues were rinsed with 3 successive aliquots of distilled water which were added to vials containing the tissue contents. These liquids were made to known volumes, and carefully shaken. Aliquots were applied to filter paper, dried, and counted with a gas-flow counter.

The absorbed dose was calculated as the difference between the radiocarbon administered and that remaining in the gastrointestinal tract.

Uniform sections of tissues were cut and placed on aluminum planchets. These were desiccated for 4 days and their activities measured with a gas-flow counter. Corrections for background and self-absorption were made. Specific activities of the tissues were calculated as the percentage of absorbed dose in counts per minute per gram of dry tissue.

One hundred-microliter aliquots of whole blood were placed on filter paper, dried, and counted with a gas-flow counter. The total radioactivity present in blood was calculated therefrom.

Other blood samples were centrifuged to separate plasma and red cells. The red cells were washed 3 times with physiological saline. Plasma proteins were precipitated with acetone, plated on filter paper discs, desiccated and counted. Aliquots of whole plasma, protein-free plasma, and red cells were applied to filter paper for counting. All measurements of radioactivity were made with a gas-flow counter. The measured activities were expressed as counts per minute per 100 microliters of sample adjusted to 9.2 million count/min fed.

Two-dimensional chromatograms of the contents of the 6 segments of the gastrointestinal tract, protein-free plasma, hydrolyzed whole plasma, and water-soluble, hexane-soluble, and hydrolyzed protein fractions of all tissues were run in water-saturated phenol and butanol:propionic acid:water. The procedures for chromatography and fractionation of tissues have been described in previous publications (5, 6). The label was on methionine in all segments of the gastrointestinal tract. In tissues and plasma, although metabolic activity had resulted in the formation of other radioactive compounds within the 30-minute interval of the experiment, the major portion of the activity was present as methionine.

RESULTS AND DISCUSSION

Absorption of L- *and* DL-*methionine.* In table 1, the concentrations of L- and DL-methionine-methyl-C^{14} absorbed by adult rats are presented. The means of the absorbed doses of groups receiving L- or DL-methionine-methyl-C^{14} were 94.6 and 90.2%, respectively. The standard deviations were 2.61 and 3.81, for the L- and DL-methyl-labeled compounds, respectively.

[3] The L-methionine-$C^{14}H_3$ and DL-methionine-2-C^{14} were purchased from Tracerlab, Inc., Waltham, Massachusetts, and the DL-methione-$C^{14}H_3$ from the Isotopes Specialities Company, Burbank, California.

TABLE 1

Absorption of uniform doses of L- *and* DL-*methionine-methyl-*C^{14}

L-Methionine-$C^{14}H_3$		DL-Methionine-$C^{14}H_3$		D-Methionine-$C^{14}H_3$[1]
Rat no.	Absorbed	Rat no.	Absorbed	Absorbed
	%		%	%
1	92.2	7	92.7	90.8
2	97.3	8	93.0	91.4
3	91.4	9	85.7	76.8
4	96.0	10	93.8	93.0
5	97.2	11	85.2	75.8
6	93.2	12	90.6	86.6
	Mean 94.6		Mean 90.2	Mean 85.7
	SD 2.61		SD 3.81	
	$t = 2.34$[2]			$t = 2.71$

[1] Calculated.
[2] $t_{0.025} = 2.23$.

The difference between groups was significant at the 5% level. Using the data in table 1, the absorption of D-methionine in 30 minutes is calculated as 85.7%.

If D- amino acid isomers are absorbed much more slowly by intestinal mucosa than are their enantiomorphs, this would be evidence for an "active" mechanism of absorption which favors passage of L-amino acids into the body. The relatively slower absorption of the D-isomers and the time required for inversion are factors that would be expected to reduce their value for protein synthesis.

Considering the amount of radiocarbon remaining in the alimentary tract, the quantities of L- and DL-methionine absorbed during the 30-minute interval were 17.0 and 16.2 mg, respectively.

Distribution of L- *and* DL-*methionine in blood.* After 30 minutes, significantly greater levels of radiocarbon from L-methionine than from DL-methionine were present in whole blood (table 2). The concentration of D-methionine in blood after 30 minutes is calculated as 0.14×10^5 count/min, a small fraction of that administered to the rat.

The distribution of activity in whole plasma, protein-free plasma, red cells, and plasma proteins of these rats after 30 minutes is shown in figure 1. As would be expected, larger concentrations of radiocarbon were observed in blood plasma than in red cells. Although red cells were triple-washed, it is possible that some of their activity was due to absorbed methionine, since in previous experiments we found little activity in heme and globin 30 minutes after feeding radioactive methionine (6).

TABLE 2

Radiocarbon in blood of rats fed L- *and* DL-*methionine-methyl-*C^{14}

L-Methionine-$C^{14}H_3$		DL-Methionine-$C^{14}H_3$	
Rat no.	Total count/min in blood $\times 10^{-5}$	Rat no.	Total count/min in blood $\times 10^{-5}$
1	2.25	7	0.84
2	3.16	8	1.13
3	2.89	9	1.14
4	2.56	10	1.05
5	1.02	11	1.42
6	1.11	12	1.34
	Mean 2.16		Mean 1.15
	SD 0.21		SD 0.91
	$t = 2.66$[1]		

[1] $t_{0.025} = 2.23$.

Substantially less radiocarbon supplied by the DL- in contrast with the L- form of methyl-labeled methionine was present in whole plasma after 30 minutes, suggesting more rapid elimination via the lungs or kidneys, or both, or higher tissue uptakes. Within 30 minutes, 0.90% of the absorbed DL-methionine-$C^{14}H_3$ had been lost as $C^{14}O_2$, whereas only 0.20% of the L- amino acid had been so metabolized. The calculated loss of D-methionine-methyl-C^{14} as $C^{14}O_2$ during the 30-minute interval is 1.6%.

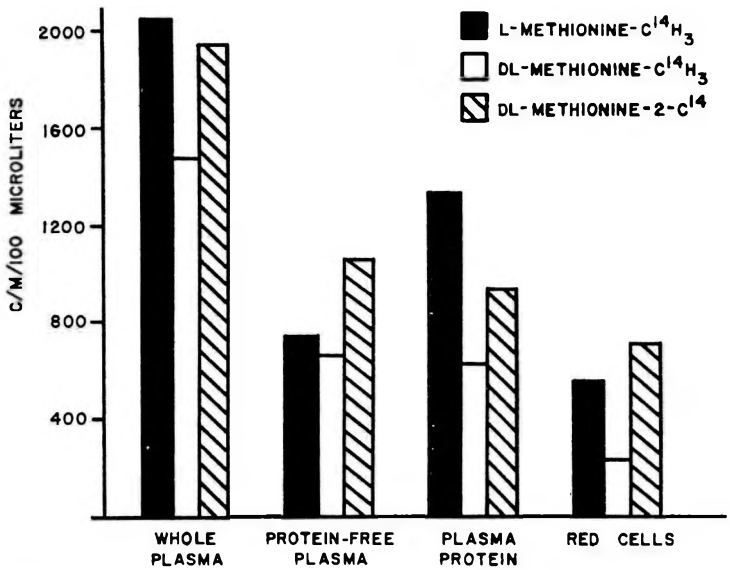

Fig. 1 Radiocarbon from uniform doses of L- and DL-methionine-methyl-C^{14} and DL-methionine-2-C^{14} in plasma and red cells.

The concentration of radiocarbon originating from the *alpha*-carbon labeled DL-amino acid was greater than that from methyl-carbon labeled DL-methionine in whole plasma after 30 minutes. This is interpreted to indicate more rapid oxidation of the methyl carbon to $C^{14}O_2$.

Differences in plasma concentrations of L- and DL-methyl-labeled methionine were due to the presence of significantly greater amounts of L- isomer-derived radiocarbon in plasma protein. Although larger amounts of the *alpha*-carbon of the amino acid were present in protein-free plasma, plasma protein also accepted more radiocarbon from this source than that arising from the methyl carbon of the DL- isomer of the amino acid.

Tissue uptake of L- *and* DL-*methionine.* In table 3, data are presented relating to the concentrations of radiocarbon from L- and DL-methionine-methyl-C^{14} and DL-methionine-2-C^{14} in 18 tissues of these adult rats after 30 minutes. The standard deviations indicate that the greatest amount of variability in the absorption of the methyl carbon of L-methionine occurred in pancreas, liver, bone marrow and thyroid.

The t values,[4] reflecting the amount of variation between means of tissues of groups of rats fed L- or DL-methionine, are shown in column 6 of table 3. Significantly greater concentrations of radiocarbon from L-methionine-methyl-C^{14} were taken up by brain, liver, heart, and lungs in contrast to that from the DL- isomer.

The conversion of both isomers of methionine to *alpha*-keto-*gamma*-methiolbutyric acid by the respective amino acid oxidases has been demonstrated (7, 8). Thus, the ability of D-methionine to support growth may be accounted for by its oxidation to *alpha*-keto-*gamma*-methiolbutyrate and subsequent transamination to L-methionine. D-Methionine can be utilized for growth in the mouse (9), rat (10–12), and for maintenance of nitrogen equilibrium in man (13).

Similar comparisons of responses of rats to the DL-*alpha*-carbon-labeled and methyl-labeled methionine (table 3) indicate that the liver accumulated significantly larger concentrations of the methyl carbon of methionine than the *alpha*-carbon ($t = 7.57$).

Concentrations of the *alpha*-carbon of methionine were strikingly similar to

[4] $$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(n_1-1)s^2 + (n_2-1)s_2^2}{n_1+n_2-2}} \times \sqrt{1/n_1 + 1/n_2}}.$$

TABLE 3

Tissue uptakes of L- *and* DL-*methionine-methyl-*C^{14} *and* DL-*methionine-2-*C^{14}

Tissue	L-Methionine-$C^{14}H_3$	DL-Methionine-$C^{14}H_3$	t^1	DL-Methionine-2-C^{14}	t^2
	% absorbed dose/g tissue	% absorbed dose/g tissue		% absorbed dose/g tissue	
Adrenal	1.79 ± 0.51[3]	1.70 ± 0.53[3]	0.30	1.79 ± 0.31[3]	0.26
Bone marrow	3.65 ± 0.75	4.57 ± 1.24	1.56	3.79 ± 1.26	0.88
Brain	0.84 ± 0.08	0.62 ± 0.10	4.40	0.60 ± 0.14	0.26
Heart	0.92 ± 0.18	0.69 ± 0.11	2.56	0.60 ± 0.17	0.98
Kidney	2.78 ± 0.51	2.33 ± 0.49	1.55	2.05 ± 0.52	0.79
Liver	4.06 ± 0.85	3.10 ± 0.33	2.59	1.22 ± 0.38	7.57
Lungs	2.42 ± 0.24	1.64 ± 0.41	3.76	1.80 ± 0.07	0.64
Muscle	0.53 ± 0.15	0.41 ± 0.05	1.71	0.48 ± 0.04	1.71
Pancreas	6.22 ± 1.95	5.00 ± 0.60	1.47	5.55 ± 0.65	1.25
Pituitary	3.29 ± 0.64	3.01 ± 0.48	0.88	3.72 ± 1.25	1.30
Prostate	1.02 ± 0.53	0.90 ± 0.27	0.45	1.10 ± 0.45	0.81
Seminal vesicle	0.67 ± 0.44	0.35 ± 0.51	1.19	0.20 ± 0.03	0.49
Skin	0.11 ± 0.11	0.24 ± 0.15	1.49	0.24 ± 0.22	0
Spleen	1.26 ± 0.15	1.08 ± 0.18	2.00	1.05 ± 0.16	0.25
Submaxillary	1.23 ± 0.12	1.05 ± 0.17	2.00	1.10 ± 0.34	0.31
Testes	1.60 ± 0.30	1.19 ± 0.34	2.16	1.74 ± 0.47	2.04
Thymus	1.25 ± 0.66	1.45 ± 0.22	0.64	1.15 ± 0.58	1.08
Thyroid	1.99 ± 0.73	1.43 ± 0.47	1.60	1.75 ± 0.39	1.00

$t_{0.025} = 2.23$ and is the value of t at the 5% level of significance.
[1] Compares means of L- and DL-methionine-methyl-C^{14}.
[2] Compares means of DL-methionine-methyl-C^{14} and DL-methionine-2-C^{14}.
[3] Mean ± SD.

those of the methyl carbon in other tissues. This observation reflects the role of hepatic tissue as the site for demethylation of methionine and suggests that the methyl group accumulates in liver whereas the methionine residue containing the *alpha*-carbon is quickly dispatched to other tissues. Approximately 6 out of every 10 methionine-derived methyl carbons in liver appear to be independent of methionine.

Larger concentrations of the residue containing the *alpha*-carbon of methionine, in contrast with its methyl carbon, were present in plasma proteins at the end of 30 minutes (fig. 1). It appears, then, that the demethylated residue containing the *alpha*-carbon of methionine accepts a methyl group from the metabolic pool prior to its incorporation into tissue protein, possibly at the point of its incorporation into plasma protein in the liver.

Relatively large concentrations of radiocarbon (6.22 and 5.00% for L- and DL-methionine, respectively) were absorbed at this early interval by pancreas. After 24 hours the mean value for pancreas decreased to 1.63% in groups fed L- and DL-methionine.

An analysis of variance was made on the data in table 3. The F value of 9.69 ($F_{0.05} = 4.9646$) indicated that there were significant differences between the true uptakes of L- and DL-methionine by these rats. After their absorption from the alimentary tract, it appears, therefore, that the response of the rat to L-methionine is significantly different from that to the DL- form.

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LITERATURE CITED

1. Gibson, Q. H., and G. Wiseman 1951 Selective absorption of stereo-isomers of amino acids from loops of the small intestine of the rat. J. Biochem., *48:* 426.
2. Delhumeau, G., G. V. Pratt and C. Gitler 1962 The absorption of amino acid mixtures from the small intestine of the rat. I. Equimolar mixtures and those simulating egg albumin, casein and zein. J. Nutrition, *77:* 52.
3. Kuroda, Y., and N. S. Gimbel 1954 Selective disappearance of stereoisomers of amino

acids from the human small intestine. J. Applied Physiol., 7: 148.
4. Cummings, A. J. 1952 Absorption of glucose and methionine from the human intestine; the influence of glucose concentration in the blood and in the intestinal lumen. J. Clin. Invest., *31:* 928.
5. Edwards, C. H., E. L. Gadsden, L. P. Carter and G. A. Edwards 1959 Paper chromatography of amino acids and other organic compounds in selected solvents. J. Chromatog., 2: 188.
6. Edwards, C. H., E. L. Gadsden and G. A. Edwards 1960 Utilization of methionine by the adult rat I. Distribution of the *alpha*-carbon of DL-methionine-2-C^{14} in tissues, tissue fractions, expired carbon dioxide, blood, and excreta. J. Nutrition, 72: 185.
7. Green, D. E., D. H. Moore, V. Nocito and S. Ratner 1944 A flavoprotein enzyme. J. Biol. Chem., *156:* 383.
8. Waelsch, H., and E. Borek 1939 The stability of the keto acid from methionine. J. Am. Chem. Soc., *61:* 2252.
9. Bauer, C. D., and C. P. Berg 1943 The amino acids required for growth in mice and the availability of their optical isomers. J. Nutrition, *26:* 51.
10. Rose, W. C. 1937 The nutritive significance of the amino acids and certain related compounds. Science, *86:* 298.
11. Wretlind, K. A., and W. C. Rose 1950 Methionine requirement for growth and utilization of its optical isomers. J. Biol. Chem., *187:* 697.
12. Jackson, R. W., and R. J. Block 1932 Metabolism of D- and L-methionine. Proc. Soc. Exp. Biol. Med., 30: 587.
13. Rose, W. C. 1949 Amino acid requirements of man. Federation Proc., 8: 546.

Effect of Barbituric Acid and Chlortetracycline upon Growth, Ammonia Concentration, and Urease Activity in the Gastrointestinal Tract of Chicks[1]

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ABSTRACT Experiments were conducted with a total of 38 lots of 15 chicks. Preliminary data from 22 lots indicate that the addition of either barbituric acid (1.28 g/kg) or chlortetracycline (0.1 g/kg) will enhance 4-week gains with diets containing either soybean meal or vitamin-free casein. An experiment with 16 lots fed a crude casein diet showed enhanced growth of 17% with barbituric acid and 25% with chlortetracycline. The combination improved gain 26%. At the time these chicks were killed, they had lowered gastrointestinal ammonia concentrations. Urease activity was also lowered in birds receiving these supplements but activity in the small intestine was variable. The data show that a cyclic urea compound can produce increased growth coincidently with decreased ureolytic activity and ammonia concentration in the gastrointestinal tract.

A number of investigators have postulated that antibacterial agents increase rate and efficiency of growth in animals and birds by suppressing production of toxic substances by gastrointestinal bacteria. Ammonia has been suggested as one of these toxins (1–5). Work at this laboratory has shown that the addition of 100 ppm of either chlortetracycline, penicillin, or arsanilic acid to a casein diet decreased *in vivo* hydrolysis of urea-C^{14} by rats and reduced *in vitro* urea splitting ability of the gastrointestinal contents (6). Gastrointestinal urease activity and *in vivo* urea hydrolysis have been reduced coincidently with increased growth and efficiency of feed utilization following active immunization with crystalline jackbean urease (7, 8).

The apparent relation of urea hydrolysis to growth and nutrition of the host, demonstrated under the conditions of the above experiments, prompted us to investigate the usefulness of chemical antagonists for studies relating gastrointestinal urease activity and its ammonia producing potential to growth of the host.

Much of the evidence that urea is not metabolically inert in simple-stomach animals exposed to a conventional environment has been reviewed (6, 9). Uric acid can be degraded in the gastrointestinal tract by bacterial enzymes to compounds including urea and thus serves indirectly as a substrate for urease and a source of ammonia (10).

Gray et al. (11, 12) have observed that certain cyclic urea compounds were capable of inhibiting bacterial and jackbean urease. In preliminary work in this laboratory, a structurally related compound, barbituric acid (malonylurea), depressed urease activity in the gastrointestinal content of chicks without impairment of appetite or growth when fed at a level of 1.28 g/kg of diet.[3] Ruminants, on the other hand, fed urea as the only source of nitrogen and supplemented with the same level of barbituric acid, lost weight. These animals showed depressed cellulolytic activity in their ruminal fluid (13). Barbituric acid has been found to be nondiabetogenic and nonhypnotic (14, 15).

The effects of barbituric acid and chlortetracycline on chick growth, when either or both were added to a purified diet fed for 4 weeks, are reported in the present paper. Data on gastrointestinal ammonia concentration and urease activity are also presented.

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[3] Visek, W. J., A. I. Jacobson, M. E. Iwert and A. P. Alvares 1961 Depression of urease activity in the gastrointestinal tract by chemical agents. Federation Proc., 20: 370 (abstract).

EXPERIMENTAL

One-day-old cockerels of the Vantress-Arboracre cross having an initial weight of 36 to 40 g were randomly divided into lots of 15. The chicks were housed throughout the 28-day experimental periods in heated battery-type cages with wire floors. Feed and water were supplied ad libitum. Body weights and feed consumption were determined at weekly intervals. Several preliminary experiments were conducted using basal diets containing soybean meal or vitamin-free casein (table 1). After completion of the preliminary experiments, the main experiment was conducted with the basal diet (diet 1) of Stokstad et al. (16) (table 2). In all studies with the exception of those with soybean meal, experimental diets had the same composition as the basal diet except for additions in grams per kilogram as follows: Diet 2, barbituric acid, 1.28; diet 3, chlortetracycline, 0.1; and diet 4, barbituric acid, 1.28, plus chlortetracycline, 0.1.

At the end of the 28-day experimental period chicks from the experiments using casein diets were fasted for 4 hours. Ten randomly selected chicks from each lot were killed for removal of their intestinal tracts plus contents. The small intestine and large intestine were separated, weighed individually, and combined by lot in appropriately marked bottles containing saline held at 4°C. The specimens were stored in this manner, homogenized, and analyzed for ammonia and urease activity using an aliquot of the pooled homogenate. The total homogenates of the small intestines and large intestines were diluted to 400 and 200 ml, respectively. Free ammonia was determined in triplicate on a 2.0-ml aliquot of homogenate following addition of 2.0 ml phosphate buffer (pH 7.0) and incubation for 5 minutes at 37.5°C. The reaction was stopped with one ml 1 N HCl and the mixture was distilled in a micro-Kjeldahl apparatus with collection of the liberated ammonia in 2.0% boric acid. The ammonium borate was titrated with 0.0125 N HCl using bromcresol green-methyl red indicator. Urease activity was determined indirectly by difference on a separate aliquot using the same procedure except that the phosphate buffer contained 3.0% urea. The latter method is that of Sumner (17) with incubations carried out at 37.5°C instead of 20°C. All of the incubations of ammonia and urea-splitting activity were completed within 72 hours after the chicks were killed. Following incubation and stoppage of the reaction with acid, the samples were frozen in a slurry of dry ice and ethanol. They were stored at − 10°C until completion of the ammonia determinations. A 20-ml aliquot was used for dry weight determinations.

RESULTS

Preliminary data (table 1) involving several experiments indicate increased growth when barbituric acid was added to diets containing either soybean or casein as the protein source. Data upon ammonia concentration and ureolytic activity of the gastrointestinal tracts from the casein-fed birds suggest that ammonia production and urea hydrolysis may be suppressed by this cyclic urea compound. This suppression concurrently with a stimulation in growth was also observed with chlortetracycline.

To obtain further evidence upon the effects observed in these preliminary experiments a study was conducted using 4 lots/treatment. The growth data are presented in table 2. During the first 2 weeks, growth was increased 6% by barbituric acid and 24% by chlortetracycline ($P < 0.05$). The data on efficiency of feed utilization are lot averages obtained by dividing the total weight gain per lot by the total feed consumed. Efficiency of feed utilization was higher during this period and the difference due to chlortetracycline was statistically significant ($P < 0.05$).

Barbituric acid influenced growth to a greater extent during the second 2-week period by increasing body weight 9% over that of the basal groups ($P < 0.05$). This improvement occurred when barbituric acid was fed alone, and no increase in growth was noted when chlortetracycline was combined with barbituric acid. Chlortetracycline increased growth by 13% ($P < 0.01$) during this period. Weight gain per gram of feed consumed was higher in all supplemented groups but the differences were not statistically significant.

TABLE 1

Summary of preliminary investigations with cockerels fed diets supplemented with barbituric acid or chlortetracycline for 4 weeks

	Additive[1]	Weight gain	Gain g/g feed
		g	
	Soybean protein diet[2]		
1	Basal (6)[3]	299.3 ± 9.4[4]	0.52 ± 0.01
2	Barbituric acid (4)	323.7 ± 13.0	0.53 ± 0.05
3	Chlortetracycline (8)	357.0 ± 12.4	0.56 ± 0.01
	Vitamin-free casein diet[5]		
1	Basal (2)[3]	289.6 ± 17.7	0.52 ± 0.01
2	Barbituric acid (2)	312.7 ± 11.6	0.55 ± 0.01
3	Chlortetracycline (2)	335.9 ± 1.6	0.58 ± 0.02
4	Barbituric acid + chlortetracycline (2)	325.8 ± 27.8	0.53 ± 0.04

		Ammonia concentration		Urea hydrolysis	
		Small intestine	Large intestine	Small intestine	Large intestine
		μg/g wet weight		*μg/g wet weight*	
1	Basal (2)[3]	721 ± 48	473 ± 57	1178 ± 78	3594 ± 26
2	Barbituric acid (2)	823 ± 36	566 ± 52	837 ± 34	3294 ± 89
3	Chlortetracycline (2)	440 ± 65	496 ± 26	817 ± 101[6]	3334 ± 37
4	Barbituric acid + chlortetracycline (2)	767 ± 36	280 ± 54	841 ± 19[6]	3016 ± 253

[1] Barbituric acid, 1.28 g/kg diet; chlortetracycline, 0.1.
[2] Solvent-extracted soybean meal, 50% protein, 40; sucrose, 48.3; corn oil plus 0.02% antioxidant, 3.0; cellulose (Cellu Flour, Chicago Dietetic Supply House, Chicago), 3.0; minerals, 5.43 and vitamin mixture, 0.27 (mineral and vitamin mixture described by Morrison et al. (21).
[3] Number of lots indicated in parentheses.
[4] Mean ± SE.
[5] Vitamin-Free Casein, General Biochemicals, Inc., Columbus, Ohio; proportion of ingredients according to Stokstad et al. (16).
[6] Chlortetracycline vs. no chlortetracycline significant at $P < 0.05$.

TABLE 2

Body weight gain and efficiency of feed utilization of cockerels fed crude casein basal diet supplemented with barbituric acid or chlortetracycline, or both

	Diet[1]	Weight gain[2]		
		0–2 weeks	2–4 weeks	0–4 weeks
		g	*g*	*g*
1	Basal	73.8 ± 9.1[3]	164.0 ± 18.2	237.8 ± 12.9
2	Barbituric acid	83.7 ± 3.5	194.1 ± 4.6[4]	277.8 ± 3.9[4]
3	Chlortetracycline	97.0 ± 4.3[5]	200.8 ± 8.9[6]	297.8 ± 4.5[6]
4	Barbituric acid + chlortetracycline	97.9 ± 8.7[5]	202.9 ± 11.0[4,6]	300.8 ± 11.1[4,6]
		Gain g/g feed		
1	Basal	0.43 ± 0.01	0.50 ± 0.02	0.48 ± 0.02
2	Barbituric acid	0.50 ± 0.02	0.55 ± 0.02	0.53 ± 0.01
3	Chlortetracycline	0.54 ± 0.02[5]	0.53 ± 0.01	0.53 ± 0.01
4	Barbituric acid + chlortetracycline	0.55 ± 0.02[5]	0.54 ± 0.02	0.54 ± 0.01

[1] Diet 1, basal: crude casein (National Casein Company, Chicago), 20; gelatin, 8; sucrose, 61.6; bone ash, 2; calcium gluconate, 5; salt mixture, 1.4; and vitamin (crystalline vitamins, Merck Institute for Therapeutic Research, Rahway, New Jersey, and Distillation Products Company, Rochester, New York) and choline mixture, 2. Diet 2, basal plus barbituric acid, 1.28 g/kg. Diet 3, basal plus chlortetracycline (Lederle Laboratories, Pearl River, New York), 0.1 g/kg. Diet 4, barbituric acid, 1.28 g/kg plus chlortetracycline, 0.1 g/kg.
[2] Average initial weight, g, by groups were: Diet 1, 36.0 ± 0.4; diet 2, 35.9 ± 0.4; diet 3, 35.7 ± 1.1; diet 4, 35.8 ± 0.2.
[3] Mean ± SE.
[4] Barbituric acid vs. no barbituric acid significant at $P < 0.05$.
[5] Chlortetracycline vs. no chlortetracycline significant at $P < 0.05$.
[6] Chlortetracycline vs. no chlortetracycline significant at $P < 0.01$.

Gain in body weight for the experimental period of 4 weeks was increased by 8% over control with the addition of barbituric acid ($P < 0.05$) and 16% with chlortetracycline ($P < 0.01$). When barbituric acid was the only additive, growth was improved by an average of 40 g (17%) over the basal diet, whereas chlortetracycline showed an increase of 60 g (25%). The combined effects of the agents on growth were not additive, there being only 63 g (26%) more growth than control with the two in combination. These data suggest that barbituric acid and chlortetracycline influence the same system(s) that affect growth.

Gastrointestinal ammonia concentration and ureolytic activity are expressed in micrograms per gram, wet weight (table 3). The overall effect of barbituric acid upon ammonia concentration in the small intestine was a depression of 26% and for chlortetracycline 18%. When fed in combination the suppression was 40% ($P < 0.05$) as compared with the basal group. Less effect was observed in the large intestine with chlortetracycline (5%); however, the reduction due to barbituric acid was 36%.

The effect on ureolytic activity was not as consistent for the small intestine as for ammonia concentration. Barbituric acid inhibited urea hydrolysis by 53% ($P < 0.05$), whereas supplementation with chlortetracycline increased urease activity 15% over the basal groups. When fed in combination the agents reversed the effect of chlortetracycline and produced a suppression of 64% ($P < 0.05$) as compared with that of the basal group (diet 1). The ureolytic activity in the large intestine was suppressed by chlortetracycline (14%) but not by barbituric acid. Thus, it appears that urease activity and ammonia production may be altered concurrently with increased growth.

DISCUSSION

The results of these experiments demonstrate that free ammonia concentration in both the large and small intestine of chicks can be depressed by supplementation of a casein basal diet with barbituric acid, alone or in combination with chlortetracycline. This reduction in ammonia concentration with antibiotics has been reported by others, but a direct correlation between ammonia concentration and growth could not be demonstrated (2, 18). The fact that barbituric acid, suggested to be an antimetabolite of uracil (19), can produce a reduction in ammonia concentration and urease activity concurrently with improved weight gain gives credence to the contention that ammonia production in the gastrointestinal tract may be related to growth and efficiency of food utilization and suggests that antibacterial agents enhance growth, in part, by affecting this system (6, 7, 18). Complete inhibition of urease activity did not occur under the conditions of these experiments and the degree of this inhibition which is optimal for growth has not been deter-

TABLE 3

Ammonia concentration and hydrolysis of urea by gastrointestinal contents of 4-week-old cockerels fed a casein diet supplemented with barbituric acid or chlortetracycline, or both (10 birds/lot; 4 lots/treatment)

Diet[1]		Small intestine		Large intestine	
		μg NH_3/g wet wt	*μg urea split/g wet wt*	*μg NH_3/g wet wt*	*μg urea split/g wet wt*
1	Basal	1151 ± 25[2]	746 ± 61	2113 ± 338	3279 ± 260
2	Barbituric acid	902 ± 150	784 ± 131[3]	1348 ± 73[3]	3043 ± 245
3	Chlortetracycline	1000 ± 82	1496 ± 43	2011 ± 87	2614 ± 401
4	Barbituric acid + chlortetracycline	685 ± 129	268 ± 52[3]	1273 ± 300[3]	2850 ± 485

[1] Diet 1, basal; diet 2, barbituric acid (1.28 g/kg); diet 3, chlortetracycline (0.1); diet 4, barbituric acid (1.28) plus chlortetracycline (0.1).
[2] Mean ± SE.
[3] Average of groups fed barbituric acid vs. no barbituric acid statistically significant ($P < 0.05$).

mined. The fact that barbituric acid and chlortetracycline in combination failed to produce greater growth than either agent alone also suggests that there is an optimal level and that they may be working through a common system. The possibility that this cyclic compound may have multiple actions upon the gastrointestinal bacteria has not been excluded (13, 19, 20).

The evidence brought forth in this paper demonstrates a third method, dietary supplementations with a cyclic urea derivative, by which ammonia concentration and urease activity of the gastrointestinal contents may be altered concurrently with increased growth of the host.

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LITERATURE CITED

1. Dintzis, R. Z., and A. B. Hastings 1953 The effect of antibiotics on urea breakdown in mice. Proc. National Academy of Sciences, 39: 571.
2. Francois, A. C., and M. Michel 1955 Action de la penicillin et de l'aureomycine sur les proprietes de la flore intestinale du porc. C. R. Acad. Sci., 240: 124.
3. Michel, M., and A. C. Francois 1955 Relation entre l'influence des antibiotiques sur la croissance du porc et l'inhibition des desaminases de la flore intestinale. Ibid., 240: 808.
4. Kornberg, H. L., and R. E. Davies 1955 Gastric urease. Physiol. Rev., 35: 169.
5. György, P. 1957 Nutrition and intestinal flora in man. Ann. Nutrition Alimentation, 11: A189.
6. Visek, W. J., J. M. Baron and D. M. Switz 1959 Urea metabolism and intestinal ureolytic activity of rats fed antimicrobial agents. J. Pharm. Therap., 126: 359.
7. Dang, H. C., and W. J. Visek 1960 Effect of urease injection on body weights of growing chicks and rats. Proc. Soc. Exp. Biol. Med., 105: 164.
8. Harbers, L. H., H. N. Bass, R. F. Hendrickson and W. J. Visek 1962 Effect of urease immunity upon rats fed vitamin A deficient diets. J. Animal Sci., 21: 998.
9. Visek, W. J. 1962 Urea hydrolysis in birds and mammals. Am. J. Vet. Res., 23: 569.
10. Sørensen, L. B. 1959 Degradation of uric acid in man. Metabolism, 8: 687.
11. Gray, C. T., J. C. Gerhart and M. S. Brooke 1959 Inhibition of urease by alloxan and alloxanic acid. Nature (London), 184: 1936.
12. Gray, C. T., M. S. Brooke and J. C. Gerhart 1961 Metabolism of alloxanic acid in a soil microorganism. J. Bacteriol., 81: 755.
13. Harbers, L. H., A. D. Tillman and W. J. Visek 1962 Effect of barbituric acid on urea diets for ruminants. J. Animal Sci., 21: 754.
14. Goldner, M. G., and G. Gomori 1944 Studies on the mechanism of alloxan diabetes. Endocrinology, 35: 241.
15. Goodman, L. S., and A. Gilman 1956 The Pharmacological Basis of Therapeutics, ed. 2. Macmillan Company, New York, New York, p. 124.
16. Stokstad, E. L. R., T. H. Jukes and W. L. Williams 1953 The growth-promoting effect of aureomycin on various types of diets. Poult. Sci., 32: 1054.
17. Sumner, J. B. 1926 The isolation and crystallization of the enzyme urease. J. Biol. Chem., 69: 435.
18. Francois, A. C. 1956 First International Conference on Antibiotics in Agriculture. National Academy of Sciences — National Research Council, Washington, D. C., p. 84.
19. Woods, D. D. 1942 The mode of action of chemotherapeutic agents. Biochem. J., 36: 3.
20. Younathan, E. S. 1962 Inhibition of the citric acid cycle by alloxan. J. Biol. Chem., 237: 608.
21. Morrison, A. B., R. Dam, L. C. Norris and M. L. Scott 1956 Further evidence on the requirement of the chick for unidentified minerals. J. Nutrition, 60: 283.

Supplementation of Cereal Proteins with Amino Acids

V. EFFECT OF SUPPLEMENTING LIME-TREATED CORN WITH DIFFERENT LEVELS OF LYSINE, TRYPTOPHAN AND ISOLEUCINE ON THE NITROGEN RETENTION OF YOUNG CHILDREN[1,2]

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ABSTRACT A lime-treated corn diet was supplemented with two levels each of lysine and of tryptophan, as well as two levels of isoleucine with a constant lysine and tryptophan supplement. Comparison of corn protein with the FAO reference protein indicates tryptophan to be the first limiting amino acid and lysine the second. In 18 3-day balance periods in two children lysine supplementation alone somewhat improved nitrogen balance, but tryptophan addition had no effect. Analysis of 12 3-day balance periods in 4 children indicated that the addition of lysine to give 180 mg/g N in the diet (0.30% L-lysine·HCl) and of tryptophan to a total of 75 mg/ g N (0.28% DL-tryptophan) approximately doubled the percentage of nitrogen retention compared with the results when the basal diet was fed. Increasing the lysine to 270 mg/g N (0.56% L-lysine·HCl) or the tryptophan to 90 mg/g N (0.35% DL-tryptophan) either singly or together, did not improve significantly the nitrogen balance compared with that obtained with these amino acids at the lower levels of supplementation. The addition of isoleucine to 270 mg/g N (0.45% DL-isoleucine) to the 270 mg lysine/g N plus 25 mg tryptophan/g N resulted in a slightly higher nitrogen balance. The addition of isoleucine to a level of 225 mg/g N gave an intermediate value for nitrogen retention. None of the amino acid combinations used to supplement corn-masa resulted in as high nitrogen retention as those obtained from feeding isonitrogenous levels of milk. The variable results with supplementation of different cereals with essential amino acids to the level in the FAO reference protein provide further evidence that reference amino acid patterns should include both upper and lower limits for the amount of each amino acid per gram of nitrogen and specify the range of protein intake over which they apply.

The effect of amino acid supplementation of a food cannot always be predicted from comparison with a reference amino acid pattern. In many instances supplementation with the first limiting amino acid to the reference level will accentuate the deficiency of the second limiting amino acid in the protein. This was clearly shown in the studies of wheat flour supplementation of Bressani et al. (1) and Rosenberg (2). The latter concluded that the amount of the most limiting amino acid to add to a deficient protein for maximal response in nutritive value should just balance the second most limiting amino acid. Since at low levels, at least, protein intake affects amino acid requirement for maximal response (3), the optimal amount of the most limiting amino acid in a given experimental situation may also depend on the protein level.

Previous studies in children (4, 5) indicated that the nutritive value of lime-treated corn could be best improved by the simultaneous addition of lysine, tryptophan and isoleucine. Similar results have been reported for adults (6) and experimental animals (7–10). In our previous studies the levels for lysine, tryptophan and isoleucine used for supplementation of lime-treated corn were those of the amino acid pattern of the FAO reference protein (11). It was the purpose of this study to find

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TABLE 1

Age and weight of subjects at start of experiment and protein and caloric intakes during the experimental periods

Case no.	Age	Weight	Protein intake	Calorie intake/ kg/day
		kg	*g/kg/day*	*Cal.*
77	2 years, 10 months	8.06	3.0	100
82	1 year, 5 months	8.70	2.0	80
102	5 years, 2 months	15.76	2.0	100
112	5 years, 1 month	13.52	2.0	100
113	3 years	10.91	2.0	100

out whether lower levels of the 3 amino acids in combination would result in nitrogen retention values as high or higher than those obtained from the use of the levels of the FAO reference amino acid pattern.

MATERIALS AND METHODS

Two children were fed lime-treated corn diets supplemented with 2 different levels of lysine alone and of tryptophan alone. One of these and three additional children were used to study the effect of feeding lime-treated corn diets supplemented with different combination of lysine and tryptophan and of lysine, tryptophan and isoleucine. The age and weight of the subjects at the start of each experiment and their protein and caloric intakes during the experimental periods are shown in table 1.

In all cases nitrogen balance studies using previously discussed techniques (1, 4–5) were carried out with a 2-day adaptation period followed by two 3-day balance periods for subjects PC-77 and PC-82 and by three 3-day balance periods for subjects PC-82, 102, 112 and 113. The basal diet in grams per 100 g consisted of lime-treated corn, 85; corn gluten, 5; L-glutamic acid, 2; glycine, 3; and cornstarch, 5. The various amino acids were substituted for an equal amount of cornstarch, the nitrogen from these replacing glycine nitrogen so that all the diets remained isocaloric and isonitrogenous.

The basal diet contained 14.4% protein and 405 Cal./100 g. Its essential amino acid composition has been described previously (4). The test diets supplied the specific level of protein and part of the calories which were adjusted to the desired level of caloric intake by the addition of sugar and vegetable fat. The children were fed 3 times a day and a vitamin and mineral capsule given daily.[4] Two levels of lysine, tryptophan and isoleucine were added to the basal diet. One level brought the amount to that in the FAO reference protein (11). The other gave a 1:4 ratio of tryptophan to lysine and a 1:3 ratio of tryptophan and isoleucine using a lower level of tryptophan as the reference base rather than the amount in the FAO reference protein (11). Corrections for the D-form of the amino acids[5] were made by doubling the amount added. The amount of lysine was corrected for the hydrochloride molecule present in the form used.

At the end of each 3-day balance period, the pooled urine and feces collections and aliquots of the diet were each analyzed for nitrogen by the Kjeldahl method. Only averages for each treatment are given since the variations among 3-day periods with the same diet combination were similar to those previously reported (1, 4–5).

RESULTS

Table 2 presents the nitrogen balance results of the individual addition of 2 levels of lysine and 2 of tryptophan to the basal diet. With subject PC-77 tryptophan was tested first and with subject PC-82 lysine was the first supplement. Lysine alone at either level improved nitrogen balance, but tryptophan addition alone did not. Table 3 presents nitrogen balance results of the children fed combinations of lysine and tryptophan or of lysine, tryptophan and isoleucine.

The addition of lysine to give 180 mg/g N in the diet (0.30% L-lysine·HCl) and

[4] Gevral, donated by Lederle Laboratories, American Cyanamid Company.

[5] Donated by DuPont de Nemours, Wilmington, Delaware.

TABLE 2

Nitrogen balance of subjects PC-77 and 82 fed lime-treated corn supplemented with two levels each of lysine and tryptophan

Diet	No. of balances	Weight	Nitrogen				
			Intake	Fecal	Urinary	Absorption	Retention
		kg	*mg/kg/day*	*% of intake*		*% of intake*	*% of intake*
			Subject PC-77				
Milk	1	8.06	486	93	320	80.9	15.0
Basal	1	8.00	474	185	349	61.0	−12.7
Basal + DL-tryptophan[1]	3	8.00	466	101	368	78.3	0.6
Basal + DL-tryptophan[2]	3	8.20	482	116	337	75.9	6.0
Basal	2	8.50	479	111	346	76.8	4.6
Basal + L-lysine·HCl[3]	2	8.50	485	131	343	73.0	2.3
Basal + L-lysine·HCl[4]	2	8.80	479	110	305	77.0	13.4
			Subject PC-82				
Milk	2	8.70	392	45	295	88.5	13.3
Basal	2	8.50	320	56	273	82.5	−2.8
Basal + L-lysine·HCl[3]	2	8.50	320	45	238	85.9	11.6
Basal + L-lysine·HCl[4]	2	8.93	350	63	276	82.0	3.1
Basal	1	8.84	346	63	287	81.8	−1.2
Basal + DL-tryptophan[1]	2	8.79	342	49	305	85.7	−3.5
Basal + DL-tryptophan[2]	2	8.80	333	56	311	83.2	−10.2

[1] To give 75 mg tryptophan/g N.
[2] To give 90 mg tryptophan/g N.
[3] To give 180 mg lysine/g N.
[4] To give 270 mg lysine/g N.

TABLE 3

Average nitrogen balances of subjects PC-77, 102, 112 and 113 fed lime-treated corn supplemented with several combinations of lysine, tryptophan and isoleucine

Diet	No. of balances[1]	Nitrogen				
		Intake	Fecal	Urinary	Absorption	Retention
		mg/kg/day	*% of intake*		*% of intake*	*% of intake*
Milk	9	338	55.4	255.5	83.6 ± 0.6[2]	24.4 ± 2.9
Basal	12	337	77.8	317.8	76.9 ± 1.1	5.7 ± 3.1
Basal + DL-tryptophan + L-lysine·HCl[3]	12	334	66.8	279.2	80.0 ± 1.0	16.4 ± 1.3
Basal + DL-tryptophan + L-lysine·HCl[4]	10	342	72.2	281.5	78.9 ± 1.4	17.7 ± 1.9
Basal + DL-tryptophan + L-lysine·HCl[5]	6	330	59.7	271.6	81.9 ± 0.4	17.7 ± 2.9
Basal + DL-tryptophan + L-lysine·HCl[6]	10	333	66.9	278.1	79.9 ± 0.9	16.5 ± 1.2
Basal	14	367	82.9	353.4	77.4 ± 2.0	3.7 ± 4.2
Basal + DL-tryptophan + L-lysine·HCl[7] + DL-isoleucine	14	363	68.6	291.5	81.1 ± 1.0	19.7 ± 2.5
Basal + DL-tryptophan + L-lysine·HCl[8] + DL-isoleucine	14	364	74.6	289.4	79.5 ± 1.0	20.5 ± 2.5
Basal	6	308	57.9	256.9	81.2 ± 1.3	16.6 ± 3.4
Milk	9	363	55.2	277.7	84.8 ± 0.9	23.5 ± 1.3

[1] Each balance was of a 3-day duration.
[2] Mean ± SE.
[3] Total tryptophan, 75 mg/g N and total lysine, 180 mg/g N in diet.
[4] Total tryptophan, 75 mg/g N and total lysine, 270 mg/g N in diet.
[5] Total tryptophan, 90 mg/g N and total lysine, 180 mg/g N in diet.
[6] Total tryptophan, 90 mg/g N and total lysine, 270 mg/g N in diet.
[7] Total tryptophan, 75 mg/g N, total lysine, 270 mg/g N and total isoleucine, 225 mg/g N in diet.
[8] Total tryptophan, 75 mg/g N, total lysine, 270 mg/g N and total isoleucine, 270 mg/g N in diet.

of tryptophan to 75 mg/g N (0.28% DL-tryptophan) approximately doubled the percentage of nitrogen retention over the results with the basal diet. Neither increasing lysine to 270 mg/g N of diet (0.56% L-lysine·HCl) nor tryptophan to 90 mg/g N (0.35% DL-tryptophan) singly or together significantly improved nitrogen balance over that obtained at the lower levels of supplementation with these amino acids. The addition of isoleucine to 270 mg/g N (0.45% DL-isoleucine) to the 270 mg lysine/g N plus 75 mg tryptophan/g N resulted in slightly higher nitrogen balance than lysine and tryptophan supplementation alone. The addition of isoleucine to the lower level of 225 mg isoleucine/g N in the diet gave an intermediate value for nitrogen retention which was not significantly different from either. None of the amino acid combinations used to supplement corn-masa resulted in as high nitrogen retention values as those obtained from feeding isonitrogenous levels of milk.

DISCUSSION

Previous work by Scrimshaw et al. (4) and by Bressani et al. (5) indicated that the single addition of either tryptophan or lysine to the basal lime-treated corn protein used in these studies did relatively little to restore or improve the nitrogen balance observed with the basal diet, and it was difficult to conclude from the data which of the two amino acids was first limiting. The present results indicate that lysine is definitely more limiting than tryptophan in lime-treated corn protein since in both children lysine at either level of supplementation gave better nitrogen retention values than tryptophan. This is contrary to the conclusion to be drawn from comparison of the amino acid pattern of lime-treated corn with that of the FAO reference protein and suggests once again that the amount of tryptophan in the FAO reference protein is too high relative to the other amino acids present.

When the protein intake was higher as in subject PC-77, the response to the addition of tryptophan resulted in positive nitrogen balance, whereas at the lower level of protein intake, as in subject PC-82, the single addition of tryptophan resulted in a negative balance which worsened with increased tryptophan. Similar results have been obtained in experiments with rats (12–14). These results are interpreted to mean that at the lower level of intake the relative deficiency of lysine in the diet became greater when tryptophan was added, resulting in greater amino acid imbalance. The fact that the extent to which an amino acid is limiting is affected by protein intake is now well recognized (3, 15, 16)[6] and is one of the important qualifications to the use of reference amino acid patterns for amino acid supplementation studies of foods. As previously pointed out (1), a reference amino acid pattern would be more useful if an upper as well as lower limit were specified for the amount of each amino acid per gram of nitrogen and if the range of protein intake to which the pattern applies were specified.

In the present study the addition of lysine and tryptophan together markedly improved the nutritive value of corn protein as reported previously by Scrimshaw et al. (4), Bressani et al. (5) in children and by Truswell et al. (6) in adults. The effect of isoleucine was small in all of these studies. The present data give no indication that levels of lysine, tryptophan and isoleucine as high as those in the FAO reference protein have any advantage over somewhat lower levels. However, the optimal levels for maximal nitrogen balance in children cannot yet be stated with confidence because the study of a sufficient number of children for statistical validity is so time-consuming and costly that it has not been made.

LITERATURE CITED

1. Bressani, R., D. Wilson, M. Béhar, M. Chung and N. S. Scrimshaw 1963 Supplementation of cereal protein with amino acids. IV. Lysine supplementation of wheat flour fed to young children at different levels of protein intake in the presence and absence of other amino acids. J. Nutrition, 79: 333.
2. Rosenberg, H. R. 1959 Supplementation of foods with amino acids. J. Agr. Food Chem., 7: 316.
3. Bressani, R. and E. T. Mertz 1958 Relationship of protein level to the minimum lysine requirement of the rat. J. Nutrition, 65: 481.

[6] Bressani, R. 1960 Optimum levels of amino acid supplementation for lime-treated corn. Federation Proc., 19: 320 (abstract).

4. Scrimshaw, N. S., R. Bressani, M. Béhar and F. Viteri 1958 Supplementation of cereal proteins with amino acids. I. Effect of amino acid supplementation of corn masa at high levels of protein intake on the nitrogen retention of young children. Ibid., 66: 485.
5. Bressani, R., N. S. Scrimshaw, M. Béhar and F. Viteri 1958 Supplementation of cereal proteins with amino acids. II. Effect of amino acid supplementation of corn masa at intermediate levels of protein intake on the nitrogen retention of young children. Ibid., 66: 501.
6. Truswell, A. S. and J. F. Brock 1961 Effects of amino acid supplements on the nutritive value of maize protein for human adults. Am. J. Clin. Nutrition, 9: 715.
7. Benton, D. A., A. E. Harper and C. A. Elvehjem 1955 Effect of isoleucine supplementation on the growth of rats fed zein or corn diets. Arch. Biochem. Biophys., 57: 13.
8. Hogan, A. G., G. T. Gillespie, O. Koctürch, B. L. O'Dell and L. M. Glynn 1955 The percentage of protein in corn and its nutritional properties. J. Nutrition, 57: 225.
9. Mitchell, H. H. and D. B. Smuts 1932 The amino acid deficiencies of beef, wheat, corn, oats and soy beans for growth in the white rat. J. Biol. Chem., 95: 263.
10. Sauberlich, H. E., W. Y. Chang and W. D. Salmon 1953 The comparative nutritive value of corn of high and low protein content for growth in the rat and chick. J. Nutrition, 51: 623.
11. Food and Agriculture Organization 1957 Protein requirements. Report of the FAO Committee, Rome, Italy, October, 1955. FAO Nutritional Studies no. 16. Rome.
12. Rosenberg, H. R., E. L. Rohdenburg and R. E. Eckert 1960 Multiple amino acid supplementation of white corn meal. J. Nutrition, 72: 415.
13. Harper, A. E. 1957–1958 Balance and imbalance of amino acids. Ann. New York Acad. Sci., 69: 1025.
14. Harper, A. E. 1959 Amino acid balance and imbalance. I. Dietary level of protein and amino acid imbalance. J. Nutrition, 68: 405.
15. Harper, A. E. 1959 Sequence in which the amino acids of casein become limiting for the growth of the rat. J. Nutrition, 67: 109.
16. Rosenberg, H. R. 1959 Amino acid supplementation of foods and feeds. In Protein and Amino Acid Nutrition, ed. A. A. Albanese. Academic Press, New York-London, pp. 381–417.

Effect of Methionine and Age of Rat on the Occurrence of Hemorrhagic Diathesis in Rats Fed a Ration Containing Irradiated Beef[1,2,3]

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ABSTRACT There was no mortality in weanling Sprague-Dawley rats fed nonirradiated beef. A significant ($P < 0.01$) number of deaths occurred in the group fed the irradiated-beef ration. Methionine reduced the mortality rate linearly ($P < 0.01$). The prothrombin rate (reciprocal of prothrombin time $\times$ 1000) of rats fed an irradiated-beef ration was lower ($P < 0.01$) than that for rats fed a ration containing nonirradiated beef. The prothrombin rate increased ($P < 0.01$) when the ration was supplemented with methionine. Growth was depressed ($P < 0.01$) by dietary methionine. Some adult Sprague-Dawley rats died of internal hemorrhages when fed a ration containing nonirradiated beef, indicating adults were more susceptible than weanling rats to hemorrhagic diathesis. Feeding irradiated beef increased the mortality ($P < 0.01$), but it was decreased ($P < 0.01$) by supplemental methionine. The methionine required to prevent hemorrhagic disease in adult rats fed nonirradiated-beef diet was less ($P < 0.01$) than that of rats fed irradiated beef. Feeding irradiated beef decreased the prothrombin rate ($P < 0.01$) and supplementation with methionine increased the rate ($P < 0.01$). There was a linear ($P < 0.01$) decrease of body weight gain with increasing supplemental methionine, which was less ($P < 0.05$) for rats fed nonirradiated beef than for those fed irradiated-beef rations.

It was reported by Carter and Warner (1) that methionine administration increased the Factor V (accelerator) activity. Tsien and Johnson (2) observed that irradiated (5.58 megarads) beef per se contained less methionine than nonirradiated beef and since Mellette and Leone (3) reported that Factor V was lowered by feeding an irradiated-beef ration to rats, we were led to investigate the possible role of methionine in the occurrence of hemorrhagic diathesis.

MATERIALS AND METHODS

Ground nonirradiated or irradiated (5.58 megarads) beef was incorporated at the level of 35% (ration solids) into a ration which also contained: (in per cent) starch, 35; sucrose, 19; cod liver oil, 1.5; wheat germ oil, 0.5; salt mix USP XIV, 4; and vitamin mix, 5. The composition of vitamin mixture was: thiamine·HCl, 50 mg; riboflavin, 50 mg; pyridoxine·HCl, 50 mg; Ca pantothenate (dextrorotatory), 400 mg; nicotinic acid, 200 mg; *p*-aminobenzoic acid, 1 g; inositol, 2 g; choline chloride, 20 g; and glucose monohydrate,[4] 976.25 g.

Beef contained 40.5% total solids, 17.1% protein, 19.8% fat and 0.8% ash (4). The experimental procedure and care of animals have been described (5). A total of 102 weanling male rats were used in 4 different experiments; however, pooled data is given. Rats were distributed into 6 groups. One group received a nonirradiated-beef ration without added methionine. The remaining 5 groups were fed an irradiated-beef (5.58 megards) ration supplemented with zero, 0.23, 1.37, 2.06 or 2.74% DL-methionine (ration solids) at the expense of sucrose. The animals were kept on the experiment for 9 weeks except in one experiment where they were fed the experimental ration for 8 weeks and

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[2] Irradiation with gamma rays was carried out at the Atomic Energy Commission's Material Testing Reactor at Arco, Idaho, by the Phillips Petroleum Company.

[3] From a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Medical Science.

[4] Cerelose, Corn Products Company, Argo, Illinois.

a group of rats fed nonirradiated beef ration was discontinued at the end of the seventh experimental week.

Seventy-seven adult rats (7.5 weeks old) were distributed into 8 groups; 6 groups received the same treatments as the 6 groups of weanling rats; remaining groups were fed nonirradiated-beef ration supplemented with 1.14 or 1.83% DL-methionine. Tsien and Johnson (2) reported that 100 g of protein of nonirradiated and 5.58 megarads irradiated beef contained 2.93 and 2.16 g L-methionine, respectively. Based on this report, the nonirradiated-beef ration contained 0.44% L-methionine, and 5.58 megarads irradiated beef ration contained 0.32% L-methionine. The irradiated-beef ration supplemented with 0.23% DL-methionine increased the amount of available methionine to 0.55%.

The weanling rats were on experiment for 9 weeks, the adults for 13 weeks. Blood samples for determination of prothrombin times (6) were taken at various intervals. Prothrombin times do not follow the normal distribution curve and hence were transformed into prothrombin rates (reciprocal of prothrombin time for statistical analysis). In the text, prothrombin rates are expressed as 1000/sec.

Rats that died of hemorrhage or after a blood sample was obtained and their prothrombin times abnormally prolonged (longer than 20 seconds) were recorded under "death due to hemorrhage." Rats that died of anesthesia or died after a blood sample was obtained, and whose prothrombin time was less than 20 seconds, were recorded as accidental deaths and were excluded from consideration in the mortality data.

RESULTS

None of the weanling rats fed nonirradiated beef died, but 79% of those receiving irradiated beef without methionine supplementation died within 4 weeks (table 1). This difference was highly significant ($P < 0.01$). Supplementing the irradiated-beef ration with 0.23% DL-methionine did not affect the mortality rate, but 1.37% DL-methionine decreased it from 79 to 12% at 4 weeks. The effect of methionine added to the ration at the level of 1.37% became less apparent with time, 60% of the rats being dead by the end of 9 weeks as compared with 89% when no methionine was added. However, when methionine was increased to 2.74%, no mortality occurred. There was a significant reduction ($P < 0.01$) in mortality by methionine at 4, 8, and 9 weeks. Eight-weeks' mortality data were tested and showed a linear effect of DL-methionine.

Adult rats fed nonirradiated beef survived for 4 weeks, but a significant ($P < 0.01$) number (80%) fed irradiated beef died (table 1). No hemorrhage or death occurred in 4 weeks when the latter ration was supplemented with 2.06 or 2.74% DL-methionine. This effect of methionine in reducing the mortality rate was highly significant ($P < 0.01$). During 8 weeks, 40% of the rats fed nonirradiated beef

TABLE 1

Effect of methionine on mortality of weanling and adult male rats

Beef irradiation	DL-methionine[1]	Mortality (weanling)			Mortality (adults)		
		Week			Week		
		4	8	9	4	8	13
megarads	%						
0	0	0/12[2]	0/6	0/6	0/10	4/10	5/10
0	1.14[3]				0/9	0/9	0/9
0	1.83				0/9	0/9	0/9
5.58[1]	0	19/24	19/24	16/18	8/10	9/10	10/10
5.58	0.23	8/10	9/9	9/9	7/10	8/10	8/10
5.58	1.37	2/17	6/16	6/10	4/10	5/10	6/10
5.58	2.06[3]	0/15	0/15	1/15	0/9	0/9	1/9
5.58	2.74[3]	0/15	0/14	0/8	0/10	0/10	0/10

[1] Effect of methionine and irradiated beef highly significant ($P < 0.01$).
[2] Number rats died/number rats per group after excluding those that died of accidents or discontinued from the experiment.
[3] Methionine reqnired by adult rats to prevent death due to hemorrhage was significantly lower ($P < 0.01$) for those fed nonirradiated than irradiated-beef ration.

died, as compared with 90% fed irradiated beef, a highly significant difference ($P < 0.01$). The addition of 1.14% DL-methionine to the nonirradiated-beef diet was sufficient to prevent the hemorrhagic disease completely. It was necessary to add 2.06% DL-methionine to the irradiated-beef ration to prevent the disease completely. The methionine requirement was significantly lower ($P < 0.01$) for rats fed a nonirradiated-beef diet than for rats fed irradiated beef. Mortality at 13 weeks confirmed the results at 8 weeks; however, 2.74% supplemental methionine prevented the occurrence of hemorrhagic diathesis to rats fed irradiated beef.

For the adult rats fed nonirradiated beef not supplemented with methionine, the average prothrombin rate was 67 (table 2), compared with 88 for stock rats (5), and significantly lower ($P < 0.01$), 43, for adult rats fed irradiated beef. Supplementing the rations with DL-methionine significantly ($P < 0.01$) increased the prothrombin rates linearly. Rats fed nonirradiated beef required less methionine to increase the prothrombin rates than did rats that received irradiated beef. When the irradiated-beef ration contained 2.06% DL-methionine, the average prothrombin rate was 87, about equal to that for stock rats. For rats fed irradiated beef, the percentage of plasma samples with prolonged prothrombin time was more than for rats fed nonirradiated beef. Methionine supplementation decreased the percentage of prolonged prothrombin times.

The average prothrombin rates of weanling rats fed nonirradiated or irradiated beef were 73 and 51, respectively, differing significantly ($P < 0.01$). Supplementing the irradiated-beef ration with 0.23% DL-methionine had no effect on the prothrombin rate. Increasing methionine to 1.37 or 2.06%, increased the prothrombin rates significantly ($P < 0.01$) to 69 and 84, respectively.

Seven per cent of the plasma samples of weanling rats fed nonirradiated beef showed prolonged prothrombin times as compared with 50% of the rats fed irradiated beef. When the latter ration was supplemented with 2.74% DL-methionine, no prolonged prothrombin times were noted. Thus, rats fed nonirradiated beef had some hemorrhagic tendency, and high levels of methionine could overcome this tendency completely, even when the rats were fed irradiated beef.

Four- and ten-weeks' body weight gains for weanling and adult rats, respectively, (table 3) were diminished significantly ($P < 0.01$) by supplementing the rations with DL-methionine. This effect was nonlinear for the weanling rats, 2.06 or 2.74% methionine decreasing the weight gains dramatically. The regression was linear for adult rats. The effect was significantly less ($P < 0.05$) for adult rats fed nonirradiated beef than for those fed irradiated beef. There was no significant difference

TABLE 2

Effect of methionine on the average prothrombin rate (1000/sec) and the percentage of plasma samples with prolonged prothrombin times

Beef irradiation	DL-methionine[1]	Weanling			Adult		
		No. samples	% Prolonged	Rate	No. samples	% Prolonged	Rate
megarads	%						
0	0	28	7	73 ± 2[2]	20	19	67 ± 4[2]
0	1.14[3]				16	0	83 ± 5
0	1.83				17	0	89 ± 4
5.58[1]	0	28	50	51 ± 3	6	83	43 ± 7
5.58	0.23	16	50	51 ± 4	9	33	64 ± 6
5.58	1.37	45	22	69 ± 3	16	31	64 ± 5
5.58	2.06[3]	28	4	84 ± 3	17	0	87 ± 4
5.58	2.74	38	0	85 ± 3	26	4	85 ± 4

[1] Effect of methionine and irradiated beef on rate significant ($P < 0.01$).
[2] SE of mean.
[3] Methionine requirement for adult rats fed nonirradiated beef was significantly ($P < 0.01$) less than those fed irradiated beef.

TABLE 3

Effect of methionine on average body weight gained by weanling and adult male rats

Beef irradiation	DL-methionine[1]	Weanling			Adult		
		No. rats	Initial wt	Wt gained (4 weeks)	No. rats	Initial wt	Wt gained (10 weeks)
megarads	%		*g*	*g*		*g*	*g*
0	0	12	95	142 ± 4.0[2]	5	263	170 ± 12.2[2]
0	1.14				9	270	165 ± 9.1
0	1.83				9	274	160 ± 9.1
5.58	0	5	79	148 ± 6.3	1	242	183 ± 27.4
5.58	0.23	2	79	154 ± 9.9	2	264	159 ± 19.4
5.58	1.37	14	76	137 ± 3.7	5	277	170 ± 12.2
5.58	2.06	15	80	113 ± 3.6	9	274	131 ± 9.1
5.58	2.74	15	71	67 ± 3.6	10	274	111 ± 8.7

[1] Methionine significantly decreased ($P < 0.01$) the body weight gains.
[2] SE of mean.

between the weight gains by the rats fed irradiated and nonirradiated beef in the absence of supplemental methionine.

DISCUSSION

Forty per cent of the adult rats fed nonirradiated beef died within 8 weeks as compared with no mortality in weanlings, indicating that the adult rats were more susceptible to the factors that cause hypoprothrombinemia, hemorrhage, and subsequent death. Mellette and Leone (3, 7, 8) and Doisy and Matshiner (9) observed adult rats to be less resistant to hemorrhagic disease as they died earlier than weanlings.

There were some defects in blood coagulation even in rats fed a nonirradiated-beef diet, and these were more severe in those fed irradiated beef. Therefore, the implication was that radiation augmented the factor or factors responsible for the prolongation of prothrombin times. Despite the effect of methionine in reducing the mortality rate, the occurrence of hemorrhage and subsequent death in rats did not take place primarily because of the destruction of methionine in beef by ionizing radiation. This was demonstrated by increasing the available methionine to 0.55%, above the amount available in the nonirradiated-beef diet, without beneficial effect. Thomas and Calloway (10) observed no appreciable change in methionine in irradiated beef. Whether the metabolism of sulfur-containing amino acids in rats fed an irradiated-beef ration was altered is not known.

There are few reports on the effect of methionine on blood coagulation and they are contradictory. Shin (11) reported a decrease in prothrombin time with oral and parenteral administration of methionine in carbontetrachloride- and chloroform-treated rabbits. In thrombocytopenic purpura, the beneficial effect of methionine in the control of spontaneous bleeding and restoration of clot retraction in man was reported by Rabinowitz (12). Methionine administered orally or intravenously prolonged both bleeding time and coagulation time in man (13). Wilson (14) observed no change in intravenous coagulation time and in prothrombin time after intravenous administration of methionine to man. However, DeGowin and co-workers (15) reported a decrease in prothrombin and Factor V in man with doubling or tripling of the normal intake of methionine. When methionine intake was increased by 10 to 15% with vitamin K present, the accelerator-activity values were elevated in one patient (1).

Miller and associates (16) observed no change in the one-stage prothrombin time in dogs fed a diet devoid of proteins for 8 to 10 weeks. The accelerator-activity in cholecystnephrostomized dogs fed protein-free and methionine-free diets was almost negligible and DL-methionine supplementation brought the activity to normal (1). There was a moderate increase of accelerator-activity in normal dogs and rats with the administration of methionine. Carter and Warner reported faster recovery of Factor V when methionine was

given to partially hepatectomized rats, but no effect on prothrombin. Intraperitoneal injections of methionine to rats fed an irradiated-beef ration improved Factor V (Ac-globulin) but decreased prothrombin to some extent (3).

Factor V appears to be necessary for the formation of the prothrombin-converting substance in blood and in tissue extract (17). Because supplementation of the irradiated-beef ration with methionine completely prevented the occurrence of hemorrhages, and prothrombin times returned to normal, it is assumed that methionine did not allow prothrombin levels to fall. Ethionine, the ethyl analogue of methionine, depressed prothrombin, Factor V, Factor VII, antithrombin and fibrinogen (18). Pool and Spaet (19) reported the reduced production of antihemophilic factor. Since ethionine is considered to interfere with methionine metabolism and to promote its functional deficiency, it thus presumably interferes with normal manufacture and activity of the clotting factors by causing functional methionine deficiency.

Carter and Warner (1) observed an increase of Factor VII in cholecystnephrostomized dogs given methionine. It is a possibility that methionine increased Factor VII which was then converted to prothrombin in rats receiving irradiated beef. The metabolism of administered DL-methionine may be different from that of methionine bound with other amino acids in natural proteins.

Another explanation of methionine's protective effect could be that it depressed growth and hence the vitamin K requirement. Only 1.14% supplemental DL-methionine prevented death due to hemorrhages in adult rats fed nonirradiated beef and had no detrimental effect on body weight gains. Addition of 1.37% DL-methionine to the irradiated-beef ration reduced the mortality from 79 to 12, and from 90 to 50%, in weanling and adult rats, respectively, without affecting the growth of the rats adversely. These data indicate that the methionine effect in preventing the occurrence of hemorrhages was not consequent upon a decrease in growth.

Growth retardation in rats fed excessive dietary methionine, reported here, has been reported previously. This effect was associated not with the D- form (20), but with metabolism of the homocysteine moiety of methionine and decreased feed intake (21).

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LITERATURE CITED

1. Carter, J. R., and E. D. Warner 1958 Correction of defects in clotting accelerator activity by administration of methionine and vitamin K and of a new sulfhydryl-substituted methylnaphthoquinone, vitamin K-S (II). J. Clin. Invest., *37:* 70.
2. Tsien, W. S., and B. C. Johnson 1959 The effect of irradiation sterilization on the nutritive value of foods. V. On the amino acid composition of milk and beef. J. Nutrition, *69:* 45.
3. Mellette, S. J., and L. A. Leone 1959 Mechanism of the hemorrhagic phenomenon produced in male rats by feeding of irradiated beef. Progress Report 2 on Contract DA-49-007-MD-951 with Office of Surgeon General, Department of the Army, Washington, D. C.
4. Reber, E. F., O. P. Malhotra, J. P. Kreier, H. W. Norton and P. D. Beamer 1960 The effects of feeding irradiated beef to dogs. I. Growth. Am. J. Vet. Res., *21:* 367.
5. Malhotra, O. P., A. V. Nalbandov, E. F. Reber and H. W. Norton 1963 Effects of rat strain, stilbestrol and testosterone on the occurrence of hemorrhagic diathesis in rats fed a ration containing irradiated beef. J. Nutrition, *79:* 381.
6. Quick, A. J. 1938 The nature of the bleeding in jaundice. J.A.M.A., *110:* 1658.
7. Mellette, S. J., and L. A. Leone 1960 Mechanism of the hemorrhagic phenomenon produced in male rats by feeding of irradiated beef. Progress Report 4 on Contract DA-49-007-MD-951 with Office of Surgeon General, Department of the Army, Washington, D. C.
8. ——— 1960 Influence of age, sex, strain of rat and fat soluble vitamins on hemorrhagic syndromes in rats fed irradiated beef. Federation Proc., *19:* 1045.
9. Doisy, E. A., Jr., and J. T. Matschiner 1960 Hemorrhagic syndrome in rats fed irradiated beef. Progress Report 4. On Contract DA-49-007-MD-996 with Office of Surgeon General, Department of the Army, Washington, D. C.
10. Thomas, M. H., and D. H. Calloway 1961 Nutritional value of dehydrated foods. J. Am. Dietet. A., *39:* 105.

11. Shin, T. 1952 Plasma prothrombin in liver-damaged rabbits. Fukuoka Acta Med., *43:* 791.
12. Rabinowitz, H. M. 1941 A study of the role of amino acids in clot retraction. Am. J. Surg., *51:* 366.
13. Sterner, J. H., and G. Medes 1936 The effect of certain sulfur compounds on the coagulation of blood. Am. J. Physiol., *117:* 92.
14. Wilson, S. J. 1949 The effect of methionine on blood coagulation. Proc. Soc. Exp. Biol. Med., *70:* 234.
15. De Gowin, E. L., R. F. Sheets, H. E. Hamilton and J. R. Carter 1955 Deficit of prothrombin and accelerator globulin in man from prolonged excessive feeding of methionine. Trans. A. Am. Physician, *68:* 118.
16. Miller, L. L., C. A. Rogers, B. G. Lamson and M. W. Lambie 1947 Protein depletion and hepatic function in the dog. Rationale of methionine in therapy. Am. J. Med. Sci., *214:* 84.
17. Wright, I. S. 1959 Concerning the function and nomenclature of blood clotting factors, with a preliminary report of the profile of blood clotting factors in young males. Ann. Intern. Med., *51:* 841.
18. Witte, S., K. Heinkel, N. Henning and P. Dirnberger 1953 Das Verhalten der Blutgerinnungsfaktoren während der Äthioninpankreatitis der Ratte. Ztschr. Ges. Exper. Med., *121:* 228.
19. Pool, J. G., and T. H. Spaet 1954 Ethionine-induced depression of plasma antihemophilic globulin in the rat. Proc. Soc. Exp. Biol. Med., *87:* 54.
20. Van Pilsum, J. F., and C. P. Berg 1950 The comparative availabilities of mixtures of the L and DL modifications of the essential amino acids for growth in the rat. J. Biol. Chem., *183:* 279.
21. Cohen, H. P., H. C. Choitz and C. P. Berg 1958 Response of rats to diets high in methionine and related compounds. J. Nutrition, *64:* 555.

Tissue Distribution and Excretion of Alpha-aminoisobutyric Acid in the Rat

I. EFFECT OF DIETARY PROTEIN LEVEL AND COMPOSITION[1]

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ABSTRACT The nature of the diet fed to rats greatly influenced the distribution ratio of injected 1-C^{14}-α-aminoisobutyric acid (AIB) between tissues and serum, as well as its pattern of urinary excretion. An increased concentration of AIB was observed in the liver of animals maintained with deficient diets while at the same time the non-hepatic tissues showed a decreased concentrative capacity. The levels of dietary protein ranging from a protein-free diet to as high as 54% casein, affected very significantly the pattern of urinary excretion and the net AIB retention, but did not affect to any great extent, the relative tissue distribution. At high protein levels the urinary loss of AIB was greatly enhanced. The large amount of stored AIB in the tissues of rats fed low protein diets over an extended period was immediately mobilized and excreted when the protein content of the diet was suddenly increased. The importance of taking into account the nature, as well as the amount, of the diet consumed during studies on amino acid distribution is stressed.

The use of unmetabolizable amino acids as a means of studying transport mechanisms both *in vivo* and *in vitro* was initially suggested by Christensen and co-workers (1). These workers also demonstrated that the distribution of these amino acids between tissues and serum can be correlated with the capacity of that particular tissue to selectively incorporate amino acids into its cells, by means of an active transport process (2).

We have previously observed (3) that the injection of labeled amino acids into animals receiving testosterone propionate resulted in an increased radioactivity in the nonprotein fraction of sex linked tissues. On the other hand a similar increase in the radioactivity of the nonprotein fraction of other tissues was observed under conditions of growth inhibition induced by toxic levels of dietary tryptophan (4).

These observations encouraged us to conduct further experiments on problems related to intracellular amino acid accumulation as a result of dietary factors. The use of an amino acid such as α-aminoisobutyric acid (AIB), which cannot be incorporated into tissue protein nor metabolized, proved very suitable for our purpose.

In the following experiments the tissue distribution of AIB and its pattern of urinary excretion under the influence of diets differing both in the quality as well as the quantity of their protein content was investigated.

EXPERIMENTAL PROCEDURE

Male albino rats of the Holtzman strain were used. The basal diet, to which different protein and amino acid supplements were added, consisted of the following in grams: cornstarch, 143; HMW salt mixture (5), 10; cottonseed oil, 10; choline·HCl, 0.4; and vitamins (6). Vitamin-free casein, zein and gelatin were used as protein sources. L-Leucine, L-isoleucine and DL-tryptophan were added at the levels indicated in table 1. The zein diet was supplemented as indicated by Geiger et al. (7) with an amino acid mixture at a 6% level. All rats were injected subcutaneously with 0.5 μc of AIB- 1-C^{14}/100 g of body weight (5 μc /ml normal saline) specific activity, 6.05 mc/mmole. The animals were killed at different intervals following the isotope injection by overexposure to ether after bleeding by cardiac puncture. The organs under investigation were removed and aliquots homogenized to a 100 mg/ml suspension in 0.1 N acetic acid

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TABLE 1

Food consumption, changes in body weight and protein efficiency ratio (PER) of rats fed different unbalanced diets and varying in their protein content

Basal diet	Avg food consumption	Average daily growth	Protein intake	PER
	g/day	*g/day*	*g/day*	
9% Casein	13.7 ± 0.56[1]	3.6	1.24	2.90
+3% Leucine	12.9 ± 0.40	2.5	1.55	1.61
+3% Leucine +0.5% isoleucine	13.4 ± 0.42	3.3	1.67	1.98
18% Casein	13.8 ± 0.47	5.5	2.48	2.21
+3% Leucine	14.1 ± 0.36	5.5	2.96	1.86
18% Zein	8.2 ± 0.32	−0.9	1.47	
+6.2% Amino acid mixture[2]	11.8 ± 0.42	1.4	2.91	0.48
18% Gelatin	8.2 ± 0.30	−1.0	1.47	
+2.5% Tryptophan	10.0 ± 0.47	0.6	1.83	0.33

[1] Mean ± SE.
[2] Amino acid mixture: L-arginine, 1 g; L-histidine, 0.5 g; L-tryptophan, 0.25 g; L-lysine, 1.2 g; L-cystine, 0.25 g; DL-threonine, 1.2 g; DL-valine, 1.6 g.

(pH 5). Samples were deproteinized by heating at 75°C, centrifuged and the supernatant plated on aluminum planchets, and finally counted with a gas-flow ultrathin window Geiger-Muller tube. These values were compared with those in serum to determine the concentrative capacity of the organ in question. The distribution ratio was calculated.

The urinary excretion studies were conducted with animals maintained in individual stainless steel metabolic cages. The animals started to be fed their respective diets two days previous to the injection of AIB. The daily urine output was filtered, diluted, and an aliquot counted for radioactivity. Values are expressed as the percentage of the injected radioactivity excreted daily.

RESULTS

The changes in body weight exhibited over the 21-day period by rats fed the different diets is shown in figure 1. These results are in general agreement with observations reported in the literature. However, this experimental approach is presently being used to study whether the results observed may not be related, at least in part, to disturbances in amino acid transport mechanisms. Thus as shown by Harper and co-workers (8) the addition of 3% leucine to a 9% casein diet caused an imbalance, as evidenced by growth inhibition, which was almost completely prevented by the further supplementation with 0.5% isoleucine. At higher protein levels (18% casein) the same amount of leucine supplementation did not cause a growth inhibition.

Growth was also inhibited in animals fed the zein or gelatin diets. However, the addition of a mixture of missing and physiologically unavailable amino acids to the zein diet (7) and tryptophan to the gelatin diet improved growth.

The food consumption and the protein efficiency ratio (PER) calculated on the basis of the body weight increment per gram of dietary protein is shown in table 1. The most efficient protein utilization occurred at the lower level of casein and was decreased in all instances by the addition of leucine to the diet.

At the end of a three-week adaptation period all rats were injected subcutaneously with 0.5 μc of AIB/100 g of body weight. They were killed 24 hours later, serum and tissues were taken and analyzed for radioactivity. No AIB whatsoever was incorporated into the protein fractions and all radioactivity was associated with the

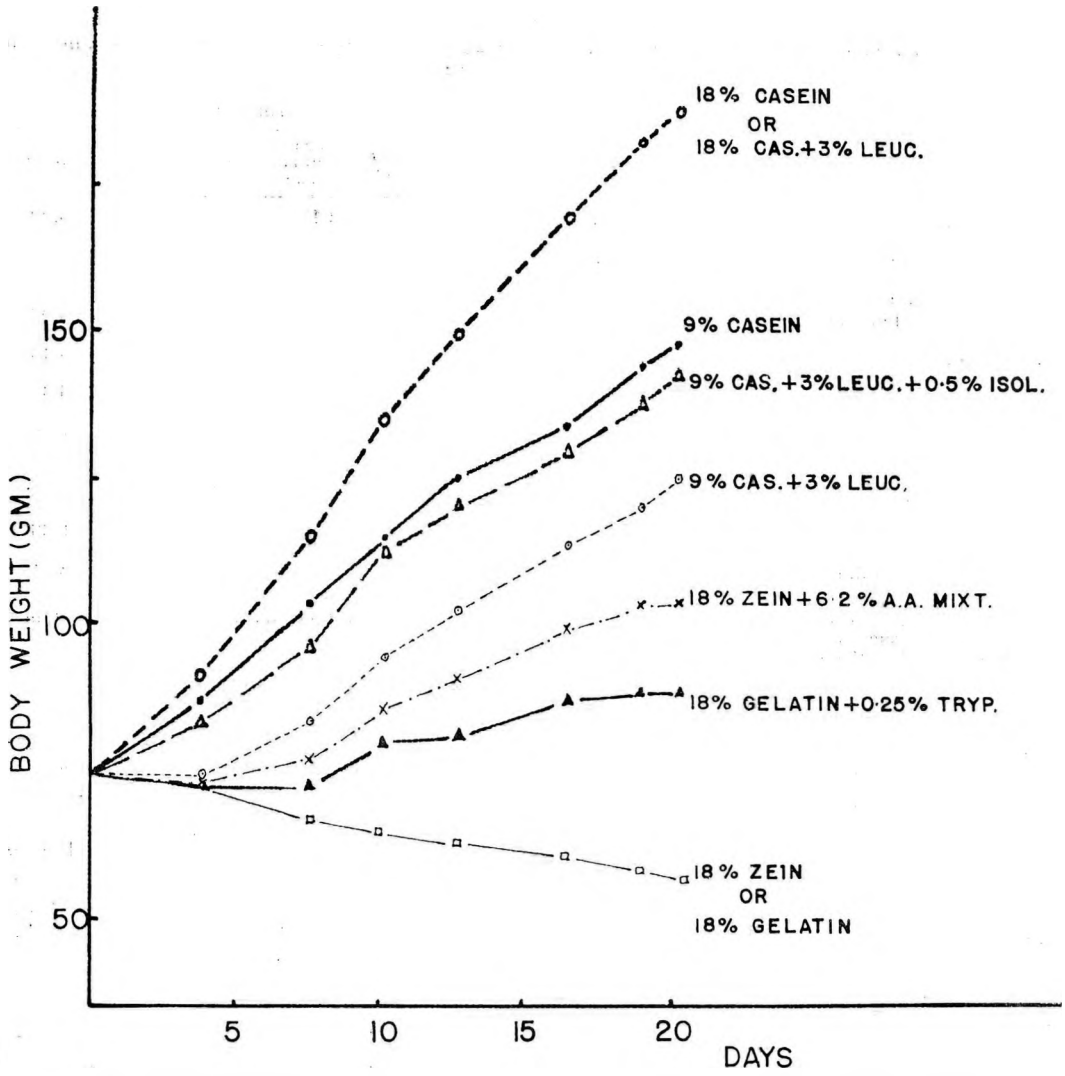

Fig. 1 Changes in body weight of rats fed different diets at various protein levels.

deproteinized tissue extract. The distribution of AIB in the various tissues 24 hours following its administration to the different groups is shown in table 2.

The serum specific activity was lowest in the casein-base diets. It was significantly increased in the serum of rats fed the diets containing the incomplete proteins zein and gelatin. With the exception of the liver, where significantly higher distribution ratios were obtained for rats fed the zein or gelatin diets, AIB was much less efficiently concentrated in tissues of rats fed diets producing poorest growth increments (table 2). This pattern of AIB distribution caused by amino acid deficiency is very similar to that reported by Riggs and Walker (9) for vitamin B_6-deficient rats. No significant differences were observed with the 9% casein diets as a result of amino acid imbalance.

The effect of the level of dietary protein on the urinary excretion of AIB following a single tracer dose of this compound is shown in figure 2. Rats fed the higher protein levels showed a marked initial peak in their excretory pattern immediately following the injection. The urinary

TABLE 2

Effect of amino acid imbalance and deficiency on the α-aminoisobutyric acid (AIB) distribution ratio in the tissues of the rat

Basal diet	Serum specific activity[2]	Distribution ratio of AIB[1]						
		Liver	Kidney	Gluteus muscle	Gastrocnemius muscle	Heart	Brain	Skin
9% Casein	1530 ± 59[3]	1.82	17.0	2.80	3.12	6.82	1.65	5.22
+3% Leucine	1480 ± 96	2.12	19.1	3.58	3.11	6.34	1.84	4.98
+3% Leucine +0.5% isoleucine	1490 ± 81	1.85	16.8	3.16	3.01	6.85	1.76	5.06
18% Casein	1410 ± 107	1.89	12.6	3.46	3.32	7.32	1.63	5.43
+3% Leucine	1200 ± 58	2.13	15.9	3.98	3.62	7.76	1.84	6.35
18% Zein	2200 ± 95	4.58	9.77	1.81	1.63	4.63	1.72	1.98
+18% Amino acid mixture[4]	1680 ± 82	2.25	10.35	2.84	2.52	5.21	1.72	4.46
18% Gelatin	2790 ± 216	3.65	7.53	1.21	1.33	3.26	1.40	1.66
+0.25% Tryptophan	2600 ± 205	2.05	12.98	1.05	1.37	3.25	1.36	2.01

[1] Each value is the average of 7 rats, 24 hours after isotope administration; distribution ratio: count/min per gram wet tissue/count/min per milliliter serum.
[2] Serum specific activity: count/min/ml serum.
[3] Mean ± SE.
[4] See footnote 2, table 1.

radioactivity gradually decreased towards the end of the experiment. Rats fed the 18% casein and gelatin diets showed a rather similar and constant rate of radioactive output in their urine. Rats consuming the protein-free diet, after a small initial peak in their excretion, showed the maximal AIB retention.

Figure 3 shows the urinary radioactive excretion of rats fed a 9% casein diet during 17 days following the tracer AIB dose, and then changed to a diet with 54% casein. Immediately following the change in diet, excretion of radioactive AIB increased in these rats through mobilization of their body stores of AIB. Preliminary experiments using a diuretic agent indicate that the increased excretion of AIB under conditions of high nitrogen intake is not due to the resulting increase in urinary volume.

The rats whose urine excretion curves are shown in figure 2 were killed at the end of the seventeenth day and serum and tissues analyzed for their specific activity. The data are presented in table 3. As protein levels in the diet increased, the body stores of AIB were rapidly depleted, as suggested by the increased urinary output and confirmed by tissue analysis. At the 54% casein level the radioactivity remaining in the blood and other tissues was almost insignificant.

The serum levels of AIB decreased very rapidly with the increasing levels of dietary protein. The tissue/serum distribution ratio did not change appreciably, indicating that the loss of radioactivity was a generalized effect. The lowest concentrating capacity of skeletal muscle was evidenced during the feeding of the 18% gelatin and protein-free diets, whereas the ratio for skin approached unity in these instances.

DISCUSSION

The nature of the diet significantly altered the distribution pattern for AIB in several tissues of the rat. Whereas brain concentrated AIB almost independently of the nutritional status of the animal, other tissues showed marked correlation between growth and their capacity to retain AIB. Serum AIB levels 24 hours following its subcutaneous administration was highest in those animals consuming the markedly deficient diets and lowest in the supplemented and casein groups.

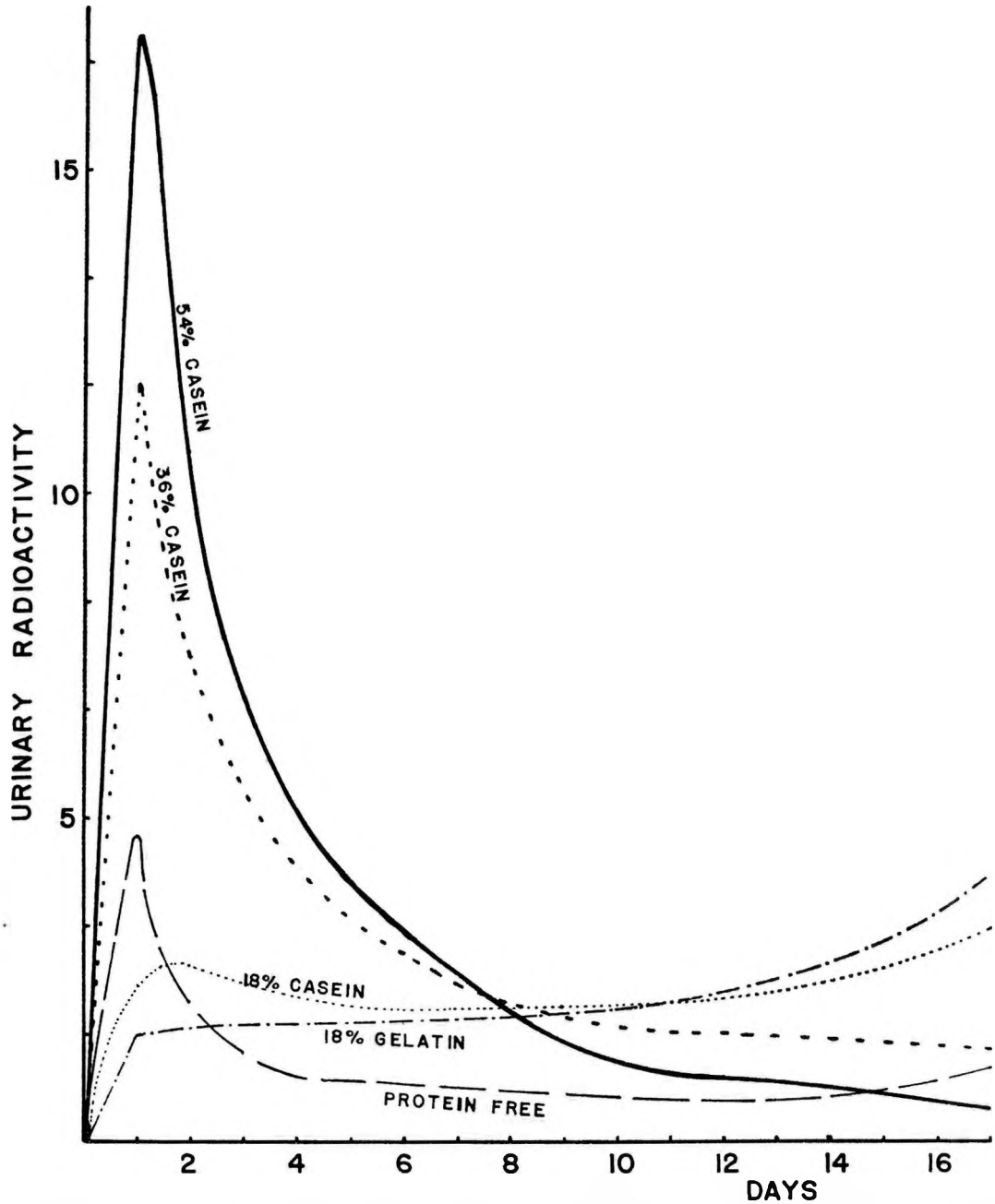

Fig. 2 Urinary excretion of C^{14} following the injection of a single dose of aminoisobutyric acid-1-C^{14} to rats fed diets differing in their protein content and quality. Values are expressed as the percentage of the injected dose excreted.

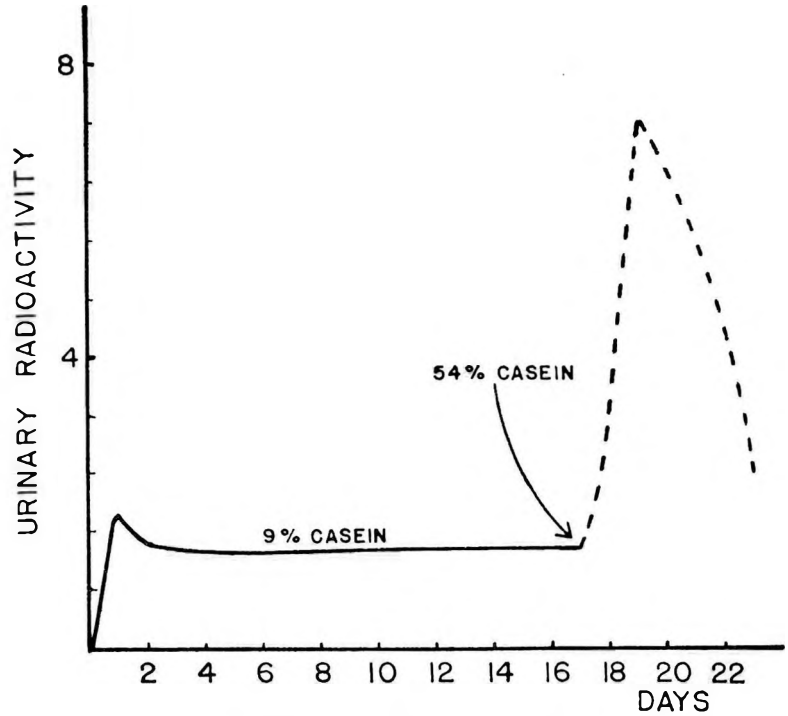

Fig. 3 Pattern of 1-C^{14}-α-aminoisobutyric acid excretion of rats fed a 9% casein diet during 17 days following a single dose of tracer amino acid, and then changed to a 54% casein diet. Values are expressed as the percentage of the injected dose excreted in the urine.

TABLE 3

Effect of different dietary protein levels on the distribution ratio of 1-C^{14}-α-aminoisobutyric acid 17 days following its administration to rats

Basal diet	Serum specific activity[1]	Distribution ratio						
		Liver	Kidney	Heart	Spleen	Brain	Gluteus muscle	Skin
Protein-free	1020 ± 95[2]	1.67	10.2	3.67	1.73	1.82	1.37	0.95
18% Casein	270 ± 30	1.73	16.7	4.75	3.02	1.92	2.68	2.95
36% Casein	60 ± 11	2.30	19.5	4.50	3.67	3.50	3.0	2.65
54% Casein	8 ± 2		20.0	3.8	3.5	1.5	4.5	
18% Gelatin	500 ± 53	2.74	14.3	4.80	2.0	1.72	1.34	0.90

[1] Count/min/ml deproteinized serum.
[2] Mean ± SE.

The increased serum radioactivity of the growth-retarded animal may be caused by a decrease in the tissue uptake or urinary output. This decreased tissue uptake was most marked for the skin, skeletal muscle and kidney. Nevertheless, and in contrast, an increased radioactivity in the liver of these animals appears to reflect catabolic activity at this site, where most of the degradation of amino acids is known to occur. Noall et al. (10) demonstrated that plasma amino acid radioactivity decreased as a result of hydrocortisone, or epinephrine, but increased in the liver. Christensen et al. (11) showed that plasma amino acids in man decreased after stress and that liver amino acids increased after surgery in rats. It thus appears reasonable that the high levels of AIB in the liver of rats fed gelatin or zein diets should correlate with the high rate of amino acids being sent there for catabolic

purposes. In general the concentrative ability of tissues was lowest in those animals receiving the unsupplemented gelatin or zein diets. The supplementation of these diets with the missing and physiologically unavailable amino acids tended to normalize the distribution.

At the lower level of dietary casein, the addition of leucine caused an imbalance that could be overcome by isoleucine supplementation. This is reflected in the body weight changes and also in the protein efficiency ratio of these animals. It nevertheless did not significantly alter the capacity of the various tissues studied to concentrate AIB. To further distinguish between quantity and quality of the dietary protein intake, the levels of protein in the diet were varied and urinary AIB excretion followed over a 17-day period. The amount of AIB excreted in this period paralleled roughly the protein levels in their diets.

The increased rate of excretion of AIB which follows the feeding of a high protein diet may reflect a faster exchange rate of amino acids between cells and extracellular fluids. An increased influx into the cell caused by the greater availability of amino acids may stimulate an increased efflux through stimulation of the transport mechanisms. It might also be a reflection of the decreased "amino acid hunger" of tissues (10) as a result of the high levels of dietary protein.

The dramatic increase in urinary AIB excretion which occurred when changing animals from a 9% casein diet to one containing 54% casein supports the view that during the flooding of the tissues with dietary amino acids, the efflux of the intracellular AIB is greatly enhanced. This would lead to a depletion of the tissue stores of AIB and to its final appearance in the urine (table 3). At the lower levels of dietary protein, the amount of AIB retained in the organism was very high compared with the almost insignificant level remaining after 17 days of feeding the high protein diets. This may also reflect the "leaching out effect" stimulated by the high levels of dietary protein. At low protein levels a sparing effect for amino acid conservation by the cells may be responsible for the AIB retention. The rats fed protein-free diets maintained high levels of serum AIB, whereas serum radioactivity was almost negligible in those fed the high protein diets. The distribution ratio within the different groups was not drastically altered. The concentrative capacity of the renal cells as measured by the distribution ratio, tended to increase with increasing protein levels of the diet. When rats were fed casein and gelatin at a similar level (18%) over a prolonged period, the latter retained significantly more AIB in their tissues at the end of the experimental period on the basis of absolute values, although their concentrative capacity was not altered as may be noticed by the similarity in their distributive ratio. The fact that higher levels of AIB are retained by the gelatin fed rats than the casein fed rats, despite their urinary excretion being very similar, may suggest differences in the fecal AIB output. In this connection, using everted intestinal sacs, Akedo and Christensen (12) observed that the intestinal mucosa actively concentrated AIB from the serosal fluid.

LITERATURE CITED

1. Christensen, H. N., A. J. Aspen and E. G. Rice 1956 Metabolism in the rat of three amino acids lacking α-hydrogen. J. Biol. Chem., *220:* 287.
2. Christensen, H. N. 1959 Active transport with special reference to the amino acids. Persp. Biol. Med., *2:* 228.
3. Nimni, M. E., and L. A. Vavetta 1961 Effect of testosterone propionate on tissue protein synthesis in the castrated male rat. Proc. Soc. Exp. Biol. Med., *106:* 738.
4. Nimni, M. E., D. Hom and L. A. Bavetta 1962 Dietary composition and tissue protein synthesis. II. Tryptophan toxicity and amino acid deficiency in collagen synthesis. J. Nutrition, *78:* 133.
5. Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. Ibid., *14:* 273.
6. Bavetta, L. A., S. Bernick and B. H. Ershoff 1957 The influence of dietary thyroid on the bones and periodontium of rats on total and partial tryptophan deficiencies. J. Dental Res., *36:* 13.
7. Geiger, E. C., G. W. Courtney and L. Geiger 1952 The physiological availability of some essential amino acids present in zein, in the

acid and in the enzyme hydrolysate of zein. Arch. Biochem. Biophys., *41*: 74.
8. Harper, A. E., D. A. Benton and C. A. Elvehjem 1955 L-Leucine, an isoleucine antagonist in the rat. Ibid., *57*: 1.
9. Riggs, T. R., and L. M. Walker 1958 Diminished uptake of C^{14}-α-aminoisobutyric acid by tissues of vitamin B_6-deficient rats. J. Biol. Chem., *233*: 132.
10. Noall, M. W., T. R. Riggs, L. M. Walker and H. N. Christensen 1957 Endocrine control of amino acid transfer. Distribution of an unmetabolizable amino acid. Science, *126*: 1002.
11. Christensen, H. N., J. T. Rotwell, R. A. Fears and J. A. Streicher 1948 Association between rapid growth and elevated cell concentrations of amino acids. II. In regenerating liver after partial hepatectomy in the rat. J. Biol. Chem., *175*: 101.
12. Akedo, H., and H. N. Christensen 1962 Transfer of amino acids across the intestine: A new model amino acid. Ibid., *237*: 113.

Ratios of Essential-to-Nonessential Amino Acids in Plasma from Rats Fed Different Kinds and Amounts of Proteins and Amino Acids[1]

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ABSTRACT Weanling rats fed diets containing amounts of proteins and amino acids that were suboptimal for growth showed a reduction in the plasma ratio of essential-to-nonessential amino acids (EN ratio) when this ratio was compared with that of rats fed 18% casein diets. For young adult rats fed 18, 8 and 4% casein, there was a decreased plasma EN ratio in rats fed the low casein diets. Glycine supplements in the diet also decreased the EN ratio in the plasma of both weanling and young adult rats. Glutamic acid supplements fed to young adult rats did not have this effect. The decreased plasma EN ratios were the net result of a decrease in essential amino acids and, in most instances, an increase in nonessential amino acids. The limiting essential amino acid in the diet was reduced in the plasma to the greatest degree, but the levels of other essential amino acids were also affected.

A number of investigations with animals (1–3) have demonstrated relationships between the plasma amino acid concentrations and the amino acid composition of the ingested diet. A previous study (4) indicated that when nonessential nitrogen supplements were added to a low casein basal diet, weanling rats fed those diets had a reduced rate of growth and a decreased ratio of essential-to-nonessential amino acids (EN ratio) in blood plasma. In the present study molar EN ratios were determined in the plasma of rats fed different kinds and amounts of proteins and amino acids. The observation (5) that human subjects receiving low protein diets for periods of 30 days or longer showed decreased plasma EN ratios gave impetus to these experiments.

EXPERIMENTAL

Weanling male rats (Sprague-Dawley strain) weighing between 50 and 60 g were fed the experimental diets for periods of 4 weeks. The experimental procedures and the diet have been described previously (4) with the exception that in the present study the nitrogen-containing components of the diet were varied. The sources and amounts which were fed are shown in table 1.

Young adult male rats (Sprague-Dawley strain), 3 months of age and weighing approximately 350 g, were used for some experiments. They were fed the experimental diets for periods of 2 weeks. Again the nitrogen-containing components of the diet were varied and the sources and amounts which were fed are shown in table 2.

The amino acids were determined in picric acid extracts of pooled samples of blood plasma from 5 or 6 nonfasting rats prepared according to the procedures of Tallan et al. (6). The analyses were made by ion-exchange chromatography using a Beckman-Spinco instrument.

RESULTS

Values are recorded in table 1 for the total essential amino acids (including cystine and tyrosine), the total nonessential amino acids and the EN ratios derived therefrom in plasma of weanling rats receiving various amounts and kinds of dietary nitrogen. All values are presented on a molar basis. With 18% casein in the diet, the amounts of both essential and nonessential amino acids and consequently, the EN ratio, were very similar as determined in 3 separate experiments (groups 1, 4 and 7). When the amount of casein in the diet was reduced to 8%

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TABLE 1

Total amounts and molar ratios of essential and nonessential amino acids in blood plasma of weanling rats

Group	Diet treatment	Avg wt gain[1]	Total essential amino acids[2]	Total nonessential amino acids	EN ratio[3]
		g	*μmoles/ 100 ml*	*μmoles/ 100 ml*	
1	18% Casein	132±3	202	200	1.01
2	8% Casein	34±1	151	297	0.51
3	8% Casein+0.3% DL-methionine	96±7	109	188	0.58
4	18% Casein	150±2	204	223	0.92
5	18% Wheat gluten	20±1	120	280	0.43
6	18% Wheat gluten+0.5% L-lysine	74±5	138	370	0.37
7	18% Casein	144±2	195	215	0.91
8	18% Casein+7.5% glycine	111±6	105	358	0.29

[1] Mean of 6 rats ± SE for 28-day period.
[2] Includes cystine and tyrosine.
[3] Molar ratio of essential-to-nonessential amino acids.

TABLE 2

Total amounts and molar ratios of essential and nonessential amino acids in blood plasma of young adult rats

Group	Diet treatment	Avg wt gain[1]	Total essential amino acids[2]	Total nonessential amino acids	EN ratio[3]
		g	*μmoles/ 100 ml*	*μmoles/ 100 ml*	
1	18% Casein	32±4	147	198	0.74
2	8% Casein+0.3% DL-methionine	21±3	131	217	0.57
3	4% Casein+0.15% DL-methionine	−7±4	107	256	0.42
4	8% Casein+0.3% DL-methionine +7.5% glycine	−9±8	86	308	0.28
5	18% Casein+7.5% glycine	+7±6	110	260	0.42
6	18% Casein+14% L-glutamic acid	+33±4	141	194	0.73

[1] Mean of 6 rats ± SE for 14-day period. Rats weighed approximately 350 g at beginning of experiment.
[2] Includes cystine and tyrosine.
[3] Molar ratio of essential-to-nonessential amino acids.

(group 2), the growth rate was lower and the plasma EN ratio was also lower. When the 8% casein diet was supplemented with methionine, the most limiting amino acid (group 3), the growth rate was increased, but not to the level of that obtained with the 18% casein diet, and the plasma EN ratio remained low. With the 18% wheat gluten diets (groups 5 and 6) the growth rate was below that obtained when 18% casein diets were fed and the plasma EN ratios were also lower. Adding a glycine supplement to the 18% casein diet caused a slight reduction in growth rate (group 8) and a low plasma EN ratio.

In all instances of lowered plasma EN ratios, there was a reduction in total essential amino acids and in most instances, an increase in the nonessential amino acids. However, neither the decrease in EN ratio nor the decrease in total essential amino acids was proportional to the decrease in growth rate. Possible explanations for this can be found in a consideration of the values for the individual essential amino acids in plasma from rats being fed the different diets (table 3). It can be seen that the concentrations of many of the amino acids are altered in animals fed the various diets when these concentrations are compared with those from rats fed 18% casein. In all instances, the amino acid limiting for growth appears to be reduced to the greatest degree. Hence, the total concentration of essential amino acids is dependent not only on the net result of the changes in individual amino acid levels, but also on

TABLE 3

Essential amino acids in blood plasma of weanling rats fed diets differing in protein and amino acid content (all values are μmoles/100 ml)

Essential amino acid	Diet fed					
	18% casein	8% casein	8% casein + 0.3% DL-methionine	18% wheat gluten	18% wheat gluten + 0.5% L-lysine	18% casein + 7.5% glycine
Threonine	47.3	28.1	5.6	47.0	9.2	12.5
Valine	25.2	16.5	10.7	13.8	18.1	16.0
½ Cystine	3.5	0.4	1.9	2.2	3.8	1.4
Methionine	7.4	3.4	5.5	3.1	5.1	5.8
Isoleucine	10.1	6.8	5.4	7.0	11.1	7.2
Leucine	17.8	11.6	8.6	12.9	17.9	12.0
Tyrosine	13.4	7.9	6.3	5.5	11.5	5.2
Phenylalanine	6.8	5.4	3.2	5.3	7.9	3.8
Lysine	55.6	50.2	50.2	4.1	28.3	36.1
Tryptophan	3.7	1.3	1.5	0.4	2.7	1.5
Histidine	5.4	9.6	5.5	9.2	8.6	2.7
Arginine	8.3	10.4	7.7	9.2	14.2	3.8

the identity of the limiting amino acid, and these various factors are not necessarily correlated with the rate of growth.

Evidence for the limiting dietary amino acid being most reduced in plasma is shown in the case of the 18% wheat gluten diet where the limiting dietary amino acid, lysine, was reduced to the greatest degree as compared with values obtained with the 18% casein diet (table 3). When lysine was added to the diet, the second limiting dietary amino acid, threonine (7), was most reduced in blood plasma. Similar relations hold for the 8% casein diet where the sulfur-containing amino acids were limiting and the 8% casein diet supplemented with methionine where threonine was limiting (8). From this relationship it appears that when glycine is added to the 18% casein diet, threonine becomes the limiting amino acid for growth because it was the amino acid reduced to the greatest degree in plasma.

The increase in the nonessential amino acids which was associated with lowered EN ratios in most of the diets studied (table 3) reflected a general increase in individual amino acid concentration (not shown in table) except in the case of the glycine-supplemented diet. With this diet, only glycine and serine were increased in the plasma and the other nonessential amino acids were reduced.

In table 2 are given EN ratios of young adult rats receiving diets containing different amounts of casein for 2-week periods. Here again a decrease in the amount of casein fed resulted in a reduced EN ratio as the net result of a decrease in essential amino acids and an increase in nonessential amino acids. However, the 8% casein-methionine supplemented diet did not produce as great a change in plasma amino acid concentrations as occurred with the weanling rats (table 1). The addition of glycine to either an 8% casein–0.3% methionine diet or an 18% casein diet caused a decrease in EN ratio with a substantial reduction in essential amino acids. The addition of glutamic acid to an 18% casein diet had no effect on the EN ratio.

Again in these experiments with young adult rats (table 2) the limiting amino acid, threonine, was the most reduced of the essential amino acids in plasma when the EN ratio was decreased. It was reduced from a value of 32.5 μmoles/100 ml with the 18% casein diet (group 1) and to 22.7 μmoles with the 8% casein–0.3% methionine diet (group 3). When glycine was added to the 8% casein-methionine supplemented diet (group 4) the plasma threonine value was 8.8 μmoles/100 ml. Also, the increase in nonessential amino acids associated with the low EN ratios was the result of a general increase in all nonessential amino acids except in the instance of the glycine supplement where the increase was confined to glycine and

serine. These results with young adult rats are therefore similar in many respects to results obtained with weanling rats, with the exception that the level of casein in the diet of the young adult must be lower to produce comparable changes in plasma amino acids.

DISCUSSION

The experiments reported here add to the increasing evidence that plasma amino acids can serve as valuable indicators in studies of protein nutrition. The results confirm those of other investigators (1–3) that the limiting dietary amino acid is most reduced in the plasma and support the hypothesis that measurement of the plasma amino acids will reveal the amino acid limiting in the diet.

In addition, it has been shown that reduced EN ratios are associated with suboptimal protein intakes in the growing rat and also with low protein diets in the young adult rat. These decreased EN ratios occurred not only when the ratio of essential-to-nonessential amino acids in the diet was lowered by nonessential amino acid supplementation but also when the dietary ratio remained the same as in the experiments involving different dietary levels of casein. These results with experimental animals confirm and extend observations obtained with human subjects (5) which show an association of low protein diets and low EN ratios. It appears that the plasma EN ratio might prove to be a sensitive measurement of protein nutritional status and that the use of this criterion and possibly other plasma amino acid ratios should be further explored.

The increase in nonessential amino acids, which usually occurs when the EN ratio is reduced, can perhaps be explained on the basis of continued synthesis of these compounds in the face of restricted protein synthesis. This process is necessarily dependent on adequate amounts of dietary nitrogen. In table 1, plasma nonessential amino acids were higher with the 18% wheat gluten diets than with the 8% casein diets.

The reduction in essential amino acid plasma levels associated with glycine supplementation is apparently not a characteristic effect of all nonessential nitrogen sources since administration of isonitrogenous amounts of glutamic acid did not produce these results. This effect of dietary glycine on plasma amino acids cannot readily be interpreted. It may be that glycine interferes with the absorption of amino acids, but it is also possible that high concentrations of glycine or serine in plasma might affect the activity of certain enzyme systems.

LITERATURE CITED

1. Richardson, L. R., L. D. Blaylock and C. M. Lyman 1953 Influence of dietary amino acid supplements on the free amino acids in the blood plasma of chicks. J. Nutrition, *51:* 515.
2. Denton, A. E., and C. A. Elvehjem 1954 Availability of amino acids *in vivo*. J. Biol. Chem., 206: 449.
3. Longenecker, J. B., and N. L. Hause 1959 Relationship between plasma amino acids and composition of ingested protein. Arch. Biochem., *84:* 46.
4. Swendseid, M. E., J. B. Hickson and B. W. Friedrich 1962 Effect of nonessential nitrogen supplements on growth and on the amino acid content in plasma and muscle of weanling rats fed a low protein diet. J. Nutrition, *78:* 115.
5. Swendseid, M. E., W. H. Griffith and S. G. Tuttle 1963 The effect of a low protein diet on the ratio of essential to nonessential amino acids in blood plasma. Metabolism, *12:* 96.
6. Tallan, H. H., S. Moore and W. H. Stein 1954 Studies on the free amino acids and related compounds in the tissues of the cat. J. Biol. Chem., *211:* 927.
7. Rosenberg, H. R., E. L. Rohdenburg and R. E. Eckert 1960 Supplementation of bread protein with lysine and threonine. J. Nutrition, *72:* 423.
8. Harper, A. E. 1959 Sequence in which the amino acids of casein become limiting for the growth of the rat. J. Nutrition, *67:* 109.

Modifying Effect of Diet on Daily Variations of Certain Liver Constituents[1]

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ABSTRACT The sequential effect on certain liver enzymes and on liver glycogen of high-carbohydrate or carbohydrate-free meals was studied in rats either adapted to high-carbohydrate or carbohydrate-free diets. The pattern of glycogen deposition and repletion on the various treatments was indicative of a rapid takeover of gluconeogenic reactions to maintain appreciable glycogen stores during a carbohydrate-free dietary regimen. This ability disappeared following a single high-carbohydrate meal. The activity of glutamic-pyruvic transaminase (GPT) varied significantly with respect to time after feeding. This effect was somewhat modified by the carbohydrate-free, high-fat regimen. Glucose-6-phosphatase (G-6-Pase) activity showed some evidence of substrate induction 24 hours after high-carbohydrate meals, but this also disappeared during carbohydrate-free feeding. Glucose-6-phosphate dehydrogenase (G-6-PDH) exhibited no definite 24-hour pattern, but its activity was depressed considerably during the carbohydrate-free regimen. At 24 hours after a meal of the high-carbohydrate diet, however, G-6-PDH activity was significantly increased over previous values obtained during carbohydrate-free feeding.

In studies of pemmican as a survival ration our interest has been directed toward the maintenance of blood glucose levels (1) and glycogen storage in the liver (2). We, as well as others (3) have shown that animals fed carbohydrate-free diets can maintain appreciable levels of liver glycogen. It is well known, however, that these entities fluctuate widely as a result of ingestion of food. Changes in enzyme activity related to time after feeding also have been reported (4). Consequently, in comparing some of the reactions which may be involved in the regulation of gluconeogenesis during various dietary regimens, it appeared to us that more valid information could be obtained by training animals to eat meals than by allowing them to nibble freely. In the experiments reported here we studied the activities of glutamic-pyruvic transaminase (GPT), glucose - 6 - phosphatase (G-6-Pase) and glucose-6-phosphate dehydrogenase (G-6-PDH) — as well as levels of liver glycogen — as related to time after ingestion of high-carbohydrate and carbohydrate-free (pemmican) meals in rats either adapted or not adapted to the pemmican diet.

METHODS

Male, Sprague-Dawley rats were trained to eat their daily ration within a 2-hour period. The high-carbohydrate diet contained 18% casein, 68% sucrose, 10% vegetable oil, 4% USP salt mixture no. 2, and all required vitamins. The carbohydrate-free diet consisted of Quartermaster Meat Food Bar (hereafter called pemmican) supplemented with bone meal to give levels of 46% protein, 46% fat and 8% salts. Rats adapted to pemmican were maintained for at least 6 weeks with this diet — the high-carbohydrate rats were maintained for at least three weeks with their diet. When killed, body weights were 250 to 300 g.

Animals were killed 4, 8, 16 and 24 hours after the final meal. Methods used for the assay of liver G-6-Pase, G-6-PDH, and glycogen have been described previously (2, 5). The GPT was assayed by the method of Wróblewski and LaDue (6). Enzyme activities are expressed as micro-

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TABLE 1

Response of various liver constituents to experimental treatments

Meal	Time after meal	Glycogen	Liver wt/ 100 g body wt	Glutamic-pyruvic transaminase	Glucose-6-phosphatase	Glucose-6-phosphate dehydrogenase
	hours	*% of fresh wt*		*μmoles/100 g body weight*		
			Adapted to high carbohydrate			
High carbohydrate	4	4.65±0.34[1]	3.88±0.13	147±13	46.0±3.2	24.8±4.1
	8	8.09±0.38	4.54±0.12	174±7 (8)[2]	45.3±2.8	29.1±2.8
	16	4.58±0.65	3.92±0.22	203±10(8)	41.3±1.5	34.3±3.3
	24	1.05±0.15	3.45±0.17	160±17(8)	47.6±1.3	33.9±6.5
Carbohydrate-free (pemmican)	4	0.43±0.21	3.24±0.10	171±6	43.7±2.9	23.5±3.0
	8	0.90±0.15	3.63±0.08	179±11(8)	46.6±2.9	28.7±4.5
	16	2.39±0.30	3.70±0.03	220±11(8)	44.7±1.4	33.3±5.6
	24	1.75±0.19	3.61±0.11	191±11(8)	48.3±3.3	26.8±3.9
			Adapted to pemmican (carbohydrate-free)			
Carbohydrate-free (pemmican)	4	1.96±0.32	4.03±0.13	179±10	40.1±2.4	4.8±1.5
	8	2.79±0.22	4.32±0.03	188±14(8)	43.6±0.9	4.4±0.5
	16	2.91±0.09	4.05±0.05	207±6 (7)	45.8±1.3	3.0±0.3
	24	2.15±0.26	3.59±0.08	176±6 (8)	40.1±2.2	3.7±0.4
High carbohydrate	4	4.13±0.36	4.13±0.24	154±24	43.3±5.7	4.9±0.5
	8	7.09±0.40	4.29±0.11	156±9 (8)	40.6±2.6	4.7±0.4
	16	5.60±0.32	4.02±0.14	214±3 (6)	41.0±1.7	3.4±0.6
	24	1.09±0.23	3.31±0.11	183±11(8)	46.9±2.9	9.6±1.1

[1] SE of mean.
[2] Animals per group for this assay. Means not marked represent 5 animals.

moles of substrate converted per minute per 100 grams of body weight, since this mode of expression seems to us to be more relevant to dietary adaptation where changes in liver to body weight ratios may be quite marked (table 1). Liver-to-body weight ratios were calculated from body weights obtained just before the respective meals were offered.

RESULTS AND DISCUSSION

Results are summarized in table 1. They are arranged so as to present a reasonable sequence of events, reading from top to bottom. First, diurnal events in the liver during the feeding of high-carbohydrate meals were measured. Then, enzyme and glycogen responses to the first 24 hours of adjustment to a carbohydrate-free meal were observed. After adaptation for 6 weeks to the carbohydrate-free meal, the diurnal responses to a pemmican meal were measured. Finally, the first stages of de-adaptation from pemmican were studied by feeding a high-carbohydrate meal.

After feeding a high-carbohydrate meal to carbohydrate-adapted rats, there was an initial rapid deposition of liver glycogen and later removal until quite low levels were reached. Upon receiving a carbohydrate-free meal, these levels decreased even further for a while, but by 16 hours synthesis of glycogen was evidently proceeding, probably from protein. At 24 hours, 1.75% remained. After the animals had received the carbohydrate-free diet for 6 weeks, liver glycogen, following a carbohydrate-free meal, did not reach an appreciable peak at 8 hours, whereas at 24 hours, over 2% still remained in the liver. Finally, upon receiving one meal of the high-carbohydrate diet, rats that were adapted to pemmican exhibited the same pattern of glycogen deposition as the rats not adapted.

Thus, rats receiving this type of carbohydrate-free diet maintain appreciable levels of liver glycogen during the 24 hours following a meal. They appear to be less dependent on glycogen for either glycolytic reactions or blood sugar formation, or both, for their glycogen levels do not fall as precipitately as with rats fed a high-carbohydrate diet. This ability to maintain glycogen levels evidently disap-

pears immediately upon receiving only one high-carbohydrate meal, for glycogen content again decreased to the low 24-hour level observed before. These observations are in line with results reported by Samuels et al. (7) and Pagé and Babineau (8) who showed that animals fed high-fat rations are able to maintain higher liver glycogen levels when fasted.

It might be expected, then, that during pemmican feeding, gluconeogenesis would be increased and that this increase would be reflected in elevated levels of G-6-Pase and GPT (9, 10). As the results show, however, the activity of GPT was not elevated significantly after ingestion of pemmican if the activities at identical times after feeding are compared. Another pertinent point is the rather wide variation in activity at different times after feeding. Especially in animals fed a high-carbohydrate meal, the highest values (16 hours) were 39% higher than the lowest (4 hours). This pattern was modified somewhat when pemmican was fed, in particular following adaptation to this diet when the peak elevation was only 16% higher than the lowest value observed.

The failure of GPT activity to increase as a result of the increased protein intake from pemmican was somewhat unexpected, since Rosen et al. (10) and Muramatsu and Ashida (11) have shown that GPT activity increases with increased protein intake. We have also observed[2] that the cold-induced increase in GPT activity is apparently a simple response to increased protein intake. It is possible that, in the present experiments, the simultaneously increased fat intake nullified the effect of increased protein as well as modified the pattern of GPT activity following a meal.

The only noticeable response in G-6-Pase activity occurred at 24 hours versus 16 hours when the animals had been fed a high-carbohydrate meal. It is our opinion that this slight increase could be a temporary response to substrate (glucose-6-phosphate) arising from glycogen mobilization. That it is not a stress response to blood glucose depletion appears to be indicated by its failure to increase further after the first meal of pemmican, when no sugar was ingested and glycogen decreased to even lower levels. In this connection, no difficulty was encountered in persuading rats to eat the unfamiliar pemmican; hence, as far as the animal as a whole was concerned, the fasting period did not extend beyond 24 hours.

Glucose-6-phosphate dehydrogenase activity decreased to virtually minimal levels after 6 weeks of the pemmican diet. We have observed previously that no depression in its activity occurred when a carbohydrate-free diet of 90% protein was fed (12). In the present experiment, however, although the fat content was only 46%, activity decreased to levels comparable to those previously observed with 70% fat diets containing much less protein (12). The retarding influence of dietary fat on absorption may be involved here, in that fat may prevent flooding of certain systems with consequent substrate induction. The reduced activity of this enzyme (13), as well as "malic enzyme" (14), reported during stock diet feeding may possibly be related to the slower rate of release of starch-derived glucose.

When fed a high-cabohydrate meal, these pemmican-adapted rats exhibited a significant increase in G-6-PDH by the end of the 24-hour period. These results are similar to those observed by Tepperman and Tepperman (15) with refeeding experiments. As shown by these authors and by Hollifield and Parson (16), the activity of this enzyme is increased by meal feeding high-carbohydrate diets per se, when compared with previous results (12) during ad libitum feeding of equivalent high-carbohydrate diets. Furthermore, once a level characteristic of a particular dietary treatment is attained, little, if any, effect of time after feeding is noticeable on the activity of G-6-PDH.

We did not study lipogenesis during these experimental treatments. It appears likely, however, that it would have been depressed, considering the fact that others (15, 17) have shown a correlation, if not a causal effect, between hexose monophosphate shunt activity and lipogenesis. Still others (18) have demonstrated lipogenic depression when high fat diets are fed. In view of the modifications arising from either increased fat intake, or absence of

[2] To be published.

carbohydrate shown here, or both, it seems pertinent to study the effect on lipogenesis and fat deposition of meal feeding diets higher in fat than are commonly fed rats, especially with respect to hypotheses proposed by some investigators (16, 19) concerning the effect of eating patterns on human obesity.

LITERATURE CITED

1. Drury, H. F., D. A. Vaughan and J. P. Hannon 1959 Some metabolic effects of a high-fat, high-protein diet during semistarvation under winter field conditions. J. Nutrition, *67:* 85.
2. Vaughan, D. A., J. P. Hannon and L. N. Vaughan 1958 Associated effects of diet, environmental temperature and duration of exposure on the major constituents of the livers of rats. Am. J. Physiol., *194:* 441.
3. Thorn, W., and H. Scheitza 1961 Glucosebildung und Glykogeneinlagerung in Gehirn, Leber, Niere, Herz und Skeletmuskel bei kohlenhydratfreier Ernährung. Pflügers Arch. ges. Physiol., *273:* 18.
4. Nielson, R. R., and H. M. Klitgaard 1961 Changes in succinic dehydrogenase activity related to feeding. Am. J. Physiol., *201:* 37.
5. Hannon, J. P., and D. A. Vaughan 1960 Effect of prolonged cold exposure on the glycolytic enzymes of liver and muscle. Ibid., *198:* 375.
6. Wróblewski, F., and J. S. LaDue 1956 Serum glutamic pyruvic transaminase in cardiac and hepatic disease. Proc. Soc. Exp. Biol Med., *91:* 569.
7. Samuels, L. T., R. M. Reinecke and H. A. Ball 1942 Liver fats and glycogen of hypophysectomized rats on high carbohydrate and high fat diets. Ibid., *49:* 456.
8. Pagé, E., and L. M. Babineau 1954 Tissue glycogen and glucose absorption in rats adapted to cold. Canad. J. Biochem. Physiol., *32:* 395.
9. Freedland, R. A., and A. E. Harper 1958 Metabolic adaptations in higher animals. II. Changes with time in the adaptive response of glucose-6-phosphatase. J. Biol. Chem., *230:* 833.
10. Rosen, F., N. R. Roberts and C. A. Nichol 1959 Glucocorticosteroids and transaminase activity. I. Increased activity of glutamic-pyruvic transaminase in four conditions associated with gluconeogenesis. Ibid., *234:* 476.
11. Muramatsu, K., and K. Ashida 1962 Effect of dietary protein level on growth and liver enzyme activities of rats. J. Nutrition, *76:* 143.
12. Vaughan, D. A., J. P. Hannon and L. N. Vaughan 1960 Effects of diet on selected glycolytic enzymes of the rat. Am. J. Physiol., *199:* 1041.
13. Tepperman, J., and H. M. Tepperman 1958 Effects of antecedent food intake pattern on hepatic lipogenesis. Ibid., *193:* 55.
14. Fitch, W. M., and I. L. Chaikoff 1961 Effect of previous diet on response of hepatic enzyme activities to a 24-hour fast. Arch. Biochem. Biophys., *94:* 380.
15. Tepperman, H. M., and J. Tepperman 1958 The hexosemonophosphate shunt and adaptive lipogenesis. Diabetes, *7:* 478.
16. Hollifield, G., and W. Parson 1962 Metabolic adaptations to a "stuff and starve" feeding program. II. Obesity and the persistence of adaptive changes in adipose tissue and liver occurring in rats limited to a short daily feeding period. J. Clin. Invest., *41:* 250.
17. Tepperman, J., and H. M. Tepperman 1961 Metabolism of glucose-1-C^{14} and glucose-6-C^{14} by liver slices of refed rats. Am. J. Physiol., *200:* 1069.
18. Hausberger, F. X., and S. W. Milstein 1955 Dietary effects on lipogenesis in adipose tissue. J. Biol. Chem., *214:* 483.
19. Cohn, C., and D. Joseph 1960 Role of ingestion of diet on regulation of intermediary metabolism. Metabolism, *9:* 492.

Effect of Protein Intake and Cold Exposure on Selected Liver Enzymes Associated with Amino Acid Metabolism[1]

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ABSTRACT Two experiments were conducted to examine the possibility that increased activities of some enzymes in cold-exposed animals could be substrate-induced rather than a direct effect of cold, since the cold-exposed animals increase appreciably their food intake.

Two groups of cold-exposed rats were allowed to eat only as much protein as their warm mates, and their extra energy requirement was met by sugar or fat. Another cold-exposed group of animals was allowed to eat the complete diet freely.

After a 4-week cold exposure, the activities of 5 liver enzymes involved in amino acid metabolism were markedly increased in all groups of cold-exposed rats. However, the activities of 3 of these enzymes, arginase, glutamic-oxalacetic and glutamic-pyruvic transaminase were increased only as a result of a cold-induced higher protein intake. In contrast, the activities of tryptophan pyrrolase and tyrosine-α-ketoglutaric transaminase were increased by cold per se. The data demonstrate that both substrate-induced and cold-induced enzymatic changes occur in cold-exposed animals.

It is well established that animals exposed to cold for a prolonged period of time undergo certain enzymatic changes associated with an increased thermal demand in the cold environment.

Major changes in the activities of specific enzymes involved in carbohydrate metabolism of cold-exposed rats were reported by Hannon and Vaughan (1, 2) and Vaughan et al. (3). These authors found that the cold-exposed rat has increased activities of liver glucose-6-phosphatase, glucokinase, malic dehydrogenase and an increase in the rate of pyruvate formation from 3-phosphoglycerate. The rates of oxidation of the Krebs cycle substrates, succinate, isocitrate, citrate, α-ketoglutarate, fumarate and malate were also found to be markedly increased as a result of a chronic cold exposure (4). In addition, a significant increase in the activities of liver succinic dehydrogenase, cytochrome oxidase, and of DPNH- and TPNH-cytochrome c reductase of cold-exposed animals were also reported (5, 6). More recently, it was reported from this laboratory that the activities of liver arginase, glutamic-oxalacetic and glutamic-pyruvic transaminase in cold-exposed rats fed a low protein diet were significantly higher than those in the comparable animals kept at room temperature (7).

However, it has been observed that the cold-exposed animals increase their food intake appreciably in order to satisfy the energy needs for both heat production and growth. Since, in the final analysis, the elevated energy metabolism is the result of increased rates of oxidation of proteins, carbohydrates and fats, perhaps the augmented flow of substrates passing over certain enzymes in the cold-exposed animals would increase the rate of synthesis of these enzymes, and thus alter their activities, according to the hypothesis proposed by Spiegelman (8).

The present experiments were conducted to study effects of diet and cold exposure on 5 liver enzymes associated with amino acid metabolism in the rat and to examine the possibility that increased activities of some enzymes in cold-exposed animals are protein-induced rather than cold-induced.

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[1] The views expressed are those of the authors and do not necessarily represent official Air Force policy. The experiments were conducted according to the "Rules Regarding Animal Care" as established by the American Medical Association (AFR 160-94).

METHODS

Male Sprague-Dawley rats, ranging in weight from 165 to 200 g were used in all experiments. The control group (group 1), kept at 25°C, was given the experimental diet ad libitum. The cold-exposed rats were kept at 7°C in a cold room. One group of the cold-exposed rats (group 2) was also fed ad libitum, whereas the other two cold-exposed groups (groups 3 and 4) were pair-fed with the warm controls. However, in order to meet a higher energy demand in cold, the animals in groups 3 and 4 were offered an unrestricted amount of either sucrose or fat, respectively, as soon as they consumed the daily portion of the experimental diet.

The experimental diet had the following percentage composition; crude casein, 20; sucrose, 72; corn oil, 4; USP salt mixture no. II, 4; the vitamin mixture supplied 2,000 units of vitamin A; 222 units of vitamin D; 11.1 mg of α-tocopherol and the following: (in milligrams) ascorbic acid, 100; inositol, 11.1; choline chloride, 166.5; menadione, 5; *p*-aminobenzoic acid, 11.1; niacin, 10; pyridoxine·HCl, 2.22; riboflavin, 2.22; thiamine·HCl, 2.22; Ca pantothenate, 40.3; also 44 μg of biotin, 200 μg of folic acid and 3 μg of vitamin B_{12}/100 g of diet. Individual wire cages were used throughout the experiment. At the end of 28 days the animals were decapitated, and the livers immediately excised, chilled in chipped ice and assayed for arginase, glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and tryptophan pyrrolase (TP), in experiment 1, and for tyrosine-α-ketoglutaric transaminase (TKGT), in experiment 2.

Arginase determination was carried out according to the procedure of Brown and Cohen (9) as used by Klain et al. (7). The GOT and GPT were measured by following the oxidation of DPNH with a Beckman DK recording spectrophotometer according to procedures similar to those used by Wróblewski and LaDue (10) and LaDue et al. (11).

The activity of TP was determined according to the procedure of Knox and Auerbach (12) and estimations of TKGT were made according to the method used by Lin and Knox (13). All enzyme assays were carried out on duplicate samples from 5 or more animals.

Aliquots of tissues were analyzed for nitrogen content by acid digestion and the nesslerization procedure. Since all livers had the same nitrogen content, the enzyme activities were expressed in units per gram of fresh tissue rather than per unit of nitrogen.

RESULTS AND DISCUSSION

Data obtained in these studies are summarized in table 1. The mean weight changes for the 28-day experimental period indicate that the amount of protein consumed by the pair-fed animals met their needs for a reasonable rate of growth (group 3 and group 4 vs. group 2).

The activities of arginase, GOT and GPT were markedly increased in the cold-exposed, ad libitum-fed animals (group 2 vs. group 1) and, remained unchanged in the pair-fed groups (groups 3 and 4 vs. group 1). This effect can be apparently related to a higher protein intake per se since the animals in group 2 consumed over 44% more of the complete diet than those in the pair-fed groups. There is ample evidence in the literature, demonstrating that activities of certain enzymes involved in protein metabolism can be readily affected by the quantity of protein consumed. Thus, arginase activity increases on high protein diets and, conversely, decreases on low protein diets (14–16). Similarly, Rosen et al. (17) reported an increase in GOT and GPT activity in livers of both intact and adrenalectomized rats fed high protein diets, showing that the adaptation was in response to a high protein intake.

In contrast, the activities of TP and TKGT were uniformly increased in all 3 cold-exposed groups, regardless of the dietary treatment. Thus, the most important conclusion to draw from the foregoing data is that both substrate-induced and cold-induced enzymatic changes occur in the cold-exposed animals.

The specific cellular mechanism responsible for cold-induced changes in the activities of TP and TKGT remains to be elucidated. However, it is possible that the adaptation could be mediated by adrenocortical hormones as it was observed that the activities of these two enzymes

TABLE 1

Weights, food intakes and activities of selected enzymes in warm- and cold-exposed rats

	Treatment group			
	1	2	3	4
	Warm	Cold	Cold pair-fed	
		Fed ad libitum	+ Sugar	+ Fat
Experiment 1				
Avg Δ body wt, g[1]	120 ±3.1	107 ±2.9	104 ± 3.0	107 ±2.5
Avg daily intake of complete diet, g	17.4	25.1	17.4	17.4
Avg daily sucrose intake, g	—	—	6.4	—
Avg daily fat intake, g	—	—	—	3.1
Arginase[2]	41.6 ±1.4	53.5* ±1.7	43.2 ± 1.2	40.4 ±2.4
Glutamic-oxalacetic transaminase[3]	194 ±7.3	245* ±9.2	190 ±12.1	209 ±5.2
Glutamic-pyruvic transaminase[4]	35.3 ±2.6	47.6* ±3.5	31.1 ± 3.5	41.2 ±2.6
Tryptophan pyrrolase[5]	1.9 ±0.1	2.8* ±0.2	2.8* ± 0.1	3.0* ±0.2
Experiment 2				
Avg Δ body wt, g[1]	118 ±4.2	114 ±3.1	108 ± 4.4	109 ±4.7
Avg daily intake of complete diet, g	17.8	25.7	17.8	17.8
Avg daily sucrose intake, g	—	—	6.1	—
Avg daily fat intake, g	—	—	—	3.9
Tyrosine-α-ketoglutaric transaminase[6]	58.1 ±3.6	72.3* ±2.8	75.4* ± 5.1	69.2* ±4.8

[1] Avg value of 10 animals ± SE.
[2] As mmoles urea/g liver/hr.
[3] As μmoles DPN/g liver/hr.
[4] As μmoles DPN/g liver/hr.
[5] As μmoles kynurenine/g liver/hr.
[6] As μmoles *p*-hydroxyphenylpyruvate/g liver/hr.
* Difference from warm groups ($P < 0.05$).

could be increased by injecting glucocorticosteroids, (12, 13, 18–21). Furthermore, it has been established that cold-exposed animals produce more corticosteroids than comparable animals kept at room temperature (22, 23).[2] The hormones could alter the enzyme activities directly by increasing the rate of enzyme synthesis or indirectly by increasing availability of nonspecific secondary inducers.

The data indicate that the enzyme activities in the pair-fed groups were not altered, regardless of whether sugar or fat were offered as an additional source of energy in cold.

LITERATURE CITED

1. Hannon, J. P., and D. A. Vaughan 1960 Effect of prolonged cold exposure on the glycolytic enzymes of liver and muscle. Am. J. Physiol., *198:* 375.
2. ——— 1961 Effect of exposure duration on selected enzyme indexes of cold acclimatization. Ibid., 200: 94.
3. Vaughan, D. A., J. P. Hannon and L. N. Vaughan 1961 Interrelationships of diet and cold exposure on selected liver glycolytic enzymes. Proc. Soc. Exp. Biol. Med., *201:* 33.
4. Hannon, J. P. 1958 Respiration of rat liver homogenates following prolonged cold exposure. Ibid., *97:* 368.
5. ——— 1960 Effect of prolonged cold exposure on components of the electron transport system. Am. J. Physiol., *198:* 740.
6. Reyenafarje, B., and R. Chaffee 1960 Pyridine nucleotide-cytochrome c reductases in acclimatization to cold. Proc. Soc. Exp. Biol. Med., *103:* 225.
7. Klain, G. J., D. A. Vaughan and L. N. Vaughan 1962 Interrelationships of cold exposure and amino acid imbalances. J. Nutrition, *78:* 359.
8. Spiegelman, S. 1946 Nuclear and cytoplasmic factors controlling enzymic constitution. Cold Spring Harbor Symposia on Quant. Biol., *11:* 256.
9. Brown, G. W., Jr., and P. P. Cohen 1959 Comparative biochemistry of urea synthesis. I. Methods for the quantitative assay of urea cycle enzymes in liver. J. Biol. Chem., *234:* 1769.
10. Wróblewski, F., and J. A. LaDue 1956 Serum glutamic-pyruvic transaminase in cardiac and hepatic disease. Proc. Soc. Exp. Biol. Med., *91:* 569.
11. LaDue, J. S., F. Wróblewski and A. Karmen 1954 Serum glutamic-oxalacetic transaminase activity in human acute transmural myocardial infarction. Science, *120:* 497.
12. Knox, W. E., and V. H. Auerbach 1955 The hormonal control of tryptophan peroxidase in the rat. J. Biol. Chem., *214:* 307.

[2] Héroux, O., E. Schönbaum and A. Desmarais 1959 Adrenal and thyroid adjustments in white rats during seasonal and thermal acclimation. Federation Proc., *18:* 264 (abstract).

13. Lin, C. C. E., and W. E. Knox 1957 Adaptation of the rat liver tyrosine alpha-ketoglutarate transaminase. Biochem. Biophys. Acta, *26:* 85.
14. Mandelstam, J., and J. Yudkin 1952 Effects of variations in dietary protein upon hepatic arginase of rat. Biochem. J., *51:* 681.
15. Ashida, K., and A. E. Harper 1961 Metabolic adaptations in higher animals. VI. Liver arginase activity during adaptation to high protein diet. Proc. Soc. Exp. Biol. Med., *107:* 151.
16. Rosenthal, O., C. S. Rogers, H. M. Vars and C. C. Ferguson 1950 Arginase, adenosinepyrophosphatase and rhodanase levels in the liver of rats. J. Biol. Chem., *185:* 669.
17. Rosen, F., N. R. Roberts and C. A. Nichol 1959 Glucocorticosteroids and transaminase activity. I. Increased activity of glutamicpyruvic transaminase in four conditions associated with gluconeogenesis. Ibid., *234:* 476.
18. Thomson, J. F., and E. T. Mikuta 1954 Effect of total body x-irradiation on the tryptophan peroxidase activity of rat liver. Proc. Soc. Exp. Biol. Med., *85:* 29.
19. Civen, M., and W. E. Knox 1959 The independence of hydrocortisone and tryptophan induction of tryptophan pyrrolase. J. Biol. Chem., *234:* 1787.
20. Lin, C. C. E., and W. E. Knox 1958 Specificity of the adaptive response of tyrosine alpha-ketoglutarate transaminase in the rat. Ibid., *233:* 1186.
21. Litwack, G., and T. I. Diamondstone 1962 Nonspecific stimulation of tyrosine alpha-ketoglutarate transaminase activity *in vivo.* Ibid., *237:* 469.
22. Schönbaum, E. 1960 Adrenocortical function in rats exposed to low environmental temperatures. Federation Proc., *19* (Suppl. 5): 85.
23. Sobel, H., M. Sideman and R. Arce 1960 Steroid excretion by guinea pigs exposed to cold for prolonged periods. Am. J. Physiol., *198:* 1107.

Phenylalanine Requirement of Women Consuming a Minimal Tyrosine Diet and the Sparing Effect of Tyrosine on the Phenylalanine Requirement[1,2]

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ABSTRACT This study was initiated to determine for 6 female subjects the minimal amounts of phenylalanine necessary for nitrogen equilibrium. When the subjects received their respective phenylalanine requirements, as determined at a 10-g nitrogen intake, the effects of increased and decreased total nitrogen levels on nitrogen balance were observed. In 3 subjects, the sparing effect of tyrosine on the phenylalanine requirement was shown. Phenylalanine requirements, determined at a 10-g nitrogen level, were 834, 924, 984, 1064 and 1184 mg. All subjects retained more nitrogen at the 15-g than at the 10-g nitrogen level, the mean increase being 0.80 g. Two of the 4 subjects who received a 5-g nitrogen intake retained less nitrogen than when they received 10 g of nitrogen; however, mean nitrogen balances for the 4 subjects were the same for the 2 periods when nitrogen levels were 5 g and 10 g. The remaining 2 subjects received a 3-g nitrogen intake and showed negative balances that were 0.45 and 0.47 g lower than respective balances for the 10-g nitrogen intake. Tyrosine exerted a sparing effect of at least 70% on the phenylalanine requirement.

The phenylalanine requirement of young men was found to be 1.10 g/day and tyrosine showed a sparing effect of 70 to 75% upon the phenylalanine requirement (1). When 900 mg of tyrosine was provided daily, 220 mg of L-phenylalanine was the lowest intake that kept all of a group of young women in the zone of nitrogen equilibrium (2). Approximately 90 mg of L-phenylalanine/kg a day satisfied the phenylalanine requirement of infants (3). The daily phenylalanine requirement without tyrosine reported for 10- to 12-year-old boys was 0.8 g or 27 mg/kg a day (4).

This study, which was conducted between November 1961 and March 1962, was initiated to determine for 6 young women 1) the phenylalanine requirement in the presence of a minimal amount of tyrosine; 2) the effects of increased and decreased total nitrogen intakes on nitrogen balance when the phenylalanine requirement was fed; and 3) the sparing effect of tyrosine on the phenylalanine requirement in women.

EXPERIMENTAL PROCEDURE

Subjects

Six young women who were college students at Tuskegee Institute served as subjects for this study. Their ages ranged from 20 to 28 years, body weights from 53 to 65 kg, and heights from 150 to 172 cm. Each subject was given a thorough physical examination and analyses were made of samples of urine and blood. The girls lived in campus dormitories and were engaged in their usual activities, which did not include strenuous activities that would cause sweating or the excessive use of calories. All meals were prepared and served in the diet kitchen of the Home Economics Building.

Food and supplements

Ordinary diet. The subjects were given ordinary foods which provided 10 g nitrogen/day the first 5 days of the study. If a subject did not go into positive nitrogen balance within the first 5 days, the ordinary diet was continued until this was obtained. Two periods during which the ordinary diet was fed were necessary be-

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cause of the break taken for the Christmas holiday.

Semipurified diets. Components of all semipurified diets were 1) a constant low-nitrogen basal diet, plus varying amounts of certain nitrogen-free foods; 2) 6.786 g of a mixture of essential amino acids (EAA); 3) varying amounts of phenylalanine; 4) 12.000 g of a mixture of nonessential amino acids (NEAA); and 5) varying amounts of glycine and diammonium citrate (DAC).

The basal diet contained 0.57 g of nitrogen and consisted of jelly, lettuce, peaches, pears, applesauce, pineapple, bananas, juices — orange, tomato and lemon — and wheat starch muffins and pudding.[4] In addition to the basal diet, gumdrops, peppermint candy, sucrose, butterfat, and certain carbonated drinks were consumed by each subject in amounts that maintained the caloric intake between 50 and 55 Cal./kg of body weight, or which prevented weight gain.

All amino acids were L- isomers and C.P. grade. Each was tested for purity and for contamination with other amino acids. The EAA mixture was phenylalanine-free, but contained the other 7 essential amino acids in amounts that corresponded to the safe allowances (table 1). A part of the methionine allowance was provided by cystine; tyrosine was omitted. The NEAA mixture was patterned after the occurrence of each nonessential amino acid in milk, except for glycine, which was omitted (table 1). One-third of the daily intakes of phenylalanine, the EAA mix and the NEAA mix were served at each meal.

TABLE 1

Composition of synthetic amino acid mixtures

Amino acids, C.P. grade	Amount fed
	g/day
Essential	
L-Isoleucine	0.954
L-Leucine	1.240
L-Lysine·HCl	1.250
L-Methionine	0.735
L-Cystine	0.367
L-Threonine	0.620
L-Valine	1.300
L-Tryptophan	0.320
Nonessential	
L-Arginine·HCl	0.846
L-Histidine·HCl	0.787
L-Alanine	0.801
L-Aspartic acid	0.786
L-Glutamic acid	4.968
L-Proline	2.504
L-Serine	1.308

Glycine and DAC solutions were given in varying amounts in order to make the total nitrogen intakes of the semipurified diets either 3, 5, 10 or 15 g. The nitrogen content of these solutions was 0.035 g/ml for glycine and 0.062 g/ml for DAC. While the total quantity of nitrogen provided by glycine:DAC solutions varied, isonitrogenous amounts of glycine and DAC with respect to one another were always fed. One-third of the daily intake of glycine:DAC was consumed at each meal.

Two multivitamin capsules were taken daily by each subject, and a 30-IU capsule of vitamin E was taken weekly. A 3.580-g quantity of mineral mixture, patterned after the one used by Leverton (5), served as the primary source of minerals.

Management of dietary periods

During the first period following the diet of ordinary food, phenylalanine was fed in an amount considered to be adequate or more than adequate for maintenance of nitrogen equilibrium in all subjects when their total nitrogen intake was 10 g. This was done to ascertain adequacy of the EAA mix. Subsequently, phenylalanine intakes were reduced stepwise until nitrogen equilibrium was obtained for each subject. The intake of phenylalanine at the time of nitrogen equilibrium (total nitrogen intake, 10 g) was considered to be the requirement for the subject. While continuing to feed the phenylalanine requirement, total nitrogen intake was increased to 15 g for all subjects and then decreased to 5 g for 4 subjects and 3 g for 2 subjects. The 3-g level of nitrogen given 2 subjects was due to miscalculation, the lowest intended nitrogen intake for all subjects being 5 g.

Collection and handling of metabolic products

Twenty-four-hour urine collections were made in bottles containing 25 ml 2% acetic acid. Nitrogen determinations were made daily in triplicate on 10-ml portions

[4] Starbake 100 (wheat starch) was a gift from Hercules Powder Company, Harbor Beach, Michigan.

of urine by the method of Kjeldahl (6). The procedure of Clark and Thompson (7) was used to determine creatinine in 1:10 dilutions of the 24-hour urine samples.

Carmine was given on the first day of each dietary period to mark the feces. Fecal samples were frozen until one or more periods were completed, after which a composite of all samples collected within a dietary period was made for each subject. Each composite was analyzed for nitrogen (6).

RESULTS

Nitrogen balances for all dietary periods, except those when ordinary food was eaten, are shown in table 2. Urinary nitrogen excretions for the first 2 days of each dietary period were omitted in calculating mean nitrogen balances, the reason for this being that urinary excretions were quite variable immediately following a change in the level of dietary N.

Creatinine excretion values for 5 subjects, excluding BP, showed ± 8% variation from period to period. Creatinine excretions for BP were considered constant among all periods. Variation within periods was generally slight, with the exception of the first and second days of the menses for certain subjects, when creatinine values were low.

All subjects, except TS, were in positive nitrogen balance when given 1184 mg phenylalanine with a total nitrogen intake of 10 g. Subject TS was given a 2.6-Cal./kg/day increase with the 1184 mg of phenylalanine and her nitrogen balance

TABLE 2

Dietary intakes, nitrogen balances and urinary creatinine excretions for different levels of phenylalanine and of total nitrogen

Subjects	No. of days	Phenylalanine intake[1]	Caloric intake	Nitrogen intake	Nitrogen balance	Urinary creatinine
		mg/day	*Cal./kg/day*	*g/day*	*g/day*	*g/day*
TS	13	1184	52.1	9.96	−0.54	1.68
	13	1184	54.7	9.96	+0.46	1.49
	10	1064	55.0	9.93	−0.50	1.62
	10	1184	54.6	14.94	+1.11	1.80
	7	1184	54.3	4.94	+0.26	1.75
SH	6	1184	48.5	9.96	+1.13	1.24
	7	934	47.5	9.96	−0.50	1.24
	17	984	53.6	9.96	+0.03	1.32
	20	984	53.2	14.96	+2.61	1.54
	14	984	53.6	4.94	+0.24	1.49
LT	6	1184	49.0	9.96	+1.51	1.24
	10	834	49.7	9.97	+0.35	1.27
	24	834	48.9	14.97	+0.40	1.50
	11	834	49.2	4.94	+0.19	1.48
AR	13	1184	56.1	9.96	+0.42	1.13
	11	1184	57.7	9.96	+0.08	1.20
	10	1064	56.4	9.93	+0.14	1.33
	10	1064	55.5	14.93	+1.27	1.48
	14	1064	62.5	4.95	+0.26	1.46
HW	6	1184	48.5	9.96	+1.07	1.16
	6	984	47.0	9.96	+0.47	1.16
	12	924	44.3	9.95	−0.04	1.27
	10	924	43.7	14.96	+0.15	1.27
	10	924	43.2	3.17	−0.49	1.49
BP	6	1184	50.0	9.96	+0.66	1.46
	7	984	51.2	9.96	−0.30	1.44
	12	984	55.6	9.96	+0.11	1.44
	10	984	54.7	14.96	+0.25	1.44
	10	984	54.2	3.18	−0.36	1.44

[1] Includes 45 mg of tyrosine and 123 mg of phenylalanine in basal diet.

TABLE 3

Caloric intakes, nitrogen balances and urinary excretions of creatinine with different levels of phenylalanine and tyrosine

Subject	No. of days	Caloric intake	Phenylalanine intake	Tyrosine intake	Nitrogen intake	Nitrogen balance	Creatinine
		Cal./kg/day	*mg/day*	*mg/day*	*g/day*	*g/day*	*g/day*
TS	7	54.2	327	861	9.96	+1.18	1.73
HW	11	43.3	313	615	9.95	+0.30	1.43
	9	42.9	275	653	9.95	+0.24	1.38
BP	10	53.7	328	660	9.96	+0.55	1.64
	10	54.2	287	701	9.96	+0.06	1.74

became positive. The EAA mixture was proved adequate for all subjects.

Phenylalanine requirements, when the total nitrogen intake was 10 g, ranged from 834 to 1184 mg/day, or 14 to 18 mg/kg/day. Nitrogen balances were + 0.46, + 0.03, + 0.35, + 0.14, − 0.04 and + 0.11 g/day. The respective phenylalanine requirements were then fed with an increase in total nitrogen to 15 g. In the same order, nitrogen balances were + 1.11, +2.61, +0.40, +1.27, +0.15 and +0.25 g /day. Feeding the same phenylalanine intakes, but decreasing total nitrogen to 5 g, resulted in nitrogen balances of +0.26, +0.24, +0.19 and +0.26, respectively, for the first 4 subjects, whereas reducing total nitrogen to 3 g caused negative balances of −0.49 and −0.36 g/day, respectively, for the last 2 subjects.

In table 3 are results for the sparing effect of tyrosine on the phenylalanine requirement of 3 subjects who participated in this part of the study. Sixty-six and 70% of the phenylalanine requirements for subjects HW and BP were supplied by tyrosine, and subject TS received only the 70% replacement. Total nitrogen intake during this phase of the study was 10 g. Mean nitrogen balances when tyrosine replaced 66 and 70% of the phenylalanine requirement were +0.30 and +0.24, respectively, for subject HW; and +0.55 and +0.06, respectively, for subject BP. Mean nitrogen balance for subject TS when 70% of her phenylalanine requirement was provided by tyrosine was +1.18 g.

DISCUSSION

Because of the positive nitrogen balances which resulted when 1184 mg phenylalanine were fed (table 2), a decrease in phenylalanine intake was given all subjects. The amounts of phenylalanine shown in table 2 include the phenylalanine of the EAA mix plus 123 mg bound phenylalanine and 45 mg bound tyrosine in the basal diet; converted gravimetrically, 45 mg tyrosine is equivalent to 41 mg phenylalanine.

Phenylalanine requirements for the 6 subjects, determined at the 10-g nitrogen intake, were 834, 924, 984, 984, 1064 and 1184 mg/day. Rose et al. (8) defined the minimum requirement of an amino acid as "the smallest amount capable of inducing distinctly positive balance." They considered the largest amount of an amino acid required by any one subject in the group studied as the minimum requirement. According to this definition, the minimum phenylalanine requirement for young women in the Tuskegee study was 1184 mg/day. This amount of phenylalanine is quite similar to the 1100 mg required by men (9).

In the studies of Leverton et al. (2), the amount of phenylalanine that permitted a nitrogen excretion within 95 to 105% of the nitrogen intake (zone of equilibrium) was regarded as the requirement. In the complete absence of tyrosine, all nitrogen balances for Leverton's young women were below the negative boundary of the equilibrium zone. In the presence of 900 mg tyrosine, mean nitrogen balances were in the equilibrium zone at phenylalanine levels of 220, 320, and 1280 mg. If one applies Rose's criterion of nitrogen equilibrium to these data, 50% of the subjects who were given the 220- and 320-mg levels and 5 of the 6 subjects who were given 1280 mg phenylalanine

were receiving less than their requirements. The tentative daily requirement of young women recommended by Leverton et al. (2) is 220 mg phenylalanine in the presence of 900 mg tyrosine. Although the total of these 2 amounts is quite similar to the 1184 mg phenylalanine obtained for young women in the Tuskegee study, if the same criterion of nitrogen equilibrium is applied to the 2 studies, the phenylalanine requirement of Leverton's young women is higher than that of the Tuskegee group.

Most of the caloric intakes were maintained between 50 and 55 Cal./kg/day. Because of the tendency to gain weight shown by subject HW, it seemed advisable in certain periods to reduce caloric intakes. Although calorie reduction stabilized body weight, the extent to which this influenced subsequent nitrogen balances became a factor of concern. The magnitude of decrease in caloric intake may appear small and insignificant, but the effect is detected by the sensitivity of the biological system and is reflected in nitrogen balance. Clark et al. (10) observed that a critical caloric intake exists for each subject, at which satisfactory retention of nitrogen occurs and below which nitrogen retention decreases proportionately to calorie reduction. They also noted that caloric restriction was reflected more promptly by change in daily nitrogen balance than by change in body weight. In our studies increases of 2.6 and 4.4 Cal./kg/day for subjects TS and BP resulted in increased nitrogen retentions of 1.00 and 0.41 g/day, respectively.

Clark et al. (10) also observed that an increase in calories as much as 20% above the critical intake did not improve nitrogen storage. In our studies the need of subject AR for a large calorie intake was shown during the first days of the study. She received more than 55 Cal./kg in all periods. During the first 8 days of the 14-day period in which subject AR received a total nitrogen intake of 5 g, an average nitrogen loss of 1.72 g/day was shown. Caloric intake was increased by 7 Cal./kg/day and her mean nitrogen balance for the last 6 days was +0.35. An intake of 50 to 55 Cal./kg/day with a semipurified diet is usually considered adequate (11). However, for subject AR, although 56 Cal./kg/day was adequate, at 10- and 15-g levels of nitrogen intake, this amount was distinctly inadequate when the total nitrogen intake was reduced to 5 g. This suggests that the critical caloric intake is related to level of total nitrogen, the critical caloric intake being higher when total nitrogen intake is low.

When the diet provided 15 g nitrogen, the mean nitrogen balance for 6 subjects was +0.98 g, whereas the comparable mean for the 10-g nitrogen intake was +0.18 g. Subject LT had difficulty eating all of the diet during the period of 15-g nitrogen intake. It was necessary to maintain her at this level for 24 days before 6 consecutive days of complete intakes, unaccompanied by regurgitation, were obtained. The extent to which subject LT's balance for the 15-g nitrogen period was influenced by her physiological condition is not known. Differences in nitrogen balances for the 6 subjects receiving the 10- and 15-g nitrogen intakes were not statistically significant, at either the one or 5% level, according to Student's *t* test.

Although 2 of the 4 subjects retained slightly more nitrogen while receiving the 10-g than with the 5-g nitrogen intake, the mean balance for the 4 subjects was the same, +0.24 g, for both the 10-g and 5-g nitrogen levels. Subjects HW and BP were in negative nitrogen balance while receiving 3 g nitrogen daily, −0.38 and −0.45 g, respectively. These negative balances may be attributed to an inadequate total nitrogen intake, or to insufficient calories. To our knowledge, the minimal amount of nitrogen that has maintained positive balance in man is 3.50 g (12).

As shown in table 3, positive nitrogen balances were obtained during both periods when a part of the phenylalanine intake was replaced by tyrosine. The results indicate that at least 70% of the combined phenylalanine and tyrosine intake may be supplied by tyrosine when the total nitrogen intake is 10 g. The mean nitrogen balance for the 3 subjects when tyrosine supplied 70% of the phenylalanine:tyrosine intake was +0.49 g, whereas the corresponding mean for the same subjects receiving phenylalanine alone at the same

nitrogen level was +0.18 g. The implication of this result is that a mixture of phenylalanine and tyrosine may be preferred over phenylalanine alone. More data are needed to verify this implication.

Throughout the study the periodic occurrence of menses influenced the data obtained for subjects LT, BP, and AR. Urinary creatinine and nitrogen excretions on the first and second days of menses were low.

LITERATURE CITED

1. Rose, W. C., and R. L. Wixom 1955 The amino acid requirements of man. XIV. The sparing effect of tyrosine on the phenylalanine requirement. J. Biol. Chem., *217*: 95.
2. Leverton, R. M., N. Johnson, J. Ellison, D. Geschwender and F. Schmidt 1956 The quantitative amino acid requirements of young women. IV. Phenylalanine with and without tyrosine. J. Nutrition, *58*: 341.
3. Snyderman, S. E., E. L. Pratt, M. W. Cheung, P. Norton and L. E. Holt, Jr. 1955 The phenylalanine requirement of the normal infant. Ibid., *56*: 253.
4. Nakagawa, I., T. Takashi, T. Suzuki and K. Kobayashi 1962 Amino acid requirements of children: minimal needs of threonine, valine and phenylalanine based on nitrogen balance method. Ibid., *77*: 61.
5. Leverton, R. M., M. R. Gram, M. Chaloupka, E. Brodovsky and A. Mitchell 1956 The quantitative amino acid requirements of young women. I. Threonine. Ibid., *58*: 59.
6. Association of Official Agricultural Chemists 1950 Official and Tentative Methods of Analysis, ed. 7. Washington, D. C., p. 12.
7. Clark, L. C., and H. L. Thompson 1949 Determination of creatine and creatinine in urine. Anal. Chem., *21*: 1218.
8. Rose, W. C., G. F. Lambert and M. J. Coon 1954 The amino acid requirements of man. VII. General procedures; the tryptophan requirement. J. Biol. Chem., *211*: 815.
9. Rose, W. C., B. E. Leach, M. J. Coon and G. F. Lambert 1955 The amino acid requirements of man. IX. The phenylalanine requirement. Ibid., *213*: 913.
10. Clark, H. E., S. P. Yang, L. L. Reitz and E. T. Mertz 1960 The effect of certain factors on nitrogen retention and lysine requirements of adult human subjects. I. Total caloric intake. J. Nutrition, *72*: 87.
11. Rose, W. C., M. J. Coon and G. F. Lambert 1954 The amino acid requirements of man. VI. The role of the caloric intake. J. Biol. Chem., *210*: 331.
12. Rose, W. C., and R. L. Wixom 1955 The amino acid requirements of man. XVI. The role of the nitrogen intake. Ibid., *217*: 997.

Effect of Methionine and Choline Deficiency on Liver Choline Oxidase Activity in Young Rats

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ABSTRACT Young male Sprague-Dawley rats were fed a water-soluble chemically defined diet containing 0.06, 0.12, 0.24 or 0.60% methionine with or without 0.25% choline for 10 to 42 days. Liver choline oxidase activity decreased in rats that ingested the choline-deficient diets (devoid of choline and low (0.06 or 0.12%) in methionine). These animals developed pathologic changes in the liver and kidney characteristic of acute choline deficiency. Liver choline oxidase activity remained normal in rats that were fed the methionine-deficient diets (low in methionine but supplemented with 0.25% choline).

Many studies (1) have demonstrated that choline deficiency in rats leads to fatty change in the liver and under some circumstances to cirrhosis and to hepatoma formation. The rat is especially susceptible to the development of choline deficiency and also is known to have a high level of liver choline oxidase activity (2).

Earlier studies have shown that liver choline oxidase activity decreases in rats fed a choline-deficient diet (3, 4), a low protein diet (4,5), or a lysine-deficient diet (6). Since the low choline diets used in these studies (3, 4) were also low in methionine, it has not been established whether the decrease of enzyme activity was due to a deficiency of choline, of methionine, or both components. The availability during the past few years of water-soluble chemically defined diets by Greenstein et al. (7) offered the possibility of obtaining a clear-cut answer to this question. Therefore liver choline oxidase activity, growth and gross and microscopic morphologic changes were observed in young rats fed for periods of 10 to 42 days a highly purified diet containing varying levels of methionine with or without choline. Our results indicating that the level of choline oxidase activity in the liver is sensitive to choline deficiency, but not to methionine deficiency, are presented and briefly discussed.

METHODS

Male albino rats of the Sprague-Dawley strain from the colony maintained at the National Institutes of Health, 3 to 4 weeks old and maintained with a commercial chow[3] diet since weaning were used. For 2 to 7 days prior to the institution of the experimental regimens, animals of approximately the same weight were fed the water-soluble chemically defined diet no. 26 of Greenstein et al. (7). The *basal* experimental diet contained only L- amino acids and contained no methionine and choline. The animals were divided into 8 groups according to the additions to the basal experimental diet as follows: (1) 0.06% methionine + 0.25% choline; (2) 0.06% methionine; (3) 0.12% methionine + 0.25% choline; (4) 0.12% methionine; (5) 0.24% methionine + 0.25% choline; (6) 0.24% methionine; (7) 0.60% methionine + 0.25% choline; and (8) 0.60% methionine. To maintain the same total S in the diets of all groups, L-cysteine ethyl ester hydrochloride was added in equivalent amounts to that of the omitted methionine. The diets and water were supplied ad libitum throughout the experiments. Fat and the fat-soluble vitamins were fed 3 times a week to each animal.

The animals weighed on the average 53 g at the onset of the experiments. They were weighed at 2- to 3-day intervals

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throughout the experiments. The animals were housed individually in wire-suspended cages in an air-conditioned room maintained at 78°F.

In one experiment the animals were killed after 10 days; in a second, after 14 days; and in a third, after 42 days. In a fourth experiment, one or two animals of each group were killed at 2, 3, 4, 5 and 6 weeks. Animals were anesthetized with ether and killed by exsanguination from the abdominal aorta. All were autopsied and pieces of tissue from selected organs, liver, kidney, testis, adrenal, spleen, thymus, stomach, small intestine, gastrocnemius muscle and heart, were fixed in Zenker-formol solution. Paraffin-embedded tissues were sectioned and routinely stained with hematoxylin and eosin. In addition, pieces of liver were fixed in 10% formalin and frozen sections were stained with oil red O.

Liver tissue for assay of choline oxidase activity was cooled immediately upon removal and was homogenized with 5 volumes of ice-cold 0.04 M phosphate buffer, pH 7.4. Choline oxidase was measured manometrically by the method of Williams et al. (8) as modified by Richert and Westerfeld (4) and by Wells (9). Since the rates of oxygen uptake decreased progressively after incubation periods of from 20 to 40 minutes, the 2 consecutive highest readings during the first 40 minutes of incubation were used to calculate the units of enzyme activity (μliters O_2 uptake/g tissue/hr.). Protein was measured by determination of Kjeldahl nitrogen (10) on the trichloroacetic acid (TCA) precipitates from aliquots of the liver homogenates. The aliquots were washed in succession with 5% TCA, 95% ethanol, ethanol-ethyl ether mixture (3:1) and ethyl ether before digestion. For total liver lipid analysis, an aliquot of liver was dried by grinding with anhydrous sodium sulfate and was extracted with chloroform for 24 hours. The residue was extracted with petroleum ether and lipid was determined gravimetrically after evaporation of solvent.

RESULTS

The growth of the rats for 24 days when fed the 8 different diets was determined from the combined data of 4 experiments (fig. 1). The presence or absence of 0.25% choline in the diet had little influence on growth. The rate of growth of the rats depended on the amount of methionine in the diet — the greatest growth increment occurred in rats receiving the highest level of methionine, and the least

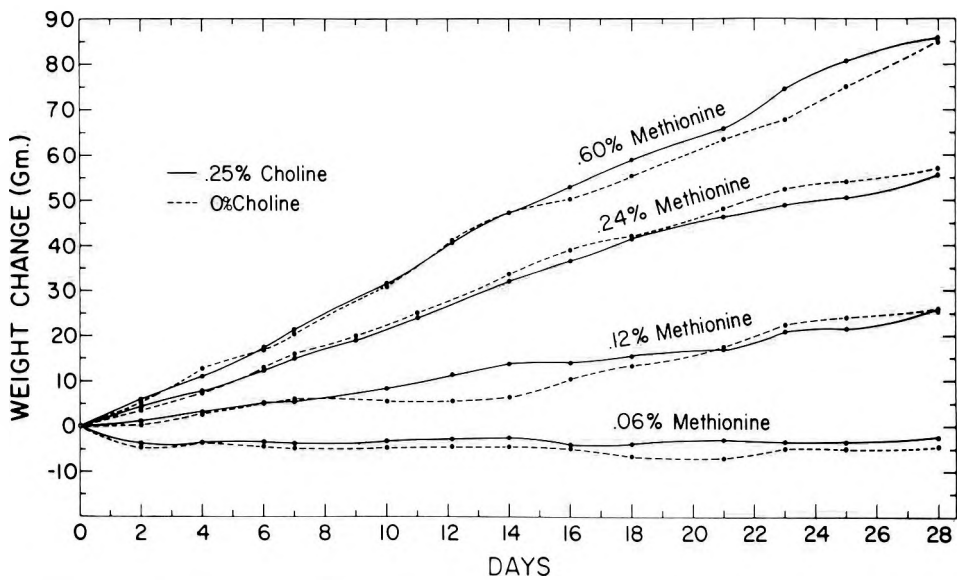

Fig. 1 Growth curves of rats fed highly purified diets containing 0.06, 0.12, 0.24 or 0.60% methionine with or without 0.25% choline.

growth in those with the lowest level. Rats consuming 0.06% methionine lost weight in all of the experiments. The average diet consumption per rat in grams of total solids of the liquid diet per day was calculated for the first 14 days of the experiments and is expressed according to the methionine content with and without 0.25% choline respectively: 0.60% methionine, 7.9, 8.2; 0.24% methionine, 7.2, 7.0; 0.12% methionine, 5.2, 4.9, and 0.06% methionine, 3.4, 3.4.

Analyses for liver choline oxidase were performed on animals of each group fed the different diets for intervals of from 10 to 42 days. The results for each group of animals at the different time intervals were essentially the same and therefore were combined. The values are recorded in table 1. Since extremely fatty livers were present in rats fed the diets containing 0.06 or 0.12% methionine without choline, it was desirable to express the activities on the basis of liver protein as well as per unit wet weight. Liver choline oxidase activity was markedly decreased in rats ingesting the diet containing 0.06% methionine and no choline. Rats ingesting the diet containing 0.12% methionine and no choline had a moderate decrease in liver choline oxidase activity. In contrast, rats ingesting the diets containing 0.25% choline, regardless of the quantity of methionine, had high levels of liver choline oxidase activity.

Morphologic findings

Rats fed diet 10 to 14 days. Rats fed the diets containing 0.06 or 0.12% methionine without choline had the major findings characteristic of acute choline deficiency, i.e., fatty change of the liver with a central lobular distribution of lipid, and renal hemorrhage. Three animals fed the diet containing 0.06% methionine without choline died within 10 days and severe renal hemorrhage was noted. Rats fed the other diets showed essentially no changes.

The livers of 2 rats fed the diet containing 0.06% methionine without choline for 2 weeks showed a remarkable alteration. Grossly the livers were yellow on the left and brown on the right (fig. 2). Microscopically, the yellow areas showed lipid accumulation in the central areas of the lobule, whereas in the brown areas the liver was essentially normal (fig. 3). The line of demarcation between these areas was sharp. The 2 different areas of liver from one rat were analyzed separately for lipid. The yellow area contained 23% lipid, and the brown area, 7% lipid. Since the lobes of the liver on the left in each of the 2 cases had fatty change and the lobes on the right were normal, it is possible to explain these findings on an anatomical basis. The left lobes of the liver receive blood predominantly from the splenic vein, whereas the right lobes receive the major part of their blood from

TABLE 1

Effects of dietary levels of methionine and choline on liver weight, lipid and choline oxidase activity

Dietary		No of rats	Liver			
Methionine	Choline		Weight	Lipid	Choline oxidase	
			g	%	$\mu l\ O_2/hr/g$	$\mu l\ O_2/hr/mg$ *protein*
0.06	0.25	11	2.34 ± 0.06[1]	3.98 ± 0.11[1]	1878 ± 120[1]	15.1 ± 1.7[1]
0.06	0	10	2.69 ± 0.26	17.57 ± 2.83[2]	917 ± 82[2]	7.7 ± 0.9[3]
0.12	0.25	10	3.57 ± 0.19	3.77 ± 0.17	2248 ± 138	16.1 ± 0.8
0.12	0	9	4.18 ± 0.25	16.40 ± 1.71[2]	1599 ± 248[3]	12.1 ± 1.5[3]
0.24	0.25	6	4.58 ± 0.33	4.21 ± 0.25	2368 ± 128	15.8 ± 0.6
0.24	0	6	4.53 ± 0.37	4.42 ± 0.60	2294 ± 268	16.2 ± 1.9
0.60	0.25	12	4.82 ± 0.34	4.38 ± 0.15	2282 ± 232	21.6 ± 1.3
0.60	0	10	5.33 ± 0.39	4.89 ± 0.20	2532 ± 293	18.9 ± 1.3

[1] Mean value ± SE of mean, calculated by the method of Mantel (11).

[2] Choline effect highly significant, $P < 0.01$.

[3] Choline effect probably significant, $0.01 < P < 0.05$.

the mesenteric veins. In a borderline deficiency such as could have been the case in these two animals, there may have been less nutrients in splenic blood than in blood from the intestines. A similar explanation has been advanced in explaining partial hepatic dietetic necrosis (12). A striking difference in the gross and anatomic accumulation of lipid has also been observed in some rats fed a purified diet containing orotic acid (13). However, in this instance the distribution is reversed — most of the lipid appearing on the right side.

Rats fed diet 21 to 42 days. The rats fed the diets containing 0.06 or 0.12% methionine without choline had severe fatty livers with a central distribution of the lipid and the kidneys of many rats had focal scarred areas with accumulations of lymphocytes and macrophages and an increase in fibrous tissue in the cortex. The liver of rats fed the other diets appeared normal. The kidneys of a few rats fed the diet containing 0.12 or 0.24% methionine with 0.25% choline had focal areas of chronic inflammation.

Rats fed the diets containing 0.06% methionine with or without 0.25% choline for 10 to 42 days had testicular changes.

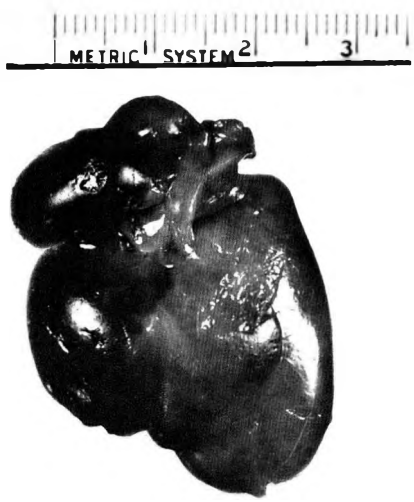

Fig. 2 Liver of rat fed the purified diet containing 0.06% methionine without choline for 14 days. Note sharp line of demarcation between dark normal liver at left and light yellow liver at right.

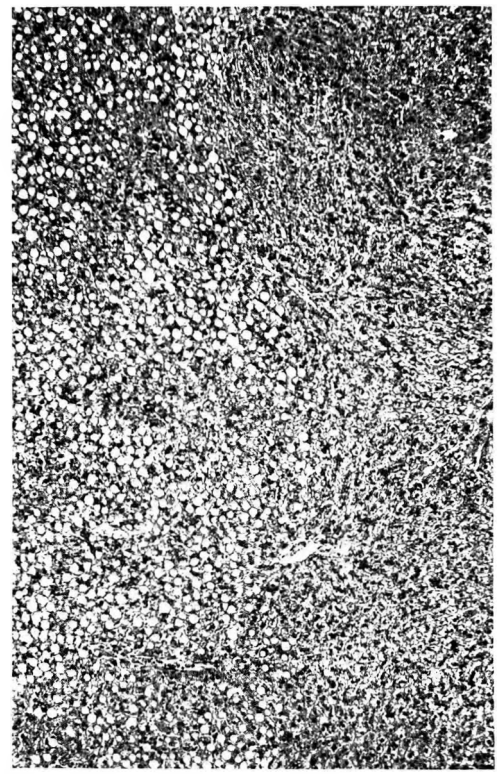

Fig. 3 Photomicrograph of liver demonstrated in figure 2. Note sharp demarcation between hepatic cells containing lipid on the left and normal hepatic cells on the right. Hematoxylin and eosin. × 75.

The seminiferous tubules were small and contained sparse germinal epithelial cells. The testes of rats fed the diet containing 0.60% methionine appeared normal. No pathologic changes were noted in the heart, spleen, thymus, gastrocnemius muscle, adrenal, stomach, and small intestine of rats fed any of the diets.

DISCUSSION

Choline oxidase is an important enzyme that provides the only known mechanism for irreversible biological degradation of choline in higher animals. This enzyme has been investigated under many experimental dietary alterations (14). Fasting (2), choline supplementation (2), a high protein diet (15), a histidine-deficient diet (16) or certain vitamin-deficient (thiamine or pyridoxine) diets induced no change in liver choline oxidase activity in the rat. On the other hand, a low protein

diet (4, 5), a lysine-deficient diet (6) or certain other vitamin-deficient (nicotinic acid, riboflavin, or choline) diets (4) led to a decrease in liver choline oxidase activity. Our present observations indicate that liver choline oxidase decreases with choline deficiency but does not change with methionine deficiency. Diet consumption and growth of the animals were of no importance in influencing enzyme activity as animals receiving low methionine but adequate choline showed no growth or even loss of weight, yet had normal levels of choline oxidase activity. Animals that received adequate levels of methionine but no choline had normal levels of choline oxidase activity indicating that in these animals sufficient choline was synthesized from methionine.

Although many studies have described choline deficiency in the rat (1), relatively few studies have dealt with methionine deficiency (17–20). In our present study of liver choline oxidase activity in choline and methionine deficiency, it was of special interest to compare the morphologic findings with these 2 deficiencies, especially since the diet used was a highly purified one containing only 1.5% total lipid. This level is far below that used in most experiments with choline deficient diets where the lipid content usually falls between 10 to 30%. Animals fed the choline-deficient diets developed early severe pathologic lesions in the liver and kidney, as previously described by many investigators (1). However, animals fed the methionine-deficient diet showed obvious retardation of growth with only one significant morphologic alteration, testicular atrophy. A similar testicular change has been described in some studies in rats fed diets containing selected proteins low in methionine (18, 20) but not in other studies in which rats were fed a purified diet devoid of methionine for 7 (19) or as long as 100 (17) days. In all of these studies on methionine deficiency, the diets were fed ad libitum. It is noteworthy that young rats receiving known amounts of a methionine-devoid diet by force-feeding for as short a period as 3 to 7 days develop obvious pathologic lesions in the liver (19, 21). This difference between animals fed ad libitum and force-fed similar regimens is probably a function of the quantity of the diet consumed.

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LITERATURE CITED

1. Griffith, W. H., and J. F. Nyc 1954 The Vitamins, vol. 2, eds., W. H. Sebrell, Jr. and R. H. Harris. Academic Press Inc., New York, p. 62.
2. Sidransky, H., and E. Farber 1960 Liver choline oxidase activity in man and in several species of animals. Arch. Biochem. Biophys., *87*: 129.
3. Handler, P., and F. Bernheim 1942 The choline oxidase activity of fatty liver. J. Biol. Chem., *144*: 401.
4. Richert, D. A., and W. W. Westerfeld 1952 The effect of diet on tissue choline oxidase. Ibid., *199*: 829.
5. Bargoni, N. 1951 Enzymatic activity and protein amounts in the liver. Experientia, *7*: 104.
6. Bothwell, J. W., and J. N. Williams, Jr. 1954 Effects of a lysine deficiency upon enzyme activity in rat liver. Proc. Soc. Exp. Biol. Med., *85*: 544.
7. Greenstein, J. P., S. M. Birnbaum, M. Winitz and M. C. Otey 1957 Quantitative nutritional studies with water-soluble, chemically defined diets. I. Growth, reproduction and lactation in rats. Arch. Biochem. Biophys., *72*: 396.
8. Williams, J. N., Jr., G. Litwack and C. A. Elvehjem 1951 Studies on rat liver choline oxidase: an assay method. J. Biol. Chem., *192*: 73.
9. Wells, I. C. 1954 Oxidation of choline-like substances by rat liver preparations. Inhibitors of choline oxidase. Ibid., *207*: 575.
10. Perrin, C. H. 1953 Rapid modified procedure for determination of Kjeldahl nitrogen. Anal. Chem., *25*: 968.
11. Mantel, N. 1951 Rapid estimation of standard errors of means for small samples. Am. Statist., *5*: 26.
12. Himsworth, H. P. 1950 Lectures on the Liver and its Diseases. Harvard University Press, Cambridge, Massachusetts, p. 26.
13. Sidransky, H. 1963 Sex difference in induction of fatty liver by dietary orotic acid. Endocrinology, in press.
14. Knox, W. E., V. H. Auerbach and E. C. C. Lin 1956 Enzymatic and metabolic adaptations in animals. Physiol. Rev., *36*: 164.
15. Hove, E. L., and J. O. Hardin 1951 Effect of vitamin E and CCl_4 on fat, respiration and choline oxidase of rat livers. Proc. Soc. Exp. Biol. Med., *78*: 858.
16. Bothwell, J. W., and J. N. Williams, Jr. 1951 Effect of histidine deficiency upon en-

zyme activity in the rat. J. Biol. Chem., *191:* 129.
17. Glynn, L. E., H. P. Himsworth and A. Neuberger 1945 Pathological state due to deficiency of the sulphur-containing amino-acids. Brit. J. Exp. Path., 26: 326.
18. Sós, J., T. Kemény, and M. Schnell 1953 Genitale Veränderungen an mannlichen Ratten verursacht durch partiellen Methioninmangel. Acta Physiol. Hung., *4:* 211.
19. Sidransky, H., and E. Farber 1958 Chemical pathology of acute amino acid deficiencies I. Morphologic changes in immature rats fed threonine-methionine-, or histidine-devoid diets. A.M.A. Arch. Path., 66: 119.
20. Pomeranze, J., S. J. Pilero, P. T. Medici and A. Plachta 1959 Deficiency diets in young growing rats. Proc. Soc. Exp. Biol. Med., *100:* 207.
21. Sidransky, H., and E. Farber 1958 Sex difference in the induction of periportal fatty liver by methionine deficiency in the rat. Ibid., *98:* 293.