Some Selenium Responses in the Rat not Related to Vitamin E [1,2,3]

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ABSTRACT Rats fed a low selenium ration containing Torula yeast with adequate vitamin E grew and reproduced normally. Their offspring were almost hairless, grew more slowly and failed to reproduce. Supplemental *dl*-methionine, sodium sulfate, or increased dosage of vitamins was without effect. Supplementing with 0.1 ppm selenium as sodium selenite restored haircoat, growth, and reproductive capabilities. Some usual biochemical tests for selenium or vitamin E deficiency status, or both, were inconclusive when compared with rats fed normal rations. Rats fed low selenium corn protein also had sparse hair and poor growth in the second generation. A low selenium ration from ewe muscle, however, supported rats for three generations without any abnormalities.

The essentiality of selenium (Se) as a nutrient with or without vitamin E has been debated (1–5). The essential role of selenium may be due to its analogue replacement of sulfur in naturally occurring compounds, or the formation of compounds not related to sulfur derivatives (6–8). Selenium may parallel the role of vitamin E acting as an antioxidant against lipid double bond peroxidation (9), or it may have a sparing effect on vitamin E itself (10). On a molar basis, selenium compounds constitute the most potent factors yet found to prevent death from liver necrosis in the rat fed a ration which has Torula yeast as its source of protein (8). Torula yeast [4] is unique in that it is very low in sulfur amino acids, vitamin E and selenium, and high in unsaturated fatty acids (11). Witting and Horwitt (12) have shown selenium growth responses in rats supplementing a casein diet with a high selenium (0.25 ppm) and a low α-tocopherol content.

It has been reported that ewes possess a carryover effect from an adequate selenium intake during the first pregnancy to the second pregnancy when the dam is existing on a low selenium ration which should otherwise produce lambs having white muscle disease (WMD), a selenium-responsive myopathy (13). The pregnant ewe evidently possesses the metabolic ability of storing minute quantities of selenium which it imparts to the lamb to prevent WMD in the next generation. McConnell and Roth (14) have shown a milk and placental selenium carryover in dogs using radiotracers. Recent evidence by Burk et al. (15) has shown that selenium is rapidly depleted from weanling rats on a Torula yeast diet, and that this loss is seemingly unaffected by vitamin E.

The object of the experiment was to determine whether rats fed a Torula yeast ration, or other low selenium protein rations with added vitamin E and without added selenium, could grow and reproduce through several generations. The results would provide an insight into the carryover effect of selenium in other animals and would present evidence that selenium does have an essential function separate from vitamin E.

EXPERIMENTAL PROCEDURE

Composition of rations used is listed in table 1. The Torula yeast ration (0.020 ppm Se) differs from that reported by Schwarz and Foltz (8) by increasing Torula yeast from 30 to 40% and sub-

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[2] Supported in part by Public Health Research Grant no. HE 09228 from the National Institutes of Health.

[3] This report constitutes a portion of a thesis submitted by Kim E. M. McCoy in partial fulfillment of the requirements for the Ph.D. degree.

[4] Lake States Yeast and Chemical Division 1959 Analysis of type B Torula yeast. St. Regis Paper Company, Rhinelander, Wisc.

TABLE 1

Composition of low selenium rations

	Torula yeast [1]	Ewe muscle [2]	Corn [3]
	%	%	%
Protein source	40.0	35.0	85.0
Sucrose	41.5	51.5	3.0
Vegetable oil [4]	5.0	0.0	5.0
HMW salt mixture [5]	5.0	5.0	5.0
Vitamin mixture [6]	1.0	1.0	1.0
Cellulose [7]	7.5	7.5	0.0
dl-Methionine	0.0	0.0	0.2
l-Lysine	0.0	0.0	0.5
Selenium conc, ppm	0.020	0.027	0.030

[1] Lake State Yeast, Rhinelander, Wisc.
[2] Freeze dried, low selenium.
[3] Low selenium corn was graciously supplied by W. H. Allaway, Agricultural Research Service, U.S. Department of Agriculture, Ithaca, N. Y.
[4] Wesson Oil, refined cottonseed oil, Wesson Sales Company, Fullerton, Calif.
[5] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutr., *14:* 273.
[6] Vitamin mixture contained: (in milligrams) thiamine·HCl, 40; Ca D-pantothenate, 200; menadione, 10; folic acid, 20; riboflavin, 25; pyridoxine·HCl, 20; biotin, 10; and (in grams) vitamin B_{12}, (1% trituration), 1; niacin, 1; choline chloride, 10; and lactose to make 100 g. Vitamin A acetate, 10 mg; *d*-α-tocopheryl acetate, 60 mg; and vitamin D_2, 100 μg were supplied in 95% ethanol/kg of diet.
[7] BW 100 Solka Floc purified cellulose, Brown Company, New York, N.Y.

stituting cottonseed oil for stripped lard as vitamin E was added to the ration. Low selenium ewe muscle protein was prepared by freeze-drying selected lean muscle from ewes which had been fed low selenium alfalfa (0.010 ppm) for at least 1 year and had produced WMD lambs. This muscle ration (0.027 ppm Se) was fed at a 20% protein level. A low selenium corn ration (0.030 ppm) was prepared using ground whole kernels and adding 0.2% *dl*-methionine and 0.5% *l*-lysine. The protein content, even with the added amino acids, was only 8.2%. A commercial ration [5] constituted the control ration. Rats used were from our closed colony of OSU Browns. Four females with their litters (average of seven pups each) were fed Schwarz's preweaning diet (16) until weaned at 21 days, when they were randomly assigned to one of the four dietary regimens. They were fed ad libitum with weekly determinations of growth and feed intake. As a prophylactic measure for the prevention of respiratory disease in long-term reproductive experiments, sulfa drugs [6] were added to the distilled water for 1 week/month. At 4 months of age, the animals were mated within each group and all were fertile. The offspring were fed their respective rations or with supplements as will be described. Selenium analyses were made on all rations using the method of Allaway and Cary (17). Protein was determined using AOAC procedures (18). Blood was obtained by heart puncture from rats anesthetized with carbon dioxide and the plasma was assayed according to Sigma for SGOT,[7] LDH,[8] and CPK [9] enzymes. Selenium-75 red blood cell uptake from radioactive sodium selenite [10] (specific activity = 6.0 Ci/g Se) was determined by the method of Wright and Bell (19), except that during incubation air was used as previous results with air did not differ from those using 95% N_2 : 5% CO_2. Individual hearts and skin samples (0.5 g) were extracted by homogenizing with chloroform and methanol using the procedures of Bligh and Dyer (20). Fatty acid esters were obtained by using 5% HCl and anhydrous methanol. The fatty acid methyl esters were chromatographed according to Lowry and Tinsley (21) using a chromatograph [11] fitted with a hydrogen flame detector. The fatty acid methyl esters were identified by the relative retention times compared with methyl stearate (C 18:0) and by reference to standards from the Hormel Institute and from data in the literature (22).

RESULTS AND DISCUSSION

There was considerable difference in growth supported by these rations (table 1), and between the first and second generations (fig. 1). Only females showed decreased growth in generation 2 on the muscle ration ($P < 0.01$), but the ration

[5] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.
[6] Harr, J. R. 1968 Department of Veterinary Medicine, Oregon State University, personal communication.
[7] The colorimetric determination of glutamic-oxaloacetic and glutamic-pyruvic transaminases at 490–520 mμ in serum or other fluids. Sigma Chemical Company, St. Louis. Tech. Bull. no. 505, revised September 1964.
[8] The ultraviolet determination in serum at 340 mμ of lactic dehydrogenase. Sigma Chemical Company, St. Louis. Tech. Bull. no. 340–UV, June 1966.
[9] The colorimetric determination of creatine phosphokinase in serum and other fluids at 620–700 mμ. Sigma Chemical Company, St. Louis, Tentative Tech. Bull. no. 661, revised May 1967.
[10] New England Nuclear Corporation, Boston, Mass.
[11] Beckman model GC-2, Beckman Instruments, Inc., Fullerton, Calif.

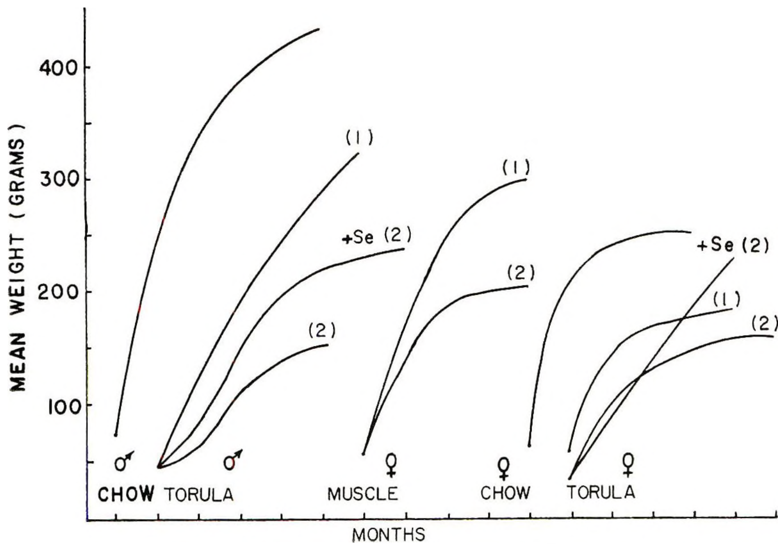

Fig. 1 Growth curves for rats on vitamin E-adequate diets. Each line is an average of four females or three males. Parentheses indicate generation of rat.

supported reproduction through three generations. Both males and females showed decreased growth in generation 2 on the Torula yeast diet ($P < 0.01$). Growth of both males and females was improved by the addition of 0.10 ppm Se as sodium selenite to the Torula ration ($P < 0.01$). Rats on the corn ration grew very slowly as could be expected from the low protein content. From weaning, generation 2 young from the Torula ($n = 24$) and corn ($n = 21$) rations all exhibited sparse hair. The young of the animals fed corn protein were all small and in poor condition from birth. The corn experiment, therefore, was terminated when these generation 2 young were 3 months old. The hair coat on all these animals looked most sparse at 2 months of age and improved only slightly the following months. To investigate if a dietary factor was involved in this hairlessness, the generation 2 Torula animals were divided into groups of four animals each with the following supplements: (1) 0.5% *dl*-methionine; (2) 0.1 ppm Se as sodium selenite; (3) double the vitamin mixture of table 1; (4) triple the vitamin A addition of table 1; (5) triple the vitamin E addition of table 1; and (6) control diet, table 1.

All rats receiving the selenium supplementation had considerable hair production within 2 weeks and normal coverage at 4 weeks, whereas the other groups showed no visible improvement (fig. 2). All rats of generation 2 fed the Torula rations were sterile except those fed the selenium-supplemented ration. Sterility was determined by a lack of breeding with fertile rats and by examining the male organs for spermatozoa. Immotile spermatozoa, with separation of heads from tails, were found in five out of eight males, and no spermatozoa were located in the other three males with no selenium supplementation. The two males and two females in the selenium-supplemented group were fertile but had litters of only one and two animals, all of which died within a few days. It would appear that selenium supplementation permitted some fertilization to occur, but only a limited number of young were carried to full term. Apparently they were not normal as they lived only a short time.

When weanling generation 2 female rats of the ewe muscle ration were fed the Torula ration, all 15 young showed the characteristic sparse hair, along with a pale iris and an apparently white retina. To identify in which chemical fraction this biological response was contained, 24 g ewe muscle ether extract or 15 g ewe muscle ash/2 kg Torula yeast ration were fed to

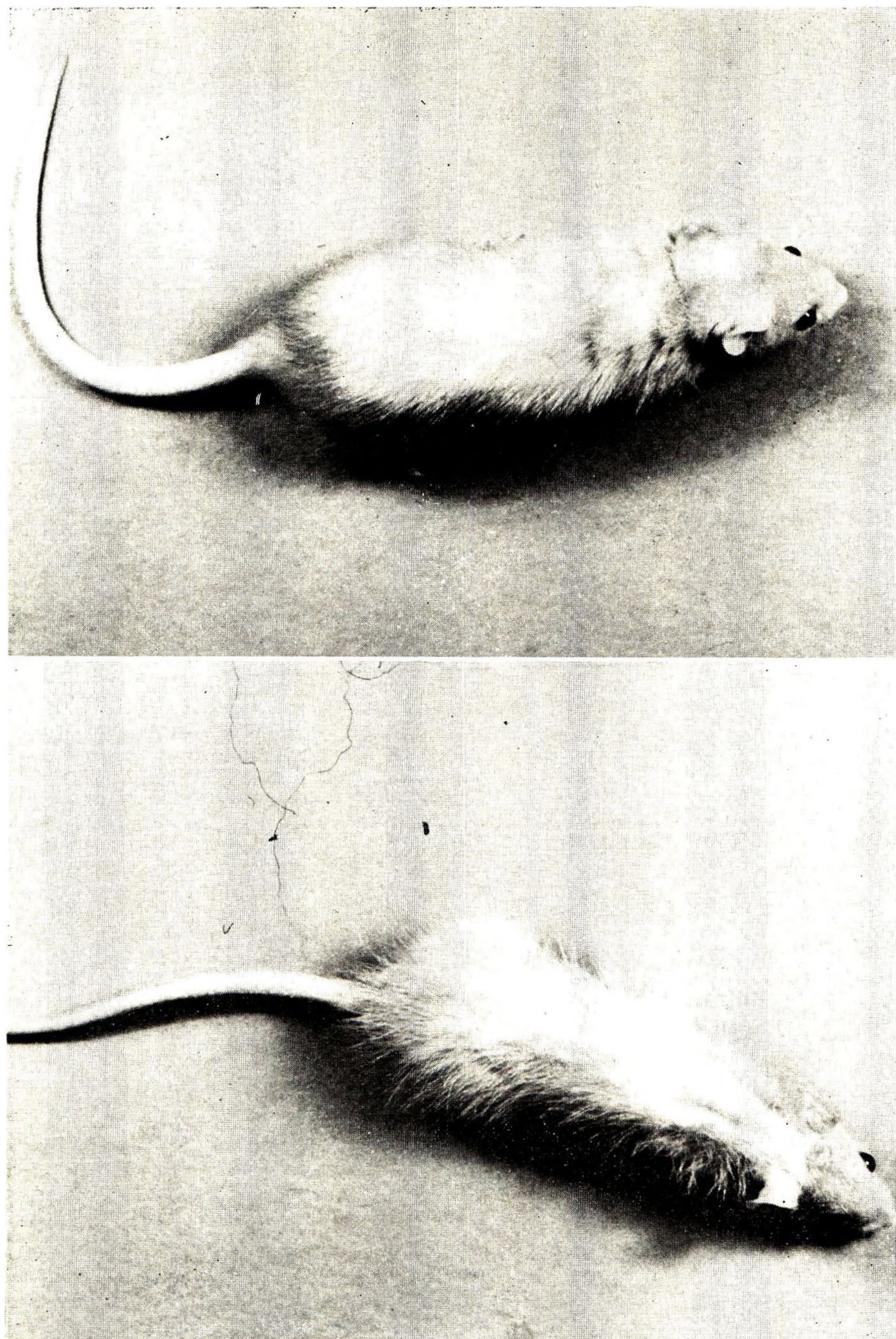

Fig. 2 Litter-mate generation 2 animals at the age of 9 weeks on the Torula yeast + vitamin E ration. The female on the top, 85 g, has had a 0.1 ppm Se supplement as sodium selenite for the last 4 weeks, whereas the male on the bottom, 65 g, has had no selenium supplement, and has sparse hair.

groups of three weanling rats that showed sparseness of hair and a lightened iris and retina. Additions of 0.1 ppm Se as sodium selenite or 1% Na_2SO_4 were also included. Based on visual observations, it was found that only the ewe muscle ether extract or selenium promoted hair growth, and selenium alone returned the eye color to normal during a 6-week period (fig. 3). It would appear that the low selenium ration from ewe muscle is not selenium deficient or that it has other substances which prevent the selenium-responsive deficiency signs that occur in generation 2 rats fed Torula ration.

To determine whether ewe muscle, yeast, and corn were biologically selenium deficient, they were exhaustively ether extracted to free them of ether-soluble substances, including vitamin E. These three protein sources were then bioassayed in duplicate for selenium according to Schwarz and Foltz (8) with the following average results for the day of death after weaning: extracted ewe muscle, 26; extracted corn, 31; extracted Torula, 26; and Schwarz's Torula assay ration, 19. All rats had grossly necrotic livers.

Certain plasma enzymes from rats, such as glutamic-oxalacetic transaminase (GOT) and lactic dehydrogenase (LDH), are increased (10 to 20 times) when no selenium or vitamin E is added to Torula yeast rations.[12] It has also been established that erythrocytes are more susceptible to oxidative hemolysis with dialuric acid in the absence of vitamin E (23). The selenium-75 uptake of erythrocytes from sheep fed low selenium diets or forages is significantly increased (19, 24). None of these diagnostic tests could distinguish the Torula yeast ration with or without selenium from the commercial ration. The plasma GOT Sigma-Frankel units ($\pm$ SD) per milliter for six animals per group all averaged 114 ± 29, and the percentage selenium-75 red blood cell uptake averaged 14.2 ± 3.1. No differences were again found for CPK, LDH, or hemoglobin values when compared with normal animals.

Preliminary work using two animals per group revealed no differences in the fatty acid analysis of the heart. However, the relative percentage of skin fatty acids $C_{18:3}$, $C_{20:2}$, $C_{20:5}$, and $C_{22:4}$ were higher in the animals fed the Torula yeast ration supplemented with selenium and in the commercial ration, compared with the nonselenium-supplemented animals. The relative percentage of these skin polyunsaturated fatty acids in the selenium group was 19.9% compared with 9.8% in the nonselenium group ($P < 0.05$).

Apparently selenium is required for reproduction, normal haircoat, and normal eye development in generation 2 rats fed a Torula yeast ration supplemented with vitamin E. Low selenium corn produced some of the same deficiency signs. The reason that low selenium ewe muscle protein supports normal growth and reproduction is not explained by a selenium carryover effect for at least three generations, whereas such an explanation is plausible for rats fed Torula yeast. Differences in polyunsaturated fatty acid composition of selenium-supplemented rats, and the capability of the ether extract of ewe muscle to cure the sparse hair suggest that the difference in metabolic action is related to lipid metabolism or protection. It is noteworthy that these selenium-responsive syndromes are observed in the presence of added vitamin E.

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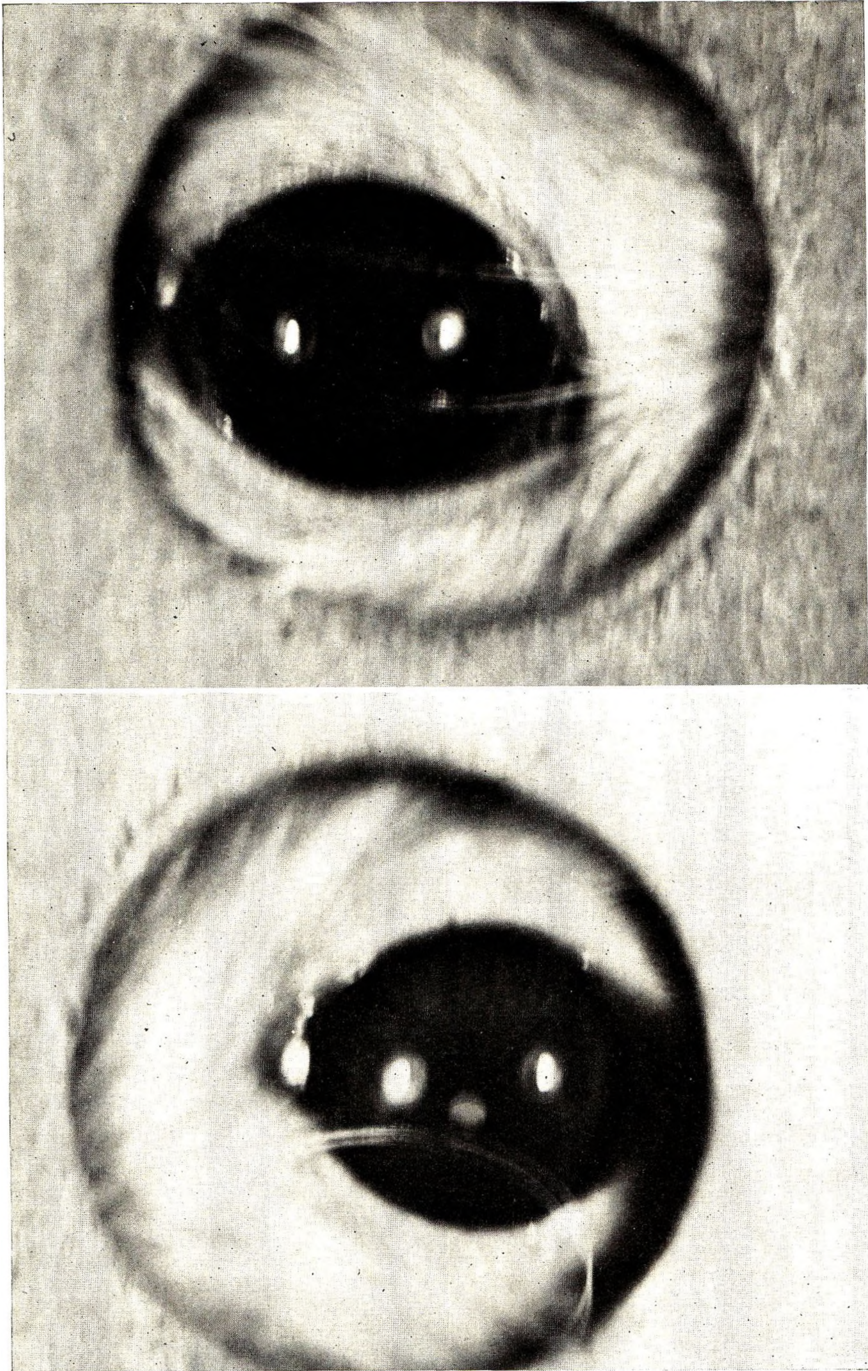

Fig. 3 When weaning generation 2 female rats of the ewe muscle ration were fed the Torula ration, their young had a pale iris and an apparently white retina (bottom). A litter-mate's eye (top) became normal after 2 weeks on the 0.1 ppm Se supplementation. The two white spots on each eye are lamp reflections.

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A Simplified Hemolysis Test for Vitamin E Deficiency [1]

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ABSTRACT A bioassay procedure for vitamin E is described in which inhibition of spontaneous hemolysis of erythrocytes incubated in isotonic saline-phosphate buffer at 37° serves as the criterion of response. The method offers advantages over the conventional dialuric acid-induced hemolysis test with respect to simplicity, sensitivity and reproducibility.

The discovery by Rose and György (1, 2, 8) that erythrocytes of vitamin E-deficient rats hemolyze in the presence of certain mild oxidizing agents has been widely used as a means of evaluating the biological activity of the various tocopherols, and of estimating the vitamin E requirement of animals and man. In determining the biopotency of certain tocopherol derivatives by the dialuric acid-induced hemolysis procedure of Friedman et al. (3), it was observed that the spontaneous hemolysis of the control samples (i.e., those exposed only to buffered saline) increased as a function of the length of time the rats were fed a vitamin E-deficient diet. This phenomenon has been observed previously by Christensen and co-workers (4). After depletion for 6 to 12 months the control values increased to such an extent that the accuracy of the assay was impaired. This observation suggested that the bioassay could be based directly on the degree of spontaneous hemolysis in buffered isotonic saline solution. This procedure has been found to be simpler, more sensitive and more reproducible than the dialuric acid test. The method is similar to that recently developed independently by Jager (5).

METHODS

Sixty weanling female rats of the Sprague-Dawley strain were fed ad libitum a vitamin E-deficient diet of the following composition: (in percent) glucose monohydrate (Cerelose), 65.4; casein ("vitamin-free"), 20.0; molecular-distilled lard, 10.0; salts 4164 (6), 4.0; vitamin premix in Cerelose (7), 0.5; and choline chloride, 0.1. Twenty-five international units of stabilized vitamin A palmitate and 2 IU vitamin D were added per gram diet. The diet was mixed every 3 weeks and kept under refrigeration.

Most of the data were obtained when the rats reached 190 to 225 g in body weight, by which time all animals exhibited essentially complete hemolysis by the dialuric acid method. Additional observations were made on rats fed the same diet for periods up to 1 year. All blood samples were subjected to the dialuric acid hemolysis procedure of Friedman and co-workers (3) as a reference method, and aliquots from the same samples were tested by the spontaneous hemolysis method outlined below. To obtain a range of comparative values, deficient rats were given oral doses of 0.75 to 5.0 mg of *dl-α*-tocopheryl acetate in 0.5 ml olive oil (3), and hemolysis determinations were made on blood samples taken at daily intervals thereafter until maximum values were again observed. (It was observed, incidentally, that weanlings exhibited positive hemolysis reactions when received from the supplier. Values ranging from 51 to 82% by the dialuric acid method were recorded for two separate shipments of animals; hemolysis decreased to normal following oral administration of vitamin E.)

Spontaneous hemolysis was evaluated as a function of incubation time, incubation temperature and concentration of saline-phosphate in the buffer. As a result the following routine procedure was adopted.

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Three drops of blood were added to 3 ml saline-phosphate buffer, pH 7.4 (3). Phosphate buffer was prepared by dissolving 14.2 g anhydrous Na_2HPO_4 in distilled water, adding 20.0 ml 1:0 N HCl and diluting to 1 liter. Saline solution contained 8.90 g NaCl made up to 1 liter with distilled water. Saline-phosphate buffer consisted of equal parts of these solutions. After centrifuging for 10 minutes at 500 × *g* the supernate was removed under mild suction and the cells were resuspended in 3 ml of buffer. One milliliter of cell suspension was transferred to each of two test tubes. To one tube was added an additional 4 ml buffer, and to the other 4 ml distilled water. The tubes were incubated in a water bath at 37° for 4 hours, and again were centrifuged. Aliquots of each supernate were transferred to a 3-ml spectrophotometer tube [2] and the optical density was read at 415 mμ against a buffer blank. The percentage hemolysis was calculated by dividing the extinction value for the tube containing the buffer by that for the completely hemolyzed tube containing added water, and multiplying by 100.

RESULTS

The relationship between the results obtained by the dialuric acid reference method (3) and by the spontaneous hemolysis procedure is illustrated in figure 1. The data show that, except at the extremities of the range, the spontaneous hemolysis values were higher than those obtained by the conventional method. This observation concurs with that of Jager (5). Both methods yielded values for extensively depleted animals of 92 to 98%. The spontaneous hemolysis procedure provides a significant advantage in sensitivity, as it yields values of about 25% before a clear response is discernible by the dialuric acid method. There is also less variation in replicate determinations carried out on the same sample.

Attempts to further simplify or shorten the procedure by increasing the temperature of incubation, using a hypotonic buffer solution or substituting whole blood for the cell suspension, were unsuccessful. The kinetics of hemolysis in saline-phosphate buffer at three different incubation temperatures are illustrated in figure 2. No difference in results was obtained at 37 and 38°, but a sharp acceleration was observed at 40°. However, temperature increments which significantly reduced the time necessary to attain extensive hemolysis of deficient cells also caused hemolysis of normal cells. Data are presented in figure 3 depicting the hemolytic behavior of normal erythrocytes at 37 and 41°. Although extension of the incubation period yields slightly higher values for extensively depleted cells, the 4-hour interval gives data which are comparable to those obtained by the dialuric acid method, and provides an adequate range to accommodate a dose-response assay (fig. 1).

Normal cells were found to be highly sensitive to dilution of the salts in the buffer medium. A dilution of 10% caused considerable hemolysis within 90 minutes at 37° (fig. 4). This observation precluded the possible use of a hypotonic solution to accelerate the rate of hemolysis of deficient cells.

The fact that the dilution of plasma in the initial cell suspension is about 50-fold suggested that it might be feasible to carry out the hemolysis test directly on the diluted sample of whole blood. Even at this

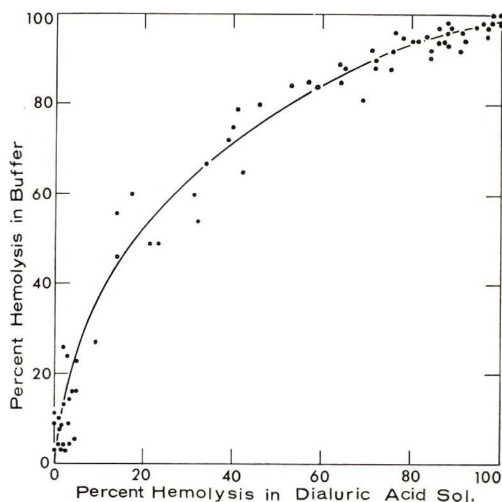

Fig. 1 Relationship between percentage hemolysis observed by dialuric acid procedure (3) and by incubation in saline-phosphate buffer at 37°.

[2] Coleman Instruments, Maywood, Ill.

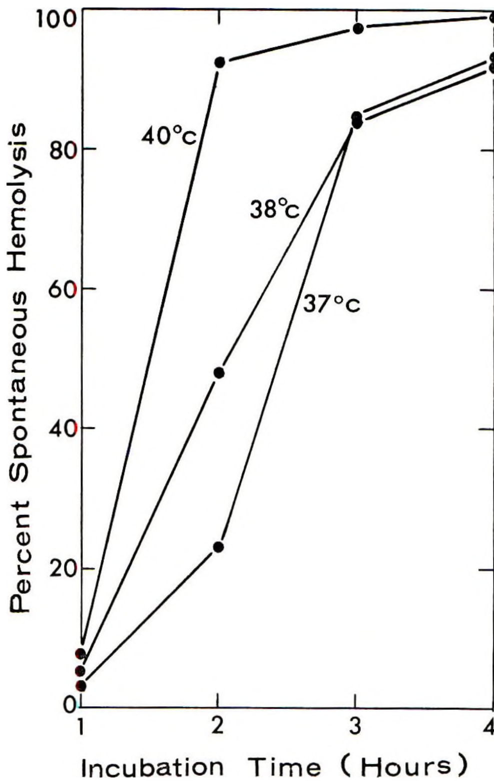

Fig. 2 Influence of incubation time and temperature on hemolysis of erythrocytes from vitamin E-deficient rats in saline-phospahte buffer. Each point is the mean for eight animals.

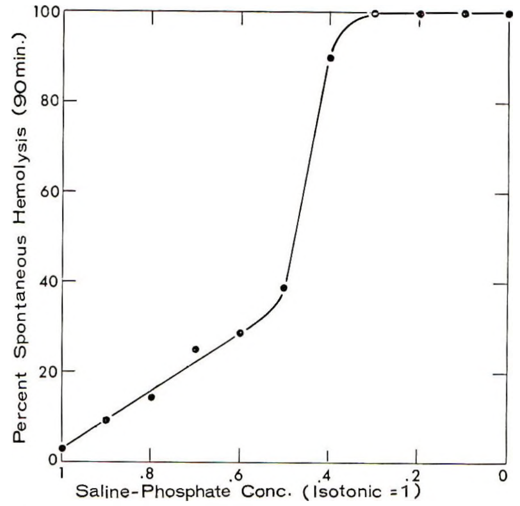

Fig. 4 Relationship between concentration of saline-phosphate buffer and hemolysis of erythrocytes from normal rats at 37°. Each point is the mean for eight animals.

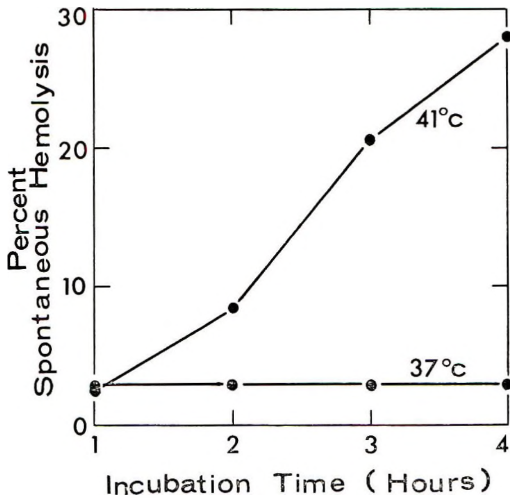

Fig. 3 Hemolysis of erythrocytes from normal rats in saline-phosphate buffer at 37 and 41°. Each point is the mean for eight animals.

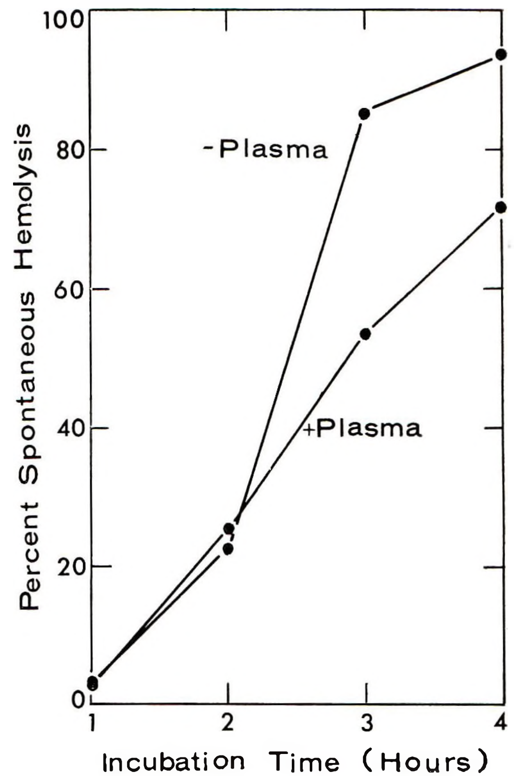

Fig. 5 Influence of plasma on the hemolysis of erythrocytes from vitamin E-deficient rats in saline-phosphate buffer at 37°. Dilution of cells and whole blood was about 50-fold. Each point is the mean for eight animals.

dilution, however, plasma inhibited spontaneous hemolysis (fig. 5). This indicates that the plasma of severely depleted rats contains factors which have a stabilizing influence on the red blood cell membrane.

DISCUSSION

The proposed hemolysis procedure requires about the same overall length of time as the dialuric acid method, but it has several operational advantages. Less actual operator time is entailed, since fewer tubes and less pipetting are required, and daily preparation of the dialuric acid solution is avoided. The test is more sensitive (and, therefore, advantageous in detecting a mild deficiency) and the results are more reproducible. In the midrange, the coefficient of variability for the spontaneous hemolysis values was about 40% of that for the dialuric acid hemolysis values. In addition, the revised procedure avoids the problem of high control values (the result of spontaneous hemolysis) which was encountered when the dialuric acid method was applied to extensively depleted rats. These advantages also apply with reference to the hydrogen peroxide-induced hemolysis procedure (8). Christensen et al. (4) noted the greater consistency with which deficient cells hemolyze in isotonic saline, and also observed that hemolysis could be prevented by prior flushing of the solution with N_2.

Observations on rats fed the vitamin E-deficient diet for periods up to 1 year indicated that the response to oral administration of vitamin E was affected by the duration of depletion beyond the time at which the animals exhibited maximum hemolysis by the dialuric acid test. During this interval the control values steadily increased. Administration of *dl*-α-tocopheryl acetate, in doses of 0.75 to 1.50 mg, was followed by a decrease in spontaneous hemolysis in the control tubes containing buffer, but no significant diminution in the hemolysis of cells exposed to dialuric acid. Data illustrating this effect are presented in table 1. The result of administering a single 1.05-mg dose of vitamin E to extensively depleted rats was to reduce hemolysis of the control samples from 59 to 5%, without appreciably reducing hemolysis of the cells suspended in buffered dialuric acid solution. These results illustrate the greater sensitivity of the spontaneous hemolysis response. A second 1.05-mg dose induced a distinct response in dialuric acid-induced hemolysis. These data show that not all rats which exhibit maximum hemolysis by the dialuric acid test are equally responsive to vitamin E. It is not feasible, therefore, to use deficient rats for bioassay purposes unless they are all in approximately the same stage of depletion, or unless a new standard dose-response curve is established at the time of each assay.

TABLE 1

Influence of tocopherol status on the response to oral administration of vitamin E according to the dialuric acid method (3)

Vitamin E status	Treatment	Dialuric acid hemolysis	Control hemolysis [1]
	mg	%	%
Normal	—	1	5
Deficient [2]	—	94	59
Deficient	1.05	91	5
Deficient	1.05 (repeat) [3]	64	7
Deficient	2.95	61	8
Deficient	2.95 (repeat) [3]	3	6

[1] Expressed as optical density for control tube/optical density for completely hemolyzed sample × 100. Control tubes contained buffered saline only.

[2] Fed deficient diet for 6 months. Dialuric acid test performed 48 hours after oral dose of *dl-a*-tocopheryl acetate. Rats weighed 370 to 390 g. Eight rats per group.

[3] Repeat dose given 48 hours after first dose.

The response to *dl*-α-tocopheryl acetate of rats depleted for 6 months (table 1) was lower, on an equal body weight basis, than that of animals fed the same diet for 2 months. The importance of employing bioassay animals at the same stage of deficiency also applies to the spontaneous hemolysis method of assessing the response.

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Amino Acid Regulation of Albumin Synthesis [1,2]

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ABSTRACT The effects of amino acids on albumin synthesis were measured in 47 studies using the isolated perfused rabbit liver. Carbon-14 carbonate was used to label the hepatic intracellular arginine pool, and hence, the carbon of urea and the guanido carbon of arginine. Amino acid levels in the perfusate and in the liver were determined. All studies were run for 2.5 hours to assure release of labeled albumin. Livers obtained from fasted rabbits synthesized 18 ± 1.1 mg of albumin 117 ± 8 mg of urea. The addition of methionine, lysine, leucine, valine, or threonine, at 10 μmoles/ml, failed to alter albumin synthesis. Tryptophan at 0.05 to 10.0 μmoles/ml increased albumin synthesis 138 to 175%, and urea synthesis by 56%. The addition of isoleucine also resulted in an 89% increase in albumin synthesis, and both isoleucine and tryptophan resulted in ribosomal reaggregation. When the donor rabbits were fed, albumin synthesis averaged 33 mg, and no increase was observed with tryptophan or isoleucine. Results of these studies are compatible with the concept that tryptophan stimulates the ribosomal reaggregation with enhanced albumin production. Isoleucine, while effecting reaggregation, may be either less efficient in this regard or less specific for albumin.

Malnutrition results in a low rate of albumin production, and upon the administration of an adequate diet, albumin synthesis rapidly returns toward normal and the albumin pool increases (1–4). Similar changes in protein production are seen in hepatic subcellular systems (5). In the absence of a source of amino acids, subcellular systems cease to incorporate amino acids into protein, but after the addition of a complete amino acid mixture including tryptophan these subcellular components again become functional (4). In this system, tryptophan has played a limiting but not unique role (5). However, these studies have not been directed toward any specific protein. Since albumin synthesis is rapidly inhibited by fasting it was felt that tryptophan might reverse this inhibition. This study reports on the effect of tryptophan and isoleucine on albumin production examined in the isolated perfused rabbit liver derived from fed and fasted animals.

METHODS

Perfusion technique. Forty-seven perfusion studies were performed using livers removed from rabbits fasted for 48 hours before surgery. Under light ether anesthesia the stomach and esophagus were ligated and severed. The bile duct was isolated and cannulated and bile was expressed to empty the gall bladder. The portal vein was cannulated and perfusion was started immediately at a rate of 15 to 30 ml/minute from a 500 ml reservoir at a height of 40 to 50 cm. The inferior vena cava was cannulated from below the liver and ligated above the diaphragm, with circulation maintained throughout surgery except for only a few seconds during the entire procedures. The liver was removed and mounted on a platform in a humidified box maintained at 37° as described previously (6). Perfusion was directed into the portal vein at a rate of 1.0 to 1.4 ml/g liver per minute. The perfusion volume of 140 to 170 ml was recirculated, and oxygenation was carried out in a disk oxygenator that received the output from the inferior vena cava. Bile was collected from the cannulated biliary duct. After 15 to 30

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minutes of perfusion, 100 μCi of ^{14}C carbonate (specific activity, 5 mCi/mmole) was injected directly into the inflow tube connected to the portal vein, and the perfusion continued for 2.5 hours at which time the total perfusate was collected.

In 25 of the 47 studies, the perfusate consisted of two parts of fresh heparinized rabbit blood to one part oxygenated Krebs-Henseleit solution which contained 50 to 90 mg/100 ml glucose. In 22 studies the red cells were washed twice with saline and resuspended in a known mixture of amino acids in Krebs-Henseleit solution. In addition, 0.69 μmole of L-glutamine/ml and 0.24 μmole of L-arginine/ml were added to the perfusates before each study. The albumin level of the perfusate was adjusted to 2.7 to 2.9 g/100 ml with rabbit albumin.[3] In 36 perfusions using livers derived from fed and fasted rabbits, excess amino acids were added to the starting perfusate. Isoleucine, methionine and tryptophan were studied each at levels of 10 mM; tryptophan at a level of 0.05 mM; and valine, leucine, lysine and threonine in combination, twice, at a level of 10 mM of each amino acid. In 14 studies the amino acid content of the perfusates before and after the study were measured. In 11 studies the amino acid content of the liver was examined at the end of the 2.5-hour perfusion (7). Amino acid analyses were performed on a Phoenix 6800 Amino Acid Analyser.[4] The method was essentially that of Moore et al. (7) with the modifications noted below. The acidic and neutral amino acids were run on a column of 0.9 cm by 60 cm Spherix[5] resin at 50°. The initial citrate buffer was 0.2 M Na^+ at pH 3.15, containing 2% *n*-propanol, followed by pH 4.25 0.2 M Na^+ after 76 minutes. The basic amino acids were analyzed on a 0.9 cm by 15 cm column of Spherix resin at 60° using pH 5.00 citrate buffer 0.33 M in Na^+. This system separated tryptophan and ornithine from lysine.

Albumin synthesis was calculated from the expression:

$$\text{Albumin synthesis (mg)} = \text{Total perfusate albumin (mg)} \times \frac{\text{albumin guanido-}^{14}\text{C specific activity}}{\text{synthesized urea-}^{14}\text{C specific activity}} \quad (6)$$

Synthesized urea was calculated from the difference between the initial and final urea content of the perfusate, and the volume of distribution within the liver and red cells (6). The specific activity of this urea carbon was assumed to equal the mean specific activity of the precursor guanido carbon of arginine (6, 8–10).

The concentration of protein in plasma or perfusate was determined with a biuret reagent (11) and protein partition with a Kern microelectrophoresis unit (12).[6]

Albumin was isolated by preparative acrylamide gel electrophoresis. Five milliliters of red cell free perfusate were dialyzed overnight in cold against 500 ml of 0.128 M glycine-tris (hydroxymethyl) aminomethane-1,3-propanediol buffer, pH 8.3. After the addition of 50 mg sucrose to the dialyzed perfusate, 3.5 ml were layered over a 5 cm by 13 cm (height × diameter) 7.5% polyacrylamide column at pH 8.9 (1.5 M Tris-HCl) in a Fractophorator™.[7] A constant current of 5 ma was applied for 30 minutes to migrate and stack the proteins into the gel. The current was increased to 10 ma and 4-ml fractions collected every 5 minutes with a fraction collector. Tubes were read at 278 mμ. The fractions of the first peak, from the midpoint of the ascending slope to the midpoint of the descending slope, were pooled and examined by qualitative polyacrylamide electrophoresis (13) and immunoelectrophoresis.

The samples prepared by this method resulted in a single sharp band when examined by quantitative gel electrophoresis, whereas the preparations isolated chemically gave four distinct bands (fig. 1) (6). In simultaneous studies, the specific activity of the albumin guanido carbon isolated by these two methods was essentially identical indicating that the apparent heterogeneity of the chemically isolated prep-

[3] Pentex Corporation, Kankakee, Ill.
[4] Phoenix Precision Instrument Company, Philadelphia, Pa.
[5] See footnote 4.
[6] Kern Instruments, Inc., Port Chester, N. Y.
[7] Buchler Instruments, Inc., Fort Lee, N. J.

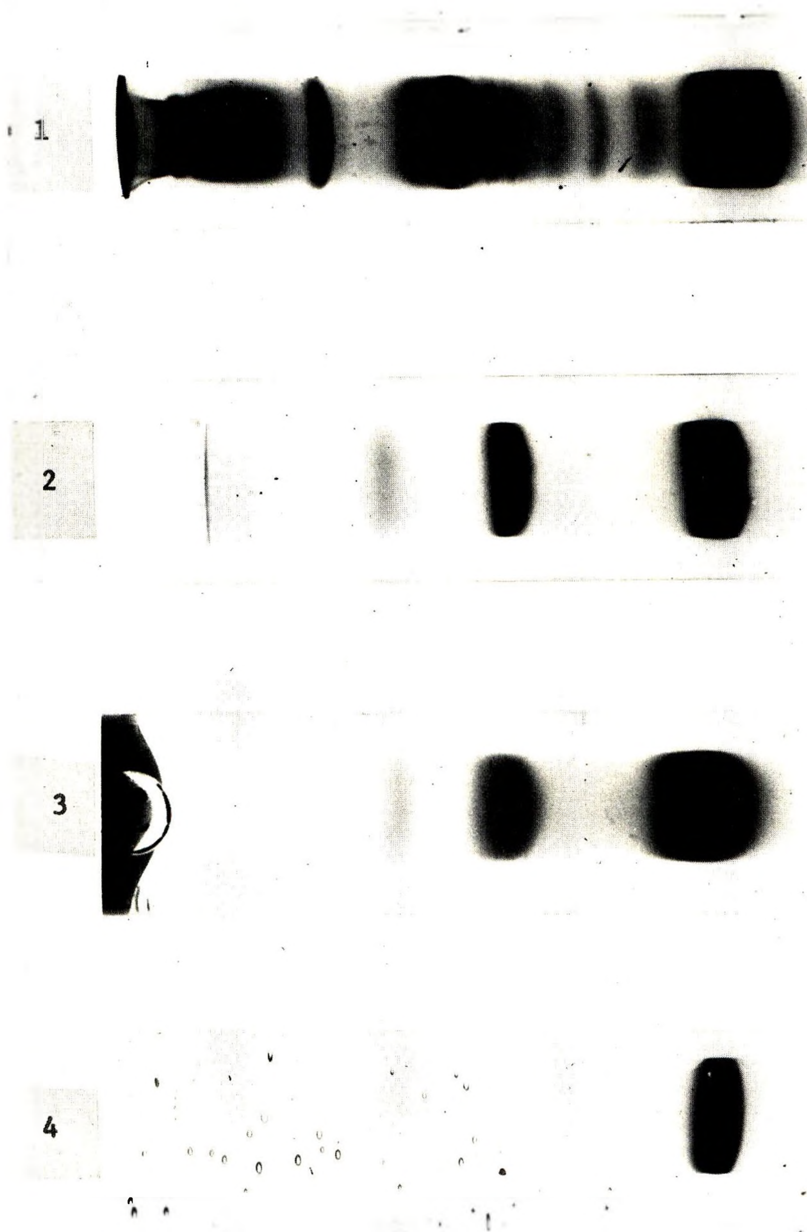

Fig. 1 Qualitative acrylamide gel electrophoresis. Reading from the top of the figure downward: tube 1, the perfusate; tube 2, commercial albumin preparation; tube 3, albumin isolated by TCA–alcohol–ether technique; and tube 4, albumin isolated by preparative acrylamide gel electrophoresis. The specific activity of albumins isolated in tubes 3 and 4 were identical.

arations was probably due to polymers of albumin. Samples of the isolated albumin were concentrated to 6% and examined by immunoelectrophoresis to guard against gross serum protein contamination (fig. 2) (14). Previous studies have compared chemically isolated albumin with simultaneously prepared albumin employing specific antisera against rabbit albumin and the resulting specific activities have agreed within 6% (6).

The albumin was hydrolyzed with 6 N HCl, neutralized and passed through a resin column according to the method of McFarlane (10) and treated consecutively with arginase and urease. An aliquot was incubated with urease according to the method of Conway and Byrne (15), the ammonia released by 45% K_2CO_3 was trapped in 2 N H_2SO_4, and the nitrogen assayed with Nessler's reagent. An identical aliquot was incubated with urease, treated with H_3PO_4, trapped in phenethylamine and dissolved in 15 ml of a scintillator (6); the ^{14}C was assayed in an ambient temperature liquid scintillation counter (6).[8] The perfusate was examined for urea carbon in the same way using the supernatant from heated, tungstic acid-precipitated samples to drive off any trapped ^{14}C-CO_2.

In eight additional studies hepatic ribosomal profiles were obtained from livers after the 2.5-hour perfusion (4). The liver was perfused with ice-cold saline, weighed and 20-g aliquots minced and homogenized in 50 ml of buffer (0.025 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$ and 0.05 M Tris, pH 7.6). The homogenate was spun at 19,500 $\times g$ for 10 minutes at 0°. Freshly prepared 10% sodium deoxycholate in 0.05 M Tris, pH 8.2, was added to the supernatant to a final concentration of 1.3%. A sample of this supernatant equivalent to 70 mg of liver was layered over 24 ml of a 0.3–1.1 M linear sucrose gradient over 1 ml of a 50% sucrose cushion. Ferritin was used as a marker. The gradient was spun for 180 minutes in an ultracentrifuge[9] using a number 25.1 rotor at 25,000 rpm. The resultant gradient was analyzed with an ultraviolet analyzer.[10] Areas under the various peaks were determined with a rotary planimeter.

Perfusion system. Lactate utilization, glucose production, bile flow and urea production were used to assay the function of the liver preparation as described previously (6). Lactate levels fell to nearly zero

[8] Nuclear-Chicago, Des Plaines, Ill.
[9] Spinco model L, Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.
[10] ISCO model UA-2, Instrumentation Specialties Company, Inc., Lincoln, Nebr.

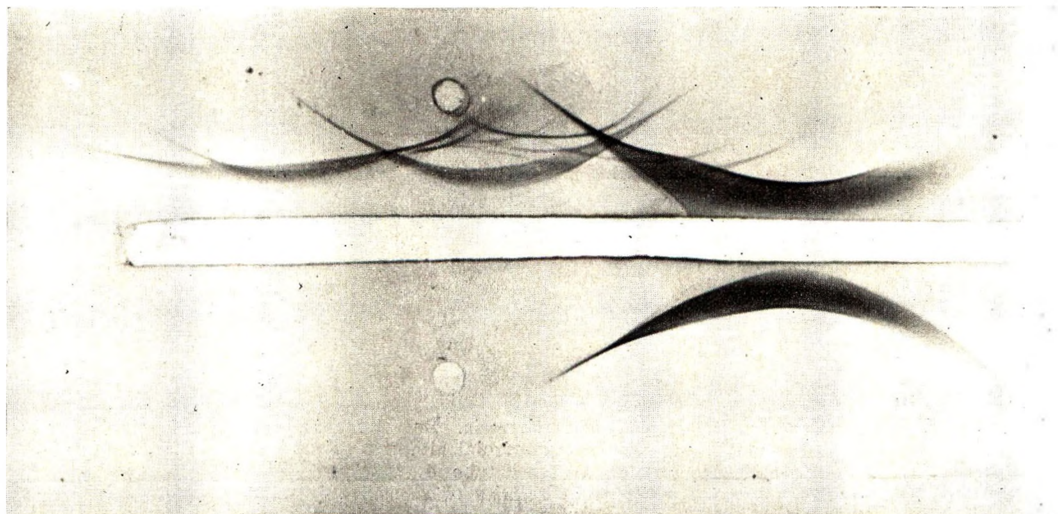

Fig. 2 Immunoelectrophoresis of polyacrylamide gel-separated albumin. Whole rabbit serum was used as the control with goat antisera against whole-rabbit serum. The serum demonstrated multiple immunologic lines whereas the isolated albumin at a 6% concentration showed a single sharp bond.

in all studies; circulating glucose levels rose from 50 mg/100 to 70 mg/100 ml or more in livers from fasted rabbits, and bile flow averaged 2 to 3 ml/hour. Urea production was equivalent to that which had been reported previously (6). Glycogen levels in the fasted livers prior to perfusion were less than 2 to 3 mg/g and were slightly higher after perfusion, 5 to 10 mg/g (16). Two and one-half hours were chosen for perfusion because previous studies have indicated the completeness of release of labeled albumin at this time (6).

RESULTS

Some of the amino acids in the perfusate were used during the perfusion and others were added to the perfusate. Arginine, methionine and alanine were used by the liver, whereas leucine, valine, isoleucine and cystine were added to the perfusate. These observations are in accord with those reported by Fisher and Kerly employing the isolated perfused rat liver (17) (table 1). Following the perfusion for 2.5 hours using the serum or washed red cell preparations, the levels of the various amino acids in the perfusates were measured and no differences were found in the amino acid levels in these two mixtures (table 1). The livers from fed rabbits had higher levels of amino acids (table 2), but the perfusate levels after perfusion were not different from the amino acid levels seen in perfusates used to study livers from fasted rabbits.

The results of the albumin studies are shown in table 3. No significant differences were observed regardless of the perfusate employed, and thus the studies using either plasma or washed red cells were pooled in terms of albumin and urea productions. The livers from fasted rabbits perfused with the basic perfusate synthesized 18.0 ± 1.1 mg albumin and 117 ± 8 mg urea/100 g of wet liver weight in 2.5 hours. The addition of methionine or a combination of valine, leucine, lysine and threonine at levels of 10 μmoles/ml, failed to increase albumin synthesis or urea production. In six studies with isoleucine, 10 μmoles/ml, albumin synthesis rose 89% to 34.1 mg. With tryptophan, 0.05 μmole/ml, albumin synthesis increased 138% to 42.8 ± 2.4 mg; with an increase in tryptophan levels to 10 μmoles/ml albumin synthesis

TABLE 1

Perfusate amino acids

Amino acids	Washed red cells		Serum	
	Start	End	Start	End
	μmoles/100 ml		μmoles/100 ml	
Essential				
Arginine	24	5 ± 0.5 [1]	40 ± 9 [1]	2.5 ± 1.0 [1]
Histidine	6	8 ± 2	10 ± 1	12.5 ± 2.0
Leucine [2]	35	75 ± 26	8 ± 1	64 ± 14
Lysine [2]	15	24 ± 2	17 ± 2	14 ± 3
Threonine [2]	20	24 ± 3	40 ± 3	43 ± 5
Tyrosine	2	7 ± 1	5 ± 1	4.5 ± 0.6
Valine [2]	30	50 ± 14	15 ± 2	66 ± 3
Isoleucine [2]	5	35 ± 10	6 ± 1	44 ± 2
Methionine [2]	3	2 ± 0.7	2.6 ± 0.3	tr ± —
Tryptophan [2]	0–2	tr [3] ± —	0–2.0 ± —	tr ± —
Phenylalanine	10	4 ± 0.6	4 ± 1	4 ± 0.3
Nonessential				
Alanine	40	15 ± 3	26 ± 2	10 ± 4
Aspartic	5	— ± —	2 ± 0.5	0.3 ± 0.2
Half-cystine	30	20 ± 5	5 ± 0.3	24 ± 5
Glycine	30	38 ± 7	71 ± 9	45 ± 7
Proline	12	9 ± 2	12 ± 2	10 ± 2
Ornithine	0	9 ± 1	tr ± —	9 ± 1

[1] ± SEM.
[2] Starting levels were adjusted to 10 mM as described in Methods. Arginine and glutamine added as described in all studies.
[3] tr = trace.

TABLE 2

Hepatic amino acids after perfusion

Amino acids	Fasted donors	Fed donors
	μmoles per 100 g	
Essential		
Arginine	tr [1]	tr [1]
Histidine	36 ± 5 [2]	36 ± 3 [2]
Leucine	36 ± 5	62 ± 9
Lysine	34 ± 3	63 ± 13
Threonine	83 ± 10	96 ± 25
Tyrosine	4 ± 0.4	16 ± 2
Valine	35 ± 4	60 ± 13
Isoleucine	22 ± 3	34 ± 6(ns) [3]
Methionine	4 ± 0.5	16 ± 4
Tryptophan	tr–0	2 ± 1
Phenylalanine	6 ± 1	15 ± 4
Nonessential		
Alanine	56 ± 6	217 ± 68
Aspartic	57 ± 6	154 ± 24
Half-cystine	8 ± 1	19 ± 9
Glutamine	68 ± 6	136 ± 12
Glycine	139 ± 11	209 ± 40
Proline	24 ± 3	70 ± 15
Ornithine	31 ± 2	28 ± 9

[1] tr = trace.
[2] ± SEM.
[3] ns = not significant.

rose 175% to 49.4 mg and urea production increased to 183 mg.

As has been shown previously, albumin synthesis averaged 33 mg/100 g per 2.5 hours when the livers were obtained from fed animals (6). This perfusate contained little or no tryptophan (less than 0.02 μmole/ml). When tryptophan or isoleucine was added to the perfusate the livers from fed rabbits showed no change in either albumin or urea synthesis. Albumin production averaged 33.1 ± 2.3 mg/100 g per 2.5 hours and was significantly less than that seen when the fasted preparation with added tryptophan was studied ($P < 0.01$). The ribosomal profiles obtained from four perfused livers are shown in figure 3. The profiles from a liver obtained from a fasted rabbit showed only a small polyribosomal peak. The profiles from a liver from a fed animal and those from fasted animals perfused with either tryptophan or isoleucine (10 mM) showed an increase in the more heavy aggregates (table 4).

DISCUSSION

The adaptation to a low nitrogen intake is vital to the total organism (18). Upon reduction of nitrogen intake albumin synthesis rapidly falls in vivo and in vitro and reflects the nutritional state directly (3, 18) Employing the ^{14}C-carbonate method, Kirsch et al. (3) demonstrated that albumin synthesis could be returned toward normal values within 1 day of refeeding, and Staehelin et al. (19) showed that the

TABLE 3

Albumin and urea synthesis

State of donor rabbit	Test amino acid	No. of studies	Albumin synthesis	P value	Urea synthesis	P value
			mg/100 g per 2.5 hr		*mg/100 g per 2.5 hr*	
Fasted	0	11	18.0 ± 1.1 [1]		117 ± 8 [1]	
Fasted	Isoleucine, 10 mM	6	34.1 ± 3.5	< 0.01	134 ± 4	< 0.1
Fasted	Tryptophan, 10 mM	8	49.4 ± 4.0	< 0.01	183 ± 10	< 0.01
Fasted	Tryptophan, 0.05 mM	6	42.8 ± 2.4	< 0.01	126 ± 6	ns [2]
Fasted	Methionine, 10 mM	3	21.7 ± 3.5	ns	99 ± 8	ns
Fasted	Lysine, leucine, valine, threonine, 10 mM	2	24.9, 17.7		108, 114	
Fed [3]	0	10	33.7 ± 2.1		91 ± 8	
Fed	Tryptophan, 0.05–10.0 mM	9	33.1 ± 2.3	ns	106 ± 14	ns
Fed	Isoleucine, 10 mM	2	31.8, 29.4		78, 88	

[1] ± SEM.
[2] ns = not significant.
[3] Previously reported (6)

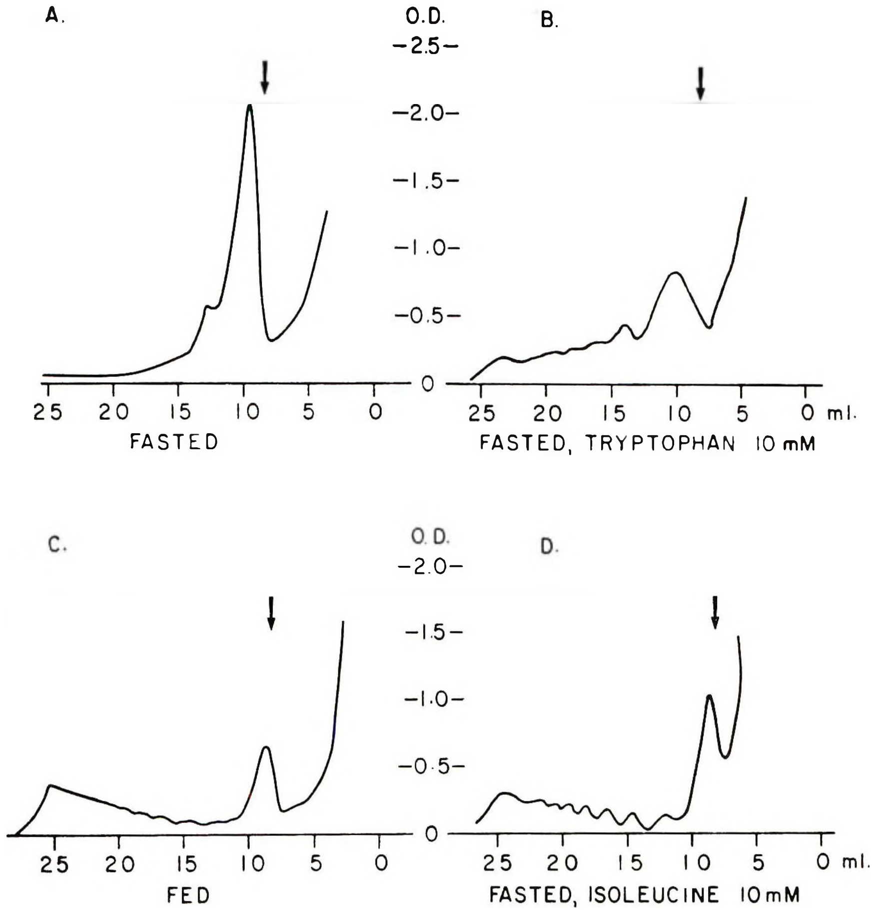

Fig. 3 Ribosomal profiles from sucrose density gradient analysis. The arrow indicates ferritin marker. A, liver from fasted animal; B, tryptophan-perfused liver from fasted animal; C, liver from fed animal; and D, perfused liver from fasted animal. The use of tryptophan and isoleucine results in a pattern approaching that seen in the liver from a fed animal.

hepatic polysomal profile can be restored toward heavy aggregates within 30 minutes following administration of a casein hydrolysate supplemented with tryptophan in vivo. In livers from tryptophan-deficient, or protein-depleted, or starved rats a larger proportion of the total ribosome mass was found to be in the free state, and these ribosomes had a decreased ability to incorporate amino acids into protein (4, 5). Fasting induced a decrease in polysomal size, resulted in a lowered rate of incorporation of amino acids into protein, and an increase in the rate of RNA breakdown. Munro (5) showed that a diet deficient in tryptophan failed to reverse these effects. Absence of isoleucine in the hepatic subcellular system derived from fasting mice (5) did not result in a depressed incorporation of labeled amino acid into protein and no requirement for isoleucine was found in this in vitro system. The present studies show that livers obtained from rabbits fasted for 48 hours and perfused for only 2.5 hours with an amino acid red cell mixture, respond rapidly in terms of albumin production to at least two amino acids in the perfusate. Increased levels of isoleucine and tryptophan resulted in an increase in albumin synthesis. The addition of either isoleucine or tryptophan did reverse the fasting polysomal profile and a larger pro-

TABLE 4
Ribosomal aggregation

Experiment	Albumin synthesis	Ribosomal aggregation	
		Heavy	Light
	mg/100 g per 2.5 hr	%	%
Fed	30	65	35
Fasted	18	24	76
Fasted tryptophan	41	44	56
Fasted isoleucine	26	47	53

portion of heavy aggregates was observed after 2.5 hours of perfusion. This finding agrees with that reported recently by Jefferson and Korner (20) who showed that isoleucine as well as other amino acids, at 10 times the plasma level, would result in an aggregated ribosomal profile using the in situ perfused rat liver. There are differences between isoleucine and tryptophan even through both result in reaggregation. Tryptophan is effective at levels of 0.05 μmoles/ml, whereas isoleucine stimulates albumin production only at high levels and to a lesser degree. Tryptophan was either not detected, or at trace levels in the livers of starved rabbits, and was at low levels in the livers derived from fed animals. Thus, the addition of tryptophan may have supplied the needed or limiting amino acid. Isoleucine was present in the livers of both groups at levels of 22 μmoles/100 g in the fasted liver, and 34 μmoles/100 g in livers from fed animals. No effect of isoleucine on urea production was noted.

Urea excretion and the urea cycle enzymes are increased upon fasting, and the levels of these enzymes have been felt to be related more to urea production than to nitrogen in the diet (21). A diet deficient only in nitrogen results in the opposite effect in vivo (21). Increased levels of tryptophan resulted in a marked stimulation of urea synthesis in the perfused liver. Because serum protein degradation is minimal in the perfused liver (22) and the nitrogen sources for urea are in constant supply, these results probably represent a further stimulation of some aspect of the urea enzyme cycle.

In the fed state the addition of tryptophan and isoleucine had no effect. If tryptophan acts to stimulate albumin production through reaggregation of ribosomes then no stimulation would be expected when the liver is obtained from a feeding rabbit, since disaggregation did not occur. The administration of tryptophan to fasted preparations resulted in a level of albumin production higher than that seen when the livers were obtained from a fed animal. This result could be due to the stability and long life of the mRNA for albumin production (23). In the starved animal whose mRNA synthesis has been inhibited by actinomycin, refeeding results in an increase in the proportion of newly made protein that is albumin. If stimulation of reaggregation occurs in the presence of a population of stable mRNA coded for albumin, then production of albumin might predominate until new, less stable, mRNA were available (24). Isoleucine, while stimulating reaggregation, did not stimulate albumin synthesis to the same degree as tryptophan. Perhaps the aggregates were not bound to endoplasmic reticulum and thus, not specific for export protein. Without additional data, however, further speculation on this point is not rewarding.

The present data show that albumin synthesis in the perfused liver can be stimulated by isoleucine and tryptophan when the livers are obtained from fasting rabbits Although the data indicate that the mechanism of tryptophan action is of polysome reaggregation (5), the mechanism of action of isoleucine may be different and remains to be clarified.

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Artificial Feeding of Infant Rats by Continuous Gastric Infusion [1]

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ABSTRACT A technique for the artificial feeding of infant rats by continuous gastric infusion is described. Using an infusion pump, a milk diet is continuously conveyed to the stomach of the animal via a gastric cannula which has been surgically implanted by gastrostomy shortly after birth. The method is designed to raise eight rats from birth to the age of weaning. Normal growth rates can be approximated. Because the technique is semiautomatic it has the advantage over other methods of artificial feeding in that it avoids the danger of trauma that accompanies repeated passage of oral-gastric feeding tubes. In addition, it eliminates the need for attendance during the night and can be readily handled by a single investigator.

Artificial feeding of infant rats and other small laboratory animals has been performed in only a few laboratories to date, mostly for the purpose of raising a first generation of germfree animals (1–3). Recently, there has been increasing interest in other problems involving artificial feeding, such as studies of the effects of nutritional factors on various aspects of early development (4, 5), or of the psychological effects of social deprivation (6), but advances in these fields have been hampered by technical difficulties associated with the hand-rearing of such animals (5, 7).

These difficulties have been partially overcome by a new method for artificial feeding in which neonatal rats are provided with nonlactating foster mothers(8). Even this method, however, requires that animals be force-fed at regular and frequent intervals, both day and night. This procedure can be traumatizing to the animal and is a major disadvantage to investigators with limited technical assistance.

The present work describes a semiautomatic technique for feeding newborn rats in which a constant infusion pump conveys milk directly into the stomach of the animal via a gastric cannula which had been surgically implanted shortly after birth.

A method for the continuous gastric infusion of adult rats has been previously described by Holtzman and Visek (9); this method is not applicable to newborn rats.

EXPERIMENTAL PROCEDURE

Milk. The ingredients of the milk used as a diet for the rats are listed in table 1. The milk was prepared in batches of 500 ml. After the ingredients were mixed for 3 minutes in a blender [4] the mixture was poured into a filter flask; it was allowed to cool in the refrigerator and then was deaerated by the gradual application of a vacuum. It was divided into a number of small lots and stored at −20° until used. The diet remained homogeneous upon thawing. Its pH was 6.2. Its osmolarity, determined by freezing point depression using an osmometer,[5] was 670 milliosmols per liter.

Table 2 lists the composition of the milk diet with respect to its major constituents; for the sake of comparison the composition of rat milk is also given.

Gastric cannula. The gastric cannula or gastrostomy tube for the constant infusion of milk was constructed of the following parts, designated A, B, C, D and E (fig. 1): A, an 18-cm length of polyethylene tubing (internal diameter, 0.28 mm;

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[4] Waring Blendor, Waring Products Company, Winsted, Conn.

TABLE 1
Ingredients of milk diet for infant rats

	ml/100 ml
Evaporated milk	75
Sterile water	17
Minerals solution [1]	1.0
	g/100 ml
Vitamin mix [2]	1.0
Corn oil	6
L-Methionine	0.1
L-Tryptophan	0.05
	mg/100 ml
Riboflavin	1
Pyridoxal·HCl	1

[1] Mineral solution: (in milligrams per milliliter) $FeSO_4 \cdot 7H_2O$, 2.7; $CuSO_4 \cdot 5H_2O$, 1.5; and $ZnSO_4 \cdot 7H_2O$, 1.6.

[2] The vitamin mix was a commercial product designated as vitamin mix (Czajka) from General Biochemicals, Inc., Chagrin Falls, Ohio. Its composition was identical with that of Dymsza et al. (4). It contained glucose as a diluent.

TABLE 2
Composition of milk diet: comparison with rat milk

	Milk diet [1]	Rat milk [2]
	g/100 ml	
Water	74	74
Fat	12	12
Protein	5.5	9.2
Carbohydrate	8.9	3.0
	mg/100 ml	
Calcium	200	330
Phosphorus	163	230
Sodium	94	66
Potassium	241	140
Magnesium	21	27
Chlorine	190	176
Iron	0.61	0.61
Copper	0.43	0.43
Zinc	1.0	1.0

[1] The values for the composition of the milk diet are calculated from data for the composition of evaporated cow's milk supplied by Carnation Research Labs., Van Nuys, Calif., with the exception of those for magnesium, chlorine, copper and zinc, which were calculated from data for whole cow's milk (10).

[2] The composition of rat milk is taken from Dymsza et al. (4), except for the values for calcium and magnesium, which are mean values calculated from figures quoted by Luckey et al. (11).

external diameter, 0.61 mm); [6] B, a 3.5-cm length of silicone rubber tubing (internal diameter, 0.305 mm; external diameter, 0.635 mm); [7] C, a 3-mm length of silicone rubber tubing (internal diameter, 0.51 mm; external diameter, 0.94 mm); [8] D, a polyethylene doughnut-shaped disc (external diameter, 2 mm). This was made by heating the end of a small piece of polyethylene tubing (internal diameter, 0.76 mm; external diameter, 1.22 mm) [9] until it began to melt, whereupon it was immediately flattened by pressing it against a smooth surface; the rest of the tubing was then cut off flush with the disc; and E, a polyethylene tip. The end of a piece of polyethylene tubing (internal and external diameters like those of A) was similarly flared after heating. The tubing was cut off 3 mm from the flared end.

The cannula was assembled as follows. The piece of silicone rubber tubing designated B (fig. 1) was temporarily expanded by soaking it in ether for about 1 minute; a 1.5-cm length of it was then immediately slipped over one end of the polyethylene tubing A. Next, the small piece of rubber tubing C was expanded, inserted over the free (proximal) end of A, and slipped over the distal join between A and B. The hole in the disc D was enlarged with a 20-gauge hypodermic needle; the disc was then inserted over the free end of A and forced past the proximal join between A and B until it finally rested against the proximal end of C. The end of the rubber tubing B was trimmed to within 1.3 cm of D, then expanded in ether to permit insertion of the polyethylene tip E up to the latter's flared end.

Finally, a 30-cm length of surgical steel wire [10] was inserted into the cannula such that a 2-mm length of it protruded from the tip, the remainder being allowed to trail from the other end. Figure 2 is a photograph of the assembled cannula.

[5] Fiske Associates, Inc., Uxbridge, Mass.
[6] Intramedic tubing PE 10, Clay-Adams, Inc., New York, N. Y.
[7] Silastic medical-grade tubing 602-101, Dow Corning Company, Midland, Mich.
[8] Silastic medical-grade tubing 602-131, Dow Corning Company.
[9] Intramedic tubing PE 60. Dow Corning Company.
[10] Ethi-Pack, size 6–0, Ethicon, Inc., Somerville, N. J.

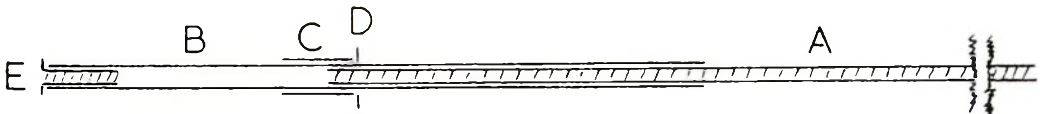

Fig. 1 Diagram of gastric cannula. For explanation of lettering see text.

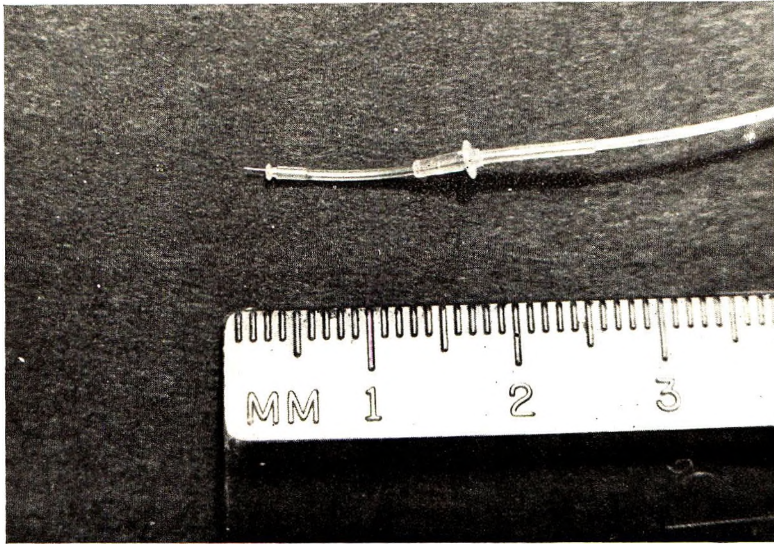

Fig. 2 Photograph of major part of assembled gastric cannula.

Implantation of gastric cannula. A newborn rat, aged from 4 to 24 hours, was anesthetized by inducing hypothermia in crushed ice for 4 minutes, and then laid on its right side in a Petri dish containing ice. Since the animal had had time to suckle, its stomach was clearly visible through the skin as a white crescent-shaped object.

A 1-cm lateromedial skin incision was made over the stomach, followed by a similar incision in the muscle tissue and peritoneum, thus exposing the stomach. A purse-string, consisting of two sutures approximately 2 mm apart, was placed in the fundal portion of the stomach using black braided surgical silk.[11] A hole was made in the stomach by careful insertion of a 20-gauge hypodermic needle between the two sutures; the stomach was then lightly grasped with tissue forceps and the tip of the gastric cannula (E, fig. 1) inserted into the hole with the aid of a twisting motion. The purse-string was tightened around the part of the cannula between E and C (fig. 1), tied, and both ends cut short. The surgical steel wire, whose function had been to stiffen the cannula during its insertion, was removed.

A 19-gauge hypodermic needle, whose base had been cut off, was inserted via the abdominal incision and guided between abdominal muscle layers to the midback of the animal, through which it was made to penetrate. This needle was then used as a guide to pass the free end of the cannula through the back of the animal. The cannula was pulled through until the polyethylene disc (D, fig. 1) reached the inner surface of the muscle layers at the needle opening.

The peritoneal cavity was irrigated with a drop of a mixture of penicillin G (1000 U/ml) and streptomycin (1 mg/ml) in sterile saline, and the abdominal incision closed with three sutures.

The entire surgical procedure on one animal took about 12 minutes. Normal sterile precautions were taken throughout.

The animal was finally dried with tissue paper and placed in the incubator, after which it usually awoke within 10 minutes.

Incubator. The rats were kept in a water-bath incubator similar to that described by Thoman and Arnold (6). The nest of the incubator was a basket made of perforated stainless steel, 42 cm by 25 cm by 8 cm, which was suspended over the water bath. The floor of the nest consisted of four layers of surgical gauze which were tightly stretched over a 1-cm high stainless steel frame which fitted into the bottom of the basket. The basket was divided into eight

[11] Cardiovascular surgical sutures, size 6-0, Ethicon, Inc.

compartments by a removable divider made of transparent plastic, which fitted into the basket over the stainless steel frame. (fig. 3). The bottom of the divider had six semicircular openings situated as shown in figure 3, each 3 cm in diameter, to accommodate the two mother surrogates (see below).

Mother surrogates. Each compartment of the nest contained a section of warm, moist dialysis tubing acting as a mother surrogate (6). This was achieved using two lengths of dialysis tubing (expanded diameter approximately 3 cm) which lay on the bottom of the nest, one on each side of the lengthwise division (fig. 3). Water at 37° was circulated through the tubing from a thermostatically controlled reservoir at a rate of about 75 ml/tubing per minute.[12] The tubing was closed off at each end by rubber stoppers held in place by rubber bands. The stoppers were perforated to accommodate short pieces of bent glass tubing which connected the mother surrogates with rubber tubing leading to the water reservoir via Y tubes.

Feeding technique. The animals were fed continuously by means of an infusion pump[13] modified to hold eight 5-ml or 10-ml syringes. Thus, a maximum of eight animals, one per compartment, could be fed at a time. The syringes and needles were of the disposable type. The gastric cannulas were connected to these syringes via polyethylene tubing[14] which, to minimize tension on the cannulas, was draped over rubber bands suspended from a thin flexible bar which was held above the incubator (fig. 3). The polyethylene tubing was connected to the syringe needles as well as to the free ends of the gastric cannulas by short (2 cm) lengths of silicone rubber tubing.[15]

Feeding was begun as soon as possible after the implantation of the gastric cannulas into eight animals. The initial rate of the infusion was 1.14 ml/24 hours. The

[12] Porta-Temp, Precision Scientific Company, Chicago, Ill.
[13] Compact infusion pump, model 975, Harvard Apparatus Company, Inc., Dover, Mass.
[14] See footnote 6.
[15] See footnote 8.

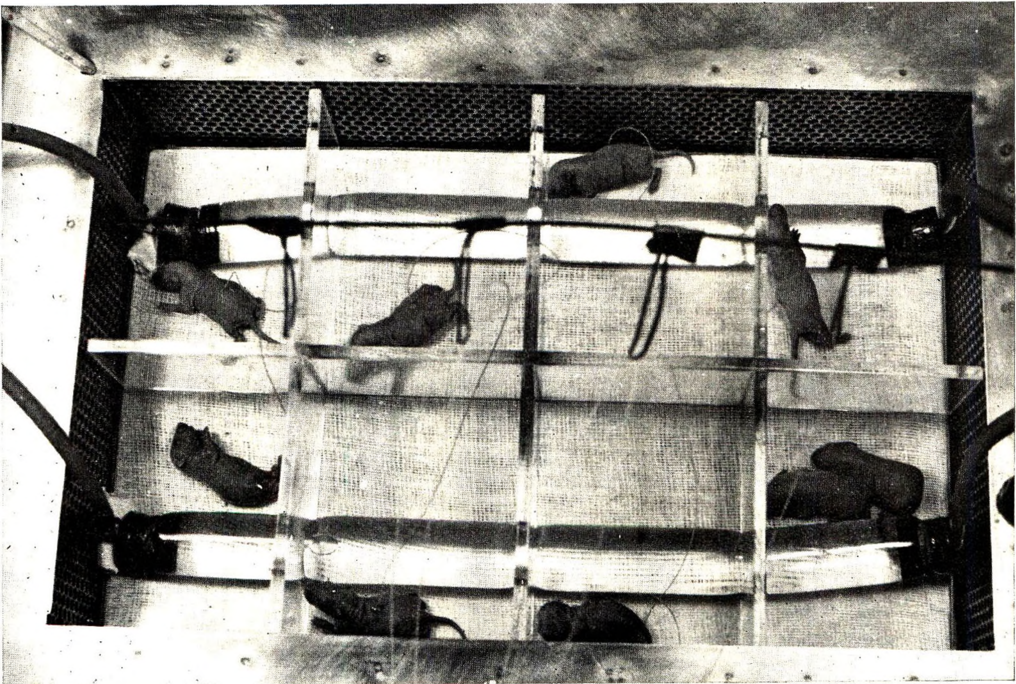

Fig. 3 Photograph of rat incubator. Rats are 3 days old. Compartment at right bottom corner contains one mother-fed control rat for comparison.

rate was then adjusted each day to approximate normal growth as closely as possible, without producing overdistension (fig. 4). The milk diet and syringes were changed every 12 hours to minimize bacterial contamination of the milk.

During the first few days, when the pumping rate was relatively slow, blockage of the gastric cannulas could usually be prevented by frequent renewal of the polyethylene leads. Blockages were cleared by rapidly injecting a small quantity of milk or water directly into the gastric cannula.

Care of the animals. It was essential to stimulate urination of the animals by light stroking of the urethral papilla (1, 2) at least once every 12 hours, preferably more often during the daytime. At the same time the animals were wiped to remove feces. The surgical gauze forming the bottom of the nest, as well as the dialysis tubing of the mother surrogates and the distilled water in the reservoir, were renewed every day.

The temperature of the water in the bath was maintained at 35° during week 1 after birth, at 33° during week 2 and 30° during week 3. It was found to be of the greatest importance to maintain the temperature inside the nest at 34 to 37° during the first 4 days; this was achieved by partially covering the top of the incubator with paper towels.

The stitches were removed from the abdominal incision on day 4 after the operation. During the first 5 days an antibiotic cream [16] was applied to the abdominal incision and to the back wound twice a day. During week 1 it was important to watch the animals closely for signs of infection (distension of the stomach, decreased activity, and the like). Intraperitoneal injection of 25 μliters of a mixture of penicillin (10,000 U/ml) and streptomycin (10 mg/ml) sometimes, but not always, cleared the infection.

After week 2 the animals became capable of puncturing the mother surrogates by biting or scratching; the surrogates were therefore removed on day 14.

From day 16 the incubator was covered with wire mesh to prevent the animals from climbing out.

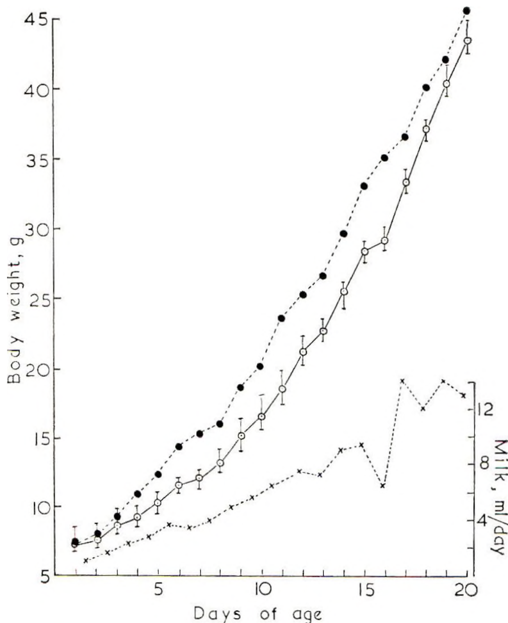

Fig. 4 Weight gains of mother-fed (⊙——⊙) and artificially fed (●——●) infant rats, and milk intake of artificially fed rats (×——×). Each group of rats comprises six animals. The bars indicate the range.

RESULTS AND DISCUSSION

The chief aim of the present experiments was to establish the feasibility of feeding infant rats by gastric infusion. Many of the early trials directed to this end were only partially successful. In the first few experiments, many animals died within 36 hours of the operation due to poor surgical technique. The main cause of failures in the initial trials, however, was a faulty design of the cannula, leading to frequent rupture of the stomach or loss of the cannula. Much time was spent therefore in attempts to improve the cannula; we consider the present design (fig. 1) to be satisfactory since the tip of the cannula is flexible, thus minimizing the risk of damage to the stomach, while the polyethylene disc fairly effectively prevents the cannula from being accidentally pulled out.

After the surgical technique and design of the cannula had been perfected, it was observed that during the first few days the stomachs of the animals were often very slow in emptying. The result was that rate

[16] Neosporin-G brand Polymyxin B-Neomycin-Gramicidin Cream, Burroughs Wellcome & Co., (U.S.A.) Inc., Tuckahoe, N. Y.

of feeding had to be kept very low, and the animals showed subnormal weight gains. This was found to be due to a failure to maintain a sufficiently high temperature in the nest (see Experimental Procedure).

In the earlier trials the milk diet did not contain additional riboflavin, pyridoxal, methionine and tryptophan (table 1); these were added when it was found that from day 6 on the animals often showed a scaly skin, reduced rate of stomach emptying, failure to gain weight, bursts of hyperactivity, tremors, partial paralysis, premature opening of the eyes, and cataracts. No such symptoms were observed after these additions to the diet had been made. It was not possible to decide which of the added constituents had reversed the presumed deficiency.

Initially, the mother surrogates had been made to pulsate as described by Thoman and Arnold (6). Since pulsation was difficult to arrange for as many as eight rats in one incubator it was abandoned in favor of nonpulsating surrogates, with no apparent ill effects.

In a recent and relatively successful experiment, gastric cannulas were implanted into eight rats of the Sprague-Dawley strain approximately 24 hours after birth. One animal died of peritonitis on day 5, while another lost its cannula during the night of day 6. The daily weight gain and milk intake of the six animals which survived to the age of weaning (20 days) are shown graphically in fig. 4. The weight gain was only slightly less than that of a litter of six mother-fed control animals which were kept in the laboratory at the same time. The total milk intake over 20 days was 131 ml/animal. In view of the relatively low protein content of the milk diet, compared with rat milk (table 2), it appears likely that the milk intake of the experimental animals was greater than that of the mother-fed animals.

In both the experimental and control animals the eyes opened from day 11 to day 14 after birth. Despite the relatively high lactose content of the milk diet (7.9%), none of the animals appeared to have cataracts. In the experimental animals the growth of hair, especially on the abdominal surface, did not seem to be quite as good as in the controls, but this may have been due, partially, to lack of grooming. The only obvious abnormality seen in the experimental animals was their somewhat sluggish behavior. When some of the animals were allowed oral feedings after they had been disconnected from the pump on day 20 they appeared to have voracious appetites.

Upon autopsy of the 20-day-old animals, a noninflamed, fistulous tract surrounding the cannula was seen in five of the six animals. The intestine was dilated and appeared to contain an abnormally large amount of fluid; this may be related to the relatively high osmolarity of the milk diet (670 milliosmols/liter) compared with that of rat milk (352 millosmols/liter; Miller and Czajka (12)). The weights of the organs (table 3) showed that the cecum was considerably enlarged and the liver and kidneys slightly enlarged in the experimental animals compared with the control animals ($P<0.01$). The mean weights of the heart, spleen, lungs and brain did not differ significantly between the two groups of animals ($P>0.05$).

We conclude from these results that it is possible to feed infant rats by continuous gastric infusion from shortly after birth to the age of weaning, and to achieve normal growth rates. Even better results might be obtained in the future with a milk diet containing less carbohydrate and more protein.

The technique has the advantage over other methods of artificial feeding in that it is semiautomatic, eliminating the need for attendance during the night. In addition, it avoids the danger of trauma that accom-

TABLE 3

Body and organ weights of artificially fed and mother-fed infant rats

	Artificially fed (6) [1]	Mother-fed (6)
	g	g
Body	43.4 ± 0.83 [2]	45.7 ± 1.2 [2]
Cecum	0.28 ± 0.039	0.14 ± 0.019
Liver	1.87 ± 0.043	1.63 ± 0.067
Kidneys	0.57 ± 0.018	0.50 ± 0.016
Heart	0.21 ± 0.007	0.23 ± 0.006
Spleen	0.18 ± 0.007	0.19 ± 0.014
Lungs	0.51 ± 0.039	0.50 ± 0.027
Brain	1.40 ± 0.034	1.46 ± 0.022

[1] Number in parentheses indicates number of animals.
[2] Mean of weights ± SE.

panies repeated passage of oral-gastric feeding tubes and it can be handled by a single investigator.

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Effects of High Protein Diets with Normal and Low Energy Intake on Wound Healing, Hair Growth, Hair and Serum Zinc, and Serum Alkaline Phosphatase in Dairy Heifers [1,2]

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ABSTRACT Effects of level of protein and energy intake on several measures were studied in 24 female Holstein calves. The four dietary treatments which provided varying amounts of protein and energy in relation to National Research Council (NRC) requirements were: a) control (100% protein, 100% energy); b) medium high protein (150% protein, 100% energy); c) high protein (200% protein, 100% energy); and d) high protein, restricted intake (same diet as high protein fed at a level to provide 60% of energy needs). Healing was more rapid in animals fed the high protein diet at 100% of energy requirement. Those given the high protein, restricted intake treatment healed at a rate comparable to that of controls, thus indicating that total protein intake had more influence on rate of healing than level of energy intake. Average daily gains were increased, and feed efficiency and hair growth rate slightly increased in those given the high protein treatments. Hair and serum zinc content, serum alkaline phosphatase, packed cell volume (PCV), and hemoglobin values were not materially affected by higher than normal dietary protein intakes. Restricting intake of the high protein feed reduced weight gains, feed efficiency, hair growth, and serum alkaline phosphatase, but did not affect hair and serum zinc, PCV, and hemoglobin values.

In the United States, more hospital beds are occupied by surgery and wound-healing patients than all others combined.[5] Accordingly, wound healing is an important area of research with small laboratory animals and man (1–10). Relatively little information, however, is available on the relation of nutrition to wound healing in ruminants (11–13).

Zinc deficiency or reduced energy intake retarded wound healing in cattle (13), but feeding higher than normal amounts of zinc had little influence (12). Subnormal protein dietary percentages or levels of energy intake reduced wound-healing rate in young growing cattle, with a combination deficiency causing a further reduction (11). Effects of higher than normal dietary protein intakes on wound healing in cattle, apparently, have not been investigated previously. In other species, the data concerning the influence of higher protein intake on wound healing are limited and nonconclusive (2, 3, 5, 7). The relationship of high protein diets on hair growth rate, hair and serum zinc and serum alkaline phosphatase had not been studied appreciably.

This experiment was conducted to study effects of high protein diets with normal and restricted intakes in young cattle on a) wound healing, b) hair growth rate, c) weight gains and feed efficiency, d) hair and serum zinc, e) serum alkaline phosphatase, f) packed cell volume, and g) blood hemoglobin.

EXPERIMENTAL PROCEDURE

Twenty-four Holstein heifers averaging 110 days and 116 kg in initial age and weight, respectively, were divided into six comparable groups on the basis of age and weight. One animal from each group was allotted at random to four experimental

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[1] Journal Series Paper no. 342, University of Georgia College of Agriculture Experiment Station, College Station, Athens.

[2] Institute of Comparative Medicine Paper no. 716. Supported in part by Public Health Service Research Grant no. AM-07367-NTN from the National Institute of Arthritis and Metabolic Diseases.

[3] Department of Dairy Science.

[4] School of Veterinary Medicine.

[5] Personal communication from Dr. William Gay, Chief, Research Grants Branch, National Institute of General Medical Sciences, NIH, Bethesda, Maryland.

dietary treatment groups. The dietary treatments included three practical-type diets of approximately equal energy content and varying protein levels. The normal and medium high protein diets were fed at 100% of NRC energy requirements (14), and the high protein diet at 100 and 60% of energy requirements. Thus, the four treatments were as follows: a) control (100% protein, 100% energy); b) medium high protein (150% protein, 100% energy); c) high protein (200% protein, 100% energy); and d) high protein, restricted intake (200% protein, 60% energy). The diets consisted of ground yellow corn, soybean oil meal, cottonseed hulls, citrus pulp plus vitamins,[6] antibiotics,[7] and minerals [8] including $CaCO_3$ to equalize the calcium and phosphorus content of the diets. Ingredient composition and chemical analyses of the diets are given in tables 1 and 2. The different levels of protein were obtained by substituting soybean oil meal for corn meal. The

[6] The authors are indebted to Dawe's Laboratories, Inc., Chicago, Ill., for vitamins A and D; to American Cyanamid Company, Princeton, N. J., for chlortetracycline; Smith-Douglas Company, for defluorinated rock phosphate; and Cotton Producers Association, Atlanta, Ga., for feed used in rearing calves.
[7] See footnote 6.
[8] See footnote 6.

TABLE 1

Composition of experimental diets

Ingredients	Diets		
	100% protein	150% protein	200% protein
	kg/100 kg	*kg/100 kg*	*kg/100 kg*
Corn grain, ground	46.1	31.7	17.2
49% soybean oil meal	15.9	30.5	45.1
Cottonseed hulls	25.0	25.0	25.0
Citrus pulp	10.0	10.0	10.0
Defluorinated rock phosphate	1.70	1.42	1.15
Trace mineralized salt [1]	1.00	1.00	1.00
$CaCO_3$	0.00	0.10	0.25
	g/100 kg	*g/100 kg*	*g/100 kg*
Vitamin A, 30,000 IU/g	200.0	200.0	200.0
Vitamin D, 27,000 IU/g	30.8	30.8	30.8
Chlortetracycline, 110 g/kg	77.0	77.0	77.0

[1] Guaranteed to contain between 97.8 and 98.8% NaCl and not less than the following: (in percent) Mn (manganous oxide), 0.200; I (calcium iodate), 0.007; Fe (ferrous carbonate), 0.160; Cu (copper oxide), 0.033; Co (cobalt oxide), 0.010; Zn (zinc oxide), 0.010.

TABLE 2

Analyses of diets

	Diets		
	100% protein	150% protein	200% protein
	%	%	%
Chemical analysis			
Dry matter	89.5	89.4	90.3
Crude protein [1]	15.6	22.0	27.9
Crude fiber [1]	11.4	11.9	12.7
Ether extract [1]	2.9	2.4	2.2
Mineral matter [1]	5.8	6.3	6.9
Calcium [1]	0.75	0.72	0.76
Phosphorus [1]	0.61	0.64	0.67
Calculated analysis			
Digestible protein [2]	11.1	16.8	22.2
Total digestible nutrients [2]	73.4	72.1	70.4

[1] Values expressed on dry matter basis.
[2] Values calculated from average coefficients (14), and expressed on dry matter basis.

energy levels were obtained by varying the amount of feed offered. Within-group differences in energy requirements were calculated on the basis of body weight in kilograms to the 0.75 power ($wt_{kg}^{0.75}$). Each animal was individually fed twice daily at the level calculated to the nearest 0.227 kg/day according to current body weights for the period. Refusals were recorded daily, and feed intakes adjusted every 2 weeks. Dietary treatments were initiated 1 week before experimental wounds were imposed and continued for a total of 7 weeks.

One day prior to imposing experimental wounds, hair was clipped from gluteal, lumbar, and rib-cage areas. At 2 and 6 weeks after presurgical clipping, hair samples were taken from the previously clipped rib-cage area for zinc analysis. Zinc content of hair was determined by atomic absorption spectrophotometry (15), with nitric–perchloric–sulfuric acid wet ashing of samples. Six weeks after the initial clipping, hair samples were taken from the lumbar area for determining rate of hair growth. The length of 10 randomly selected hairs from each animal was measured with a caliper to the nearest 0.1 mm.

Wounds were imposed on both gluteal areas by surgically removing diamond-shaped areas of skin with diagonals of 4.4 and 5.5 cm (fig. 1). Prior to incisions, skin areas were treated with a local anesthetic. Wounds were permitted to heal without sutures and medicated every other day with Furacin dressing[9] which contained 0.2% Furacin brand of nitrofurazone and 0.5% butacaine sulfate in a water-soluble base.

Wound area determinations were made 1 day following surgery and at weekly intervals for 6 weeks. This procedure has been previously described and evaluated (12, 13). Wound measurements were adjusted for slight differences in initial wound sizes assuming a regression coefficient of 1.0 (11–13).

Blood samples were obtained by gravity flow technique into 50-ml Pyrex centrifuge tubes through 16-gauge disposable hypodermic needles. Heparin salt solution was used as an anticoagulate for hemoglobin and PCV samples. Samples were obtained 1 day before the start of dietary treatments, and at 1, 3, 5, and 7 weeks after diets were begun. Serum alkaline phosphatase[10] was determined for each collection period. Serum zinc, PCV, and hemoglobin (16) were determined for the pretreatment bleeding, and at 3 and 7 weeks after diets were initiated. Serum zinc was determined by atomic absorption spectrophotometry (15), and PCV by centrifuging 3 minutes at 11,500 rpm in microcapillary hematocrit tubes.

RESULTS AND DISCUSSION

Wound healing, as measured by rate of skin regeneration (fig. 1), followed the normal healing curve including the lag phase (week 1), the accelerated phase (weeks 2 and 3), and the decelerated phase (weeks 4, 5 and 6) (fig. 2A) (3, 4, 6–8, 11–13). Wound healing was most rapid in the high protein group throughout the entire period (significant at 5% level), with the largest differences occurring in weeks 2 and 3 (the accelerated phase) (table 3, figs. 1 and 2A). Calves fed the medium high protein diet did not heal significantly faster than the controls. Animals fed the high protein diet in restricted amounts healed as rapidly as those fed the control diet in adequate amounts.

Although these data show quite conclusively that feeding a high protein diet can result in more rapid healing in young growing cattle, they do not provide conclusive evidence concerning the nature of the mechanism involved. At least two possibilities exist. A protein-deficient diet will retard wound healing in cattle and other animal species (2, 3, 6, 8, 10, 11). Accordingly, it is possible that the dietary protein requirement of growing cattle is substantially higher for maximum wound healing than for optimum growth and other criteria on which recommended requirements are based. If this were the mechanism, calves fed the medium high protein diet should have healed faster than the controls. The r ossibility is that some physiologic ınism with a

[9] A product of Eato es, Norwich, N. Y.

[10] The colorimetric . .on of phosphatase, acid, alkaline, prostatic . .m or other fluids at 400–420 mμ. Sigma Chemical Company, St. Louis, Tech. Bull. no. 104, revised, 1961.

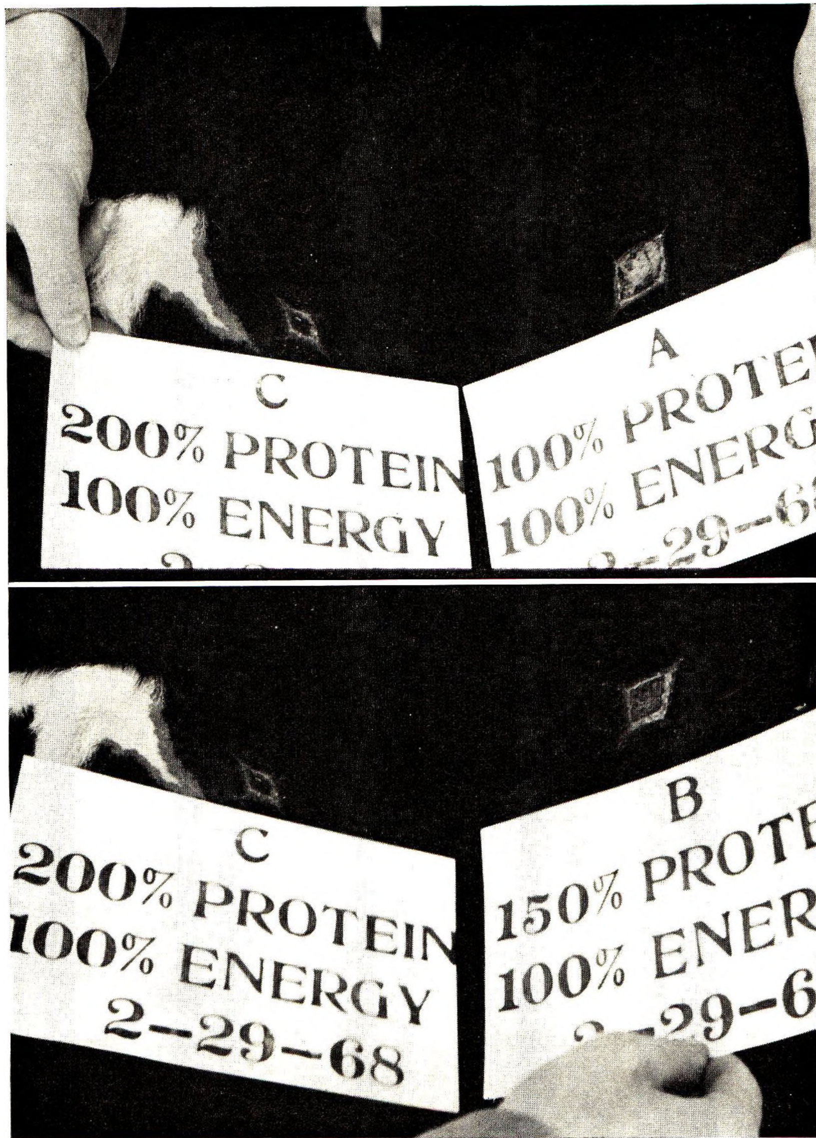

Fig. 1 Photographs taken 4 weeks after wounds were imposed showing unhealed areas on calves fed: A) control, B) medium high protein, and C) high protein dietary treatments. Note the smaller unhealed areas on calf receiving the high protein treatment.

threshold level is involved. The response of those given the medium high protein treatment would suggest such a mechanism. It is also possible, however, that the lack of a significant difference in healing rate between controls and the medium high protein group may have been due to chance. The healing rates of animals fed the medium high protein diet were not significantly (5% level) different from a rate which is half-way between that of controls and the high protein group. Reducing protein percentage of the diet below requirement levels without reducing feed intake (11) or reducing total feed intake of a diet which was just adequate in protein percentage (13) decreases wound healing rate. When these results (11, 13),

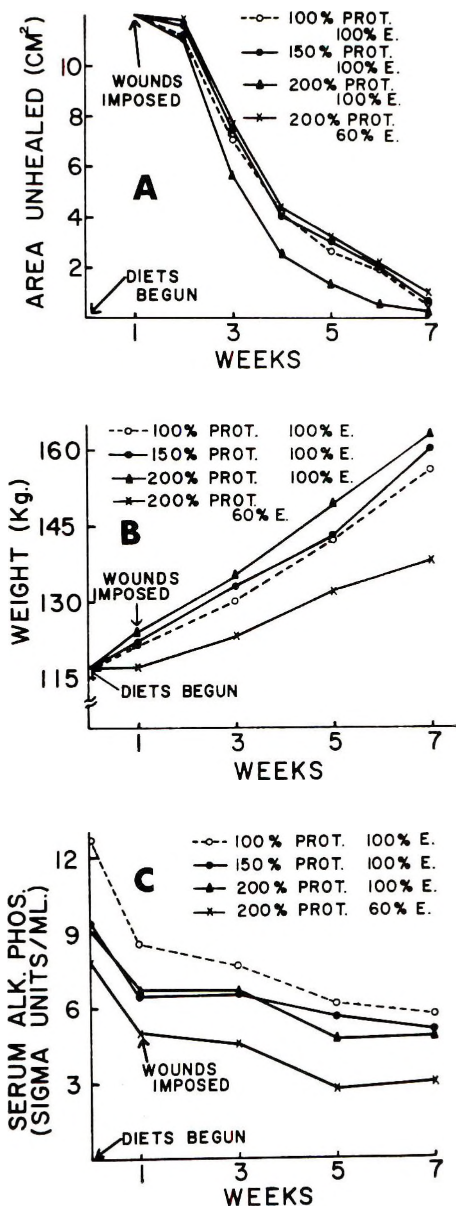

Fig. 2 Effects of a) control (100% protein, 100% energy), b) medium high protein (150% protein, 100% energy), c) high protein (200% protein, 100% energy), and d) high protein, low energy (200% protein, 60% energy) dietary treatments on: A. Rate of wound healing as measured by area of unhealed wounds; B. Body weight; and C. Serum alkaline phosphatase values. Six animals per treatment.

and those from calves fed the high protein diet in restricted amounts are considered together, it appears that total protein intake has a substantial effect on healing rate in cattle. The reduced healing rate observed when a diet containing a normal protein percentage was fed in restricted amounts (11) may have been due, at least partially, to a deficiency in amount of total protein intake. Likewise, the reduced healing rate when a normal purified diet was fed in restricted amounts may have been caused by the reduced level of total protein intake rather than by the deficiency of energy per se (13). These data, however, do not preclude the possibility that the protein-to-calorie ratio has some influence on healing rate.

The data available lead to the suggestion that total protein intake probably is more important in determining healing rate than is level of energy intake. It appears well established that the protein-to-calorie ratio is most important when formulating diets if animal performance is measured in the usual terms of gain, feed efficiency, and the like. However, these and the previous data (11, 13) cast doubt on the desirability of a constant protein-to-calorie ratio when wound healing is the important criterion.

Hair growth rate was slower in animals fed the protein, restricted intake treatment than in other groups (table 3). Rate of hair growth was slightly faster (not significant) in the high protein group. Reduced hair growth rate has been observed previously in cattle given reduced energy and protein intakes (11). A deficiency of either protein or energy reduces wool growth of sheep (17, 18). Feeding higher than normal amounts of protein has not been shown to increase wool growth.

The high protein (200% protein, 100% energy) treatment increased rate of gain (significant at 5% level) (table 3, fig. 2B). Gains of animals fed the medium high protein diet were intermediate between controls and animals fed high protein (200% protein, 100% energy). Rate of gain was greatly reduced in animals fed the high protein ration in restricted amounts (significant at 1% level). Thus, in animals given the high protein, re-

TABLE 3

Initial age and weight, and effects of dietary treatments on average daily gains, feed efficiency, and hair and skin growth rate

	100% protein 100% energy	150% protein 100% energy	200% protein 100% energy	200% protein 60% energy		Coef of variation
						%
Initial age, days	110 [a] [1]	110 [a]	109 [a]	111 [a]	3.48 [2]	7.75
Initial wt, kg	116 [a]	117 [a]	117 [a]	116 [a]	4.28 [2]	9.00
Feed intake, kg dm/day	3.19 [a]	3.26 [a]	3.32 [a]	1.92 [b]	0.137 [2]	11.43
Total digestible nutrient intake, kg/day	2.096	2.103	2.105	1.217	—	—
Protein intake, kg/day	0.498	0.717	0.926	0.536	—	—
Digestible protein intake, kg/day	0.354	0.548	0.737	0.426	—	—
Average daily gain, kg	0.78 [a]	0.84 [ab]	0.90 [b]	0.43 [c]	0.033 [2]	11.0
Kilogram dry matter per kilogram gain	4.09 [a]	3.88 [a]	3.69 [a]	4.45 [a]	0.340 [2]	22.6
Hair growth, mm/day	0.35 [a]	0.35 [a]	0.38 [a]	0.28 [b]	0.022 [2]	15.7
Skin growth rate,[3] mm^2/day	40.2 [a]	41.0 [a]	46.3 [b]	40.8 [a]	1.64 [2]	9.6

[1] Average values followed by the same letter are not significantly different at the 5% probability level.
[2] SE of treatment means.
[3] Means obtained by difference for the period week 2 through week 5.

stricted intake treatment, rate of gain was much more drastically reduced than healing rate. Feed efficiencies were in the same directions as the gains but were not statistically significant (5% level) (table 3, fig. 2B).

Since soybean oil meal contains more zinc than corn (19), zinc levels of rations increased with protein content due to substitution of soybean oil meal for corn meal. Thus, daily zinc intakes increased with higher protein treatments except for the high protein, restricted intake treatment (table 4). Even though there were substantial differences in dietary zinc intakes, there were no apparent differences, attributable to dietary treatments, in either zinc content of hair or serum at various periods. Hair zinc tended to decrease with time (table 4). Earlier studies indicated that hair zinc levels in 4-month-old Holsteins were significantly higher than in 6-month olds (11, 20). In this study, an analysis of age effects revealed no significant differences within a range from 72 to 154 days. Average hair zinc values in parts per million dry matter were as follows: 6-month olds, 125.0; 5-month olds, 123.7; 4-month olds, 127.7; and 3-month olds, 128.1. Serum zinc values were not affected by the treatments (table 4). Previously, serum zinc values in cattle were reduced when low protein rations and low energy rations were fed (11).

Serum alkaline phosphatase was lower in animals given the high protein, restricted intake treatment, but was not significantly affected by the other treatments (table 4, fig. 2C). Lower serum alkaline phosphatase has been observed in cattle on reduced intakes of both normal protein and low protein rations (11). Thus, decreased feed intake results in reduced serum alkaline phosphatase activity regardless of dietary protein content. Serum alkaline phosphatase which is much reduced in zinc-deficient animals (22) has been suggested as offering considerable potential diagnostic value for determining whether or not animals are zinc deficient under field conditions. Serum alkaline phosphatase values, however, vary widely in different herds of normal cattle (21). Consequently, before it can be used effec-

TABLE 4

Zinc contents of diets, hair, and serum and serum alkaline phosphatase, hemoglobin, and packed cell volume content of blood

	100% protein 100% energy	150% protein 100% energy	200% protein 100% energy	200% protein 60% energy		Coef of variation
						%
Zinc content of feed, ppm of dm	31.2	36.7	46.7	46.7	—	—
Zinc intake, mg/day	99.8 [a] [1]	119.9 [b]	155.6 [c]	89.1 [a]	3.5 [2]	7.3
Zinc content of hair, ppm						
Pretreatment	134.4	140.0	141.0	137.2	—	—
3 weeks [3]	130.0	136.1	122.9	126.0	—	—
7 weeks	119.9	122.9	122.2	129.1	—	—
Treatment avg	124.1 [a]	129.5 [a]	122.5 [a]	127.5 [a]	3.5 [2]	6.9
Zinc content of serum, ppm						
Pretreatment	1.31	1.11	1.27	1.11	—	—
3 weeks [3]	1.12	1.14	1.20	1.05	—	—
7 weeks	1.19	1.20	1.28	1.20	—	—
Treatment avg	1.16 [a]	1.17 [a]	1.24 [a]	1.13 [a]	0.05 [2]	9.7
Serum alkaline phosphatase [4] Sigma units/ml	7.02 [a]	5.92 [a]	5.74 [ab]	3.83 [b]	0.68 [2]	30.0
Hemoglobin,[5] g/100 ml	12.2 [a]	12.3 [a]	12.6 [a]	13.2 [a]	0.31 [2]	6.1
Packed cell volumes,[5] %	35.8 [a]	34.3 [a]	35.5 [a]	37.1 [a]	0.94 [2]	6.5

[1] Average values followed by the same letter are not significantly different at 5% probability level.
[2] SE of the treatment means.
[3] Weeks after dietary treatments begun.
[4] Average of values determined at 1, 3, 5, and 7 weeks after dietary treatments begun. Analysis by co-variance with pretreatment values as covariants.
[5] Average of values determined 3 and 5 weeks after dietary treatments initiated.

tively for diagnostic purposes under field conditions, the effects of other variables must be better understood.

Packed cell volume and hemoglobin were not changed either by dietary treatment or by duration of treatments (table 4). It has been observed that low protein intakes increase both PVC and hemoglobin (11). In another study, zinc-deficient cattle had lowered hemoglobin values, but had no effect of PCV's (22).

When considered at the fundamental level, wound healing is exceedingly complex (1, 2, 6, 23, 24). Numerous factors influence healing with several nutritional deficiencies known to retard this process (1, 2, 6). Even though effects of a protein deficiency have been widely studied, the mechanism whereby it slows healing has not been clearly delineated. The complexity of the healing process makes it difficult to determine basic mechanisms by which rates are affected. Because of their importance in collagen synthesis, a major component of connective tissue, the sulfur-containing amino acids have received major attention (2, 6). Relatively little controlled research has been published on the wound healing of ruminants (11–13). Thus, with the basic differences which exist between ruminants and nonruminants in amino acid metabolism, it is not known to what degree results would be transferable. Somewhat contrary to long generally accepted concepts, recent studies have shown benefits in wool production, weight gains, and milk production when added methionine is administered to ruminants in a way which permits it to avoid bacterial degradation in the rumen (25–27).

A variety of techniques has been used to study wound healing rates, with breaking strength of incisions being one of the most widely employed (2, 6, 23). The procedure used in this study involving

skin regeneration appears to be especially suitable to cattle (11–13). It is important to note, however, that different techniques do not necessarily measure the same phenomena (6). For example, skin removed during injury is replaced both by growth of new tissue and by wound contraction or the migration of the surrounding skin over the wound area (24). Thus, affecting rate of wound healing as measured in this work could be via any of the processes.

It is well known that many nutritional deficiencies affect both hair and skin (28). Changes in these tissues are often among the more important symptoms of nutritional inadequacy. The sulfur-containing amino acids are regarded as most important in both skin and hair growth.

It is characteristic of protein metabolism that nitrogen balance can be maintained at many different levels of protein intake, which result in alterations of metabolic patterns (29). Details of the effects of such alterations on animal health and performance are very inadequate. This research indicates an important beneficial effect of a high protein level on wound healing in cattle. Whether other desirable effects might also exist remains open to speculation.

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Effect of Vitamin B_6 Deficiency on Leucine Transaminase Activity in Chick Tissue [1,2]

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ABSTRACT To test the effect of vitamin B_6 deficiency on levels of leucine transaminase activity in various tissues of the chick, 1-day-old male chicks were reared on a purified diet supplemented with zero or 1.2 mg pyridoxine·HCl/100 g diet. After 8 days, chicks fed the deficient diet showed evidence of severe vitamin B_6 depletion: anorexia, retarded growth, ataxia, hyperactivity and convulsions. Leucine transaminase activity was significantly decreased in kidney of chicks fed the deficient diet for 1.5 days. In liver and heart, significant decreases in leucine transaminase activity were detected after 4 days; in brain, after 9 days. No depression in leucine transaminase activity occurred in pair-fed controls. Pyridoxal phosphate added to enzyme reaction mixtures stimulated activity, but it did not restore leucine transaminase activity in homogenates from deficient tissue to control levels.

Although a deficiency of vitamin B_6 has long been known to depress transaminase activity in various species (1–10), no investigation has been reported of the effect of vitamin B_6 deficiency on the activity of the leucine transaminases (EC.2.6.1.6) which catalyze the transamination of the branched-chain amino acids essential for animals with alpha-ketoglutaric acid. Purification of the leucine transaminases from pig heart has recently been reported (11–15). The enzymes seemed of particular interest for nutritional studies because of the marked stimulation observed in work with pig heart when pyridoxal phosphate was added to enzyme reaction mixtures (12). We have carried out studies with vitamin B_6-depleted chicks to determine whether either leucine transaminase activity or the degree of stimulation of leucine transaminase activity by in vitro additions of pyridoxal phosphate might prove a useful index of vitamin B_6 nutrition. Details of our observations are presented below.

MATERIALS AND METHODS

Animals and diet. Experiment 1: One-day-old male chicks (Arbor Acres broilers weighing 40 ± 5 g) were housed in temperature-controlled battery brooders with wire-screen floors in a laboratory maintained at 25° and fed ad libitum the purified diet (16) whose composition is given in table 1. Twenty chicks received only the purified diet; 20 received the purified diet mixed with 1.2 mg pyridoxine·HCl/100 g diet. The supplemented diet contained about 10 times the minimum daily requirement of vitamin B_6 for the chick (17). After 9 days, when chicks fed the deficient regimen showed symptoms characteristic of vitamin B_6 deficiency (growth depression, anorexia, ataxia, hyperirritability and convulsions (17, 18), they were decapitated. Liver, heart, brain and kidney were removed immediately; they were frozen and stored at −15°. All tissues were analyzed within 2 months after the chicks were killed.

Experiment 2: One-day-old male chicks of the same strain were reared under the conditions described in experiment 1 with several modifications. All chicks were fed the pyridoxine-supplemented purified ration for 4 days. The chicks were then divided into three groups: controls (45 chicks), fed the pyridoxine-supplemented ration; deficients (75 chicks), fed the purified ration containing no vitamin B_6 supplement; and pair-feds (45 chicks), fed

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TABLE 1

Composition of basal diet

	%
Glucose [1]	47.8
Isolated soybean protein [2]	32.0
Soybean oil [3]	10.0
Mineral mix [4]	7.3
Vitamin mixture [5]	1.1
Glycine	1.0
Methionine	0.7
Antioxidants [6]	0.02

[1] Anhydrous Cerelose, Corn Products Company, Argo, Ill.

[2] C-1 Assay Protein, Archer-Daniels Midland, Minneapolis, Minn.

[3] Edible soybean oil, Pacific Vegetable Oil Corporation, Richmond, Calif.

[4] Mineral mixture supplied: (in milligrams per 100 g diet) $CaHPO_4 \cdot 2H_2O$, 3600; $CaCO_3$, 1000; K_2HCO_3, 900; Na_2HPO_4, 700; NaCl, 400; KCl, 300; $MgSO_4$, 300; $MnSO_4 \cdot H_2O$, 25; Fe citrate, 50; $ZnCO_3$, 2; KIO_3, 1; $NaMoO_4$, 0.8; Co acetate, 0.18; and $NaSeO_3$, 0.022.

[5] Vitamin mixture supplied: (in milligrams per 100 g diet) niacin, 10; calcium pantothenate, 4; riboflavin, 2; thiamine·HCl, 1; folacin, 0.3; menadione, 0.3; biotin, 0.04; vitamin A palmitate, 1000 IU; vitamin D_3, 150 ICU; alpha-tocopheryl acetate, 3 IU; cerelose, 800; cyanocobalamin, 0.005; and choline chloride, 200.

[6] A 50:50 mixture of butylated hydroxytoluene (BHT) and diphenyl-p-phenylenediamine (DPPD).

the pyridoxine-supplemented ration but with food intake restricted to amounts consumed by chicks fed the deficient ration. After 8 days on the experimental diet, 24 of the chicks which had been fed the vitamin B_6-deficient ration received a supplement of 1 mg pyridoxine·HCl/day. Food intake of these repleted chicks (referred to hereafter as "repleted pair-feds") was restricted to that of chicks fed the vitamin B_6-deficient ration. Chicks were killed after 1.5, 4, 7, 9, 11 and 13 days on the experimental diets. Tissues were removed and stored at $-15°$ and analyzed within 10 months of the time of killing. There was no loss of leucine transaminase activity in kidney during 10 months of storage. Leucine transaminase activity in heart and liver was stable for 2 months and then steadily decreased. Brain lost about 30% of its leucine transaminase activity after 1 month of storage; thereafter, activity remained stable. To make possible meaningful comparisons of leucine transaminase activity in tissues stored several months, enzyme assays from control, deficient, pair-fed and repleted pair-fed chicks killed on the same day were carried out as a single group of determinations. That tissue storage does not interfere with comparisons of tissue analyzed after the same storage time is shown by the narrow range of values for various experimental groups killed on a given day (see table 5). Leucine transaminase activity in animals killed on day 1 (analyzed first) may not, however, be compared with that in the same tissue of animals analyzed on day 13 (analyzed last) because of the effects of storage.

Analytical procedures. To prepare chick tissue for enzyme assays, the tissue was thawed at 5°; it was homogenized in ice-cold 0.05 M K_2HPO_4 buffer, pH 7.0, and centrifuged at $37{,}750 \times g$ at 0° for 20 minutes. Ratios of buffer to tissue wet weight (milliliters per gram) were as follows: Liver, 3:1; brain, 10:1; kidney, 10:1; heart, 15:1. The supernatants were stored at $-15°$.

Leucine transaminase activity was measured by the 2,4-dinitrophenylhydrazine procedure of Taylor and Jenkins (11) at pH 8.3 in the presence of 300 μmoles mercaptoethanol. L-Leucine and alpha-ketoisocaproate were used as substrates. The activity of valine and isoleucine as substrates was not tested. Both are transaminated by leucine transaminase from pig heart (11, 14, 15). Enzyme activity is expressed as micromoles of alpha-ketoisocaproate formed in 15 minutes at 37° per gram wet weight of tissue. To test the effect of added pyridoxal phosphate on enzyme activity, 50 μmoles was added to the reaction mixture prior to adding enzyme.

Materials. Chemicals used in analytical determinations were reagent grade and were purchased from commercial sources.

RESULTS

Experiment 1 showed the effect of severe vitamin B_6 deficiency on leucine transaminase levels in the chick. After 9 days on the experimental diet, the chicks were killed. At this time, three of the deficient chicks had died after convulsive seizures and the remainder showed hyperirritability, anorexia and severe growth depression. (For food intake and growth data, see table 2).

The effect of vitamin B_6 deficiency on leucine transaminase activity is shown in table 3. Leucine transaminase activity in liver, kidney and heart from chicks fed the

TABLE 2

Growth, survival and food consumption of chicks fed vitamin B_6-deficient and control diets (exp. 1)

Group	No. of chicks	Mean body wt at day 9	Avg daily wt gain	Avg daily food intake	Feed efficiency [1]	Survival
			g/day	g/day		%
Control	21	137.9	11.0	11.0	1	100
Deficient	20	60.9	2.4	5.1	0.47	85

[1] Ratio of weight gain to food intake.

deficient diet for 9 days was significantly lower than in controls. Levels in brain of deficient chicks were lower, but the difference was not statistically significant. Additions of pyridoxal phosphate to the enzyme reaction mixtures stimulated activity in preparations from deficient kidney and heart more than in those from control kidney and heart, when expressed as a percentage increase. In brain and liver, pyridoxal phosphate stimulated activity of both control and deficient tissues to about the same extent. In controls, the stimulation of enzyme activity by additions of pyridoxal phosphate was much greater in heart (about 33%) than in liver, brain and kidney (about 10% or less).

Experiment 1 did not rule out the possibility that reduced food intake in the deficient chicks might have reduced leucine transaminase activity. Nor did it determine when the first statistically significant decrease in leucine transaminase activity could be detected in chicks fed the unsupplemented ration. We, therefore, carried out another experiment using pair-fed controls and repleted pair-fed chicks (see Materials and Methods) and killed chicks after 1.5 to 13 days on the experimental diet. Although all chicks were fed the pyridoxine-supplemented rations for 4 days before the start of the experiment, results in table 4 and figure 1 show that the growth depression and reduced food intake characteristic of vitamin B_6 deficiency developed rapidly. A significant decrease in food intake occurred in chicks fed the unsupplemented diet for 3 days; the first significant growth depression occurred after 4 days on the deficient ration.

Increased excitability was observed in deficient chicks fed the experimental diet for 5 days. Convulsive seizures were first observed in deficient chicks fed the deficient diet for 7 days. No nervous disorders were observed in controls, pair-fed controls or in repleted pair-fed chicks.

Results of leucine transaminase assays on tissue from control, deficient, pair-fed and repleted pair-fed chicks are shown in table 5. An observation of particular interest is that changes in leucine transaminase activity occur before gross symptoms of vitamin B_6 deficiency (appetite depression, weight loss and hyperirritability) can be detected. A statistically significant depression in leucine transaminase activity in kidney occurs in chicks fed the deficient diet for 1.5 days. Significant depressions of activity occur in heart and liver after 4 days; in brain, after 9 days.

As in experiment 1, leucine transaminase activity in tissue from both control and deficient chicks could be stimulated by in

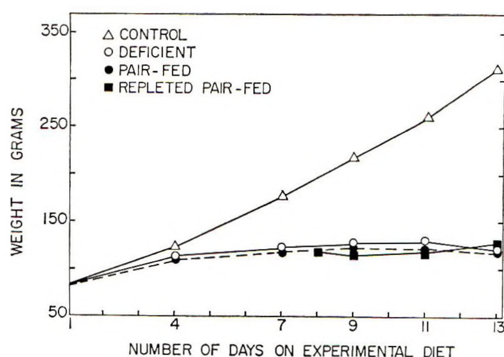

Fig. 1 Growth of control, vitamin B_6-deficient, pair-fed and repleted pair-fed chicks from experiment 2.

TABLE 3

Leucine transaminase activity in tissues of control and B_6-deficient chicks (exp. 1)

Group	Liver – PALP [2]	Liver + PALP	Brain – PALP	Brain + PALP	Kidney – PALP	Kidney + PALP	Heart – PALP	Heart + PALP
	Leucine transaminase activity [1]							
Control	4.0 ± 0.2 [3] (11) [4]	4.3 ± 0.2 (11)	6.7 ± 0.6 (10)	7.3 ± 0.5 (10)	46.7 ± 2.7 (3)	49.7 ± 3.3 (3)	15.8 ± 1.8 (8)	21.0 ± 1.7 (8)
Deficient	1.7 ± 0.2 [5] (14)	1.9 ± 0.2 [5] (14)	5.7 ± 0.4 (12)	6.4 ± 0.4 (12)	17.5 ± 3.3 [5] (6)	26.3 ± 1.9 [5] (6)	4.2 ± 0.5 [5] (10)	6.4 ± 0.8 [5] (10)
	% stimulation of leucine transaminase activity by PALP							
Control	7.5		9.0		6.4		33.0	
Deficient	12.0		12.3		50.0		52.3	

[1] Enzyme activity is expressed as micromoles alpha-ketoisocaproate formed in 15 minutes at 37° per gram wet weight of tissue.
[2] Assays were carried out with (+) and without (–) the addition of 50 μmoles pyridoxal phosphate (PALP) to the reaction mixture.
[3] Mean ± SE.
[4] Figures in parentheses indicate number of chicks in each group.
[5] $P < 0.001$ compared with ad libitum controls.

vitro additions of pyridoxal phosphate The effect was most pronounced in kidney where stimulation ranging from 20 to 135% was observed in enzyme preparations from deficient animals as compared with 6 to 12% for controls. Similar though smaller effects were observed in liver and heart: in liver, the stimulation in response to added pyridoxal phosphate was 10 to 25% for deficient chicks compared with 3 to 10% for controls; in heart, from 40 to 90% for controls compared with 65 to 110% for deficients. In brain, the magnitude of the pyridoxal phosphate effect was the same for deficient and control tissue. As in experiment 1, heart showed a greater response to additions of pyridoxal phosphate than any other control tissue, 40 to 90% increases in activity compared with 5 to 15% increases in activity in kidney, brain and liver.

Restricting food intake did not depress leucine transaminase activity. In pair-fed controls, leucine transaminase activity was significantly higher than in ad libitum fed controls in kidney by day 11. Increases in activity in liver and heart from pair-fed controls were smaller but were significantly greater than in ad libitum controls by day 13.

When chicks depleted of vitamin B_6 for 8 days were given a supplement of pyridoxine·HCl, leucine transaminase activity increased sharply. In chicks killed 1 day after receiving the dose of pyridoxine·HCl, leucine transaminase activity was significantly greater than in unsupplemented deficient chicks in kidney, liver, heart and brain. After 5 days of repletion, leucine transaminase activity in heart, liver, kidney and brain was equal to or greater than that in chicks fed the supplemented ration ad libitum. It should be noted that the increase in leucine transaminase activity was not due to increased food intake. Repleted chicks were starved so that their food intake matched that of deficient chicks. Increases in leucine transaminase activity occurred in the presence of continuing acute growth depression (see fig. 1).

DISCUSSION

Leucine transaminase activity in kidney is a highly sensitive indicator of vitamin B_6

TABLE 4

Food consumption and growth of chicks fed vitamin B_6-deficient and control diets (exp. 2)

Group	Avg 24-hr food intake						Mean daily food intake	Mean daily wt gain	Feed efficiency [1]
	Day 1	Day 4	Day 7	Day 9	Day 11	Day 13			
	g	*g*	*g*	*g*	*g*	*g*	*g*	*g*	
Control	12.8 (48) [2]	16.8 (45)	22.7 (40)	28.6 (33)	38.5 (25)	49.0 (16)	28.1	18.3	0.65
Deficient	12.3 (75)	12.5 (71)	6.0 (64)	6.2 (35)	5.4 (23)	3.7 (13)	7.7	3.7	0.48

[1] Ratio of weight gain to food intake.
[2] Figures in parentheses indicate number of animals in each group.

nutrition in the chick. Gross symptoms of vitamin B_6 deficiency such as appetite failure, weight loss and hyperirritability do not occur until 2 to 5 days after a significant drop occurs in kidney leucine transaminase activity. Earlier work on the response of pyridoxal phosphate enzymes to vitamin B_6 depletion indicated that transaminases are less affected by deficiency than are certain pyridoxal phosphate-dependent enzymes concerned with the metabolism of sulfur-containing amino acids (10, 19). The response of kidney leucine transaminase to vitamin B_6 depletion, however, is as rapid as that of cysteic acid decarboxylase which has been reported to decrease markedly in livers of rats fed a vitamin B_6-deficient diet for only 2 days (20). Significant decreases in leucine transaminase activity in heart and liver of deficient chicks appear at about the same time as the onset of deficiency symptoms; in brain, activity drops only after 9 days when animals are severely depleted. Reasons for the differences in the rate of response of leucine transaminase activity in various organs are not known. The slower changes in brain may reflect a slower turnover of protein. It is also possible that different proteins which vary in their ability to bind pyridoxal phosphate catalyze the transamination of branched-chain amino acids in different organs. Although leucine transaminase activity is widely distributed in animal tissues [4] (14), it has been highly purified only from pig heart (11–15). Two different proteins catalyze the transamination of branched-chain amino acids in pig heart: one a supernatant enzyme with a K_m for pyridoxal phosphate of 6.7×10^{-5}; the other a mitochondrial enzyme with a K_m for pyridoxal phosphate of 1.4×10^{-5} (15).

Addition of pyridoxal phosphate to assay mixtures stimulates activity of enzyme preparations from both control and deficient tissue, but it does not restore activity of deficient tissue to control levels. These results indicate that leucine transaminase apoenzyme is depleted in all tissues. In kidney, both coenzyme and apoenzyme are depleted in vitamin B_6-deficient chicks, but the cofactor appears to be lost more rapidly than is the enzyme protein, judging from degree of stimulation observed in the presence of in vitro additions of pyridoxal phosphate. The striking increase in activity of kidney leucine transaminase from deficient chicks, when assayed in the presence of saturating amounts of pyridoxal phosphate, suggests that cofactor stimulation of leucine transaminase may have potential value in clinical nutrition. A search needs to be made for blood fractions whose sensitivity to deficiency equals that of kidney.

Because of the characteristic appetite depression which occurs in vitamin B_6 deficiency, it was important to show that food restriction does not decrease leucine transaminase activity. Our data show that near starvation does not depress leucine transaminase activity. In fact, leucine transaminase activity is somewhat higher (and, in some cases, significantly higher) in pair-fed controls than in ad libitum fed controls. Although this was an unexpected finding, the same phenomenon has been reported by other investigators in studies on glutamic oxaloacetic transaminase levels in rat liver (3, 21), and pyruvic trans-

[4] Shiflett, J. M. 1969 Master's thesis, University of California, Davis, California.

TABLE 5

Progressive changes in leucine transaminase activity during vitamin B_6 depletion in the chick (exp. 2)

Group	Days on diet	Leucine transaminase activity [1]						Leucine transaminase activity [1]					
		Kidney			Brain			Heart			Liver		
		− PALP [2]	+ PALP	No. of chicks	− PALP	+ PALP	No. of chicks	− PALP [2]	+ PALP	No. of chicks	− PALP	+ PALP	No. of chicks
Control	1.5	45.4 ± 1.7 [3]	49.0 ± 2.1	(5)	6.2 ± 0.2	6.5 ± 0.1	(5)	8.9 ± 1.0 [3]	12.5 ± 1.0	(5)	4.2 ± 0.4	4.4 ± 0.4	(5)
	4	45.0 ± 3.6	48.1 ± 3.8	(5)	4.7 ± 0.3	5.1 ± 0.2	(5)	3.6 ± 0.2	5.7 ± 0.4	(5)	3.7 ± 0.4	3.9 ± 0.4	(5)
	7	36.6 ± 2.8	41.0 ± 3.1	(7)				4.3 ± 0.7	6.8 ± 0.8	(7)	2.9 ± 0.1	3.1 ± 0.1	(7)
	9	36.4 ± 3.1	40.2 ± 2.4	(7)	4.4 ± 0.2	4.8 ± 0.1	(8)	5.5 ± 0.8	9.8 ± 1.6	(8)	3.9 ± 0.6	4.0 ± 0.6	(7)
	11	47.3 ± 2.6 [4]	50.2 ± 3.1 [4]	(8)									
	13	54.5 ± 2.8 [5]	60.1 ± 2.9 [5]	(6)	4.4 ± 0.2	5.0 ± 0.3	(6)	2.7 ± 0.2 [5]	5.2 ± 0.4	(6)	2.1 ± 0.0 [5]	2.3 ± 0.1 [5]	(6)
Deficient	1.5	28.3 ± 1.7 [6]	34.0 ± 2.6 [7]	(4)	5.3 ± 0.6	5.8 ± 0.7	(4)	5.0 ± 1.1	8.2 ± 1.0	(4)	3.1 ± 0.3	3.7 ± 0.4	(4)
	4	22.3 ± 2.3 [6]	32.2 ± 1.7 [7]	(5)	4.0 ± 0.2	4.4 ± 0.2	(5)	1.6 ± 0.1 [6]	2.8 ± 0.2 [6]	(5)	1.8 ± 0.1 [7]	2.1 ± 0.1 [7]	(5)
	7	8.0 ± 0.8 [6]	18.8 ± 1.2 [6]	(6)				0.7 ± 0.0 [6]	1.5 ± 0.2 [6]	(4)	1.1 ± 0.0 [6]	1.2 ± 0.0 [6]	(7)
	9	11.0 ± 1.5 [6]	18.9 ± 1.6 [6]	(9)	3.1 ± 0.2 [6]	3.3 ± 0.2 [6]	(7)	1.3 ± 0.1 [6]	2.3 ± 0.1 [6]	(9)	1.1 ± 0.0 [6]	1.3 ± 0.0 [6]	(7)
	11	14.7 ± 1.6 [6]	22.3 ± 2.2 [6]	(9)									
	13	19.0 ± 2.2 [6]	31.6 ± 2.8 [6]	(10)	3.5 ± 0.2 [7]	4.0 ± 0.2	(10)	1.1 ± 0.1 [6]	2.6 ± 0.3 [6]	(9)	1.2 ± 0.1 [6]	1.5 ± 0.1 [6]	(9)
Pair-fed [8]	1.5												
	4	42.4 ± 4.5 [9]	45.7 ± 5.1	(5)	4.2 ± 0.1 [10]	5.0 ± 0.1	(5)	4.2 ± 1.0	5.9 ± 1.6	(5)	3.5 ± 0.2 [9]	3.7 ± 0.2 [9]	(5)
	7	33.6 ± 3.7 [9]	40.1 ± 4.1 [9]	(7)				3.4 ± 0.4 [9]	5.7 ± 0.8 [9]	(5)	3.0 ± 0.2 [9]	3.3 ± 0.2 [9]	(7)
	9	44.5 ± 3.1 [9]	47.9 ± 2.3 [9]	(7)	4.4 ± 0.3 [10]	4.6 ± 0.2 [10]	(8)	7.4 ± 0.9 [9]	11.1 ± 1.2 [9]	(8)	3.0 ± 0.4 [9]	3.2 ± 0.3 [9]	(7)
	11	62.1 ± 2.2 [9]	67.9 ± 4.0 [9]	(8)									
	13	66.8 ± 2.8 [9]	74.2 ± 2.8 [9]	(6)	5.0 ± 0.1 [9]	5.5 ± 0.2 [9]	(8)	4.1 ± 0.3 [9]	7.6 ± 0.8 [9]	(8)	2.9 ± 0.2 [9]	3.0 ± 0.2 [9]	(8)
Repleted pair-fed [11]	9	34.6 ± 3.2 [9]	40.0 ± 3.0 [9]	(7)	4.1 ± 0.3 [10]	4.6 ± 0.2 [9]	(7)	3.3 ± 0.7	4.8 ± 0.8 [10]	(7)	2.0 ± 0.1 [7,9]	2.2 ± 0.1 [7,9]	(7)
	11	58.5 ± 2.8 [7,9]	62.8 ± 2.8 [7,9]	(7)									
	13	68.3 ± 2.8 [7,9]	75.2 ± 2.7 [7,9]	(8)	4.2 ± 0.2 [10]	4.5 ± 0.2	(8)	3.2 ± 0.2 [9]	5.2 ± 0.4 [9]	(8)	3.3 ± 0.4 [7,9]	3.5 ± 0.4 [7,9]	(8)

[1] Enzyme activity expressed as µmoles alpha-ketoisocaproate formed in 15 minutes at 37° per gram wet weight of tissue.
[2] Assays were carried out with (+) and without (−) the addition of 50 µmoles pyridoxal phosphate (PALP) to the reaction mixture.
[3] Mean ± SE. The number of tissues analyzed in each group is shown in parentheses. Only kidney was analyzed on day 11.
[4] $P < 0.001$ compared with pair-feds.
[5] $P < 0.01$ compared with pair-feds.
[6] $P < 0.001$ compared with ad libitum controls.
[7] $P < 0.01$ compared with ad libitum controls.
[8] Pair-fed tissue was not analyzed on day 1.5.
[9] $P < 0.001$ compared with deficients.
[10] $P < 0.01$ compared with deficients.
[11] Deficient animals supplemented with pyridoxine·HCl daily from day 8 to 13 but pair-fed to remaining deficients (see Materials and Methods).

aminase in rat liver (3,21,22) and rat kidney (22).

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Tryptophan Metabolism of Rats Fed a Threonine-free Amino Acid Diet [1,2]

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ABSTRACT This study was conducted to clarify metabolic changes in rats given an amino acid diet devoid of one essential amino acid from the complete amino acid diet. At the beginning of the study, rats were force-fed the threonine- or tryptophan-free diet and the complete amino acid diet to observe the body weight changes, the urinary nitrogen and N^1-methylnicotinamide (MNA), and changes in the liver enzyme activities, tryptophan pyrrolase (TPase) and tyrosine-α-ketoglutarate transaminase (TKase), and the like. It was observed that the excretion of the urinary MNA of rats force-fed a threonine-free diet was higher than that of animals fed the complete amino acid diet, and that increase of the liver TPase activity three hours after feeding was larger in the latter. It was presumed from the result that tryptophan in the diet, which is normally used for protein synthesis, was converted to nicotinic acid when protein formation was limited by the omission of threonine, and excreted in urine as MNA. There was also a remarkable increase of TKase activity 3 hours after feeding either the threonine-free diet or the complete amino acid diet, but a greater increase was observed with the deficient than with the complete diet. The mechanism of the induction of TPase and TKase activities after force-feeding of the different patterns of amino acid diets is discussed.

Krehl et al. (1–3) observed that the growth retardation caused by the inclusion of 40% corn grits in a low protein ration was counteracted by the addition of tryptophan or nicotinic acid to the diet, suggesting that tryptophan might function as a biological precursor of nicotinic acid. Since then, a number of studies have shown that tryptophan and its metabolites are precursors of niacin in animals and *Neurospora* (4).

Koeppe and Henderson (5) found that tryptophan served more effectively as a niacin source when a relative lack of some other amino acid limited its availability for protein formation. It has been reported from our laboratory that the excretion of N^1-methylnicotinamide (MNA), a major metabolite of nicotinic acid, by rats fed a threonine-free, valine-free, or isoleucine-free amino acid diet was much higher than that by rats fed the complete amino acid diet, while no such difference in the excretion of thiamine, riboflavin, and pyridoxine was observed (6). This result led us to the assumption that tryptophan, which was not incorporated into protein because the diet was lacking in an essential amino acid, must be metabolized in part to nicotinic acid and excreted in the urine as MNA.

The present study was undertaken to test this hypothesis by investigation of the relationship between the amount of excreted MNA and liver enzyme activities in rats fed a threonine-free, tryptophan-free, or complete amino acid diet. It was observed that the tryptophan pyrrolase (TPase) (EC. 1.13.1.12) activity of rats fed the threonine-free diet was increased much more 3 hours after feeding than of those receiving the complete amino acid diet, but the activity in the group receiving the tryptophan-free diet was less than that of the control group. Changes in TPase activity of liver after feeding were in proportion to the changes in MNA excretion in the urine.

EXPERIMENTAL

Animals and diet. Male rats of the Wistar-Donryu strain, weighing 150 to 190 g, were used. Animals were fed ad libitum a 20% casein diet containing niacin until they were used for the experiment. Rats of the control group were fed the diet shown in table 1, containing either amino acid

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[2] Presented in part at the annual meeting of the Agricultural Chemical Society of Japan, Nagoya City, 1968.

TABLE 1

Composition of complete amino acid diet and of amino acid mixtures

Complete amino acid diet		Amino acid mixture [1]		
			A [2]	B [3]
	%		*g/100 g of diet*	
Amino acid mixture	10.0	L-Arginine·HCl	0.32	0.37
Sucrose	80.0	L-Histidine·HCl·H_2O	0.32	0.41
Corn oil	5.0	L-Isoleucine	0.38	0.53
Salts B [4]	4.0	L-Leucine	0.64	0.77
Niacin-free vitamin mixture [5]	1.0	L-Lysine·HCl	0.70	0.78
		L-Methionine	0.38	0.37
		L-Phenylalanine	0.70	0.66
		L-Threonine	0.46	0.56
		L-Tryptophan	0.13	0.17
		L-Valine	0.45	0.66
		L-Cystine	—	0.21
		L-Tyrosine	—	0.21
		L-Alanine	0.57	0.43
		L-Aspartic acid	1.43	0.88
		L-Glutamic acid	2.28	1.88
		Glycine	0.57	0.31
		L-Proline	0.85	0.43
		L-Serine	—	0.43

[1] Amino acids were purchased from Tanabe Amino Acid Research Foundation, Osaka, Japan.
[2] Reported at the 6th International Congress on Nutrition held in Edinburgh, Scotland, 1963, Yoshida, A. and K. Ashida 1969 Agr. Biol. Chem., 33: 43.
[3] Reported at the 31st Meeting of the Essential Amino Acid Committee, 1965, by M. Miyazaki.
[4] Harper, A. E. 1959 J. Nutr., 68: 405.
[5] The vitamin mixture was made with sucrose and provided: (in milligrams per 100 g diet) thiamine, 1; riboflavin, 1; pyridoxine, 1; vitamin B_{12}, 0.001; folic acid, 0.15; Ca pantothenate, 2.5; biotin, 0.01; choline chloride, 100; α-tocopherol, 5; vitamin K, 0.1; vitamin A, 2000 IU; and vitamin D, 250 IU.

mixture A or B; for the experimental groups the diets were modified by omitting threonine or tryptophan from the complete amino acid mixture and replacing the omitted amino acid by a mixture of the nonessential amino acids.

Experiment 1. Rats were divided into four groups: group 1 was fed the complete amino acid diet with amino acid mixture A; group 2 was fed the threonine-free diet; group 3 was fed the threonine- and tryptophan-free diet; and group 4 was fed the threonine-free diet with 0.26% tryptophan (twice the amount in the control diet). Rats of the other three groups were pair-fed with those fed the threonine-free diet. Urine was collected from the animals which were maintained in individual cages. Collection period 1 was for 2 days, just before the experimental feeding. Collections 2, 3 and 4 were for consecutive 2-day periods of the experimental feeding; collection 5 was for 1 day only.

Experiment 2. In experiment 1, owing to the low food consumption, even the rats fed the complete amino acid diet decreased in weight. It might be inferred from the reduction in body weight that there could be catabolism of amino acids. Therefore, force-feeding adequate amounts of diet was used in the following experiments to exclude effects due to low food consumption and to make it possible to compare directly the amounts of excreted MNA and changes in activities of amino acid-catabolic enzymes in rats fed the experimental diets. For tube-feeding, the diet was homogenized with water in a blender so that 1 ml contained 0.7 g of solids. The force-feeding was done three times daily at 5-hour intervals; the amounts of diet administered at each feeding were 3.5, 3 and 3.5 g. As several days were required to accustom the rats to the procedure of force-feeding, they were subjected to a preliminary 4-day period with the complete amino acid diet supplemented with 10 mg niacin/100 g diet. The rats were then divided into two groups. Group 1 was composed of six rats given the complete amino acid diet with amino acid mixture B and killed 3 hours after feeding 1 on an arbitrary day during the experimental period. Group 2 was composed of 18 rats fed the threonine-free diet; 6 animals of this group were sacrificed 3 hours after feeding 1 on days 3, 5, and 7

of the experimental feeding period, respectively. The urine collection periods in the preliminary feeding and the experimental feeding were as in experiment 1.

Experiment 3. Rats were fed as in experiment 2. They were divided into four groups: groups 1 and 2 received the complete amino acid diet with amino acid mixture B; groups 3 and 4 received the threonine-free diet. One group of each pair was killed just before feeding 1, the other group 3 hours after feeding 1 on day 5 of the experimental feeding period.

Experiment 4. Rats were subjected to a preliminary period of force-feeding as in the previous experiments, and divided into three groups: group 1 received the complete amino acid consisting of amino acid mixture B; group 2 received the tryptophan-free diet; group 3 received the tryptophan-free diet containing 0.01% niacin. In each case, rats were killed 3 hours after the first administration on day 5 of experimental feeding; urine collection periods were the same as in experiment 2.

Analytical procedures. The determination of nitrogen was made by the semimicro-Kjeldahl method, and the amount of MNA in the urine was determined by Nose and Ueda's modification (7) of the acetone-condensing method of Huff and Perlzweig (8). Liver fat was estimated in the dried and ground livers pooled from rats in each group by ethyl ether extraction in a Soxhlet extractor for 20 hours. TPase activity was determined by the method described by Knox and Auerbach (9). Tyrosine-α-ketoglutarate transaminase (TKase) (EC. 2.6.1.5) activity was measured according to the procedure of Canellakis and Cohen (10). Estimation of glutamate-pyruvate transaminase (GPT) (EC. 2.6.1.2) was made according to Caldwell and McHenry (11).

RESULTS

Experiment 1. Urinary nitrogen and MNA of rats fed a threonine-free, threonine- and tryptophan-free, threonine-free and high tryptophan, or complete amino acid diet. Daily food consumption of rats during the experimental period was 7.0 to 7.3 g. Changes in the body weight are shown in figure 1a. The body weight of rats fed any of the experimental diets gradually decreased during the experimental period, but the decrease in group 1 (complete amino acid diet) was the least. Because there was no nicotinic acid in the amino acid diets, and the level of nitrogen in these diets was lower than that in the casein diet given to rats before the experimental period, levels of urinary nitrogen and MNA in all the groups dropped sharply after rats were shifted to the experimental

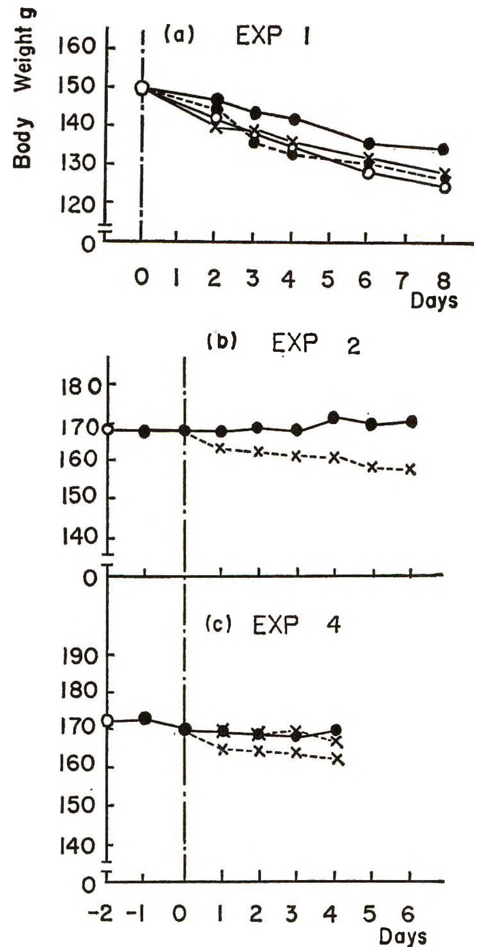

Fig. 1 Body weight changes in rats fed experimental amino acid diets. Each point represents the average of five to six rats: (a) ●——● complete amino acid diet; ●.....● amino acid diet of threonine-free and 0.26% tryptophan; ○——○ amino acid diet of threonine-free and 0.13% tryptophan; ×——× threonine- and tryptophan-free amino acid diet. (b) ●——● complete amino acid diet; ×.........× threonine-free amino acid diet. (c) ●——● complete amino acid diet; ×——× tryptophan-free amino acid diet; ×.........× tryptophan-free amino acid diet with niacin.

diets as shown in figures 2 and 3, respectively. Thereafter, the urinary nitrogen levels of group 2 (threonine-free diet), group 3 (threonine- and tryptophan-free diet), and group 4 (threonine-free and high tryptophan diet) were clearly higher than that of group 1. On the other hand, urinary MNA excretion in group 4 was several times as high as the levels of any other group throughout the experimental period. Among the other groups, the level of urinary MNA in group 3 was the least, and that of group 2 was significantly higher than that of group 1.

Experiment 2. Urinary nitrogen and MNA, and activities of liver TPase of rats force-fed a threonine-free or complete amino acid diet. Changes in body weight are shown in figure 1b. The body weight of rats administered the complete amino acid diet remained constant or slightly increased during the experimental period; the weight of rats administered the threonine-free diet, however, gradually decreased during the experimental period. As shown in table 2, the level of urinary nitrogen in the complete amino acid group was constant, corresponding to the stability of body weight, whereas the urinary nitrogen level of the threonine-free group almost doubled. The levels of urinary MNA in both groups sharply decreased just after the beginning of the experimental period as it did in experiment 1. Thereafter, the level of MNA in the group given the threonine-free diet increased, whereas that in the control

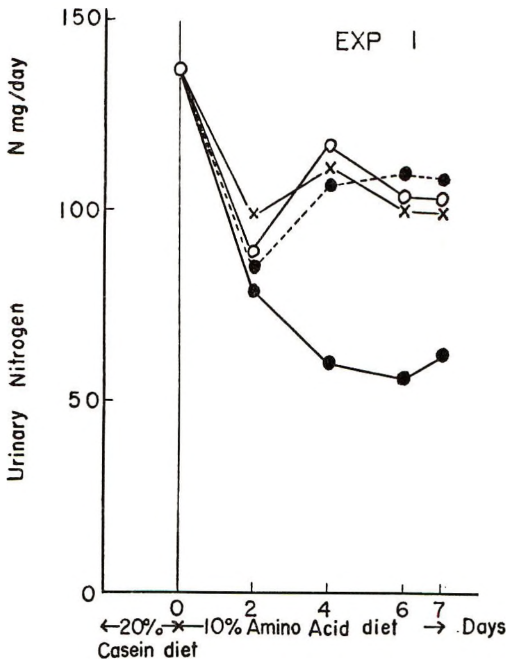

Fig. 2 Urinary nitrogen of rats fed amino acid diets. Urinary nitrogen (milligrams per day) was measured in 2-day-urine collections (except last 1 day) of each rat in the four experimental groups. Each point represents the average of six rats: ●———● complete amino acid diet; ●........● amino acid diet of threonine-free and 0.26% tryptophan; ○———○ amino acid diet of threonine-free and 0.13% tryptophan; ×———× threonine- and tryptophan-free amino acid diet.

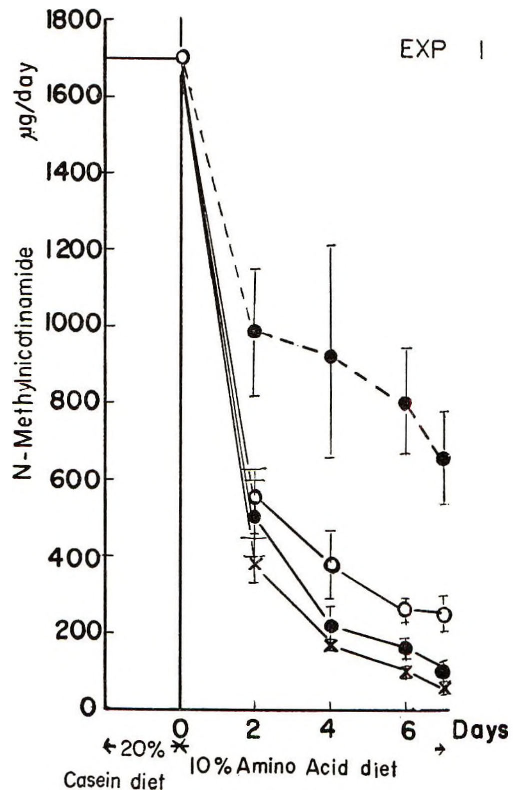

Fig. 3 Urinary N^1-methylnicotinamide of rats fed amino acid diets. Urinary N^1-methylnicotinamide (micrograms per day) was measured in 2-day-urine collections (except last 1 day) of each rat in the four experimental groups. Each point and bar represents the mean of six rats and standard deviation, respectively: ●———● complete amino acid diet; ●.....● amino acid diet of threonine-free and 0.26% tryptophan; ○———○ amino acid diet of threonine-free and 0.13% tryptophan; ×———× threonine- and tryptophan-free amino acid diet.

group maintained the decreased level as shown in table 2. The liver TPase in the experimental group showed the highest level on day 5 of the experiment, but abruptly decreased on day 7 as shown in table 3. The increase of TPase and the fat content of liver are highest on day 5 as indicated in table 3.

Experiment 3. Activities of liver TPase, and TKase of rats force-fed a threonine-free or a complete amino acid diet. On day 5 of the experimental feeding of the threonine-free or complete amino acid diet, after the preliminary period of force-feeding, enzyme activities in rats just before, and 3 hours after, administration of each diet were determined. The results are shown in table 4. TPase activity in rats fasted for 14 hours was about the same in control and experimental groups. The activity was significantly increased 3 hours after administration of the threonine-free diet, but was not much changed by recent feeding in the control group. The TKase

TABLE 2

Changes in levels of urinary nitrogen and N^1-methylnicotinamide (MNA) in rats administered a threonine-free diet (T) and complete amino acid diet (C)

	Diet	No. of rats	Collection period of urine during experimental feeding		
			Day 1–2	Day 3–4	Day 5–6
Urinary nitrogen,[1] mg/day	C	6	55 ± 13 [2]	60 ± 14	56 ± 7
	T	6	61 ± 17	116 ± 20	105 ± 9
Urinary MNA,[1] μg/day	C	6	42 ± 14	47 ± 19	57 ± 25
	T	6	116 ± 27	219 ± 47	236 ± 48

[1] Levels of urinary nitrogen and MNA in the preliminary feeding were 66 ± 11 mg/day and 628 μg/day, respectively.
[2] Mean ± SE.

TABLE 3

Effect of a threonine-free diet on tryptophan pyrrolase activity and fat content of liver

	Day of experimental feeding		
	3	5	7
Tryptophan pyrrolase [1]	9.3 ± 0.9 [2] (6) [3]	10.7 ± 0.6 (6)	8.5 ± 1.5 (6)
Fat [4]	343	685	321

[1] As micromoles kynurenine per liver per hour. The value of the control group during the experimental period was 7.7 ± 1.4 (6). Probability of error in significance of the difference between the value of TPase on day 5 of the group fed the threonine-free diet and the value of the control group was 5 to 10%.
[2] Mean ± SE.
[3] Numbers in parentheses are number of rats.
[4] Expressed as milligrams per liver. Fat content of liver in the control group during the experimental period was 283. Each value was obtained from the pooled livers of six rats.

TABLE 4

Changes in liver enzyme activities of rats just before and 3 hours after administration of diet

	Diet	Administration of diet	
		Just before	3 hr after
Tryptophan pyrrolase [1]	complete	4.9 ± 1.16 [2] (5) [3]	5.7 ± 1.14 (5)
	threonine-free	4.8 ± 1.22 (4)	8.6 ± 0.85 (4)
Tyrosine-α-ketoglutarate transaminase [4]	complete	186 ± 29 (5)	435 ± 78 (5)
	threonine-free	386 ± 22 (4)	1195 ± 105 (4)

[1] As micromoles kynurenine per liver per hour.
[2] Mean ± SE.
[3] Numbers in parentheses are numbers of rats.
[4] As micromoles p-hydroxyphenyl pyruvate per liver per hour.

activity in rats on day 5 just before administration of diets was clearly higher in the group given the threonine-free diet than that given the complete amino acid diet. The activities were increased by feeding either diet, but the increase was remarkably greater in the threonine-free group than in the control group.

Experiment 4. Urinary nitrogen and MNA, and activities of liver TPase, TKase, and GPT of rats force-fed a tryptophan-free, tryptophan-free with niacin, or complete amino acid diet. Changes in body weight from the preliminary force-feeding for 2 days to day 5 of the experimental force-feeding are shown in figure 1c. The body weight of rats given the complete amino acid diet (group 1) and the tryptophan-free with niacin (group 3) remained constant, but rats given the tryptophan-free diet (group 2) lost weight during the experimental period. Even though there was no change in body weight in group 3, the amount of urinary nitrogen, like that in group 2, showed a remarkable increase from that in the preliminary force-feeding period as shown in table 5. Table 5 also shows changes in the urinary MNA level. The urinary MNA in group 3 was slightly higher than that in the preliminary feeding period, whereas in groups 1 and 2 it sharply decreased after the beginning of the experimental feeding. TPase activities in groups 2 and 3 were slightly lower than those in group 1, but TKase and GPT activities in groups 2 and 3 were significantly higher than those in group 1 as shown in table 6.

DISCUSSION

N^1-Methylnicotinamide is one of the major metabolites of nicotinic acid in man, rat, horse, calf and pig, and nicotinic acid can be replaced with tryptophan for the growing animals. Since Koeppe and Henderson (5) reported that tryptophan served more effectively as a niacin source when a relative lack of some other amino acid limited its use for protein formation in rats, it was supposed that an appreciable amount of the excess tryptophan in rats fed the threonine-free diet was converted into

TABLE 5

Changes in levels of urinary nitrogen and MNA in rats administered the tryptophan-free diet (TR) with or without niacin, and the complete amino acid diet (C)

	Preliminary feeding	Diet	Collection period of urine during experimental feeding	
			Day 1–2	Day 3–4
Urinary nitrogen [1]	100	C	109	125
		TR	187	194
		TR-niacin [2]	194	200
Urinary MNA [1]	100	C	24	11
		TR	16	10
		TR-niacin [2]	126	110

[1] These values are shown as the ratio to the level in the preliminary feeding, as 100, and are the average of six rats, respectively.
[2] The diet contained 10 mg niacin/100 g.

TABLE 6

Effect of tryptophan-free diets on activities of enzyme in liver of rats

Diet	Complete amino acid	Tryptophan-free	Tryptophan-free + niacin
No. of rats	5	5	5
Tryptophan pyrrolase [1]	4.6 ± 1.44 [2]	3.0 ± 0.51	2.6 ± 0.26
Tyrosine-α-ketoglutarate transaminase [3]	303 ± 34	664 ± 48	701 ± 76
Glutamate-pyruvate transaminase [4]	2.7 ± 0.54	4.2 ± 0.42	4.9 ± 0.79

[1] As micromoles kynurenine per liver per hour.
[2] Mean ± SE.
[3] As micromoles p-hydroxyphenyl pyruvate per liver per hour.
[4] As millimoles pyruvate per liver per hour.

nicotinic acid and excreted in the urine as MNA. The supposition is confirmed by the results in this study. Namely, the level of urinary MNA in experiment 1 was highest in rats fed the threonine-free and high tryptophan diet, and was higher in rats fed the threonine-free diet than in rats fed the threonine- and tryptophan-free or the complete amino acid diet, in contrast to the fact that amounts of urinary nitrogen increased to the same extent in all groups except those receiving the complete amino acid diet. Moreover, the level of urinary MNA increased remarkably in rats given the threonine-free diet in contrast to the control group, but little in rats receiving the tryptophan-free diet, as observed in experiments 2 and 4, respectively. The extremely high level of urinary MNA in rats given the tryptophan-free diet containing niacin in experiment 4 was due primarily to the nicotinic acid added to the diet.

Knox and Mehler (12) and Knox (13) demonstrated that TPase activity in the liver of rats could be induced by giving hydrocortisone or a large amount of tryptophan; it was distinguished as hormone induction and substrate induction, respectively. TPase in the liver of rats receiving the threonine-free diet in experiment 2 increased progressively until day 5 of the feeding, and its change and the changes in urinary MNA and nitrogen are in the same direction. Moreover, it was observed in experiment 3 that the increase of TPase activity 3 hours after feeding the threonine-free diet was greater than that after feeding the complete amino acid diet. However, TPase activity increased very little in rats given the tryptophan-free diet or the diet with niacin in experiment 4. Therefore, it is presumed that changes in TPase activities in these experiments may be induced by the relative excess of tryptophan in a diet free of an essential amino acid except tryptophan.

On the other hand, it has been reported that the activity of TKase in the liver of rats is induced by hydrocortisone or tyrosine, which is an effective inducing factor only if the adrenal gland or hydrocortisone is present (14), and the activity of GPT in the liver of rats is affected by the level of protein in diets or administration of hydrocortisone (15). The activities of TKase and GPT in the liver of rats given the tryptophan-free diet in experiment 4 significantly increased, but the activity of TPase did not. Increase of TKase was also observed 3 hours after feeding the complete amino acid diet or the threonine-free diet in experiment 3, in which the increase was greater in the latter case. In addition, some observations in our laboratory have indicated that TKase activity increased when rats were administered a phenylalanine-free diet whether it contained tyrosine or not. These changes in activities of TPase and TKase could be considered to be in close agreement with the relationship between the activities of these enzymes and administration of their substrates or hydrocortisone reported by Knox et al. (12–14) mentioned above.

Changes in TPase activity in rats administered the threonine-free diet were in the same direction with changes in the urinary MNA in experiment 2, and a similar relationship might be inferred from the results of experiment 4. Therefore, TPase activity in liver might be useful as an index of changes in tryptophan metabolism in such feeding conditions.

Samuels et al.,[3] Sidransky and Farber (16) and Van Pilsum et al. (17) all reported fatty liver as an effect of force-feeding diets devoid of a single essential amino acid. The results in this study showed clearly the acute accumulation of lipid in the liver of rats administered the threonine-free diet for only 4 days. The phenomenon may be explained by the conversion of deaminated amino acids into fat as mentioned by Samuels et al.[4]

Fisher (18) demonstrated that rats lost weight on a threonine-free diet, and that the food intake was less than one-half that consumed on the complete diet. In the present study the low food intake of rats fed the threonine-free diet was observed in experiment 1. The low food intake might be caused by an adaptation to prevent metabolic disorders due to feeding the threonine-free diet. These metabolic disorders may be elucidated by the results of the force-feeding in experiment 2; namely, the abrupt changes in the TPase activity, the

[3] Samuels, L. T., H. C. Goldthorpe and T. F. Dougherty 1951 Metabolic effects of specific amino acid deficiencies. Federation Proc., *10:* 393 (abstract).
[4] See footnote 3.

fat content of the liver, and the plasma threonine concentration [5] of rats force-fed the threonine-free diet on day 7 of the experimental period were noticeable, and these changes might be a metabolic disorder that occurred by force-feeding the threonine-free diet for 6 days.

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[5] The lowest concentration of plasma threonine, which was reached on day 3 to day 5, was suddenly increased on day 7 (unpublished data, plasma threonine was 8.85, 0.48, 0.48 and 2.34 mg/100 ml, respectively, on days zero, 3, 5, and 7). Therefore, it is considered that the metabolic disorder corresponds directly to the plasma free threonine concentration.

Evidence for a Nonabsorptive Antihypercholesterolemic Action of Phytosterols in the Chicken [1]

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ABSTRACT Soy sterols were administered orally (1% of diet) and subcutaneously (20 mg/day) to chicks with hypercholesterolemia induced by feeding a low protein, cholesterol-containing diet (8% protein, 5% medium-chain triglycerides (MCT), and 0.5% cholesterol). Sterol levels and patterns in plasma, tissues, and excreta were determined by spectrophotometric and gas-liquid chromatographic analysis. Strong evidence for a nonabsorptive antihypercholesterolemic action of soy sterols included: similar liver cholesterol reductions for both oral and subcutaneous soy sterol administration, and reduction by subcutaneous soy sterol injection of endogenous hypercholesterolemia due to ova resorption induced by feeding 0.04% Nicarbazin to laying hens. Estimation of absorptive effects from intestinal wall concentrations of cholesterol, and subtraction of these effects from total antihypercholesterolemic effects, indicated approximately equal absorptive and nonabsorptive activity from oral soy sterols and a predominantly nonabsorptive effect of subcutaneously administered soy sterols. A comparison of the oral administration of 1% soy sterols and 1% wheat germ sterols to chicks given a hypercholesterolemic diet (25% whole egg powder) resulted in a greater antihypercholesterolemic response from soy sterols which contain more campesterol (36%) than from wheat germ sterols (25% campesterol). Campesterol appears to be the major active component of soy and wheat sterols in relation to their antihypercholesterolemic activity.

A brief review of the antihypercholesterolemic effects of phytosterols and evidence for their absorption has been presented in a previous article (1). Several reports have suggested that these sterols may have an effect on cholesterol metabolism after they have been absorbed from the digestive tract, since no increase in cholesterol excretion was found in animals fed low levels of corn oil sterols in comparison to others fed no phytosterols (2–4). The injection studies of Gerson et al. (5–7) which were designed to by-pass the absorptive process, provided evidence for an increased rate of cholesterol turnover in rats injected with β-sitosterol. Unpublished work from this laboratory [3] showed that daily injection of 0.5 mg corn oil sterols for 10 days into cockerels fed a hypercholesterolemic diet resulted in a 20 mg/100 ml plasma cholesterol decrease compared with control-injected birds ($P < 0.1$). The present investigation represents an expansion of these phytosterol-injection studies in the domestic fowl.

METHODS

General. White Leghorn hens were used in one experiment, and 1-day-old, cross-bred (Columbian ♀ × New Hampshire ♂) male or female chicks in two others. The hens were fed a standard corn-soybean laying diet. The composition of the chick diets is given in table 1. These diets were hypercholesterolemic through inclusion of crystalline cholesterol plus oil (medium-chain triglycerides (MCT)) or whole egg powder.

Terminal blood samples were taken by cardiac puncture; the birds were then killed with chloroform and samples taken of the liver and other tissues.

Preliminary tests of phytosterol-injection procedures showed that the more conveniently administered subcutaneous in-

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[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, based in part upon a thesis presented by the senior author in partial fulfillment of requirements for the M.S. degree to Rutgers University, New Brunswick, New Jersey.

[2] NDEA Title IV Fellow.

[3] Boorman, K. N. 1965 The absorption of dietary plant sterols by the fowl. M.S. thesis, Rutgers University, New Brunswick, New Jersey.

jections in the back of the neck produced effects very similar to those from intravenous injections; this technique was therefore adopted for use in these studies.

Experiment 1. Twenty-three White Leghorn hens in normal egg production, which had been fed a standard laying mash and which weighed approximately 1.5 kg each, were used. Nicarbazin,[4] a coccidiostat which induces ova resorption and resultant hypercholesterolemia in laying hens (8), was mixed into the diet at a level of 0.04%, and the hens stopped laying within 2 weeks. After 3 weeks of this treatment, a 1-ml blood sample was taken from the brachial vein of each hen. They were kept on the Nicarbazin-supplemented diet for 3 months, and then 12 hens were selected at random to form two injection groups of 6 birds each. Prior to the start of injections, a 1-ml blood sample was taken from each hen. One group received subcutaneous injections [5] of buffer containing 8.3 mg/ml lecithin [6] and 25 mg/ml soy sterols; [7] the other group received the same buffer-lecithin mixture containing 50 mg/ml soy sterols. Each hen received daily a 1-ml injection for 10 days while they were fed the Nicarbazin-containing feed throughout the experiment. Terminal blood and liver samples were taken 3 days after the last injection. Spectrophotometric analyses for total sterols (calculated as cholesterol) were carried out on these samples.

Experiment 2. One-day-old female chicks were fed a standard chick starter ration until 8 days of age. They were then fed a 20% protein diet (see table 1 for composition) until 3 weeks of age. The chicks were then weighed, and 48 of average weight divided into three treatment groups. The control group received an 8% protein diet (for composition see table 1) and a control injection (buffer, containing 40 mg/ml of lecithin); group 2 received the same diet supplemented with 1% soy sterols and the same buffer-lecithin injection; group 3 also received

[4] Trade name for 4,4'-dinitrocarbanilide-2-hydroxy-4, 6-dimethylpyrimidine, generously supplied by Merck & Company, Inc., Rahway, N. J.

[5] Sterol injection mixtures were prepared with 0.05 M phosphate buffer (pH 7.3) containing 0.2% benzoic acid as a preservative. Lecithin was suspended in a small amount of buffer with the aid of heating and stirring. Sterols were mixed with the undiluted, chilled lecithin-buffer mixture using a mortar and pestle. Additional buffer solution was used to rinse the mortar and pestle, and to bring the suspension to the desired concentration. Four 2-minute mixings were made of the sterol suspension in a high speed blender with care taken to avoid heating the mixture.

[6] Soy lecithin refined, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[7] Sterol 115, generously supplied by General Mills Kankakee, Ill.

TABLE 1

Percentage composition of diets

Ingredient	Amount		
	Exp. 2		Exp. 3
	%	%	%
Whole egg powder (feed grade) [1]	—	—	25.0
Soybean meal (50% protein)	40.0	16.0	25.0
Medium-chain triglyceride [2]	5.0	5.0	—
Fiber [3]	3.0	3.0	3.0
Limestone with trace minerals [4]	1.0	1.0	1.0
Dicalcium phosphate	2.0	2.0	2.0
Sodium chloride	0.5	0.5	0.5
Cholesterol	0.5	0.5	—
Vitamin mix [5]	0.2	0.2	0.2
Choline chloride (70%)	0.2	0.2	0.2
DL-Methionine (feed grade)	0.2	0.2	0.1
Santoquin [6]	0.02	0.02	—
Glucose [7,8]	to 100	to 100	—
Cornstarch [8]	—	—	to 100

[1] Supplied 10% protein, 12% fat, and 0.6% cholesterol.
[2] Saturated, primarily C_8 and C_{10} fatty acids, generously supplied by Drew Chemical Corporation, Boonton, N. J.
[3] Solka Floc, Brown Company, Berlin, N. H.
[4] Mico concentrate, Limestone Corporation of America, Newton, N. J.
[5] For composition see Fisher and Griminger (9).
[6] 6-Ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, Monsanto Chemical Company, St. Louis, Mo.
[7] Cerelose, Corn Products Company, New York, N. Y.
[8] Additions of protein, fat, and sterols to the diet were made at the expense of glucose or starch.

the same diet but for the injection the buffer-lecithin mixture was supplemented with 100 mg/ml soy sterols. Each chick received a daily subcutaneous injection of 0.2 ml for 13 days. After 1 week, 24-hour excreta collections were made, and feed intake was measured during this period. At the end of 2 weeks, blood, liver, and intestinal wall segment [8] samples were taken for lipid extraction, followed by spectrophotometric analysis of total sterols and gas-liquid chromatographic analysis of sterol patterns.

Experiment 3. Forty-eight day-old male chicks were divided into three groups; group 1 received the basal egg powder-containing diet (see table 1 for composition); group 2 received the same diet supplemented with 1% wheat germ oil sterols; [9] and the remaining group received the same diet supplemented with 1% soy sterols. Twenty-four hour excreta collections were made at the end of 2 weeks, and at 3 weeks, terminal blood and liver samples were taken for analysis as in experiment 2.

Analytical procedures. The tissues [10,11] for analysis were homogenized for 2 minutes in a blender [12] with a volume of chloroform–methanol (2:1, v/v) equal to 17 to 20 times the weight of the tissue in grams. The homogenate was allowed to settle, and was finally filtered into a volumetric flask with additional solvent used to rinse out the solid residue and to bring the solution to volume.

Aliquots of the lipid extracts equivalent to between 10 to 25 mg of total sterol (determined by spectrophotometric analysis of separate samples) were placed in saponification flasks and the solvent was completely evaporated in a flash evaporator.[13] Two milliliters 50% aqueous potassium hydroxide and 10 ml 95% ethanol were added to the lipid residue which was then saponified for 1 hour under a water reflux condenser, on a steam bath. After cooling, the saponificate was tranferred to a separatory funnel with the aid of 10 ml deionized water. The saponificate was extracted with two 40-ml portions of petroleum ether, and the combined extracts were washed with 20 ml deionized water. Two additional washings were made with 20-ml portions of 1 N NaOH and deionized water, respectively. The petroleum ether extract was then poured through a funnel containing about 10 g anhydrous sodium sulfate (held in place by a loosely packed glass wool plug) into a flask for flash evaporation of the solvent. Two 2-ml portions of chloroform were used to rinse the sterol residue from the flask through a small funnel into a small conical test tube. The chloroform was evaporated under a stream of nitrogen at room temperature, and a measured volume of chloroform (0.2 to 0.4 ml), sufficient to make a 5% sterol solution, was added prior to gas-liquid chromatography.[14]

Whenever possible, chromatograms of samples were compared with those of standard sterol mixtures of similar composition which were obtained on the same day. Peak areas were measured with a planimeter, and the concentration of each sterol component was calculated by comparing its area response in the sample with its area response in the appropriate standard.

The spectrophotometric method used for tissue sterol analyses (total sterols calculated as cholesterol) was that of Zlatkis et al. (10), modified after Searcy and Berquist (11). The quantity of lipid extracts was adjusted to give spectrophotometric readings within the range of the standards (50 to 200 μg of cholesterol). All the plasma, liver, and intestinal wall

[8] The portion of the small intestine lying between the junction with the ceca and the tips of the ceca, when the tips of the ceca pointed toward the gizzard.

[9] Generously supplied by Viobin Corporation, Monticello, Ill.

[10] Fresh excreta samples were dried at 90° for 3 days, and ground to a fine powder. Five-gram samples of the powder were extracted with 100 ml chloroform-methanol (2:1, v/v) by shaking for 24 hours at room temperature.

[11] Plasma extracts were prepared as outlined in the reference cited for the spectrophotometric analysis procedure.

[12] Waring Blendor, Waring Products Company, Winsted, Conn.

[13] Buchler Instruments, Fort Lee, N. J.

[14] Barber-Colman model 5320-100 flame ionization gas chromatograph was used with 183 cm by 6 mm od stainless steel coiled column packed with 3% SE-30 on 80/100 Gas Chrom Q (pre-packed column was purchased from Applied Science Laboratories, Inc., State College, Pa.). The column was operated at 235 to 242° with the injector 30° above the column and the detector 25° above the column. Gas flow rates were as follows: (in milliliters per minute) nitrogen, 125; hydrogen, 30; air 400 ± 40. The recorder range was 1 to 5 mv with the attenuator set at 1000X or 300X for cholesterol peaks, and at 30X or 10X for phytosterol peaks. One to five microliters of 5% solutions of sterols in chloroform were introduced into the chromatograph. Approximate retention times were as follows: (in minutes) cholesterol, 22; campesterol, 29; stigmasterol, 32; β-sitosterol, 36.

samples analyzed contained more than 95% cholesterol and less than 5% phytosterols, and thus the error introduced (by the reduced chromogenicities of the phytosterols compared with cholesterol) in calculating total sterols as cholesterol was less than 1% for these samples. The sterols found in the excreta of the dietary phytosterol groups were composed mainly of phytosterols, and it was necessary to correct the total sterol values determined for these samples for the reduced chromogenicity. The correction, in each instance, was determined by spectrophotometric analyses of standard mixtures similar in composition to those of the excreta mixtures (whose composition was determined by gas-liquid chromatography).

RESULTS AND DISCUSSION

Experiment 1. The mean plasma cholesterol of the hens was 529 mg/100 ml after 3 weeks of Nicarbazin treatment, and increased during the period prior to the start of injections (after 13 weeks of treatment), when it was 632 mg/100 ml in the 12 experimental hens. Significant decreases in plasma cholesterol were observed following injections of both levels of phytosterol (table 2). Although no comparison can be made between liver sterol levels before and after phytosterol injection, it is of interest to note that the hens injected with the higher dose of soy sterols had a slightly lower liver sterol concentration despite a higher percentage of liver fat than the hens injected with half the amount of soy sterols. This difference becomes more apparent when the liver sterol concentration is expressed as a percentage of total liver lipid concentration. On this basis the former group had 4.02 ± 0.91% sterol compared with 5.51 ± 1.20% sterol in the latter group ($P = 0.3$). Previous studies in this laboratory (9, 12), as well as by others (13), have shown a direct relationship between total liver lipid and liver cholesterol in the chicken.

The results of this experiment provided the following evidence for an antihypercholesterolemic effect of phytosterols not involving the absorptive process: a) an increasing hypercholesterolemia was reversed after the soy sterol injections, and it is unlikely that this effect might have occurred spontaneously since Weiss (8) found that the plasma cholesterol of Nicarbazin-fed hens remained elevated at levels between 600 to 900 mg/100 ml from 4 to 32 weeks of Nicarbazin treatment; b) the mode of administration bypassed the digestive tract; c) an absorptive effect was ruled out because the dosage levels of soy sterol in this experiment (17 and 33 mg/kg body weight) were considerably lower than the dosage used in experiment 2 where the absorptive effect of injected soy sterols was found to be negligible. Although the hypercholesterolemia was induced by ova resorption, this technique

TABLE 2

Effects of two levels [1] of subcutaneous soy sterol (SS) injections on plasma and liver sterol, and liver fat of laying hens with Nicarbazin-induced ova resorption (exp. 1) [2]

Phytosterol injections	Total sterol concentration		Liver fat
	Plasma	Liver	
	mg/100 ml	*mg/g*	*%*
Before	656 ± 48 [3]	—	—
After 25 mg SS/day [4]	364 ± 57	6.07 ± 0.19	13.8 ± 3.4
Before versus after	$P < 0.001$	—	—
Before	608 ± 101	—	—
After 50 mg SS/day [4]	352 ± 68	5.72 ± 0.26	19.1 ± 4.7
Before versus after	$P < 0.05$	—	—
25 mg versus 50 mg	ns [5]	ns	ns

[1] Six hens were given each level of injection.
[2] Normal laying hens were fed 0.04% Nicarbazin in their diet and had ceased laying eggs for 3 months prior to the start of injections.
[3] Mean ± SE.
[4] Injections were made daily for 10 days, and terminal blood and liver samples were taken 3 days after the last injection.
[5] ns = not significant.

has general applicability since Andrews et al. (14) found that the livers of laying hens synthesize most of the egg cholesterol rather than the ovary, which was previously considered to have been the site of this synthesis.

Experiment 2. Oral and subcutaneous administration of soy sterols produced significant and similar reductions in liver cholesterol (table 3). The plasma cholesterol reduction due to oral administration was significantly greater than that due to subcutaneous injection, although the latter was highly effective. Furthermore, the concentration of intestinal wall cholesterol was significantly lower in the orally treated group compared with the control group, whereas the latter and the subcutaneously treated groups were not significantly different from each other. These observations support the hypothesis that oral administration of plant sterols mediate their action, at least in part, through an effect on the absorptive process, while subcutaneous administration exerts its action via a different, as yet unrecognized pathway.

Cholesterol levels in excreta were similar for all three treatment groups (last column, table 5), which made it difficult to use this measurement as an index of cholesterol absorption (the soy sterol treatments may have caused reductions in the amounts of cholesterol resecreted into the gut). The above observations, as well as changes in intestinal wall cholesterol concentration observed in a preliminary experiment,[15] suggested that these concentrations were a more reliable index of cholesterol absorption than cholesterol excretion. When reductions in intestinal wall cholesterol were compared with reductions in the plasma and liver cholesterol pool (table 4), it appeared that decreased cholesterol absorption could account for only part of the soy sterol effect. The magnitude of the nonabsorptive effect might be related to the difference between the percentage reduction in the cholesterol pool and the percentage reduction in intestinal wall cholesterol. Based upon this interpretation, it appears that the nonabsorptive effects of both treatments were approximately equal. It is, therefore, tempting to view the similarity of plasma, liver, and intestinal campesterol levels found in the oral and injected soy sterol groups (table 5) as being associated with the nonabsorptive effects. The interpretation is reinforced by the demonstration that campesterol is absorbed to a greater extent than β-sitosterol (1), as shown in this experiment by the high campesterol-to-β-sitosterol ratios for the group given oral soy sterol, in com-

[15] Control-injected and soy sterol-injected groups of chicks were each subdivided into fed and fasted lots 17 hours before killing. The cholesterol-containing feed was removed from the fasted lots, but was given to the fed lots up to the time of killing. Fasting produced equal depressions of intestinal wall cholesterol in the control-injected chicks (from 3.60 to 3.13 mg/g) compared with the soy sterol-injected chicks (from 3.38 to 2.92 mg/g).

TABLE 3

Comparisons of the effects of orally and subcutaneously administered soy sterols on plasma, liver, and intestinal wall cholesterol, and liver fat in chicks fed a diet containing 8% protein, 5% MCT, and 0.5% cholesterol (exp. 2) [1]

Phytosterol treatment	Total sterol			Total liver lipid
	Plasma	Liver	Intestinal wall	
	mg/100 ml	*mg/g*	*mg/g*	*g*
None (16) [2]	363 ± 38 [3]	22.2 ± 2.0 [4]	6.04 ± 0.22 [4]	1.19 ± 0.04 [4]
Oral [5] (16)	183 ± 14	14.8 ± 2.0	4.94 ± 0.23	1.05 ± 0.06
Subcutaneous [6] (15)	252 ± 21	15.6 ± 1.8	5.78 ± 0.21	1.34 ± 0.06
None versus oral	$P < 0.001$	$P < 0.05$	$P < 0.01$	ns [7]
None versus subcutaneous	$P < 0.001$	$P < 0.05$	ns	$P = 0.05$
Oral versus subcutaneous	$P < 0.001$	ns	$P < 0.05$	$P < 0.01$

[1] See table 1 for complete composition of the diet.
[2] Number in parentheses indicates number of chicks per treatment.
[3] Mean ± SE (one sample per chick).
[4] Mean ± SE for eight pair-pooled samples.
[5] Diet was supplemented with 1% soy sterols.
[6] Thirteen daily injections of 20 mg soy sterols.
[7] ns = not significant.

TABLE 4

Percentage reductions in plasma and liver cholesterol pool, and in intestinal wall cholesterol from administration of oral and subcutaneous soy sterols (exp. 2)

Phytosterol treatment	Final body wt	Plasma (P) cholesterol	Liver wt	Liver (L) cholesterol	P and L cholesterol pool [1] ÷ body wt	Intestinal wall cholesterol
	g	*mg/100 ml*	*g*	*mg/g*	*mg/g*	*mg/g*
None	286 [2]	363	10.1	22.2	1.04	6.04
Oral	299	179	10.1	14.6	0.632	4.81
Subcutaneous	308	242	12.2	15.2	0.773	5.65
Reduction by oral soy sterols					39.2%	20.4%
Reduction by subcutaneous soy sterols					25.6%	6.55

[1] Plasma + liver cholesterol pool = [Plasma cholesterol × 7% × body weight] + [Liver cholesterol × liver weight], according to Leveille and Sauberlich (15).
[2] Treatment mean.

TABLE 5

Effects of oral and subcutaneous soy sterols on plasma, tissue and excreta sterol patterns of chicks fed a diet containing 8% protein, 5% MCT, and 0.5% cholesterol (exp. 2)

Phytosterol treatment	Plasma	Liver	Intestinal wall	Excreta
	mg/100 ml	*mg/g*	*mg/g*	*mg/g*
None				
Cholesterol	363 [1]	22.2	6.04	27.0
Campesterol [2]	—	—	tr [3]	0.1
β-Sitosterol	—	—	tr	0.4
Oral				
Cholesterol	179	14.6	4.81	25.2
Campesterol	3	0.2	0.09	26.8
β-Sitosterol	1	0.1	0.04	34.6
Campesterol: β-Sitosterol ratio	3.0	2.0	2.2	0.8
Subcutaneous				
Cholesterol	242	15.2	5.65	27.4
Campesterol	4	0.2	0.08	0.8
β-Sitosterol	6	0.2	0.06	1.2
Campesterol: β-Sitosterol ratio	0.7	1.0	1.3	0.7

[1] Mean of duplicate pooled samples analyzed by gas-liquid chromatography.
[2] Includes trace amounts of stigmasterol.
[3] tr = trace.

parison with those given sterol by injection (table 5).

As in experiment 1, the liver fat content was greater for the group given subcutaneous soy sterols in comparison with the control group, despite a significantly lower cholesterol concentration for the former.

Experiment 3. This experiment was undertaken to compare the antihypercholesterolemic activities of soy sterols with those of wheat germ oil sterols; the latter supply campesterol in a ratio of 1.3 relative to β-sitosterol, whereas the former have a campesterol-to-β-sitosterol ratio of 1:1.6.[16] Significant reductions in both plasma and liver cholesterol were obtained with both soy and wheat germ sterols compared with the control diet (table 6). Liver cholesterol reduction was significantly greater in the group fed soy sterol than in the group fed

[16] Duplicate gas-liquid chromatographic analyses of each sterol mixture showed that the soy sterols represented 36% campesterol, 7% stigmasterol, and 57% β-sitosterol; the wheat germ oil sterols contained 25% campesterol and 75% β-sitosterol.

TABLE 6

Comparison of dietary wheat germ sterol and soy sterol on plasma, liver and excreta sterols of chicks fed a diet containing 25% whole egg powder (exp. 3)

Phytosterol	Plasma sterol [1]	Liver sterol			Excreta sterol		
		Total	Cholesterol	Campes-terol	Cholesterol	Campes-terol	β-Sitosterol
% of diet	*mg/100 ml*	*mg/g*	*mg/g*	*mg/g*	*mg/g*	*mg/g*	*mg/g*
None [2]	250 ± 13.2 [3]	24.2 ± 1.0 [4]	24.2 [5]	tr [5,6]	6.2 [5]	0.5 [5]	0.7 [5]
Wheat germ oil, 1	216 ± 5.3	10.2 ± 1.1	10.1	0.05	9.4	12.5	37.6
Soy, 1	196 ± 5.4	7.4 ± 0.2	7.3	0.14	14.2	21.0	29.6
None versus wheat germ oil	$P < 0.01$	$P < 0.001$					
None versus soy	$P < 0.001$	$P < 0.001$					
Wheat germ oil versus soy	ns [7]	$P < 0.02$					

[1] Total sterols calculated as cholesterol.
[2] The basal diet contained soybean meal which supplied trace amounts of sterols (1).
[3] Mean ± SE (16 chicks/treatment).
[4] Mean ± SE (four pooled lots per treatment).
[5] Mean of duplicate lots analyzed by gas-liquid chromatography.
[6] tr = trace.
[7] ns = not significant.

wheat germ sterol; there was almost three times as much liver campesterol in the former group compared with the latter. A greater increase in cholesterol excretion was also noted for the soy sterol group. This increase appears to be directly related to the higher campesterol content of the dietary soy sterol since the total phytosterol concentration in the excreta was the same for the soy and wheat germ sterol groups.

These findings suggest that both absorptive and nonabsorptive antihypercholesterolemic effects are mediated primarily by campesterol rather than by β-sitosterol. Campesterol is structurally more similar to cholesterol than β-sitosterol and might therefore be expected to exert greater interference with cholesterol absorption and metabolism. Alfin-Slater has suggested that the high sterol content of wheat germ oil (5.4% compared with 0.4 to 1.0% in other vegetable oils (16)) might explain the observation that, in comparison with other vegetable oils of similar fatty acid composition, wheat germ oil produced a greater reduction in liver cholesterol of cholesterol-fed rats (9.4 versus 32.5 to 61.4 mg/g (17)). This worker further reported the concurrent finding of similar plasma cholesterol levels for the rats fed the various vegetable oils. This may be interpreted as providing additional evidence for a nonabsorptive effect of wheat germ sterols, since an antihypercholesterolemic effect based solely upon interference with cholesterol absorption should be reflected in similar plasma and liver cholesterol reductions. This may be deduced from the results of experiment 2, in which subcutaneous and oral soy sterols exerted similar effects on liver cholesterol reduction, whereas plasma cholesterol reduction with oral administration was considerably greater than with subcutaneous administration.

GENERAL COMMENTS

These studies provide considerable evidence for an antihypercholesterolemic action of plant sterols distinct from interference with the absorptive process: a) the antihypercholesterolemic effects of both oral and subcutaneous soy sterol treatments could only partially be accounted for by reductions in intestinal wall cholesterol; b) substantial and equal reductions in liver cholesterol in both oral and injected groups were accompanied by similar plasma and tissue campesterol levels; c) campesterol-to-β-sitosterol ratios in the oral soy sterol group indicated that campesterol was absorbed to a much greater extent than β-sitosterol, and d) campesterol appeared to be associated with the greater increase in

cholesterol excretion due to soy sterols when compared with wheat germ sterols.

These results are also interpreted to suggest that campesterol is the major active component of soy and wheat germ sterols. Further work is clearly needed to elucidate the metabolic role of campesterol relative to cholesterol metabolism.

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Calcium and Phosphorus Requirements for Maximal Growth and Mineralization of the Rat

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ABSTRACT This study was conducted to determine calcium and phosphorus requirements for both maximal growth and maximal mineralization under identical dietary conditions including defined levels of other minerals. A diet was fed which contained adequate amounts of vitamin D and all other essential organic nutrients and a 20% excess of the required amounts of other essential minerals. The estimates obtained for the daily calcium and phosphorus requirements of young male rats were: 22 mg calcium and 20 mg phosphorus for maximal growth, and 48 mg calcium and 34 mg phosphorus for maximal mineralization. The mineralization values are lower than the National Research Council estimate based on the same criterion. Relative to the calcium requirement, more phosphorus was required for growth (Ca/P = 1.1) than for mineralization, Ca/P = 1.4. A moderate reduction of the Ca/P ratio caused a slight increase in the estimates of calcium requirements for growth and mineralization.

Diets supplying the mineral requirements listed by the National Research Council (NRC) (1) were found adequate for maximal growth of young rats (2). We compared the NRC estimates of several minerals needed for growth in both the weanling rat and the human infant and found a fairly constant relationship between the requirements. It was not possible, however, to calculate this relationship for calcium and phosphorus as the usual criterion for determining these requirements in the rat, which is also the NRC criterion, is the level of each needed for maximal mineralization of bone, rather than the lower levels needed for maximal growth. As a review of the literature was uninformative, the requirement of these minerals for maximal growth was determined. Further, interest in the ratios of amounts needed for both criteria led to estimations, under the same conditions, of the calcium and phosphorus needed for maximal mineralization. The effects of moderately low Ca/P ratios on calcium requirements were also studied.

EXPERIMENTAL

Animals and diets. Male rats of the Sprague-Dawley strain, individually caged in screen-bottom, suspended cages kept in a constant-temperature room, were fed the experimental diets and distilled water ad libitum for a 3-week period. Weights and individual food intakes were determined. In the several experiments, the average initial group weights varied from 44 to 61 g and the ages varied from 24 to 28 days.

The basal diet contained: (in grams per 100 g) acid and water-washed lactalbumin,[1] 18; corn oil, 10; cellulose,[2] 5; vitamin mixture, 0.1; (2) choline chloride, 0.1; and glucose,[3] 66.8. The calcium and phosphorus content of this diet was 2 and 10/mg/100 g, respectively. A basal diet was prepared in bulk for each experiment. Diets for different groups were then prepared by adding a salt mixture to sufficient basal diet to make 1 kg.

The mineral mixtures used were based on the composition of a mixture described previously, mixture B (2). The diets contained 120% of the NRC requirements of all mineral elements except calcium and phosphorus. Calcium and phosphorus levels were obtained by adding the maximum possible amount of $CaHPO_4$ and either $CaCO_3$ or $NH_4H_2PO_4$ as required. The calcium and phosphorus contents of the diets

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[1] Lactalbumin, Sheffield, Norwich, N. Y.
[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.
[3] Cerelose, Corn Products Company, Argo, Ill.

were confirmed by analysis. The vitamin D_2 in the diets (300 IU/100 g) allowed normal calcium and phosphorus metabolism (1).

Analysis. The right femurs were cut out and stored for several weeks in chloroform–methanol, 3:1, to extract the fat. Adhering tissue was then carefully removed. The femurs were dried at 110° to a constant weight. Ashing was carried out by heating at 500° overnight. After partial cooling, 5 drops of concentrated HNO_3 were added to the ash and the ashing was continued for 1 hour. The ash was dissolved in hydrochloric acid and diluted to appropriate volumes. For carcass analysis the animals were weighed, killed, opened and dried to a constant weight at 110° in a hot-air oven. After grinding the entire carcass to a coarse powder, 5-g samples were ashed and prepared for analysis as described above. Calcium was determined by absorption spectrophotometry,[4] and phosphorus, by the Technicon automated procedure.[5]

RESULTS

Data from all experiments on weight gains, food, calcium and phosphorus intakes and food efficiency ratios are listed in table 1.

Experiment 1. Calcium requirement for maximal mineralization and growth. Diets containing several levels of calcium and phosphorus with similar Ca/P ratios (1.1 to 1.2) were fed. The phosphorus and calcium content of the carcass was determined. Plots of weight gain and carcass calcium content versus calcium intake are shown in figure 1.

[4] Analytical Methods for Atomic Absorption Spectrophotometry 1968 Perkin-Elmer Corporation, Norwalk, Conn.

[5] Autoanalyzer Methodology, N-4A 1963 Technicon Corporation, Ardsley, N. Y.

TABLE 1

Effect of calcium and phosphorus intakes on growth and food intake of rats

Exp. no.	Group	Diet content		Weight gain	Daily intake			Ca/P	FER [1]
		Ca	P		Food	Ca	P		
		mg/100 g		*g/day*	*g*	*mg*	*mg*		
1 (7) [2]	A	602	510	5.90 ± 0.15 [3]	13.0 ± 0.25 [3]	78	66	1.2	0.44
	B	362	310	5.84 ± 0.14	13.0 ± 0.28	47	40	1.2	0.45
	C	152	135	5.48 ± 0.11	13.0 ± 0.35	20	18	1.1	0.42
	D	110	100	4.75 ± 0.15	11.7 ± 0.36	13	12	1.1	0.40
	E	77	73	4.08 ± 0.10	11.7 ± 0.13	9.0	8.5	1.1	0.35
2 (8)	A	362	510	6.12 ± 0.12	13.4 ± 0.26	48	68	0.71	0.46
	B	362	310	5.96 ± 0.20	13.1 ± 0.22	47	41	1.1	0.46
	C	362	210	5.87 ± 0.21	12.7 ± 0.46	46	27	1.7	0.46
	D	362	160	5.60 ± 0.17	12.9 ± 0.27	47	21	2.2	0.43
	E	362	110	4.80 ± 0.11	12.4 ± 0.25	45	14	3.2	0.39
	F	362	73	4.23 ± 0.17	12.3 ± 0.37	44	9.0	5.0	0.34
3 (8)	A	202	210	5.96 ± 0.08	14.8 ± 0.20	30	31	0.97	0.40
	B	202	190	5.90 ± 0.07	14.8 ± 0.16	30	28	1.1	0.40
	C	202	130	5.77 ± 0.15	14.8 ± 0.16	30	19	1.6	0.39
	D	202	100	5.21 ± 0.15	14.2 ± 0.30	29	14	2.1	0.37
	E	202	73	4.43 ± 0.16	13.6 ± 0.27	27	10	2.7	0.33
4 (8)	A	752	510	5.83 ± 0.15	14.0 ± 0.46	105	71	1.5	0.42
	B	602	510	5.86 ± 0.20	14.1 ± 0.24	85	72	1.2	0.41
	C	402	510	6.06 ± 0.10	13.9 ± 0.22	56	71	0.79	0.43
	D	362	510	5.80 ± 0.16	14.0 ± 0.45	51	71	0.72	0.41
	E	272	510	5.72 ± 0.15	14.1 ± 0.25	38	72	0.53	0.40
	F	182	510	5.63 ± 0.16	13.7 ± 0.10	25	70	0.36	0.41
5 (7)	A	362	210	5.90 ± 0.06	13.8 ± 0.24	50	29	1.7	0.43
	B	242	210	6.10 ± 0.17	13.7 ± 0.34	33	29	1.1	0.43
	C	164	210	5.83 ± 0.10	13.7 ± 0.28	22	29	0.76	0.43
	D	118	210	5.41 ± 0.23	13.1 ± 0.43	15	28	0.54	0.41

[1] Food efficiency ratio, gram gain per gram food.
[2] Animals per group.
[3] Mean ± SE.

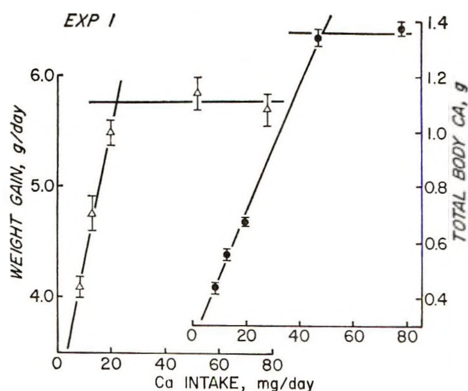

Fig. 1 Effect of various intakes of calcium on weight gain and total body calcium content of young male rats.

The plots shown in figure 1 give estimates of the calcium required for maximal growth (22 mg/day) and for maximal mineralization (48 mg/day) with corresponding phosphorus intakes of 20 and 40 mg. A plot of the average total carcass phosphorus (873 ± 14, 853 ± 20, 588 ± 9, 493 ± 7 and 419 ± 8 mg for groups A through E, respectively), versus the phosphorus intake shown in table 1 for these groups yielded an estimate of 34 mg/day as the phosphorus requirement for mineralization. As 40 mg of phosphorus was the daily intake when the calcium intake was sufficient for maximal mineralization, it was assumed that calcium was the first limiting factor.

A similar estimate of the calcium required for maximal mineralization, 50 mg/day, was obtained upon plotting calcium intake against the calcium per unit of body weight. The values for calcium, milligrams per 100 g body weight, for groups A through E, respectively, were 784 ± 23, 747 ± 29, 395 ± 10, 365 ± 20 and 314 ± 12.

Experiment 2. Phosphorus requirement for maximum mineralization. As the design of experiment 1 did not allow an estimate of the amount of phosphorus required for maximal mineralization, in experiment 2 all diets fed contained 0.36% calcium, the amount found in experiment 1 to be adequate for maximum mineralization. Phosphorus levels were varied. The extent of mineralization was estimated by femur analysis.

The plots of phosphorus intake versus total femur calcium and total femur phosphorus are shown in figure 2. The estimate of the daily phosphorus intake required for maximum mineralization, based on femur phosphorus content, was 34 mg/day, Ca/P = 1.4; the estimated requirement based on femur calcium content was similar (33 mg/day). A plot of the average femur ash (127 ± 2, 133 ± 6, 114 ± 6, 86 ± 3, 66 ± 3 and 49 ± 3 mg for groups A through F, respectively) versus phosphorus intake shown in table 1 for these groups also indicated the phosphorus requirement for mineralization was 33 mg/day. The amount of phosphorus estimated to be necessary for maximal growth was 27 mg/day; the corresponding calcium intake was 44 mg/day, Ca/P = 1.7.

Similar estimates of the daily phosphorus requirement for maximal mineralization were obtained upon plotting phosphorus and calcium per unit of bone against the phosphorus intakes of groups A through F, respectively. The estimates obtained by these plots were: from phosphorus content, 32 mg (milligrams per gram of bone, 103 ± 1, 103 ± 2, 98 ± 1, 89 ± 2, 83 ± 2 and 74 ± 2); from calcium content, 31 mg (milligrams per gram of bone, 218 ± 2, 220 ± 4, 207 ± 4, 186 ± 2, 170 ± 4 and 150 ± 5). A plot of dry femur weights against phosphorus intakes yielded an estimate of 32 mg for the daily phosphorus requirement (femur

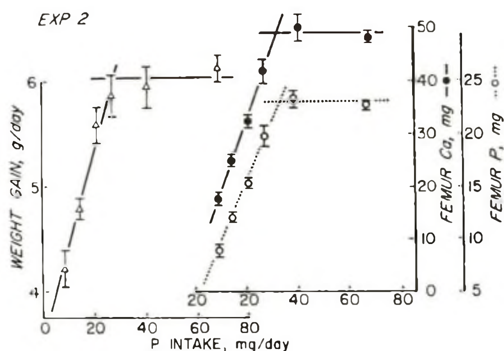

Fig. 2 Effect of various intakes of phosphorus on weight gain and femur calcium and phosphorus contents of young male rats.

weight, milligrams, 221 ± 4, 229 ±12, 203 ± 10, 173 ± 6, 146 ± 6 and 119 ± 8).

Experiment 3. Phosphorus requirement for maximum growth. In experiment 1, when varying amounts of calcium and phosphorus were fed at a constant ratio, the phosphorus intake of maximally growing animals was 20 mg/day when the corresponding daily calcium intake was 22 mg/day, Ca/P = 1.1. In experiment 2, when diets with a constant calcium content adequate for maximal mineralization were fed, the estimated phosphorus requirement for growth was 27 mg/day (Ca/P = 1.7).

To obtain a more exact estimate of the phosphorus requirement for growth, diets with various phosphorus contents, all containing a level of calcium slightly in excess of that found in experiment 1 to be necessary for maximal growth (200 mg/100 g), were fed. A plot of weight gain versus phosphorus intake (table 1, exp. 3) indicated that the phosphorus requirement was 20 mg/ day; the corresponding calcium intake was 30 mg/day; Ca/P = 1.5. This estimate agreed with the maximum requirement for phosphorus for maximal growth obtained in experiment 1.

Experiment 4. The effect of a low Ca/P ratio on calcium requirement for maximum mineralization. All diets fed contained 50 mg phosphorus/100 g of diet, the NRC requirement. Calcium levels were varied. The calcium content of the femurs was determined and the results obtained are shown in figure 3. The estimated calcium requirement was 54 mg/day. The corresponding phosphorus intake was 70 mg/day, and the Ca/P ratio was 0.77.

Experiment 5. The effect of a low Ca/P ratio on the calcium requirement for maximum growth. All diets fed contained 210 mg P/100 g of diet, an amount slightly higher than that required for maximum growth, with various levels of calcium. As shown in figure 3, the estimated calcium requirement was 24 mg/100 g of diet when the intake of phosphorus was 29 mg/day, Ca/P ratio = 0.83.

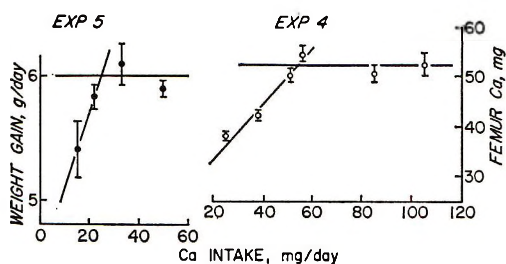

Fig. 3 Effect of various intakes of calcium in diets having Ca/P ratios of less than 1 on weight gain and femur calcium content of young male rats.

DISCUSSION

The estimated dietary requirement of a species for any essential nutrient depends upon the age, and often the sex of the animals, the criterion of adequacy and the diet.

If a criterion of maximum weight gain is used, a valid estimate requires that a diet be fed which will supply sufficient amounts of all other essential nutrients needed to support maximal growth. When rats were fed a diet which was inadequate in protein, allowing only 81% maximum growth, the mineral requirement was reduced to 70% of that required by another group growing at a maximal rate (2). In the present experiments, the groups receiving adequate calcium and phophorus gained 41 to 43 g/week, which is close to the maximal rate for young male Sprague-Dawley rats fed dry semipurified diets (2–4).

Also, diets containing excessive amounts of other nutrients which influence the requirement of the nutrient under study should obviously be avoided. As several interactions between mineral nutrients have been pointed out (5), the diets used in these tests contained only a 20% excess over NRC requirements of all other minerals except calcium and phosphorus. Further, the design of these experiments took into account the interaction between calcium and phosphorus.

The estimated requirements for calcium and phosphorus obtained in this study are listed in table 2. The average food intake of the rats receiving adequate amounts of these minerals was 14.0 g/day, the caloric density of the diet was 3.8 kcal/g and the average calorie intake was 53 kcal/day.

The daily requirements of calcium (48 mg) and phosphorus (34 mg) for maxi-

TABLE 2

Calcium and phosphorus requirements of 24- to 28-day-old rats

Criterion	Mineral	Per day	Per 100 g/diet	Per 100 kcal
Maximal weight gain	Ca, mg	22	157	41
	P, mg	20	143	38
	Ca/P = 1.1			
Maximal mineralization	Ca, mg	48	342	90
	P, mg	34	243	64
	Ca/P = 1.4			

mum mineralization in the rat (table 2) are lower than the NRC requirements of calcium (60 mg) and phosphorus (50 mg). The estimated requirements per 100 g/diet of 342 mg Ca and 243 mg P with a diet intake of 14 g/day, are much lower than those listed by the NRC, namely 600 mg Ca and 500 mg P, based on the assumption of an intake of 10 g/day.

As pointed out by Harris and Nizel (6), ordinary mixed human diets supply considerably more phosphorus than calcium (Ca/P = 0.59 to 0.84). In humans, the addition of phosphate to the diet has only a slight effect on calcium absorption (7). The FAO/WHO Expert Group concluded that the low Ca/P ratios of habitual diets are of no practical importance in human nutrition (8). The results of experiments 4 and 5 indicate that diets containing more phosphorus than calcium have only a slight effect on calcium requirements of the rat. The estimates of the calcium requirement for maximal mineralization were 54 mg/day at a Ca/P ratio of 0.77, and 24 mg/day for maximal growth when the Ca/P ratio was 0.88.

The data indicate that the ideal Ca/P ratios for growth and for mineralization may be different. The estimated Ca/P ratio for growth (1.1) was considerably lower than that required for mineralization (1.4). Thus, approximately equal amounts of phosphorus and calcium were required for maximum growth even though there is about twice as much calcium as phosphorus in bone. The explanation for this is probably related to the fact that much more phosphorus than calcium is required for the growth of soft tissues. The data of experiment 1 allowed the calculation that maximal growth resulted when the body content of phosphorus was 74% and calcium 58% of the amounts of these minerals present in the maximally mineralized rats.

The physical condition of rats receiving enough calcium and phosphorus for maximal growth, but not for maximal mineralization, was indistinguishable from rats receiving adequate amounts to achieve both. This is in accord with the conclusion of Mitchell (9) that calcium and phosphorus intakes adequate for growth, but not for maximal mineralization, have no effect on the maintenance of health. Henry and Kon (10) found that the bones of rats receiving amounts of calcium inadequate for mineralization were mineralized, at a later time, to the same adult level as those fed higher levels. Hence, in growth studies it is considered unnecessary to feed diets containing more calcium and phosphorus than are required to obtain maximal growth.

That bone mineralization in breast-fed infants is less than maximal is well recognized. Human milk supplies an average of 49 mg Ca and 22 mg P/100 kcal (Ca/P = 2.2) (11). As average growth performance of full-term infants fed at the breast of well-nourished mothers has not been exceeded by that of full-term infants consuming any artificial formulation (12), human milk obviously supplies enough calcium and phosphorus for maximal growth. However, the rate of bone mineralization is greater in artificially fed infants receiving more calcium and phosphorus (13). The relative lack of phosphorus in relation to the calcium content of human milk is indicated by the observation that administration of extra phosphate to the breast-fed infant decreases urinary calcium excretion (14), and that human milk supplies enough calcium but an inade-

quate amount of phosphorus for normal bone formation in rapidly growing premature infants (15).

In relation to the requirement of the rat listed in table 2, human milk would supply the rat with enough calcium for maximal growth but not enough for maximal mineralization. The amount of phosphorus supplied is inadequate even for maximal growth of the rat. Calculations derived from studies of the growth and calorie intake of weanling rats (16) and infants (17, 18) indicate that during the period of most rapid growth of both species, per kilogram of body weight, the weanling rat grows 6.7 times faster and consumes 4.1 times more calories than the infant. If the assumptions are made that utilizations and requirements per gram of growth of calcium and phosphorus are equal in the two species, the requirements for these minerals per 100 kcal would be 4.1/6.7=0.61 as much for the infant as for the rat. Application of this factor to the estimated requirements per 100 kcal for the rat listed in table 2, yields the predictions that the infant's requirements are 25 mg Ca and 23 mg P/100 kcal for maximal growth and 55 mg Ca and 39 mg P/100 kcal for maximal mineralization. These predictions suggest that human milk supplies approximately enough calcium for maximal mineralization (50 compared with 55 mg/100 kcal) but only enough phosphorus for maximal growth (22 compared with 23 mg/100 kcal).

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Zinc Deficiency in the Maternal Rat During Gestation, and Zinc, Iron, Copper, and Calcium Content and Enzyme Activity in Maternal and Fetal Tissues [1,2]

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ABSTRACT The effect of a zinc-deficient diet (0.75 ppm Zn) fed ad libitum to the maternal rat during gestation on zinc, copper, iron, and calcium contents, and activities of succinic dehydrogenase, lactic dehydrogenase, and ceruloplasmin in maternal and fetal tissues was studied. Compared with rats fed 9 ppm Zn, rats fed 0.75 ppm Zn showed the following effects. Feed intake of the maternal rat was reduced beginning at day 17 of fetal age. No anatomical malformations were found in 16- and 22-day-old fetuses. Dry weights of whole animal, body (whole animal less liver and heart), and liver, but not heart, were less in the 22-day-old fetuses. No significant changes were found in minerals and enzymes in maternal and fetal animals at day 16. The following changes were found at day 22. Lactic dehydrogenase activity was not altered in maternal liver, heart, and brain and fetal liver and heart, but was elevated in maternal serum. Succinic dehydrogenase activity was increased in maternal and fetal heart, but unaltered in maternal and fetal liver. Maternal serum ceruloplasmin activity was reduced. Fetal liver zinc was elevated, but copper and iron were reduced. Zinc, copper, and iron were reduced in whole animal and body. The concentration of calcium was higher in whole animal, liver, and body. Calcium was unaltered in fetal heart. Dry matter content was less in liver, adrenals, kidneys, spleen, gastrocnemius muscle, and thymus of the maternal animal. No change occurred in heart, brain, and bone. The concentration of zinc was reduced in liver, heart, and serum. Copper concentration was reduced in kidneys, spleen, and serum. The concentration of iron was lower in brain, spleen, and serum. Calcium concentration was reduced in spleen and brain, but elevated in serum and thymus.

Changes in the young rat caused by a zinc-deficient regimen have been well documented. These include reduced zinc content in tissues, reduced growth, testicular atrophy, esophageal parakeratosis, alopecia, dermal lesions, increased protein and RNA catabolism in testes, and a diminution in the activity of several enzymes (1–7).

Consequences in maternal and fetal rat as the result of feeding a zinc-deficient diet to the maternal animal during gestation have not been as extensively investigated. Hurley et al.[3] (8) found that the extent of change in fetal development was related to the degree of zinc deficiency in the maternal animal. No change in zinc content of the rat fetus nor congenital malformations were found during a mild zinc deficiency in the maternal rat. However, full-term fetuses from female rats with a more specific zinc deficiency contained less zinc and showed gross congenital malformations in a wide variety of organ systems. In a subsequent study (9), dietary zinc deficiency resulted in a fall of 45 to 60% of zinc in the plasma of pregnant female rats after 1 day on the regimen. Apgar (10) found that zinc stores in the female rat were inadequate for the stress of pregnancy and parturition and a zinc deficiency developed when a low zinc diet was fed during gestation.

The present investigation was designed to determine the effect of a zinc-deficient regimen for the maternal rat during gestation on zinc, copper, iron, and calcium content, and activity of succinic dehydro-

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[2] Supported by Public Health Service Research Grant no. HD-02103 from the National Institute of Child Health and Human Development.

[3] Hurley, L. S., H. Swenerton and J. T. Eichner 1964 Reproduction and bone zinc in zinc-deficient rats. Federation Proc., 23: 292 (abstract).

genase, lactic dehydrogenase, and ceruloplasmin in maternal and fetal tissues.

EXPERIMENTAL PROCEDURE

Female rats[4] were individually housed in stainless steel cages. Glass double-distilled water was provided in polyethylene bottles with stainless steel drinking tubes. Diets were fed ad libitum. Composition of the basal diet was as follows: (in percent) soybean protein,[5] 30; sucrose, 56.3; corn oil, 8; salt mixture (11), 2.7 DL-methionine, 0.7; vitamin mixture,[6] 1; and cellulose,[7] 1.3. The zinc-deficient diet was prepared by omitting zinc citrate in the salt mixture and demineralizing soybean protein with disodium ethylenediaminetetraacetic acid (12). Zinc content, on an as-fed basis, of the basal diet was 9 ppm and that of the zinc-deficient diet an average of 0.75 ppm.

Experiment 1. Twelve rats, ranging in weight fom 50 to 60 g, were fed the basal diet until a weight of 180 g was attained, and then were mated as previously described (13). The day sperm were found in the vaginal smear was designated as zero day age of the fetus, and rats were randomly assigned, six to each diet. Animals were killed at day 16 of fetal age, and tissues taken for enzyme analyses.

Experiment 2. Twelve rats, ranging in weight from 50 to 60 g, were treated according to the same regimen as stated in experiment 1 except that animals were killed at day 22 of fetal age, and tissues taken for enzyme analyses.

Experiment 3. Twelve rats, ranging in weight from 85 to 92 g, were subjected to the same regimen as stated in experiment 1 except that animals were killed at day 16 and tissues were taken for mineral analyses.

Experiment 4. Twelve rats, ranging in weight from 50 to 60 g, were treated according to the same regimen as stated in experiment 1 except that animals were killed at day 22, and tissues taken for mineral analyses.

At the appropriate fetal age, mothers were stunned by a blow on the head, decapitated and exsanguinated. Fetuses were removed via abdominal incision; they were stunned, decapitated and exsanguinated.

In experiments 1 and 2, litter-pooled samples of 16-day-old fetal livers, of 22-day-old fetal livers, and of 22-day-old fetal hearts were chilled in cold, 0.1 M phosphate buffer (pH 7.6). Individual liver, heart, and brain of the maternal animal were chilled in the same manner. Samples were removed from the buffer, blotted free of moisture, weighed, and homogenized in 0.9% (w/v) NaCl. Individual maternal tissues and litter-pooled fetal livers and hearts were analyzed for succinic dehydrogenase (EC. 1.3.99.1) (14) and lactic dehydrogenase (EC. 1.1.1.27) (15) activities. Maternal serum was assayed for lactic dehydrogenase (15) and ceruloplasmin (16, 17) activities.

In experiment 3, the liver of each of the 16-day-old fetuses and the maternal liver were taken for analyses. In experiment 4, liver and heart of each of the 22-day-old fetuses and liver, heart, spleen, thymus, both adrenals, both kidneys, gastrocnemius muscle, brain, bone (tibia and fibula combined), and serum of maternal animal were taken for analyses. Maternal tissues, other than serum, and fetal liver, heart, and bodies[8] were individually dried to a constant weight at 100°, and wet-digested with nitric and sulfuric acids. Tissue digests were analyzed for zinc, copper, iron, and calcium by the atomic absorption[9] technique. Minerals in maternal serum were determined without prior acid digestion. Data were analyzed statistically by the *t* test (18).

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[4] CFE strain of Sprague-Dawley rats obtained from Carworth, New City, N. Y.

[5] Soya assay protein obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

[6] Vitamins in corn starch: (amount per kilogram of diet) vitamin A, 20,000 IU; vitamin D, 2,200 IU; (in milligrams) ascorbic acid, 1017; vitamin E as α-tocopheryl acetate, 486; inositol, 110; choline dihydrogen citrate, 3715; menadione, 49.6; *p*-aminobenzoic acid, 110; niacin, 99.2; riboflavin, 22; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca pantothenate, 66; biotin, 0.44; folic acid, 1.98; and vitamin B_{12}, 29.7 μg (obtained from General Biochemicals, Inc., Chagrin Falls, Ohio).

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

[8] In this report, the term body is used in experiment 3 to mean the whole animal minus the liver, and in experiment 4 to mean the whole animal minus the liver and heart. Values for whole animal are the mathematical sum of the determined values for liver and body or liver, heart, and body.

[9] Perkin-Elmer model 303 atomic absorption spectrophotometer, Perkin-Elmer Corporation, Norwalk, Conn.

TABLE 1

Effect of zinc-deficient regimen on maternal weight gain, feed intake, and feed efficiency, and number of fetuses and fetal dry weight at 16 and 22 days of fetal age

Diet	Fetal age	Maternal wt gain	Daily feed intake	Feed efficiency [1]	Young per litter	Fetal dry wt			
						Liver	Heart	Body	Whole animal
		g	*g*			*mg*	*mg*	*mg*	*mg*
				Exp. 1					
Basal	16	59 ± 3 [2]	14.5 ± 0.2	0.26	11				
Zinc-deficient	16	49 ± 3 **	13.2 ± 0.5 *	0.21 *	11				
				Exp. 2					
Basal	22	90 ± 12	12.2 ± 0.3	0.33	10				
Zinc-deficient	22	16 ± 6 **	10.7 ± 0.6	0.09 **	11				
				Exp. 3					
Basal	16	78 ± 18	14.3 ± 0.3	0.34	11	6.1 ± 0.2		34.8 ± 2.6	40.9 ± 2.9
Zinc-deficient	16	42 ± 4	13.2 ± 0.4 *	0.20	9	5.6 ± 0.8		31.6 ± 3.5	37.2 ± 5.4
				Exp. 4					
Basal	22	85 ± 5	13.0 ± 0.3	0.30	10	35.6 ± 1.5	4.1 ± 0.2	565.1 ± 18.8	620.4 ± 10.2
Zinc-deficient	22	10 ± 5 **	10.1 ± 0.5 **	0.04 **	10	21.8 ± 1.2 **	4.2 ± 0.2	415.0 ± 14.0 **	424.9 ± 18.6 **

[1] Weight gain (g) per feed consumed (g).
[2] Mean and SE.

* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

RESULTS

A zinc-deficient regimen initiated at day zero of fetal age caused a smaller weight gain of the maternal rat at days 16 and 22 of fetal age (table 1). A comparison (fig. 1) of the weekly weight gains shows normal weight gain at days 7 and 14, but the mothers gained less, and even lost weight, during the period of rapid fetal development, namely, between days 16 and 22 of fetal age. Smaller weight gain of the mother was a reflection of reduced average daily feed intake and lower feed efficiency. A marked reduction in daily feed intake, however, did not occur until day 17 of fetal age (fig. 1). Feed efficiency was much less at day 22 than at day 16.

No significant difference was found in number of young per litter. Gross anatomical malformations were not observed in fetuses from mothers fed the zinc-deficient diet. Though dry weights of liver, body, and whole animal were less at day 16 of fetal age, the differences were not significantly different (table 1). Dry weights of liver, body, and whole animal were significantly less in the 22-day-old fetuses of zinc-deficient treatment. No difference was found in dry matter content of the fetal heart.

Data illustrating the effect of the zinc-deficient regimen on activity of lactic dehydrogenase, succinic dehydrogenase, and ceruloplasmin in maternal tissues, and lactic dehydrogenase and succinic dehydrogenase in fetal liver and heart are shown in table 2.

Activities of the enzymes were not altered in maternal and fetal tissues at day 16. Lactic dehydrogenase activity was not altered in maternal liver, heart, and brain, but was significantly increased in the serum at day 22 of fetal age. Although succinic dehydrogenase activity was not changed in maternal liver, activity was significantly increased in the heart. Maternal serum ceruloplasmin activity was significantly reduced by zinc deficiency. Activities of lactic dehydrogenase and succinic dehydrogenase were not

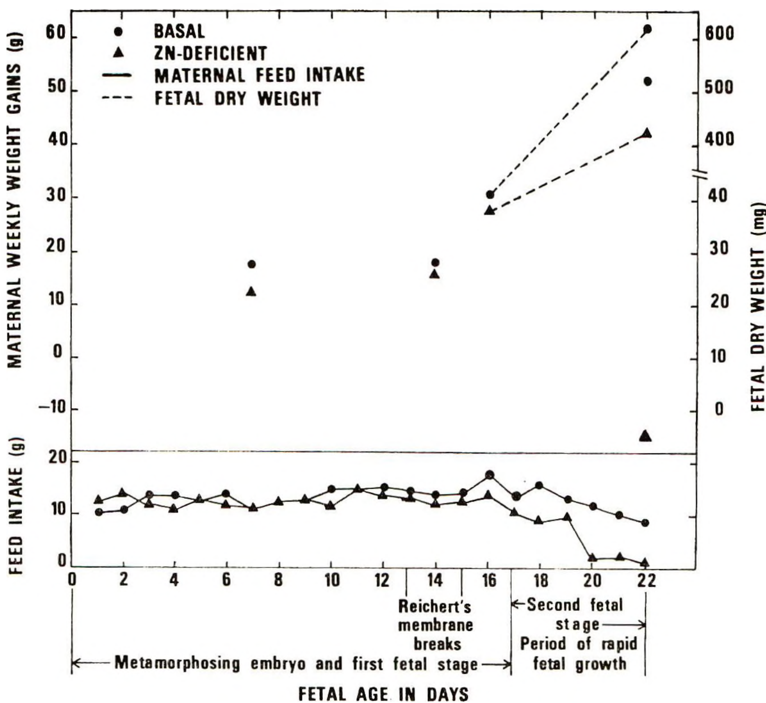

Fig. 1 Summary comparison of daily feed intake, weekly maternal weight gains, and fetal dry weight with information of the development of the rat fetus.

TABLE 2

Effect of maternal zinc-deficient regimen on lactic dehydrogenase (LDH), succinic dehydrogenase (SDH), and ceruloplasmin activities in maternal and fetal tissues

Diet	Fetal age	Liver		Heart		Brain	Serum	
		LDH	SDH [1]	LDH	SDH	LDH	LDH	Ceruloplasmin
		IU		IU		IU	IU	IU
Maternal								
Basal	16	49.4 ± 3.8 [2]	49.1 ± 2.3	32.3 ± 2.3	109.1 ± 6.7	7.1 ± 0.4	0.9 ± 0.2	47.3 ± 4.3
Zinc-deficient	16	53.8 ± 2.8	49.8 ± 4.5	27.3 ± 1.5	108.0 ± 6.4	6.4 ± 0.4	1.0 ± 0.3	56.3 ± 5.4
Basal	22	73.8 ± 5.3	49.3 ± 6.7	31.1 ± 2.1	71.9 ± 7.2	8.3 ± 1.0	1.0 ± 0.2	39.1 ± 5.1
Zinc-deficient	22	89.2 ± 3.2	46.3 ± 2.7	41.1 ± 4.9	111.4 ± 16.7 *	6.7 ± 1.2	1.7 ± 0.3 *	17.0 ± 5.7 **
Fetal								
Basal	16	12.1 ± 0.8	5.3 ± 0.6					
Zinc-deficient	16	10.8 ± 1.1	6.1 ± 0.8					
Basal	22	21.9 ± 0.8	13.2 ± 3.8	22.9 ± 2.1	23.2 ± 5.1			
Zinc-deficient	22	22.3 ± 1.1	10.9 ± 1.5	24.0 ± 0.8	38.8 ± 2.7 **			

[1] Microliters O_2 per milligram dry weight per hour.
[2] Mean and SE.
* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

changed in the fetal liver. Activity of succinic dehydrogenase was significantly higher in the fetal heart, but lactic dehydrogenase activity remained unaltered.

Data showing the effect of the zinc-deficient regimen on the concentration (parts per million) on a dry weight basis of zinc, copper, iron, and calcium in tissues of fetal and maternal animals at fetal ages of 16 and 22 days are tabulated in tables 3 and 4, respectively.

No significant change occurred in zinc, copper, iron, and calcium in whole animal, liver, heart, and body at day 16 of fetal age. A trend of a lower concentration of iron was found in the fetal animal; zinc, copper, and calcium did not show this trend.

Pertinent changes were found in the concentration of zinc, copper, iron, and calcium in the 22-day-old fetus. Zinc was significantly reduced in whole animal, heart and body, but was significantly increased in the liver. No change was found in copper in whole animal, liver, and body. Though copper was reduced in the heart, the reduction was not statistically significant. Iron was significantly reduced in whole animal, liver, and body, but no significant change was found in iron in the heart. Calcium was significantly elevated in whole animal, liver, and body, but was unaltered in the heart.

No significant change was found in dry matter content and in the concentration of zinc, copper, iron, and calcium in the maternal liver at day 16 of fetal age. However, a trend of reduced concentration of iron and zinc was found.

At day 22 of fetal age, pertinent changes were found in the content of minerals and dry matter in maternal tissues. Dry matter content was significantly less in liver, adrenals, kidneys, spleen, gastrocnemius muscle, and thymus. No change occurred in dry weight of heart, brain, and bone. The concentration of zinc was significantly reduced in liver, heart, and serum. No change was found in the zinc content in the other tissues. Copper was significantly reduced in kidneys, spleen, and serum. Though a reduction in copper was found in liver and brain, the changes were not statistically significant. In contrast, the

TABLE 3

Effect of maternal zinc-deficient regimen on zinc, copper, iron, and calcium concentration on dry weight basis in fetal tissues

Diet	Fetal age	Whole animal	Liver	Heart	Body
		ppm	*ppm*	*ppm*	*ppm*
			Zinc		
Basal	16	85.6 ± 7.2 [1]	46.6 ± 9.1		39.0 ± 2.2
Zinc-deficient	16	117.6 ± 20.0	69.4 ± 17.1		47.9 ± 3.1
Basal	22	65.0 ± 11.0	61.2 ± 1.3	246.2 ± 19.7	64.1 ± 1.9
Zinc-deficient	22	54.8 ± 5.0 *	155.7 ± 76.7 **	115.2 ± 19.5 **	32.6 ± 3.5 **
			Copper		
Basal	16	59.4 ± 4.4	51.0 ± 1.2		8.4 ± 0.9
Zinc-deficient	16	80.4 ± 30.0	75.5 ± 26.3		15.3 ± 5.7
Basal	22	13.5 ± 6.0	57.4 ± 2.6	10.6 ± 4.5	10.8 ± 1.5
Zinc-deficient	22	12.9 ± 1.8	47.7 ± 7.1	5.3 ± 2.3	10.1 ± 1.5
			Iron		
Basal	16	508.1 ± 100.5	401.5 ± 91.1		106.6 ± 11.7
Zinc-deficient	16	377.6 ± 143.0	303.7 ± 122.3		73.9 ± 22.1
Basal	22	224.9 ± 44.9	956.4 ± 48.4	596.2 ± 98.8	175.7 ± 7.5
Zinc-deficient	22	115.6 ± 7.7 **	536.9 ± 80.3 **	517.5 ± 97.6	90.4 ± 5.3 **
			Calcium		
Basal	16	152.4 ± 31.7	43.2 ± 7.0		109.2 ± 27.4
Zinc-deficient	16	199.0 ± 53.2	52.2 ± 44.7		146.8 ± 16.8
Basal	22	8.1 ± 2.1 [2]	111.5 ± 7.4	363.6 ± 39.4	8.6 ± 0.4
Zinc-deficient	22	11.1 ± 0.6 **	145.7 ± 10.8 **	385.1 ± 50.2	12.4 ± 0.6 **

[1] Mean and SE.
[2] Calcium concentration in whole animal and body are given in milligrams per gram.
* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

concentration of copper in the thymus showed a trend of an elevation. Copper was not detected in adrenals by the technique employed. Iron was significantly lower in brain, spleen, and serum. No significant change occurred in the iron content of the other tissues. Calcium concentration was significantly lower in spleen and brain, but significantly higher in serum and thymus. No significant change was found in the calcium content of other tissues.

DISCUSSION

Feeding a zinc-deficient diet to the female rat beginning at zero-day age of the fetus caused a reduction in feed intake, lower feed efficiency, and lower weight gain at days 16 and 22 of fetal age. As illustrated in figure 1, however, no marked difference was found in feed intake between mothers fed the basal and experimental diets until about day 17 of fetal age. Furthermore, weekly body weight gains of mothers were similar until day 14 of fetal age. Only during the last trimester of gestation was there a pronounced reduction in body weight gain, which coincided with the decrease in feed intake. These changes paralleled the initiation of rapid fetal growth. The poor weight gain was reflected by reduced dry matter content in liver, adrenals, kidneys, spleen, gastrocnemius muscle, and thymus. No change was found in heart, brain, and bone.

Though growth was retarded in the fetuses, no anatomical malformations were found in fetuses from mothers fed 0.75 ppm Zn. In contrast, Hurley and Swenerton (8) found gross congenital malformations in a wide variety of organ systems of full-term fetuses from mothers fed a diet containing 0 ppm Zn. Lack of agreement may be due to differences in zinc content of the zinc-deficient diets, and subsequent severity of the deficiency. Other differences between the studies that may

TABLE 4

Effect of zinc-deficient regimen on zinc, copper, iron and calcium concentration on dry weight basis in maternal tissues at day 22 of fetal age and maternal liver at day 16

Diet	Dry wt	Zinc	Copper	Iron	Calcium
	g	*ppm*	*ppm*	*ppm*	*ppm*
			Liver		
Basal [1]	3.590 ± 0.194 [2]	59.0 ± 2.9	14.6 ± 1.4	255.2 ± 35.3	
Zinc-deficient [1]	3.130 ± 0.168	57.8 ± 7.3	14.2 ± 2.1	170.2 ± 36.0	
Basal	2.447 ± 0.153	67.8 ± 3.3	31.1 ± 10.5	97.0 ± 12.4	8.0 ± 1.8
Zinc-deficient	1.824 ± 0.038 **	34.6 ± 9.9 **	15.6 ± 3.1	88.7 ± 14.4	8.1 ± 1.3
			Heart		
Basal	0.165 ± 0.005	47.0 ± 5.7	32.3 ± 9.9	265.1 ± 26.4	46.2 ± 8.6
Zinc-deficient	0.154 ± 0.007	32.3 ± 2.4 *	15.5 ± 2.5	319.9 ± 24.9	45.8 ± 4.7
			Adrenals		
Basal	0.018 ± 0.001	10.2 ± 6.0	0	224.2 ± 33.7	84.5 ± 12.3
Zinc-deficient	0.024 ± 0.002 **	7.3 ± 5.6	0	131.6 ± 24.8 *	86.3 ± 38.8
			Kidneys		
Basal	0.419 ± 0.012	38.6 ± 6.2	37.8 ± 10.4	140.0 ± 26.1	996.6 ± 262.4
Zinc-deficient	0.344 ± 0.019 **	42.4 ± 4.1	18.0 ± 1.2 *	145.0 ± 22.7	1157.1 ± 479.0
			Spleen		
Basal	0.095 ± 0.018	68.3 ± 17.7	9.3 ± 2.3	2127.7 ± 324.1	93.5 ± 35.2
Zinc-deficient	0.039 ± 0.003 **	46.0 ± 9.6	4.1 ± 0.9 *	781.0 ± 205.6 **	8.2 ± 1.0 *
			Gastrocnemius muscle		
Basal	0.430 ± 0.009	36.7 ± 4.7	5.0 ± 2.3	39.0 ± 5.5	22.1 ± 2.5
Zinc-deficient	0.314 ± 0.008 **	37.9 ± 9.5	4.7 ± 1.3	49.2 ± 4.2	24.7 ± 4.4
			Bone		
Basal	0.379 ± 0.005	65.4 ± 5.3	5.6 ± 3.0	18.0 ± 2.2	15.4 ± 3.5 [3]
Zinc-deficient	0.375 ± 0.006	59.8 ± 6.8	0.2 ± 0.1	14.3 ± 1.4	13.7 ± 1.3
			Thymus		
Basal	0.067 ± 0.003	38.4 ± 15.6	6.8 ± 1.6	154.9 ± 22.5	66.9 ± 13.9
Zinc-deficient	0.041 ± 0.007 **	17.5 ± 3.3	21.8 ± 9.2	160.6 ± 48.8	104.7 ± 14.9 *
			Brain		
Basal	0.302 ± 0.043	26.4 ± 7.5	21.5 ± 8.6	75.6 ± 5.5	103.0 ± 22.3
Zinc-deficient	0.306 ± 0.020	27.7 ± 5.5	12.2 ± 1.2	50.6 ± 11.0 *	46.5 ± 0.6 *
			Serum [4]		
Basal		0.6 ± 0.1	0.7 ± 0.03	1.7 ± 0.2	19.2 ± 1.3
Zinc-deficient		0.3 ± 0.1 *	0.4 ± 0.03 **	1.0 ± 0.1 **	24.4 ± 1.7 *

[1] Mineral and dry weight values of liver at day 16 of fetal age.
[2] Mean and SE.
[3] Concentration of calcium in bone is given in milligrams per gram.
[4] Micrograms per milliliter.
* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

contribute to nonconformity in results were composition of salt mixture, dietary regimen, and strain of rat.

An observation of interest in this study was that feeding 9 ppm Zn resulted in normal gestation of the female rat. Hurley and Swenerton (8) considered 9 ppm Zn a marginally deficient diet. Forbes and Yohe (19) reported that deficiency symptoms occurred in the male rat fed an isolated soybean protein diet containing 7 ppm Zn. Prasad et al. (3) found that 10 ppm of dietary zinc caused zinc-deficiency symptoms in the young rat. Swenerton and Hurley (7) suggested that rat diets containing isolated soybean protein should supply at least 100 ppm Zn, assuming no extraneous source of zinc. The conflicting evidence for the need of zinc by the rat can be partially explained

by differences in dietary regimen and age and strain of rat.

Lactic dehydrogenase activity was not altered in maternal liver, heart, and brain and fetal liver and heart. Lactic dehydrogenase activity was reduced in testes and bone (3), but unaltered in the heart (20) of zinc-deficient rats. Swenerton and Hurley (7) found a decrease in enzyme activity in the liver of weanling rats fed a zinc-deficient diet for a period of 4 to 6 weeks. An elevation of lactic dehydrogenase activity, concomitant with a decrease in zinc, was found in serum of mothers fed a zinc-deficient diet. These latter findings are pertinent since decreased zinc concentration and elevated lactic dehydrogenase activity were found (21) in plasma of humans with myocardial infarctions.

Increased succinic dehydrogenase activity was found in maternal and fetal heart, whereas maternal and fetal liver had no change in activity. Activity of succinic dehydrogenase was previously reported (3, 20) to be unaltered in tissues of the zinc-deficient rat. Since Beutler and Blaisdell (22) found a diminution in succinic dehydrogenase activity of heart and kidney, but not liver, in the iron-deficient rat, a similar pattern could have been expected in maternal and fetal animals with altered iron content. Skulachev et al. (23) have recently reported an inhibition of succinic dehydrogenase activity in rabbit heart mitochondria by addition of zinc ions. This suggests that maximal activity of the enzyme may not occur at the physiological concentration of zinc found in animal tissue, and offers an explanation for the increase in enzyme activity in the zinc-deficient animal. An analogous explanation has been advanced by Gallagher (24) for the increase of isocitric dehydrogenase activity found in the copper-deficient rat. He suggested that the enzyme is inhibited in tissues by the normal physiological concentration of copper, and the increased activity found during copper deficiency is the result of removing the inhibition as tissue copper is lowered.

Though a number of investigations [10] (1, 3–5, 9, 25) have studied changes in zinc, and in some cases iron, copper, and calcium content in tissues of the male and nonpregnant female rat fed a zinc-deficient diet, only data for zinc content in maternal serum (9) and fetal animal (8) are available.

Reduction of zinc in fetuses from mothers fed a zinc-deficient diet has been reported (8). Though body zinc was reduced, zinc was elevated in the fetal liver suggesting an aberration of zinc metabolism in the fetus. Reduction of zinc in the heart without a decrease in dry matter content suggests an absolute reduction and not an apparent decrease due to a diminution in dry matter content. Concentration of calcium was elevated in whole animal, liver, and body. What role the high concentration of calcium may play on the future biological development of the animal requires further study.

Though zinc, copper, and iron were generally reduced in certain maternal tissues, variations were noted in the mineral pattern. The effect of zinc deficiency of the content of these minerals in tissues of male and nonpregnant female rats has been studied. Liver zinc was reported to be either reduced (4) or not changed (3, 5, 25). Copper in tissues was found to be either unaltered (3, 5) or elevated.[11] However, copper was found (3) to be reduced in the heart. Iron was found [12] to accumulate in tissues. Though some agreement was found in the effect of zinc deficiency on tissue zinc, copper, and iron between the previous studies on male and nonpregnant female rats and the present study on the pregnant rat, there was also nonconformity. There was also disagreement in the effect of zinc deficiency on the minerals between the studies using male and nonpregnant female rats, which can be explained by differences in age, sex, strain, and dietary regimen. Lack of agreement with the present study may be related to the pregnancy status, with its accompanying stresses, of the rat. It was interesting to note that a reduction in serum zinc was previously found (9) in the maternal rat fed a zinc-deficient diet.

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[10] Moses, H. A., and H. E. Parker 1964 Influence of dietary zinc and age on the mineral content of rat tissues. Federation Proc., 23: 132 (abstract).

[11] See footnote 10.

[12] See footnote 10.

Changes found in calcium content in maternal tissues were in variance to that found for the other minerals. The concentration of calcium was lower in spleen and brain, whereas a high calcium concentration was found in serum and thymus. An increase was found [13] in calcium content of heart, kidney, liver, lung, muscle, testes, and plasma. Prasad et al. (3) reported a decrease in liver calcium. Rather than a reduction in brain calcium, elevated calcium was found [14] in the brain of maternal rats fed a diet containing excess zinc.

Since aberrations were found in minerals and enzymes in maternal and fetal animals, it became necessary to ascertain the primary cause, i.e., decreased feed intake or zinc deficiency per se. No marked reduction occurred in maternal body weight until the last trimester of the gestation period, which coincided with a reduction in feed intake. Though food intake was less in the zinc-deficient rats beginning at day 17, a very marked reduction did not occur until day 20 (fig. 1). Similarly, fetal dry weight was not altered at day 16 of fetal age, but was significantly reduced at day 22. These changes coincided with both breaking of Reichert's membrane, which allows for a more intimate association between mother and fetuses, and time of rapid fetal growth (fig. 1). A zinc-deficient regimen has been shown to cause a rapid decrease in serum zinc in sheep (26) and maternal rats (9), the latter change occurring within 1 day on the diet. This suggests that zinc metabolism is being adversely affected in the maternal rat before any marked reduction in feed intake. Since metabolic interrelationships are known (27) to exist between zinc, copper, iron, and calcium, it may be assumed that interactions occurred between these minerals relatively early in gestation. The lower, although not statistically significant, fetal iron and maternal liver iron and zinc at day 16 affirms this assumption. However, not until the stress imposed upon the mother by the rapid growth of the fetuses was there a significant change in minerals. Observations at day 22 of a) reduced copper and iron, but increased calcium in the same tissue; b) alterations in mineral content in brain and heart, that did not have lower dry matter content; and c) elevation of copper and iron in certain tissues rather than a decrease imply aberrations in the metabolism of these minerals during zinc deficiency. It is also difficult to relate an adverse effect of the maternal mineral stores to a lower feed intake of a short duration. Freedland (28) reported a decrease of lactic dehydrogenase and succinic dehydrogenase activities in the liver of adult rats after 1 and 4 days of starvation. In contrast, these enzymes were unaltered in the maternal liver, and succinic dehydrogenase was elevated in the maternal heart. The data obtained in this study suggest that the primary cause of the aberrations found in minerals and enzymes was zinc deficiency per se rather than a reduction in feed intake.

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[13] See footnote 10.

[14] Cox, D. H., S. A. Schlicker and R. C. Chu, unpublished data.

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Excess Dietary Zinc for the Maternal Rat, and Zinc, Iron, Copper, Calcium, and Magnesium Content and Enzyme Activity in Maternal and Fetal Tissues [1,2]

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ABSTRACT Excess dietary zinc (0.4%) was fed to the maternal rat during gestation to study its effect on the content of zinc, copper, iron, calcium and magnesium and the activity of various enzymes in maternal and fetal tissues. Succinic dehydrogenase activity was not altered in maternal and fetal liver and heart during gestation. Cytochrome oxidase activity was reduced in fetal liver, but not heart, and maternal liver and heart at day 16 of fetal age only. Xanthine oxidase activity was undetected in fetal liver and heart at day 22, irrespective of treatment. Activity of xanthine oxidase was lower in maternal heart, but not liver, at day 22. Ceruloplasmin activity was undetected in maternal serum at day 22. Changes in mineral content of fetal liver and body and maternal liver, heart, adrenals, kidneys, spleen, gastrocnemius muscle, thymus, and brain at day 22 of fetal age are as follows. Zinc was increased and copper was reduced in fetal body and liver. Iron was lower in the body, but unchanged in the liver. Total calcium and concentration of calcium were higher in fetal liver, but were lower in the body. Magnesium concentration, but not total, was elevated in liver and body. Total zinc and concentration of zinc were elevated in maternal liver, kidneys, and brain and total zinc was higher in thymus. Zinc content was unaltered in the other tissues. Copper was reduced in the liver only. No change was found in iron content of the tissues. Total calcium and concentration of calcium were higher in heart and brain, but reduced in kidneys. No change in calcium content was noted in the other tissues. Total magnesium and concentration of magnesium were lower in spleen; magnesium concentration was reduced in kidneys. The other tissues had no change in magnesium. The changes found in zinc and calcium in the maternal brain suggest further work in the area of brain metabolism and performance. The relation of reduced serum ceruloplasmin to iron metabolism in the maternal and fetal animal on an excess zinc regimen is discussed.

The effect of excess dietary zinc for the maternal rat during gestation on zinc, iron, and copper content of the fetus has been reported (1). Concomitant with an elevation of zinc content in the fetus were alterations in iron and copper levels, which resembled those reported (2–5) for the weanling and adult rat fed excess zinc.

In addition to altered tissue iron and copper in weanling rats fed excess zinc, changes in calcium, phosphorus, and magnesium have been reported. Sadasivan (6) found that normal deposition of calcium and phosphorus did not occur in bones of young rats. Stewart and Magee (7) confirmed Sadasivan's findings, and showed that the changes were alleviated with dietary supplements of calcium and phosphorus. They also observed a decreased retention of magnesium; however, bone magnesium was not affected.

Activity of various enzymes has been found to be altered in tissues of rats fed excess zinc. Reduction in liver catalase and in cytochrome oxidase (8), a depression in heart cytochrome oxidase (3), a decrease in intestinal alkaline phosphatase (9), an increase in liver and kidney alkaline phosphatase (8, 9), a reduction in liver xanthine oxidase (10), and a lowering of serum ceruloplasmin [3] (11) have been re-

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[3] Lee, D. D., and G. Matrone 1968 Restoration of serum ceruloplasmin synthesis in serum of rats with zinc induced copper deficiency. Federation Proc., 27: 482 (abstract).

ported. No data are available on the effect of excess dietary zinc during gestation on the activity of enzymes in maternal and fetal tissues.

The present investigation was designed to study the effect of excess dietary zinc for the maternal rat during gestation on the content of zinc, iron, copper, calcium, and magnesium and the activity of succinic dehydrogenase, cytochrome oxidase, xanthine oxidase, and ceruloplasmin in maternal and fetal tissues.

EXPERIMENTAL PROCEDURE

Nulliparous female rats,[4] ranging in weight from 195 to 245 g, were individually housed in galvanized wire cages and received feed and distilled water ad libitum. Composition of the basal diet was as follows: (in percent) casein, 20; sucrose, 63.3; cellulose,[5] 3; corn oil, 10; salt mixture (with sulfate) (12), 2.7; and vitamin mixture,[6] 1. The excess zinc diet was formulated by the incorporation of 0.4% zinc as zinc oxide into the basal diet. Zinc content of the basal diet was 9 ppm.

Experiment 1. Ten rats were mated as previously described (13). The day sperm were found in the vaginal smear was designated as zero-day fetal age, and rats then were randomly assigned to the basal and 0.4% zinc diet. At fetal age 16 days, all rats were killed.

Experiment 2. Ten rats were subjected to the same regimen as described in experiment 1 except that animals were killed at fetal age 21 days.

Experiment 3. Twelve rats were treated according to the same regimen as described in experiment 1 except that at fetal age 22 days, six basal and six experimental rats were killed.

At the appropriate age of the fetuses, mothers were stunned by a blow on the head, decapitated and exsanguinated. Fetuses were removed via abdominal incision, and were stunned, decapitated and exsanguinated. Fetal livers and hearts were removed and pooled separately according to litter. Fetal livers and hearts and individual maternal liver and heart were chilled in cold, 0.039 M phosphate buffer (pH 7.4) prepared with reagent grade KH_2PO_4 and glass-distilled water. Tissues were removed from the buffer, blotted free of moisture, weighed, and homogenized in a Potter-Elvehjem glass homogenizer with cold phosphate buffer. Homogenates were analyzed for succinic dehydrogenase (EC. 1.3.99.1) (14), cytochrome oxidase (EC. 1.9.3.1) (14), and xanthine oxidase (EC. 1.2.3.2) (15). Sera of mothers were analyzed for ceruloplasmin (16, 17). Hemoglobin level in maternal blood was measured by the acid-hematin technique.

Adrenals, kidneys, spleen, gastrocnemius muscle, thymus, and brain were removed from maternal animals at day 22 of fetal age. These maternal tissues and body[7] of the 22-day-old fetus were dried to a constant weight at 100°. Samples for mineral analyses of maternal liver and heart at day 22 of fetal age and litter-pooled livers of the 22-day-old fetuses were obtained from homogenates used for enzyme analyses, and were dried to a constant weight at 100°. Dried samples were wet-digested with nitric and sulfuric acids and analyzed for zinc, copper, iron, calcium, and magnesium by the atomic absorption[8] technique. Data were analyzed statistically by the t test (18).

RESULTS

Data illustrating the effects of feeding 0.4% Zn to the maternal rat on dry weight and enzyme activity of fetal tissues and enzyme activity and hemoglobin level in maternal tissues are tabulated in table 1.

No change was found in dry weights of the liver and heart of 16-, 21-, and 22-day-old fetuses from mothers fed excess zinc. Dry weight of body was significantly less in the 22-day-old fetuses from these mothers.

Succinic dehydrogenase activity was not altered in the liver of 16-, 21-, and 22-day-old fetuses or in the heart of 21-day-old fetuses from mothers fed 0.4% Zn. Activity of cytochrome oxidase was significantly

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[4] CFE strain of Sprague-Dawley rats obtained from Carworth, New City, N. Y.

[5] Alphacel.

[6] Vitamins in cornstarch: (amount per kilogram of diet) vitamin A, 20,000 IU; vitamin D, 2,200 IU; (in milligrams) ascorbic acid, 1017; vitamin E as α-tocopheryl acetate, 485; inositol, 110; choline dihydrogen citrate, 3715; menadione, 49.6; p-aminobenzoic acid, 110; niacin, 99.2; riboflavin, 22; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca pantothenate, 66; biotin, 0.44; folic acid, 1.98; and vitamin B_{12}, 29.7 μg (obtained from General Biochemicals Inc., Chagrin Falls, Ohio).

[7] In this report, the term body is used to mean the whole animal minus the liver and heart.

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

reduced in the liver of 16-day-old fetuses, but not at other fetal ages, from mothers fed high zinc; heart cytochrome oxidase was unaltered in 16- and 21-day-old fetuses. Xanthine oxidase activity was undetected in liver and heart of 22-day-old fetuses from each treatment.

Activity of succinic dehydrogenase was not changed in liver and heart of maternal rats fed 0.4% Zn during gestation. Cytochrome oxidase activity was significantly reduced in liver and heart at day 16, but not at day 21, of maternal rats fed high zinc. At day 22, the activity of xanthine oxidase was significantly lower in the heart, but not liver, of mothers fed excess zinc. Excess dietary zinc caused a significant reduction in the activity of serum ceruloplasmin of maternal rats at day 22; in fact, activity was undetected. At day 22, hemoglobin was significantly lower in the maternal rat fed 0.4% Zn.

Data showing the effect of 0.4% dietary Zn for the maternal rat on total amount (micrograms) and concentration (parts per million) on a dry weight basis of zinc, copper, iron, calcium, and magnesium in fetal liver and body and maternal tissues at day 22 of fetal age are given in table 2.

Liver of fetuses from mothers fed 0.4% Zn contained significantly more total zinc and concentration of zinc than liver of fetuses from mothers fed the basal diet. A significantly higher concentration of zinc, but not total, was found in the body of fetuses from maternal rats fed excess zinc. Total copper and concentration of copper were significantly reduced in liver and body of fetuses from mothers fed 0.4% Zn. Total iron and concentration of iron were significantly lower in the body, but unchanged in the liver, of fetuses from mothers fed excess zinc. Total calcium and concentration of calcium were significantly higher in the liver, but were significantly lower in the body, of fetuses from 0.4% Zn group compared with those from the basal groups. Magnesium concentration only was significantly elevated in liver and body of fetuses from mothers fed 0.4% Zn.

No significant differences were found between treatments in the dry matter content of maternal tissues. Significant elevations occurred in total zinc and concentration of zinc in liver, kidneys, and brain of maternal animals fed 0.4% Zn. Total zinc was significantly higher in the thymus of these animals. No change was found in the zinc content of other tissues examined. The liver of maternal animals fed excess zinc contained a significantly lower total copper and concentration of copper than the liver of mothers in the basal groups. Copper was not detected in adrenals, spleen, and thymus of animals from each treatment by the technique employed. Copper was not quantitatively altered in other tissues. No significant change was found in the iron content of tissues of mothers fed 0.4% Zn. Total calcium and concentration of calcium were significantly higher in heart and brain, but significantly reduced in kidneys, of maternal rats fed excess zinc. No change was noted in the calcium content of the other tissues from these mothers. Significant reductions were found for total magnesium and concentration of magnesium in the spleen and for magnesium concentration in kidneys of mothers fed the diet containing additional zinc.

DISCUSSION

It has been documented [9] (2–11) that excess dietary zinc for the rat produces an antagonism to iron, copper, calcium, magnesium, and various enzymes. Settlemire and Matrone (19) suggested that zinc affects iron metabolism by a) impairing the incorporation of iron into or release from ferritin, which would influence iron absorption and storage, and b) shortening the life span of erythrocytes, which would cause a faster turnover of iron. Van Campen (20) postulated that zinc alters copper metabolism by impairing its absorption as mediated primarily via the direct effect of zinc either in or on the intestine. Data presented by Stewart and Magee (7) indicate that zinc decreases the normal deposition of calcium in bone and reduces both the absorption and utilization of magnesium. Lowered activity of cytochrome oxidase (3, 8) and ceruloplasmin[10] (11) can be attributed to a zinc-induced copper deficiency since feeding copper restored enzyme activity and reduced copper content was found (8) in hearts with low cytochrome oxidase activity.

[9] See footnote 3.
[10] See footnote 3.

Succinic dehydrogenase activity was not reduced in maternal and fetal liver and heart during gestation, whereas cytochrome oxidase was reduced in activity at day 16 of fetal age, but not at day 21. These findings were not consistent with the view that iron and copper metabolism are altered in the maternal and fetal animal. Reduced copper was found (1) in fetal liver at day 18 of fetal age and in maternal liver and heart at day 16. A similar change in copper status was found at day 22 in this study. Iron was reduced in the fetal body, but not liver, at days 16 and 18 (1), and in the body, but not liver, at day 22 in the present study. Iron in the maternal liver was not altered. As will be discussed later, however, iron in both fetal and maternal liver may not be available for utilization. Beutler and Blaisdell (21) found a diminution of succinic dehydrogenase activity in the heart, but not liver, of the iron-deficient rat. Schultze (22) reported that cytochrome oxidase activity was not reduced in the liver and heart of the iron-deficient rat. A reduced cytochrome oxidase activity was found in the heart (22) and liver (22, 23) of rats fed a copper-deficient diet beginning at weanling age. However, though the activity of cytochrome oxidase was reduced in the liver, no change was detected in the heart of copper-deficient adult rats (24). Of particular relevance to the present study, reduced cytochrome oxidase activity was found in the heart (3), which had reduced copper content, and liver (8) of rats fed excess zinc beginning at weanling age. In both cases, feeding copper caused the activity to return to either normal or greater than normal values. Therefore, from the data relating altered enzyme activity to either excess dietary zinc, iron deficiency, or copper deficiency in the rat and the status of copper and iron in the maternal and fetal animal, findings of reduced succinic dehydrogenase activity and a more consistent diminution in cytochrome oxidase were expected. Other than the fact that the changes in enzyme activity were observed when weanling rats were used, no tenable explanation can be ascribed for the lack of agreement in this study.

The absence of xanthine oxidase in the fetal liver at day 22, which occurred irrespective of dietary treatment, is in accord with earlier studies (25, 26). In contrast to an earlier report (10), xanthine oxidase was not lowered in the liver of the maternal rat fed excess zinc; the discordance may be attributed to age difference in the animals since weanling rats were used in the earlier study. Of relevance to the role of a high zinc regimen to the utilization of iron was the reduction of xanthine oxidase in the heart and the absence of ceruloplasmin in the serum of the maternal rat. An absence of ceruloplasmin has been found[11] (11) in the nonpregnant rat fed excess zinc. Ceruloplasmin has been reported (27) to be involved in promoting iron saturation of transferrin and possibly other iron-containing proteins and enzymes, and consequently the turnover of iron in the body. This suggests, therefore, that the reduced xanthine oxidase in the heart may be attributed to decreased iron utilization as the result of the absence of ceruloplasmin. The fact that ceruloplasmin is scarcely measurable (28) at the time of birth of the rat also explains the reduction of iron in the fetal body, but not liver at day 22, and adds substance to the concept that ceruloplasmin plays an important role in iron turnover in the body. In addition to the suggestion (19) that zinc alters iron metabolism by interfering with its incorporation into or release from ferritin, consideration should be given to the alteration of ceruloplasmin when explaining the action of zinc antagonism to iron metabolism. In this connection, not only is the level of ceruloplasmin affected by impaired copper metabolism but possibly by zinc, since when zinc was added to an in vitro system at the concentration found in serum, the ascorbate oxidase activity of ceruloplasmin was inhibited (29).

Calcium was elevated in the fetal liver, but lowered in the body. In contrast, magnesium concentration, but not total, was higher in the fetal liver and body. For the maternal rat, zinc was elevated in the kidneys, brain, and thymus; calcium was higher in the heart and brain, but lower in the kidneys; and magnesium was lower in spleen and kidneys. An elevation of liver calcium was found (30) in calves fed a high level of zinc. Excess dietary zinc was

[11] See footnote 3.

found to decrease the deposition of calcium in bone (6, 7) and the absorption and utilization of magnesium (7). Though it is evident that zinc interferes with calcium and magnesium metabolism, the specific mechanism is not clear. Hormones are involved in the homeostasis of calcium and magnesium, and since zinc is known (31) to be involved with hormonal balance, further study in this area is warranted.

Of particular interest were the changes found in the content of zinc and calcium in the maternal brain. Although zinc apparently does not readily cross the blood-brain barrier in mice and dogs (32), under the condition of a high zinc regimen, zinc was elevated in the brain of the maternal rat and small but consistent linear increases occurred (33) in the brain of lambs. Intracerebral or subcutaneous injections of zinc for mice caused convulsive seizures, which were prevented by intracerebral injection of calcium (34). Though the authors did not analyze the brain for zinc or calcium, they suggested that the seizures were due to calcium depletion in neuronal membranes. Rather than a decrease, calcium was increased, concomitant with an elevation of zinc, in the brain of the maternal rat fed excess zinc. The lack of reduced brain calcium in this study may be due either to the method of increasing zinc in the brain or to the amount of zinc in the brain. The observation of an increased brain calcium in the maternal rat fed excess zinc indicates further investigation in the areas relative to brain metabolism and performance. In this connection, calcium was reduced in the brain of the maternal rat fed a zinc-deficient diet (35). A study to determine if the same phenomenons occur in the brain of the fetus and postnatal animal would be of particular interest.

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Biochemical and Physiological Changes in the Rat during Riboflavin Deprivation and Supplementation [1,2]

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ABSTRACT A study has been made of the time course of changes in organ weight, organ protein content and concentration, and the succinic dehydrogenase activity for the heart, kidneys and liver of the rat during the development of riboflavin deficiency and during the recovery process. The rate and quantity of ^{14}C-2-riboflavin uptake by the mitochondria and the specific incorporation of the vitamin into the protein of succinic dehydrogenase were studied in the liver during the recovery process. Though the deficiency caused a fall of approximately 50% in the enzyme activity of the liver of the deficient group, the restriction of food consumed by the weight-controlled pairmates caused an increase in enzyme activity of about 33% in the control group. Increases in the size and protein content of the liver provide a measure of protection for the deficient animal. The development of riboflavin deficiency appears to result in the production of increased numbers of liver mitochondria of reduced protein and flavin content. Recovery from the deficiency involves an extremely rapid uptake of protein by the mitochondria and a slower but steady incorporation of riboflavin by the protein of the enzyme. The heart is insensitive and the kidney relatively insensitive to riboflavin deprivation or supplementation.

Succinic acid dehydrogenase (SDH) is a mitochondrial enzyme. The protein portion of the enzyme is believed to be an integral part of, or firmly bound to, the structural elements of the mitochondrion. The prosthetic group of this enzyme is flavin-adenine dinucleotide (FAD) but its exact form is not known at present. The enzyme is unique among flavoprotein enzymes because it is the only well substantiated case in which the prosthetic group is attached to the protein by means of a covalent bond. The exact nature and position of the bond are unknown at present but as a result of the monumental studies made by Kearney and Singer and their associates (1, 2) the main flavin component was isolated and degraded to a hexapeptide with the flavin attached to the C-terminal group. In the case of other flavoprotein enzymes the flavin may be separated from the protein portion by mild treatment with ammonium sulfate in an acidic medium. In the case of SDH the FAD-like flavin can be removed only by extensive proteolysis (1). The enzyme, once fragmented by such means cannot be reconstituted. The biodegradation and biosynthesis of SDH are enzyme dependent and resemble protein degradation and synthesis more than the usual processes of resolution of a holoenzyme into its coenzyme and apoenzyme and their recombination.

Several enzymes within the intact mitochondrion utilize FAD as a coenzyme. When the mitochondria are isolated by suitable techniques, they contain the necessary FAD to satisfy these several flavoprotein enzymes for metabolic purposes. Acid extraction of the mitochondria removes all of the FAD except that portion which is covalently bound to the SDH.[5] This procedure provides means for the specific study of the flavin of SDH.

Recently it was reported from this laboratory (4) that the SDH activity of the

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[5] It has been reported by W. R. Frisell and C. G. MacKenzie that proteolysis is required to liberate part of the flavin bound to sarcosine dehydrogenase and dimethylglycine dehydrogenase (3). This subject has not been pursued nor the findings confirmed.

heart, kidneys and liver of the rat fell when 7-ethyl-8-methyl-10-(1-D-ribityl)isoalloxazine(7-ethyl-8-methyl-flavin) (5) was the only flavin available to the animal for metabolic purposes. It was also shown that the liver, alone of these tissues, showed depressed activity when the animal was deprived of riboflavin although the fall was far less severe than when 7-ethyl-8-methyl-flavin was used. The additional observation that the regeneration time of the enzyme in the liver was very rapid when riboflavin was subsequently administered, focused attention on the time course of many of the biochemical and physiological changes taking place during the induction of the deficiency state and during the recovery process. For a better understanding of the mechanism of action of our vitaminlike analogues of riboflavin, more information was required on the changes and rates of these changes during the development of a riboflavin deficiency and during the recovery from this state.

This is a report of the changes with time in the organ weight, organ protein content and concentration, and the SDH activity per milligram of protein and per organ of the heart, kidneys and liver of the rat during the time the animal is developing riboflavin deficiency and during the recovery process resulting from riboflavin administration. To gain greater insight into the process of regeneration of the SDH activity of the liver, the rate and quantity of ^{14}C-2-riboflavin uptake by the mitochondria and the specific incorporation of the riboflavin into the protein of SDH were studied. This information was supplemented by a study of the rate of uptake of protein by the mitochondria as a result of the supplementation with riboflavin and food. Information on most of these subjects, particularly as related to time, has not been reported.

MATERIALS AND METHODS

Whole organ studies. Female weanling rats of the Wistar strain,[6] weighing between 35 and 40 g, were used; all were caged individually and the conditions under which they were maintained have been described before (6). Fifty-four of the animals were paired on the basis of body weight. The deficient member (D) of a pair was permitted to eat ad libitum a riboflavin-deficient diet previously described (7). The riboflavin-supplemented member (R) of each pair was fed the same diet supplemented with 20 mg riboflavin/kg by the weight-control technique.[7]

On day "zero" three pairs of animals were taken for analyses. Although they were equivalent, the D members were treated as one group and the R animals as another. The animals were weighed, then decapitated and exsanguinated. The hearts, kidneys and livers were removed immediately and placed in cold 0.25 M sucrose solution. For each group the hearts were combined, the left kidneys from the animals (total weight known) were combined and approximately 1-g (weight known) portions taken from the right median lobe of each liver were combined. These tissues were converted, as nearly as possible, to 10% homogenates and the SDH activity and protein concentration of each homogenate determined as described before (4). On days 14, 28, 42 and 53, six pairs of animals were used for assays as above so that two sets of three rats from each group afforded two completely independent assays, each done in duplicate.

An additional 36 weanling rats were started on the riboflavin-deficient diet on day zero. These animals were permitted to eat the diet ad libitum for 42 days. On day 42, the diet of these deficient rats was changed to the one supplemented with riboflavin. Two groups of three animals were used for assay as described above on days 1, 2, 3, 5, 7 and 9 of supplementation.

The enzyme activities are expressed as micromoles of oxygen consumed per minute per milligram of protein and per organ. Organ weights are expressed as percentage of body weight. Protein content of the organs is expressed as milligram per gram organ and per organ. The values for the protein content were determined on the homogenates used for the enzyme assays. Since the homogenates were always strained and a very small amount of fibrous material removed by the cheesecloth, the extrapolation of the observed values to those for whole organs yields values which

[6] CFN Rats, Carworth, Inc., New City, N. Y.

[7] The deficient animal was permitted to eat at liberty; the riboflavin-receiving pair-mate was given only enough food to permit it to maintain approximately the same body weight as the deficient pair-mate.

are slightly low but which are consistently low for all groups.

Mitochondria — ^{14}C-2-riboflavin studies. An additional 36 weanling rats were fed a riboflavin-deficient diet for a period of 42 days. At this time four animals were killed for zero-time values; they were processed as described below.

The morning of day 43, 9:30 AM, was zero time for the ^{14}C-2-riboflavin incorporation studies and within 15 minutes, the remaining 32 deficient animals received by stomach tube 0.5 ml of an aqueous solution containing 40.5 µg ^{14}C-2-riboflavin (8). During the remainder of the supplementation period, all surviving animals were given the same standard quantity of the riboflavin solution each day at the same time. On days zero, 2 and 5 identical 0.5-ml samples of the ^{14}C-2-riboflavin solution were delivered into volumetric flasks so that counts per minute could be determined for the quantity of solution administered. This value was found to be 67880 ± SE 88 cpm/0.5 ml. Twenty-four hours after the first dose of radioactive riboflavin was given (9:30 AM) and at the same time at the end of 2, 4, 7 and 9 days, four animals were killed and processed as follows:

The rats were killed as described above and all subsequent steps were performed in a cold room, ice bath or refrigerated centrifuge. The livers, collected in cold 0.25 M sucrose solution, were freed of extraneous tissue, blotted, and the weights determined. The pooled livers, cut into small pieces and divided into convenient portions (6 to 7 g), were homogenized in about 30 ml of 0.25 M sucrose by making six passes of the rotating (500 rpm) Teflon pestle in the 50-ml glass homogenizer tube.[8] The combined homogenate plus washings (0.25 M sucrose used) was filtered through four layers of fine cheesecloth, and in portions of 30 ml, added to a 40-ml Dounce homogenizer. While the homogenizer tube was immersed in an ice and water bath, three passes were made with the loose-fitting plunger. The combined homogenate and washings were made to a volume equal to 10 times the weight (percent) of the liver tissue used by the addition of 0.25 M sucrose, mixed and poured into as many 50-ml centrifuge tubes as required. The tubes were centrifuged at 2500 × g for 10 minutes.[9] The supernatant suspensions were decanted, combined and stored in an ice bath. The pellets were resuspended in 0.25 M sucrose, combined and rehomogenized in a Dounce homogenizer as above. The rehomogenized material was made to 50 ml with 0.25 M sucrose and centrifuged as above; the supernatant solution was combined with that obtained above and the sedimented pellet discarded.

The combined supernatant solution was poured into as many 50-ml centrifuge tubes as required and centrifuged at 9000 × g for 10 minutes.[10] The supernatant solution was discarded and the pellets resuspended in 0.25 M sucrose and transferred to a Dounce homogenizer; when all were combined and the washings added, the contents were rehomogenized by two passes with the tight-fitting pestle. The material was transferred with liberal use of 0.25 M sucrose into a beaker, made to approximately 200 ml, poured into centrifuge tubes and centrifuged at 9000 × g for 10 minutes as above. The resulting pellets were combined by transferring them to one centrifuge tube and resedimenting them. The final pellet was transferred to a 25-ml volumetric flask and the contents made to volume with water.[11]

The mitochondria were thoroughly suspended before each of the following samples was removed: 0.10 ml was added to a 10-ml volumetric flask for protein determination and 2.00 ml were added to a 10-ml volumetric flask for "plating out" for radioactivity determinations. The remaining 22.9 ml of mitochondrial suspension was recentrifuged at 9000 × g for 15 minutes and the resulting pellet extracted with trichloroacetic acid as described by King et al. (10), to remove all flavin except the covalently bound FAD. The acid insoluble mitochondrial protein (AIMP) was col-

[8] It is helpful to familiarize oneself with the procedures for the isolation of mitochondria as collected in volume 10 of Methods of Enzymology. Particularly helpful is the procedure described by Johnson and Lardy (9).

[9] International Equipment Company B-20 centrifuge with no. 870 head was used. Speed setting 2.5 and 4500 rpm.

[10] International Equipment Company B-20 centrifuge with no. 870 head was used. Speed setting 5.0 and 9500 rpm.

[11] We are indebted to Dr. Dean Danner, Saint Jude's Research Hospital for parts of this procedure. It provides a rapid and reproducible recovery of the mitochondria. No effort was made to accomplish a quantitative recovery of the mitochondria.

lected by centrifugation at 9000 × *g* for 15 minutes and the pellet quantitatively transferred to a 25-ml volumetric flask; the contents were made to volume with water. Samples were removed as described above for protein determinations and for radioactivity determinations.

All radioactivity measurements were made with a gas-flow counter using the proportional region. The procedure of Berson and Yalow (11) was modified slightly and used to correct for self-absorption. One-milliliter aliquots of the suspensions of mitochondria and AIMP were placed in stainless steel planchets, dried at 70° and each counted for a time sufficient to give 2000 to 3000 total counts and a counting rate, "A." To these same planchets were added 1.00 ml of a pure standard ^{14}C-2-riboflavin solution with a concentration of 1.62 μg/ml. The counting rate obtained with the standard ^{14}C-2-riboflavin solution added to the sample gave a counting rate, "B." Then A/B − A × S × D, where S is the amount of ^{14}C-2-riboflavin added in micrograms and D is the dilution factor, gives the amount of labeled riboflavin in micrograms that would give the observed counting rate, "A," corrected for self-absorption. All samples were assayed in quadruplicate and the Chauvenet criterion (12) was used to reject a determination when the deviation from the mean of the four determinations by a suspected result was greater than 1.54 times the standard error of the counting rate (determined from the square root of the average total number of counts). Less than 1% of the determinations failed to meet this criterion.

RESULTS

Whole organ studies. The influence of riboflavin deficiency on the SDH activity per milligram of protein for the liver is related to time in figure 1. The figure shows the impressive loss of SDH activity from the livers during the induction of riboflavin deficiency and the extremely rapid regeneration of activity during administration of the vitamin. The pair-mates of the riboflavin-deficient rats showed a rapid and sustained increase in SDH activity. The administration of adequate riboflavin and food to the riboflavin-deficient rat caused a rapid rise in the SDH activity in the liver,

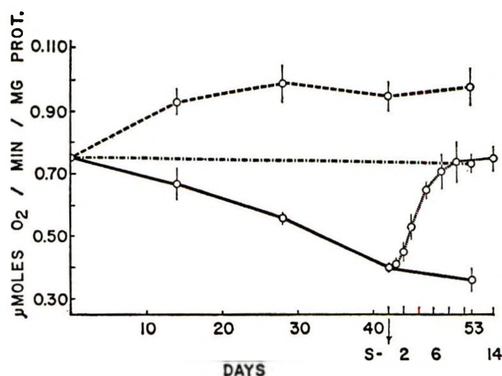

Fig. 1 Succinic dehydrogenase activity of the liver per minute per milligram of protein in homogenate. Deficient animals (——) were fed ad libitum; control animals (– –) were weight controlled to pair-mates in deficient group; normal animals (–·–) were fed an adequate diet ad libitum; supplemented animals (····) were deficient animals fed the adequate diet ad libitum from day 42. Arrow on abscissa indicates start of supplementation period. Numbers following S– on abscissa indicate days of supplementation.

not to the level of activity of the paired, weight-control animal, but to the level of activity of the animal receiving adequate riboflavin and food. Although it is not shown in this figure, when rats which have been fed adequate riboflavin but a restricted food intake such that the liver SDH activity is elevated, are permitted free access to food, the SDH activity promptly returns to the level of activity shown by animals fed adequate riboflavin and food.

The SDH activities for the heart and kidneys were not affected by riboflavin deficiency, food restriction or supplementation. The activities throughout these periods were: for the heart an average of 0.192 μmole oxygen/minute per milligram of protein with no value differing from this by more than 0.016 μmole, and for the kidneys an average of 0.162 μmole oxygen/minute per milligram of protein with no value differing from this by more than 0.006 μmole.

The changes in the total SDH activities of the organs are related to time in figure 2. Riboflavin deficiency did not influence the total SDH activity for the heart or the kidneys, as was the case for the SDH activity per milligram of protein. Riboflavin deficiency did, however, have an early and pronounced effect on the total SDH activ-

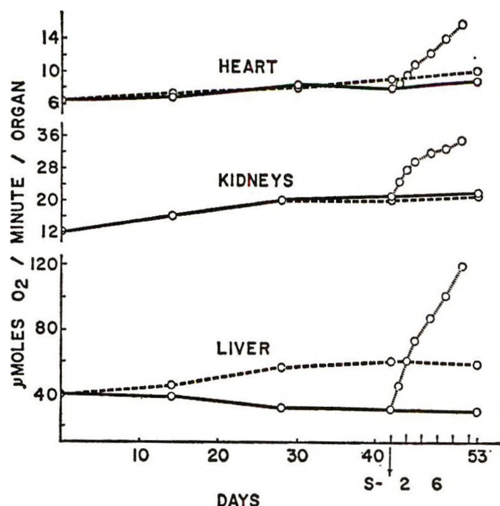

Fig. 2 Succinic dehydrogenase activity of the whole organs indicated. Deficient animals (——) were fed ad libitum; control animals (– –) were weight controlled to pair-mates in deficient group; supplemented animals (····) were deficient animals fed an adequate diet ad libitum from day 42. Arrow on abscissa indicates start of supplementation period. Numbers following S– on abscissa indicate days of supplementation.

ity of the liver. This was also the case for the SDH activity per milligram of protein. The increases in the total activities of SDH in the heart and kidneys during the supplementation period were immediate and they were maintained during the period of study. The response in total SDH activity for the liver when adequate riboflavin and food were available was an increase of 100% during the first 2 days, 200% during the first 5 days and 300% during the 9 days of the study.

The weight of the heart expressed as a percentage of the body weight was not influenced by riboflavin deficiency. The weights of the kidneys and liver as percentage of body weight were significantly affected by the deficiency as shown in figure 3. The deficient animals developed relatively larger kidneys and livers and when the deficiency was of sufficient duration, the enlargement was considerable. Thus, on day 53 of the study the kidneys and livers of the deficient animals were, respectively, 36% and 60% larger than those of the weight-controlled, riboflavin-supplemented mate. Once the deficient animal had access to riboflavin and unlimited food the relative weight of the kidneys of the previously deficient rat fell very rapidly to the value for the relative weight for the rats receiving riboflavin but restricted food. The change in the weight of the liver when the deficient animal had access to riboflavin and unlimited food was striking. After 2 days of supplementation, the weight of the liver as percentage of body weight increased 97%, but in terms of actual weight the increase was 150%. During day 1 of supplementation the average increase in liver weight was 3.7 g or almost 75% of the average total body weight increase of 5 g but also, this was equivalent to a 93% increase in the liver weight.

Figure 4 shows the changes in the concentration of protein for the kidneys and liver during the development of riboflavin deficiency and during the supplementation period. Recovery of the kidney was associated with a small, transient concentration of protein whereas that of the liver by a considerable, although equally transient, dilution of protein.

Mitochondria — ^{14}C-2-riboflavin studies. Figure 5 is composed of two parts; one part, consisting of two curves, shows the rate of increase of the total mitochondrial protein (MP) and the total AIMP per average liver during supplementation. The other part, also consisting of two curves, shows the total radioactive riboflavin up-

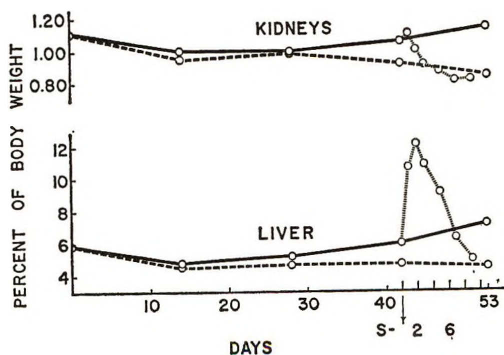

Fig. 3 Weights of the kidneys and livers as percentage of body weight (including organ). Deficient animals (——) were fed ad libitum; control animals (– –) were weight controlled to pairmates in deficient group; supplemented animals (····) were deficient animals fed an adequate diet ad libitum from day 42. Arrow on abscissa indicates start of supplementation period. Numbers following S– on abscissa indicate days o' supplementation.

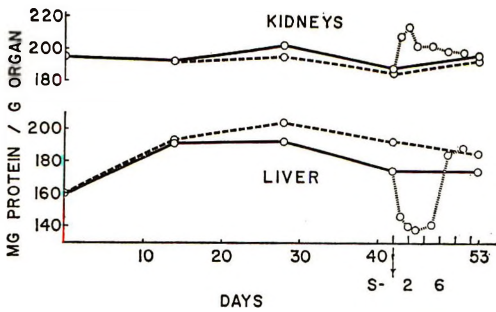

Fig. 4 Concentration of protein in the kidneys and livers. Deficient animals (——) were fed ad libitum; control animals (– –) were weight controlled to pair-mates in deficient group; supplemented animals (····) were deficient animals fed an adequate diet ad libitum from day 42. Arrow on abscissa indicates start of supplementation period. Numbers following S– on abscissa indicate days of supplementation.

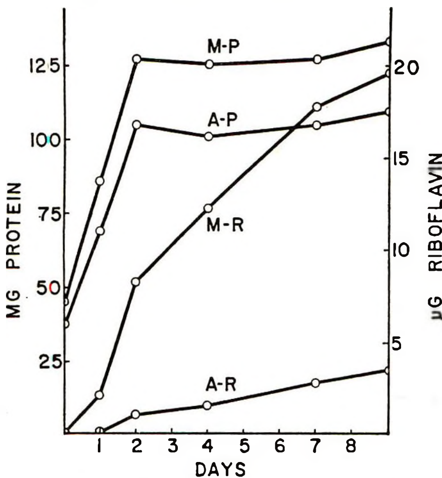

Fig. 5 Rate of increase of mitochondrial protein and riboflavin per average liver during supplementation of the deficient animals. M-P, protein in the total sample of mitochondria from an average liver. A-P, acid-insoluble protein in the total sample of mitochondria from an average liver. M-R, radioactive riboflavin uptake by the MP. A-R, radioactive riboflavin uptake by the A-P. (The values for an average liver were obtained by dividing the values for the total samples by four.)

take in terms of all of the mitochondria and in terms of all of the acid-insoluble mitochondrial residue per average liver. The increase in MP was confined to the first 2 days of supplementation. The uptake of riboflavin by whole mitochondria started immediately riboflavin became available and it continued during the period of study. The binding of the riboflavin by the AIMP showed a delay of 1 day after which time it also showed a continuous uptake during the period of study.

Figure 6 is also composed of two parts; one part, consisting of two curves, shows the rate of radioactive riboflavin uptake per milligram of MP and also per milligram of AIMP. The other part, consisting of one curve, shows the rate of increase of the SDH activity plotted as percentage of total rise of activity for the test period. In the case of the latter curve the point representing 100% (day 9) was made to superimpose on the point of maximum radioactive riboflavin uptake for the AIMP (day 9). The decision to do this was based on the obvious fact that the SDH activity is related to the covalently bound flavin. The curve expressing SDH rise and covalently bound flavin rise are virtually identical.

DISCUSSION

It should be emphasized that the induction of a riboflavin deficiency in the rat is due to a combination of vitamin deprivation and a drastically reduced food intake. Once a rat becomes deficient its weight remains relatively constant for varying

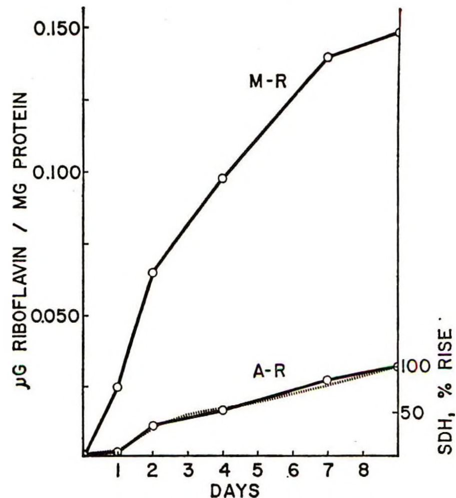

Fig. 6 Rate of uptake of radioactive riboflavin per milligram of liver MP and its covalent incorporation per milligram of liver AIMP during supplementation of the deficient animals. The dotted line relates to the right ordinate; it is the rate of increase of SDH activity for the liver expressed as percentage of total rise for the same period. M-R, riboflavin uptake by the MP. A-R, riboflavin incorporation into the AIMP.

lengths of time, but from 6 to 8 weeks are not uncommon. During this time the food intake of the animal is remarkably constant also, although it is greatly reduced.[12]

It is impossible to maintain a completely satisfactory control for an animal developing riboflavin deficiency. If the control animal receives adequate riboflavin but a quantity of food equal to that of his deficient pair-mate (paired-feeding), the former will gain weight because of the increased utilization of food due to the adequacy of the riboflavin intake. If the control is fed only enough food to keep his weight equal to that of his deficient pair-mate (weight-control feeding) the former will receive considerably less food than the latter and this may lead to deficiencies and imbalances not encountered by the deficient animal. For the purposes of this study it was thought that paired weight-control mates would provide the most meaningful controls.

The heart is insensitive to riboflavin deficiency as far as can be judged from the criteria used in this study. Once supplementation is initiated, both the protein content and the SDH activity of the whole organ show immediate and related increases, and as a consequence the SDH activity per milligram of protein is not influenced.

The kidneys also are relatively insensitive to riboflavin deficiency except that the deficient animals develop enlarged kidneys relative to their body weights (fig. 3). That an enlargement occurred after prolonged deficiency had been noted before (13), but our time-course study revealed that 3 days after supplementation was begun the kidneys had returned to their normal relative weight. The return to normal relative weight was, at least in part, due to a transient dehydration, although the concentration of protein in the deficient kidneys does not indicate that the larger kidney is enlarged due to greater hydration (fig. 4).

By far the most striking changes revealed by this study were those occurring in the liver. We were quite prepared for the loss of SDH activity in the liver during riboflavin deficiency. Two earlier studies by Axelrod and co-workers (14, 15) showed only a small loss of succinoxidase activity in the liver after prolonged riboflavin deficiency. The measurement of succinoxidase is not as meaningful under these conditions as a study of SDH; the two procedures do not measure reaction sequences with the same limiting step. More recently it had been shown by Burch and co-workers (16) that prolonged riboflavin deficiency caused a marked reduction in SDH activity in the liver of the rat, but again, the rate at which the loss occurred was not known. We did not expect that the ingestion of adequate riboflavin but inadequate food would cause a prompt and large increase in the activity of the liver SDH (fig. 1). We have observed repeatedly that the SDH activity for the liver of rats receiving both riboflavin and food in adequate amounts remains essentially constant regardless of age or weight. Whether the elevation of SDH activity due to food restriction applies to the values for the deficient animal resulting in an observed value which is too high cannot be answered at present. It is clear that the widely practiced procedure of expressing an enzyme activity of an experimental preparation as percentage of the control would have been misleading in this instance.

The rapidity with which the liver recovered its SDH activity was a point of special interest in this study. When it was discovered that the activity had returned to normal in approximately 8 days (fig. 1) of supplementation, it was realized that the synthesis of new mitochondria would not adequately account for all of the observations. The value for the half-life of rat liver mitochondria is somewhat uncertain and different methods give values ranging from 8.5 days (17) to 14 days (4). If the half-life is as short as 8.5 days and if mitochondria production were the only factor involved in the regeneration of the SDH activity, it appears that at best not more than a 50% increase could be expected. This led to the realization of the need for information relative to mitochondrial protein synthesis and riboflavin uptake during supplementation of the deficient animal.

During the induction of riboflavin deficiency the liver became enlarged (fig. 3). This confirmed an observation which had been made before (16). This enlargement

[12] This restricted intake cannot be expressed as a fraction of a normal value. The only normal value would be the food consumed by a young animal receiving adequate riboflavin and consuming food at will but not gaining weight.

is not adequate to compensate fully for the decrease in the activity of the SDH per milligram of protein as shown in figure 2 where the SDH activities of the whole liver, kidneys and heart for the two groups are compared. The enlargement, however, may be of considerable value to the deficient animal since the SDH activity of the whole liver of the deficient animal falls only 25% below normal (normal being the value for the animal receiving adequate riboflavin and food and not the weight-control mate) while it falls to less than 50% of normal when its activity per milligram of protein is used for comparison.

When the riboflavin-deficient rat is permitted to consume adequate riboflavin and food, no response observed in this study takes place as rapidly as the increase in the liver weight. After 2 days of supplementation the weight of the liver had increased 150%; since the total animal weight also increased, this is only a 97% increase relative to the body weight. Much of the initial increase in the body weight was due to the increase in the weight of the liver. On day 42 of the deficiency, the average liver weight was 4.05 g and the average body weight was 69 g. The liver represented 6% of the weight of the body without the liver (65 g). After 2 days of supplementation, the average liver weight was 9.87 g and the average body weight was 82 g; the liver represented 14% of the weight of the body without the liver (72 g). Approximately one-half of the increase of the body weight for these 2 days was due to the increased weight of the liver.

Of the nearly 6-g increase in the liver weight during the first 2 days of supplementation, approximately 0.7 g was made up of protein as shown in figure 7. Though some of the increase may have been due to lipid, it is clear that most of it must have been due to a rapid uptake of water. This conclusion is supported by the data given in figure 4 which shows the rapid decrease in liver protein concentration during this time. This decrease in concentration occurs despite the rapid increase in liver protein at the same time (fig. 7). No net increase in liver protein occurs between day 2 and day 7. The concomitant steady increase in the weight of the body during the remainder of the period is primarily responsible

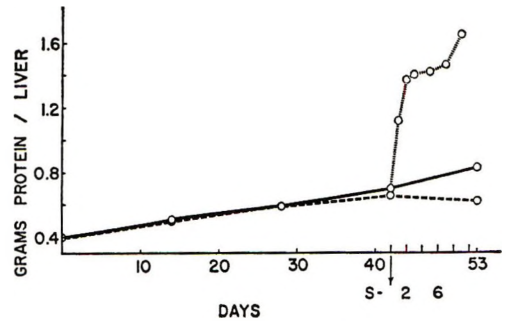

Fig. 7 Protein content of the total average liver. Deficient animals (——) were fed ad libitum; control animals (– –) were weight controlled to pair-mates in deficient group; supplemented animals (····) were fed an adequate diet ad libitum from day 42. Arrow on abscissa indicates start of supplementation period. Numbers following S– on abscissa indicate days of supplementation.

for the fall in liver weight as percentage of body weight after day 2 of supplementation.

As stated above, the rate of regeneration of the SDH activity in the liver was too rapid to be due exclusively to the formation of new mitochondria. This does not mean that an increase in the number of mitochondria is not involved, but there must be other mechanisms which can respond more rapidly. Among these might be the synthesis of new protein by the liver and the incorporation of new protein into existing mitochondria, probably including SDH apoenzyme, and the incorporation of riboflavin in the form of covalently bound FAD. The implication is that the reduced SDH activity might be due to a reduced quantity of protein in the mitochondria or a loss of flavin from the mitochondria without loss of the apoenzyme or the loss of both protein and flavin.

Figure 7 shows that the rate of increase of protein content does not parallel the rate of increase of SDH activity. The SDH activity, however, is not a function of the general protein of the liver but of the MP, and more specifically, the AIMP. Figure 5 shows that the increase in MP per average liver is remarkably like the increase in total protein per average liver. It is further shown that the AIMP per average liver parallels the MP. Since the SDH apoenzyme is a portion of the AIMP, this parallelism supports the idea that SDH apoenzyme is

synthesized very rapidly since it is unlikely to be specifically excluded from the process. The findings also support the concept that the SDH apoenzyme need not be synthesized only as part of a new mitochondrion. The portion of figure 5 related to protein rules out the possibility that the synthesis of new MP is exclusively responsible for the rapid recovery of SDH activity.

The other portion of figure 5 shows that the riboflavin uptake by the MP of the average liver starts immediately the vitamin is available and it continues at a regular rate throughout the period of study. The riboflavin incorporation by the AIMP of the average liver does not begin until after 1 day's delay but from that time the uptake also continues at a regular rate throughout the test period. During the 9 days of the test period the MP had taken up 33% of the total flavin administered while only 6% was covalently bound to SDH.

Figure 6 shows that the parallelism between the riboflavin uptake per milligram of AIMP and the rate of regeneration of SDH activity is very good; the agreement between the riboflavin uptake by the MP and the rate of regeneration of SDH activity is reasonably good. This latter agreement is probably due to the fact that the ratios of AIMP to MP for days 1, 2, 4, 7 and 9 are, 6, 17, 17, 19 and 21%, respectively.

In the excellent report published by Burch and her associates (16) an observation of considerable importance to our study was reported. The observation was: "It was consistently found that the yield of mitochondria was greater from deficient livers than from normal or calorie-restricted livers." In connection with another study we have confirmed this finding. This response of the rat to a riboflavin deficiency provides us with the necessary "facilities" to account for the rapid regeneration of the SDH activity.

The availability of increased numbers of mitochondria makes it possible to present a reasonable description of the process for the regeneration of the SDH activity of the liver. Once riboflavin and food become available to the riboflavin-deficient liver of the rat, the mitochondria replenish their protein stores extremely rapidly. The actual restoration of the SDH activity is then dependent on the rate at which the riboflavin can be covalently bound to the SDH apoenzyme.

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Studies on Blood Glucose and Hepatic Glycogen in Rats Force-fed a Threonine-devoid Diet [1]

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ABSTRACT This study was designed to determine whether young rats force-fed a purified diet devoid of a single essential amino acid develop alterations in blood glucose tolerance. The results indicate that young rats force-fed for 1 to 3 days a threonine-devoid diet in comparison with those force-fed a complete diet have an impaired glucose tolerance response following the intravenous administration of glucose. Also, 16 to 18 hours after the last feeding, the animals force-fed the threonine-devoid diet have a two- to threefold elevation of hepatic glycogen over that in animals force-fed the complete diet. The hepatic glycogen in both control and experimental animals becomes depleted to a similar degree after the administration of epinephrine, glycine, insulin or glucagon. The administration of ^{14}C-glucose intraperitoneally to animals force-fed the deficient or complete diet for 3 days resulted in greater incorporation into hepatic glycogen by experimental than of control animals.

In earlier studies from this laboratory (1–3) we found that young rats force-fed a purified diet devoid of a single essential amino acid developed pathologic changes that closely resembled many of those found in infants with kwashiorkor (4). The pathologic changes in the experimental animals consisted of fatty liver with increased glycogen, atrophy of the stomach, thymus and spleen and atrophy of the acini of the pancreas, submaxillary gland and parotid (1–3).

In view of a number of clinical reports that infants with kwashiorkor have an impairment in blood glucose tolerance (5–10), we decided to investigate whether our experimental animals which develop kwashiorkor-like pathologic lesions have an alteration in the regulation of blood glucose. In an earlier study (11) we reported that the fasting blood sugar levels were similar in rats force-fed a threonine-devoid diet and in those force-fed a complete diet. However, at the same time the hepatic glycogen content was two- to threefold higher in the experimental than in the control animals (11–13). Hepatic glucose 6-phosphatase activity was similar in both groups of animals (11).

In this study we investigated the blood glucose tolerance responses following intravenous administration of glucose to animals force-fed a threonine-devoid or a complete diet for 1, 2, or 3 days. The results indicate a rapidly developing disturbance in the rate of disappearance of blood glucose in the experimental animals. Also, in this study we investigated the levels of hepatic glycogen and blood glucose in animals at different times after force-feeding a complete or threonine-devoid diet.

EXPERIMENTAL METHODS

Male and female rats of the Sprague-Dawley strain,[3] 1 month old, weighing on the average 75 g, were used in most experiments. In two experiments (table 3 and exp. W-1 of table 4) rats weighing 136 g were used. The animals were fed a commercial diet [4] for several days before the experiments were begun. In all experiments, groups of animals, each of the same sex, age and weight were used.

The basal diet was the same as that used in our earlier experiments (3). The percentage of dietary components was as follows: essential amino acids, 9.2; nonessential amino acids, 8.1; vitamin-sucrose mixture, 5; salt mixture, 4; corn oil,[5] 5; cod

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[2] Present address: Punjab Agricultural University, Ludhiana-I, Punjab, India.
[3] Sprague-Dawley, Inc., Madison, Wis.
[4] Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.
[5] Mazola, Corn Products Company, New York, N. Y.

liver oil,[6] 1.5; and dextrin, 67.2. Dextrin was substituted for the threonine in the threonine-devoid diet. In one experiment (table 3) a phenylalanine-devoid diet was used. The diets were blended with distilled water so that each milliliter of diet mixture contained 0.5 g of diet and was in a suitable form for administration by stomach tube. Rats were tube-fed three times daily at 8:00 AM, 1:00 PM and 5:00 PM. The animals received an average daily feeding of 1 g diet/10 g of initial body weight. The complete diet was force-fed to the control rats throughout and to the experimental animals for a few days prior to beginning the deficient diet. All rats had free access to water. Rats were housed in individual wire cages with raised bottoms and kept in an air-conditioned room.

Rats were weighed at the beginning and at the termination of each experiment. The animals were anesthetized with ether and exsanguinated. The liver was rapidly removed and weighed. Equal pieces from the median and left lateral lobes, that weighed together about 1 g, were immediately placed in 30% KOH for glycogen determination, which was performed according to the method of Seifter et al. (14). In these experiments the same method was used also for determination of blood sugar, using a tungstic acid filtrate of blood samples obtained from the abdominal aorta of ether-anesthetized animals.

In studies to test for blood glucose tolerance, the animals, 16 to 18 hours after the third feeding (5:00 PM) the preceding day, were injected into the tail vein with a 50% *d*-glucose solution. The dose was 125 mg (0.25 ml) glucose/100 g body weight. Blood samples were taken from intraorbital venous plexus of each rat at zero time and at 5, 10, 20, 30, 60, and 120 minutes following intravenous glucose injection. Blood was measured by the ultramicroadaptation of the method of Keston[7] and Teller.[8]

In one experiment animals were force-fed the complete or phenylalanine-devoid diet for 3 days, then on the morning of day 4 were treated with one of the following: epinephrine (adrenalin chloride),[9] 0.05 mg/rat given intraperitoneally each hour for three doses and then killed 1 hour after the last dose, or glycine, 5 ml of a 20% solution (1 g) by stomach tube and killed 2 hours later.

In one experiment rats were force-fed the complete or threonine-devoid diet for 3 days, then on the morning of day 4 they were divided into four groups and treated intraperitoneally with one of the following: 1) insulin,[10] 4 units/rat 2 hours before killing; 2) glucagon,[11] 200 μg/rat 3 hours before killing; 3) hydrocortisone 21-phosphate,[12] 6 mg/rat 4 hours before killing, and 4) saline, 2 ml 2.5 hours before killing.

In several experiments ^{14}C-*d*-glucose (uniformly labeled) in doses of 10 or 20 μCi (15.5 mCi/mmole) was administered intraperitoneally to control and experimental animals at 0.5 to 2 hours before killing. Carbon-14-incorporation into hepatic glycogen was measured. In these experiments hepatic glycogen was obtained by the method of Seifter et al. (14) and was further purified by resuspension and precipitation. Aliquots were plated on stainless steel planchets and counted in a windowless gas flow counter.

RESULTS

Blood glucose tolerance tests. In a series of experiments, groups of rats were force-fed the complete (C) or threonine-devoid (TD) diet for 1, 2, or 3 days. The following mornings, 16 to 18 hours after the last tube-feeding, blood was taken from each animal of both groups for fasting blood sugar values. These values, in milligrams per 100 ml, were for control and experimental animals, respectively, as follows: 1 day, 89, 89; 2 days, 75, 95; and 3 days, 88, 83. All animals were then given 125 mg glucose/100 g body weight intravenously and venous blood samples were taken after 5, 10, 20, 30, 60, and 120 min-

[6] Cod liver oil liquid, Mead Johnson Laboratories, Evansville, Ind.

[7] Keston, S. A. 1956 Specific colorimetric enzymatic analytical reagents for glucose. In: Papers of the 129th Meeting of the American Chemical Society, April 1956, p. 31C (abstract).

[8] Teller, J. D. 1956 Direct, quantitative colorimetric determinations in serum or plasma glucose. In: Papers of the 130th Meeting of the American Chemical Society, September 1956, p. 69C (abstract).

[9] Adrenalin chloride, Parke, Davis & Company, Detroit, Mich.

[10] Regular insulin, Iletin, Eli Lilly & Company, Indianapolis, Ind.

[11] Crystalline glucagon, Eli Lilly & Company, Indianapolis, Ind.

[12] Hydrocortone phosphate, Merck, Sharp & Dohme, West Point, Penna.

utes. The logarithm of the blood glucose concentration (mg/100 ml) was plotted against time for each animal and the best fitting straight line drawn through the points. The rate of glucose utilization, Kt, or percent per minute (table 1), was calculated from this line for each animal of both groups of animals (15). The results of all experiments are summarized in table 1. It is readily apparent that the intravenous glucose tolerance was impaired in rats force-fed the TD diet in comparison with those force-fed the C diet. After 1 or 2 days, the difference or decrease in glucose utilization was 33 to 40%, and after 3 days, 72% in experimental in comparison with control animals. Figure 1 reveals a comparison of the blood glucose curves in response to glucose administered intravenously in rats force-fed the TD diet and those force-fed the C diet for 1 and 3 days in a typical experiment.

Studies on hepatic glycogen and blood glucose at different intervals after force-feeding diet. In a series of experiments animals were force-fed the C or TD diet for one to four feedings and then groups of animals were killed at different intervals and hepatic glycogen and blood sugar were measured. The results of these experiments are summarized in table 2. In the first series of experiments (exp. nos. 119, 123 and 187) rats received one tube-feeding at 8:00 AM and were killed after 2, 4, 5, or 6 hours. The results indicated that hepatic glycogen levels increased after feeding and that levels were similar in C and TD groups. There was, however, a 47 to 63% greater increase in blood glucose levels in the TD in comparison with control animals at intervals up to 5 hours. In the second series of experiments (exp. nos. 116 and 187) rats received two tube-feedings (8:00 AM and 1:00 PM) and were killed after 4 or 6 hours. The results indicated that hepatic glycogen and blood glucose values were quite similar in both groups. In the third series of experiments (exp. nos. 106 and 216), rats received three tube-feedings (8:00 AM, 1:00 PM and 5:00 PM) and were killed after 2, 3, 5, 6, or 16 hours. Hepatic glycogen contents were similar up to 6 hours but at 16 hours the levels were several-fold greater in the TD than in the C groups of animals. Blood sugar levels were from 39 to 132% greater in the TD than in the C animals up to 6 hours and became similar at 16 hours. In the fourth series of experiments (exp. nos. 109, 206, and 219) rats were tube-fed three times one day (8:00 AM, 1:00 PM, 5:00 PM) and then a fourth time the following morning (8:00 AM) and were killed after 2 or 5 hours. Before receiving feeding 4, hepatic glycogen was approximately 217% greater and blood glucose was similar in the TD animals in comparison to C animals. After 2 hours, a 36 to 63% increase of hepatic glycogen was present in the TD over that in the C animals. This difference, however, disappeared at 5 hours in two or three experiments. Blood glucose

TABLE 1

Intravenous glucose tolerance of rats force-fed the complete (C) or threonine-devoid (TD) diet for 1, 2 or 3 days

Duration	No. of exp.	Group	No. of rats	Kt [1]	
days				*% /minute*	*% of control* [2]
1	4	C	8	2.48 ± 0.41 [3]	100
		TD	8	1.48 ± 0.24	60 ± 5 [4]
2	2	C	4	2.68 ± 0.46	100
		TD	3	1.55 ± 0.19	67 ± 25
3	2	C	4	3.83 ± 0.34	100
		TD	4	1.06 ± 0.13	28 ± 3 [5]

[1] $Kt = \frac{2.3 (\log C_1 - \log C_2)}{t_2 - t_1} \times 100$ = percentage glucose utilized per minute; C_1 and C_2 = glucose concentration at times t_1 and t_2 after intravenous injection. Each value is the mean of the average values of two animals in each experiment for the number of experiments indicated.

[2] Values are means ± SE values in percent compared with controls in each experiment.

[3] Mean ± SE.

[4] $P < 0.01$.

[5] $0.05 > P > 0.01$.

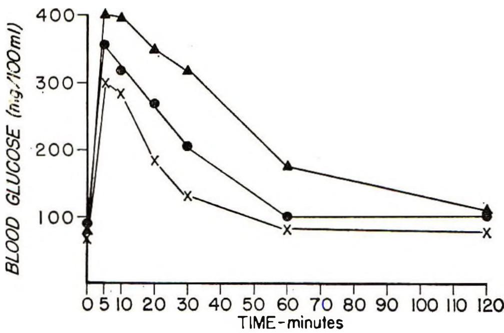

Fig. 1. Blood glucose tolerance curves of rats force-fed a complete or threonine-devoid diet for 1 or 3 days. Sixteen to 18 hours after the last tube-feeding the animals received intravenously 125 mg glucose/100 g body weight. ×——×, rats force-fed the complete diet for 1 or 3 days. Since the curves for 1 and 3 days were identical, one curve is presented and each point represents the mean of four control animals. ●——●, rats force-fed the threonine-devoid diet for 1 day. ▲——▲, rats force-fed the threonine-devoid diet for 3 days. Each point represents the mean of two experimental animals.

values were higher after 2 and 5 hours in the TD than in the C animals.

To determine whether there may be an alteration in gastrointestinal absorption under our experimental conditions, the gastrointestinal contents in one experiment (exp. 109, table 2) were removed when the animals were killed. The contents were assayed for carbohydrate by the anthrone method (14). The results expressed as mean ± SE (milligrams glucose) for the gastrointestinal carbohydrate contents of C and TD rats (three animals per group) respectively, were as follows: before receiving feeding 4, 56 ± 4, 106 ± 18; 2 hours after feeding 4, 853 ± 100, 772 ± 41; and 5 hours after feeding 4, 174 ± 35, 246 ± 12. The results indicate that more carbohydrates (89%) remained at 16 to 18 hours after feeding (before feeding 4) in the TD than in the C group; 2 hours after the morning feeding the values were high in both groups; and after 5 hours the values decreased but somewhat less marked in the TD than in the C group. It is conceivable that in the TD group there is some delay in absorption from the intestinal tract which may influence the hepatic glycogen levels after 16 to 18 hours.

Hepatic glycogen response to glycogenolytic agents. In one experiment we determined whether the increased hepatic glycogen of animals force-fed an essential amino acid devoid diet would respond to glycogenolytic agents, epinephrine and glycine (16). In this experiment, animals were force-fed for 3 days a phenylalanine-devoid (PD) or complete (C) diet. On the morning of day 4, some animals were treated with epinephrine or glycine before killing. The results of this experiment are summarized in table 3. Following epinephrine administration, the C and PD rats, respectively, had a 81 and 95% decrease in liver glycogen with a 59 and 125% increase in blood glucose. Following glycine administration, the C and PD rats, respectively, had a 99 and 98% decrease in liver glycogen and a 38 and 48% decrease in blood glucose. Thus, the response to the two agents was similar in the C and PD rats with the exception that blood glucose increased to a much greater degree in the PD rats following epinephrine. When aliquots of livers of C and PD rats were left at room temperature for 2 hours, there was a 43% decrease in liver glycogen of PD rats and a 37% decrease in liver glycogen of C rats in comparison to aliquots measured at zero time.

In one additional experiment, rats were force-fed the C or TD diet for 3 days and then the following morning they were divided into four groups of three rats each and treated intraperitoneally with one of the following: 1) insulin, 4 units/rat 2 hours before killing; 2) glucagon, 200 μg/rat 3 hours before killing; 3) hydrocortisone 21-phosphate, 6 mg/rat 4 hours before killing; and 4) saline, 2 ml 2.5 hours before killing. Hepatic glycogen (milligrams glycogen per total liver) was measured. The results in percent difference of each group compared with the control (4th) group for the C and TD animals, respectively, were as follows: insulin, −63%, −59%; glycogen, −53%, −57%; and hydrocortisone, +96%, +76%.

^{14}C-glucose incorporation into hepatic glycogen. In several experiments rats were force-fed the C or TD diets for 1 day and the following morning they received ^{14}C-glucose 0.5, 1, or 2 hours before being killed. Incorporation into hepatic glycogen

TABLE 2

Blood glucose (BG), mg %, and liver glycogen (LG)

No. of feedings [1]	Exp. no.	Group	Body wt	0 hr		2 hr		3 hr	
				BG	LG	BG	LG	BG	LG
			g						
1	119	C	75	98 ± 18 [2]	1 ± 0 [2]	171 ± 21	95 ± 17		
		TD	75			256 ± 10	126 ± 14		
	123	C	80						
		TD	75						
		C [3]	80						
		TD [3]	79						
	187	C	63						
		TD	64						
2	116	C	66						
		TD	68						
	187	C	61						
		TD	64						
3	216	C	68					147 ± 6	274 ± 1[illegible]
		TD	68					168 ± 10	274 ± 1[illegible]
	106	C	79			190 ± 40	379 ± 29		
		TD	79			441 ± 139	342 ± 40		
4	219	C	75	103 ± 12	29 ± 18	236 ± 84	115 ± 7		
		TD	75	142 ± 23	119 ± 0.2	304 ± 58	187 ± 26		
	109	C	70	103 ± 0.3	137 ± 40	136 ± 13	228 ± 15		
		TD	70	103 ± 8	305 ± 78	127 ± 11	309 ± 25		
	206	C	80			130 ± 9	329 ± 60		
		TD	81			283 ± 153	446 ± 4		

[1] Rats receiving one feeding received 4 ml of diet at 8:00 AM; rats receiving two feedings received 4 ml o[illegible] 3 ml and 4.5 ml of diet at 8:00 AM, 1:00 PM and 5:00 PM, respectively; and rats receiving four feedings[illegible] respectively. Three rats were used in each group.

[2] Mean ± SE.

[3] Rats received one feeding of 9 ml of diet at 8:00 AM.

…er in hours after last feeding

4 hr		5 hr		6 hr		15 hr	
G	LG	BG	LG	BG	LG	BG	LG
		139 ± 17	227 ± 8				
		204 ± 22	202 ± 26				
± 0	165 ± 29						
± 57	103 ± 32						
± 27	172 ± 32						
± 79	102 ± 18						
				158 ± 7	131 ± 9		
				131 ± 8	128 ± 8		
± 10	226 ± 38						
± 20	172 ± 31						
				135 ± 24	212 ± 8		
				138 ± 7	256 ± 22		
				115 ± 11	285 ± 13	82 ± 3	8 ± 4
				262 ± 88	239 ± 35	103 ± 5	121 ± 28
		257 ± 94	496 ± 40				
		357 ± 89	467 ± 70				
		157 ± 27	270 ± 18				
		224 ± 19	278 ± 55				
		124 ± 8	220 ± 31				
		125 ± 14	384 ± 33				
		136 ± 8	361 ± 27				
		184 ± 83	355 ± 26				

8:00 AM and 1:00 PM, respectively; rats receiving three feedings received 4.5 ml, 5 ml, 3 ml, 5 ml and 5 ml of diet at 8:00 AM, 1:00 PM, 5:00 PM and 9:00 AM,

TABLE 3

Effect of administration of epinephrine or glycine on liver glycogen and blood glucose of rats force-fed a phenylalanine-devoid (PD) or complete (C) diet for 3 days

Group [1]	Treatment	No. of rats	Liver glycogen	Blood glucose
			mg/liver	*mg/100 ml*
C	None	2	88 ± 5 [2]	97 ± 4 [2]
C	Epinephrine [3]	2	17 ± 7	154 ± 34
C	Glycine [4]	2	1 ± 0	60 ± 0
PD	None	2	132 ± 25	110 ± 3
PD	Epinephrine [3]	3	7 ± 4	247 ± 19
PD	Glycine [4]	2	2 ± 0	57 ± 1

[1] Rats weighed 136 g at the beginning of the experiment.
[2] Mean ± SE.
[3] Adrenalin chloride, 0.05 mg/rat given intraperitoneally each hour for three doses and killed 1 hour after the last dose.
[4] Glycine, 5 ml of a 20% solution (1 g) by stomach tube and killed 2 hours later.

measured. The results are summarized able 4. In four experiments there were eases (19% after 2 hours, 109 and % after 1 hour and 54% after 0.5 r) in ^{14}C-incorporation into total hepat- lycogen of the TD groups in compari- with the C groups. Even though the nimals had a greater pool of hepatic ogen, an average 169% increase over in C animals, the TD animals had ter incorporation into total hepatic gly- n. These data are difficult to evaluate e the pool sizes of glucose and its me- lites in the liver of C and TD rats are known.

DISCUSSION

n recent years a number of studies (5–17) have been reported on aspects of bohydrate metabolism in kwashiorkor. he majority of these reports (5–10) an airment in blood glucose tolerance has n described in these patients. In addi- , in a few reports, a fair amount (18) an elevated amount (19) of hepatic cogen has been described.

eview of experimental studies with shiorkor-like models utilized by other oratories has revealed several reports cribing a disturbance in blood glucose rance (15, 20, 21) as well as increased atic glycogen levels (15, 20–24) in experimental animals. In these studies other investigators the changes in car- ydrate metabolism were found in ex- iments which lasted days or weeks. In own present study we observed that nges can occur within 1 day after force- ding young rats a purified diet devoid of one essential amino acid. Thus, in animals force-fed a calorically adequate intake of an imbalanced diet, this altered physiological response can occur rapidly.

In earlier studies (1–3, 11–13) we reported an increase in hepatic glycogen in rats force-fed for 3 to 7 days a single essential amino acid-devoid diet. In these studies the hepatic glycogen levels were measured in the morning, 16 to 18 hours after the last tube-feeding. Therefore, it was not clear whether the increased hepatic glycogen content of the experimental animals was due to increased accumulation, decreased depletion, or a combination of both. Based on our present study, it appears that following an evening (3rd) feeding, hepatic glycogen content increases equally for up to 6 hours in control and experimental animals. After 16 to 18 hours, however, the decrease in hepatic glycogen content is less in the experimental than in control animals. Thus, the relative increase in hepatic glycogen in the experimental animals is only present after a long period following the last meal. At this time the glycogen, when examined after histological staining, was diffusely distributed in the liver (1–3). Also using electron microscopy the hepatic glycogen was randomly distributed throughout the lobule, but most often tended in any single hepatic cell to be located around the nucleus and cell periphery, and in addition many lysosomes contained large amounts of glycogen (25). In view of the latter ultrastructural finding, one might speculate that the presence of glycogen in lysosomes might possibly be related to some interference

TABLE 4

Incorporation of ^{14}C-glucose into hepatic glycogen of rats force-fed a complete (C) or threonine-devoid (TD) diet for 1 day

Exp. no.	Group	No. of rats	Body wt	Dosage of ^{14}C-glucose	Time of intra-peritoneal admini-stration [1]	Liver glycogen	Incorporation into liver glycogen	
			g	*μCi*	*minutes*	*mg/liver*	*cpm/mg glycogen*	*cpm/liver glycogen*
W-1	C	2	136	10	120	118 ± 25 [2]	6 ± 2.0 [2]	722 ± 349 [2]
	TD	3	136	10	120	423 ± 69 [3]	2 ± 0.4	862 ± 58
W-2	C	3	68	10	60	136 ± 39	8 ± 2.3	850 ± 89
	TD	2	71	10	60	294 ± 2 [3]	8 ± 2.4	2390 ± 737
W-15	C	3	77	10	60	60 ± 13	16 ± 2.8	982 ± 262
	TD	3	74	10	60	131 ± 35	18 ± 5.4	2053 ± 398
W-4	C	3	73	20	30	141 ± 25	22 ± 6.5	2883 ± 404
	TD	3	73	20	30	396 ± 37 [4]	11 ± 0.2	4426 ± 531

[1] Carbon-14 glucose administered 16 to 18 hours after last feeding.
[2] Mean ± SE.
[3] $0.05 > P > 0.01$.
[4] $P < 0.01$.

with the breakdown process (25). Further studies are being conducted in an attempt to characterize the glycogen and enzymes present within the hepatic lysosomes.

In a few other experiments we investigated several aspects of the metabolism of hepatic glycogen in rats force-fed a single essential amino acid-devoid diet. Hepatic glycogen of control and experimental animals responded to the administration of glycogenolytic agents (epinephrine, glycine, insulin and glucagon) to a similar degree. Iodine spectrum analysis on hepatic glycogen revealed no differences in control and experimental animals.[13] Liver activities of glucose 6-phosphatase (5), phosphorylase [14] and glycogen synthetase [15] were similar in control and experimental animals. Our findings for hepatic glucose 6-phosphatase are in disagreement with those of Sekhara Varma and Hanumantha Rao (26) who reported a decrease in animals force-fed a threonine-devoid diet. Recently we have again assayed for hepatic glucose 6-phosphatase using maleate buffer (27) in place of citrate buffer (11) as used earlier. Our results revealed no difference in hepatic levels of this enzyme in control and experimental animals. Therefore, we are unable to reconcile our results with those of Sekhara Varma and Hanumantha Rao (26). The latter workers also described comparatively unaltered activities of phosphoglucomutase, glucose-phosphate isomerase and glucose 6-phosphate dehydrogenase (26). Thus, based on the data of the hepatic enzymes listed above, as well as on the unimpaired glycogenolysis due to epinephrine, glycine, insulin and glucagon, we are unable to speculate as to the role of enzymes in the elevated hepatic glycogen levels in rats fed a threonine-devoid diet.

In reviewing some of the aspects that might play a part in the increased hepatic glycogen in rats force-fed an essential amino acid-devoid diet in comparison with those force-fed the complete diet, several experimental observations should be mentioned. The increase in hepatic glycogen occurred within 1 day and is present up to 1 to 2 weeks (3, 11). This increase developed with a purified diet of entirely different composition and devoid of an essential amino acid (13), one different from that used in most of our previous studies (1–3, 11, 12, 25). It did not develop in animals force-fed for 3 days an amino acid- or protein-free diet (13). In other studies dealing with protein deficiency rather than with specific amino acid deficiencies, rats force-fed protein-deficient diets revealed an increase in hepatic glycogen (28). It was found in animals force-fed a threonine-devoid diet which contained a low or high carbohydrate content (12), one which contained a low or high fat content (29), or one which contained a low or high amino

[13] Epstein, S. M., and H. Sidransky, unpublished data.
[14] Sidransky, H., D. S. Wagle, M. Bongiorno and E. Verney, unpublished data.
[15] See footnote 14.

acid content (30). Elevated hepatic glycogen was not observed when rats were force-fed a threonine-deficient diet (a diet containing 0.25% threonine) for 1 to 4 weeks (31), unlike rats force-fed a threonine-devoid diet (a diet containing no threonine) for 3 to 7 days (1, 11). It was observed in adrenalectomized (32) and in hypophysectomized (33) rats force-fed a threonine-devoid diet for 1 to 3 days. It is of interest that high levels of liver glycogen were not observed in animals fed ad libitum a threonine-, methionine-, valine- or lysine-devoid diet for 3 to 6 days (1, 2, 11) where the diet intake was voluntarily decreased. It has been observed in rats force-fed diets devoid of each of eight different single essential amino acids (1, 2, 3, 26), except with the purified diet devoid of methionine (11, 34). In earlier studies (3, 30) we have observed that rats force-fed a single essential amino acid-devoid diet show a rapid loss of skeletal muscle protein and this alteration may play a role in the overall carbohydrate metabolism of the experimental animal.

In an attempt to explain the alteration in blood glucose tolerance response in the rats force-fed the threonine-devoid diet in comparison with rats force-fed the complete diet, at least two factors can be considered. First, it is possible that there is an altered response of insulin following glucose administration in the experimental animals. In other studies an altered reaction to insulin has been reported in dogs (20) and pigs (21) fed a protein-deficient diet. Also, in other studies, it has been reported that certain amino acids may affect the response of insulin which influences blood glucose levels (35). Whether such alterations in the response of insulin may be the case in our experimental kwashiorkor-like model is as yet unknown. However, in three experiments where animals were force-fed the complete or threonine-devoid diet for 3 days and killed the morning of day 4, plasma insulin levels[16] were identical in both groups. Secondly, it is possible that since the hepatic glycogen levels were higher in the experimental than in control animals when the glucose tolerance tests were performed, less blood glucose was needed to be converted to hepatic glycogen and therefore more remained in the blood.

Although impaired intravenous glucose tolerance has been reported as an early sign of dietary necrotic liver degeneration (36), this was not the case under our experimental conditions. There was no morphologic evidence for such degeneration (1–3, 25). Likewise, studies on chemical alterations in RNA (11, 37), protein (11, 38) and lipid (11) metabolism in the livers of the experimental animals were not consistent with necrotic degeneration. However, since a number of metabolic alterations were observed (11, 37, 38), concomitantly with the changes in carbohydrate metabolism, it is conceivable that they may be interrelated. Because of this, the unraveling of the pathogenesis of this disturbance in hepatic carbohydrate metabolism may be indeed complex. Nevertheless, it is hoped that this experimental model can be used in further studies designed to gain an understanding of the pathogenesis of the nutritionally induced imbalanced state.

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[16] Insulin assays were performed by courtesy of Dr. James Field.

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Invitation for Nominations for 1970 American Institute of Nutrition Awards

Nominations are requested for the 1970 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) *A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1969,* to be considered for the 1970 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made during the banquet at the annual meeting.

Borden Award in Nutrition [1]

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Foundation Inc. The Award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritional significance of any food or food component. The Award will be made primarily for the publication of specific papers during the two previous years but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time. Employees of Borden Inc are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

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Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation,

[1] Sponsors of nominees for this award should note that a change has been made in the area of research which this award recognizes.

Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made a most significant recent contribution or has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

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Mead Johnson Award for Research in Nutrition [2]

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 40th birthday at the time the award is presented. Selection by the Jury of Award will be based either on a single outstanding piece of recent research in nutrition or on a series of recent papers on the same subject.

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The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

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1968 – W. H. Sebrell, Jr.
1969 – F. J. Stare

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Send nominations to:

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Department of Foods & Nutrition
Michigan State University
East Lansing, Michigan 48823

[2] Sponsors of nominees for this award should note the change which has been made in the age limitation.

Invitation for Nominations for 1970 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

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The following persons have been elected previously as Fellows of the Society:

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Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

Committee on Honorary Memberships:

L. C. NORRIS, *Chairman*
A. E. SCHAEFER
P. L. DAY

Send nominations to:

L. C. NORRIS
Department of Poultry Husbandry
University of California
Davis, California 95616

The following persons have been elected previously as Honorary Members of the Society:

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ERRATA

Vipperman, P. E., Jr., R. L. Preston, L. D. Kintner and W. H. Pfander. Role of calcium in the nutritional etiology of a metabolic disorder in ruminants fed a high grain ration. J. Nutr., 97: 449. On pages 457 and 458, figures 5 and 6 were printed upside down.

You may correct these errors in number 4 of volume 97 by cutting along the lines of the reprinted captions below and pasting over the captions in your copy.

Fig. 5 Illustration of extremes noted in bone ash from lambs in trial 3 which received rations containing 0.20% Ca and 0.45% P (bottom), and 0.44% Ca and 0.45% P (top).

Fig. 6 Illustration of extremes noted in thickness of metacarpal shaft from lambs in trial 3 which received rations containing 0.20% Ca and 0.45% P (bottom), and 0.44% Ca and 0.45% P (top).

Index, Volume 98

A

ABRAHAM, S. See Sabine, John R., 312.
Alcohol and amino acid transport in the human small intestine, 222.
Alkaline phosphatase in epiphyseal cartilage of normal and zinc-deficient chicks, 83.
AL NEJJAR, Z., AND F. W. HEGGENESS. Effect of spontaneous activity on response to intermittent feeding, 177.
Amino acid composition and nutritive value, effects of severe alkali treatment of proteins, 45.
— — mixtures, imbalanced, metabolic derangements in response of rats to ingestion, 255.
— — regulation of albumin synthesis, 395.
— — retention and balance in the young rat fed varying levels of casein, 168.
— — retention and balance in the young rat fed varying levels of lactalbumin, 159.
— — transport and alcohol in the human small intestine, 222.
Amino acids and liver xanthine oxidase, influence of zinc and vitamin D in rats, 351.
— —, dietary, and ambient temperature, effect on carcass fat deposition in rats, 344.
α-Aminoisobutyric acid, effect on arginine metabolism in chicks, 225.
ANILANE, J. K. See Simonson, M., 18.
Antibody formation, circulating, in scorbutic guinea pigs, 41.
Atherosclerosis and associated physiological factors in cockerels, effect of restricted energy and protein intake, 335.
Authors, Guide for, 127.
AXELROD, A. E. See Kumar, Mahendra, 41.

B

BAKER, D. H. See Sugahara, M., 344.
BAKKE, OLAV M. Urinary simple phenols in rats fed diets containing different amounts of casein and 10% tyrosine, 217.
BAKKE, OLAV M. Urinary simple phenols in rats fed purified and nonpurified diets, 209.
BALLOUN, S. L. See Rose, R. J., 335.
BARBORIAK, JOSEPH J., AND ROBERT C. MEADE. Impairment of gastrointestinal processing of fat and protein by ethanol in rats, 373.
BARRON, GEORGE P. See Ketcheson, Marion R., 303.
BENEDICT, FRANCIS GANO. A biographical sketch, 1.
BERNHART, F. W., S. SAVINI AND R. M. TOMARELLI. Calcium and phosphorus requirements for maximal growth and mineralization of the rat, 443.
Bile salt enhancement of riboflavin and flavin mononucleotide absorption in man, 288.
BITTER, R. A., C. J. GUBLER AND R. W. HENINGER. Effects of forced-feeding on blood levels of pyruvate, glucocorticoids and glucose and on adrenal weight in thiamine-deprived and thiamine antagonist-treated rats, 147.
BLACKMON, D. M. See Miller, W. J., 411.
Blood glucose and hepatic glycogen in rats force-fed a threonine-devoid diet, 477.
Bone mineralization in young chicks, effect of dietary magnesium and fluoride, 271.
BONGIORNO, M. See Sidransky, H., 477.
BUNCE, G. E., AND K. W. KING. Amino acid retention and balance in the young rat fed varying levels of casein, 168.
BUNCE, G. E., AND K. W. KING. Amino acid retention and balance in the young rat fed varying levels of lactalbumin, 159.

C

Calcium and phosphorus requirements for maximal growth and mineralization of the rat, 443.
CAMPBELL, T. C. See Weatherholtz, W. M., 90.
Carbohydrate-containing and "carbohydrate-free" diets fed to chicks, effect of various sources of nonessential nitrogen in promoting growth, 297.
Casein, amino acid retention efficiency with, 168.
—, tyrosine and urinary phenols in rats, 217.
CHENG, CHUAN HUAN, MARY KOCH AND ROBERT E. SHANK. Dietary regulation of transketolase activity in liver, 64.
Chick, growing, effect of dietary protein on hepatic lipogenesis, 356.
— tissue, effect of vitamin B_6 deficiency on leucine transaminase activity, 420.
Chicken, nonabsorptive antihypercholesterolemic action of phytosterols in, 435.
Chicks, effect of α-aminoisobutyric acid on arginine metabolism, 225.
—, fasted and fasted-refed, in vivo fatty acid and cholesterol synthesis, 367.
— fed carbohydrate-containing and "carbohydrate-free" diets, effect of various sources of nonessential nitrogen in promoting growth, 297.
—, fed copper-deficient diets, effect on bone collagen and selected bone enzymes, 57.
—, liver xanthine dehydrogenase depletion and repletion, 193.
—, normal and zinc-deficient, histochemical studies of alkaline phosphatase in epiphyseal cartilage, 83.
—, young, effect of dietary magnesium and fluoride on bone mineralization, 271.
—, zinc-deficient, pathological defects in the epiphyseal cartilage, 76.
CHOLAKOS, B. V. See Scrimshaw, N. S., 9.
Cholesterol and fatty acid synthesis, in vivo, in fasted and fasted-refed chicks, 367.

— -lowering action of phytosterols, 435.
—, synthesis and distribution, and effect of diet at liver endoplasmic reticula and plasma membranes in lean or obese rats, 105.
Choline-deficient rats, hepatic ATP and triglyceride levels in, with and without dietary orotic acid supplementation, 188.
CHOW, B. F. See Simonson, M., 18.
CHU, RICHARD C. See Cox, Dennis H., 449, 459.
Cockerels, effect of restricted energy and protein intake on atherosclerosis and associated physiological factors, 335.
Copper deficiency, effect on chick bone collagen and selected bone enzymes, 57.
—, zinc and iron in the postnatal rat, 303.
Coprophagy, effect on experimental iron absorption in the rat, 95.
COX, DENNIS H. See Ketcheson, Marion R., 303.
COX, DENNIS H., RICHARD C. CHU AND SANDRA A. SCHLICKER. Zinc deficiency in the maternal rat during gestation, and zinc, iron, copper, and calcium content and enzyme activity in maternal and fetal tissues, 449.
COX, DENNIS H., SANDRA A. SCHLICKER AND RICHARD C. CHU. Excess dietary zinc for the maternal rat, and zinc, iron, copper, calcium, and magnesium content and enzyme activity in maternal and fetal tissues, 459.
CSALLANY, A. SAARI. See H. H. Draper, 390.

D

DALLMAN, PETER R. See Messer, Michael, 404.
DDT effect on rats raised on alpha-protein rations: growth and storage of liver vitamin A, 319.
DE GROOT, A. P., AND P. SLUMP. Effects of severe alkali treatment of proteins on amino acid composition and nutritive value, 45.
Diet, complete purified equine, composition, 330.
—, effect at liver endoplasmic reticula and plasma membranes, and influence of cholesterol synthesis and distribution, in lean or obese rats, 105.
—, threonine-devoid, force-fed to rats, studies on blood glucose and hepatic glycogen, 477.
—, threonine-free amino acid, fed to rats, tryptophan metabolism, 427.
Dietary amino acids and ambient temperature, effect on carcass fat deposition in rats, 344.
— fat and the inhibition of hepatic lipogenesis in the mouse, 312.
— -induced depletion and repletion of avian liver xanthine dehydrogenase, 193.
— protein, effect on hepatic lipogenesis in the growing chick, 356.
Diets, high protein, effects with normal and low energy intake on wound healing, hair growth, hair and serum zinc, and serum alkaline phosphatase in dairy heifers, 411.
DRAPER, H. H., AND A. SAARI CSALLANY. A simplified hemolysis test for vitamin E deficiency, 390.

E

Enzymes and minerals in maternal zinc deficiency, 449.
— and minerals, maternal and fetal, 459.
—, bone, and bone collagen formation in copper-deficient chicks, 57.
Equine diet, complete purified, composition, 330.

F

Fat and protein, impairment of gastrointestinal processing by ethanol in rats, 373.
—, dietary, and the inhibition of hepatic lipogenesis in the mouse, 312.
Fatty acid and cholesterol synthesis, in vivo, in fasted and fasted-refed chicks, 367.
— liver, influence of orotic acid as an inducing factor, in rats, 33.
FEATHERSTON, W. R. See Scholz, R. W., 193.
Feeding, artificial, of infant rats by continuous gastric infusion, 404.
—, intermittent, effect of spontaneous activity on response to, 177.
FELDMAN, STUART. See Mayersohn, Michael, 288.
FILER, L. J., JR. See Fomon, Samuel J., 241.
FILLIOS, LOUIS CHARLES, OSAMU YOKONO, ANDREJ PRONCZUK, IRA GORE, TADASHI SATOH AND KATSUTO KOBAYAKAWA. Synthesis and distribution of cholesterol, and the effect of diet, at the liver endoplasmic reticula and plasma membranes from lean or obese rats, 105.
FISHER, HANS. See Konlande, James E., 435.
FISHMAN, L. See Rothschild, M. A., 395.
Fluoride and magnesium, dietary, effect on bone mineralization in young chicks, 271.
FOMON, SAMUEL J., L. J. FILER, JR., LORA N. THOMAS, RONALD R. ROGERS AND ANN M. PROKSCH. Relationship between formula concentration and rate of growth of normal infants, 241.
Formula concentration and growth in infants, 241.
Formulas, infant soybean, fed to baby pigs, growth, 279.
FOWLER, P. R. See Miller, W. J., 411.

G

GEEVER, ERVING, DORINNE KAN AND STANLEY M. LEVENSON. Effect of coprophagy on experimental iron absorption in the rat, 95.
GERSHOFF, STANLEY N. See Runyan, Thora, 113.
GIBALDI, MILO. See Mayersohn, Michael, 288.
GIFFORD, ELIZABETH DYAR. See Riley, Doris Romine, 351.
GLICK, Z. See Joslyn, M. A., 119.
Glycine metabolism in rats, effect of vitamin B_6 deficiency and deoxypyridoxine treatment, 113.
GORE, IRA. See Fillios, Louis Charles, 105.
Growth and mineralization of the rat, calcium and phosphorus requirements, 443.

— in rats fed tannins and phenolic compounds, 119.
— of baby pigs fed infant soybean formulas, 279.
— of chicks fed carbohydrate-containing and "carbohydrate-free" diets, effect of various sources of nonessential nitrogen in promoting, 297.
GRUBER, CHARLES. See Levenson, Stanley M., 99.
GUBLER, C. J. See Bitter, R. A., 147.
Guinea pigs, scorbutic, circulating antibody formation, 41.

H

HAHN, E. W., AND F. W. HEGGENESS. Preconception irradiation of dam and composition of offspring, 181.
HARMON, B. G. See Sugahara, M., 344.
HARRILL, INEZ. See Riley, Doris Romine, 351.
HASKELL, BETTY E. See Shiflett, J. M., 420.
HEGGENESS, F. W. See Al Nejjar, Z., 177.
HEGGENESS, F. W. See Hahn, E. W., 181.
Heifers, dairy, effects of high protein diets with normal and low energy intake on wound healing, hair growth, hair and serum zinc, and serum alkaline phosphatase, 411.
HENINGER, R. W. See Bitter, R. A., 147.
Hepatic ATP and triglyceride levels in choline-deficient rats with and without dietary orotic acid supplementation, 188.
— glycogen and blood glucose in rats force-fed a threonine-devoid diet, 477.
— lipogenesis in the growing chick, effect of dietary protein, 356.
— lipogenesis in the mouse, effects of dietary fat, 312.
— tyrosine transaminase rhythm, interaction of environmental lighting, food consumption and dietary protein content, 71.
Heptachlor, toxicity and metabolism, effect of dietary protein levels, 90.
HILL, D. C. See Shao, Tsang-Cheng, 225.
HOEKSTRA, W. G. See Westmoreland, Nelson, 76, 83.
HUANG, P. C. See Scrimshaw, N. S., 9.
Hydrocephalus and vitamin B_{12}, 139.

I

Infant soybean formulas fed to baby pigs, growth, 279.
Infants, normal, relationship between formula concentration and rate of growth, 241.
Iron absorption in the rat, effect of coprophagy, 95.
—, zinc and copper in the postnatal rat, 303.
Irradiation, preconception, of dam and composition of offspring, 181.
ISRAEL, Y., J. E. VALENZUELA, I. SALAZAR AND G. UGARTE. Alcohol and amino acid transport in the human small intestine, 222.

J

JENSEN, A. H. See Sugahara, M., 344.
JOSLYN, M. A., AND Z. GLICK. Comparative effects of gallotannic acid and related phenolics on the growth of rats, 119.

K

KAN, DORINNE. See Geever, Erving, 95.
KAN, DORINNE. See Levenson, Stanley M., 99.
KENNEY, MARY ALICE. Development of spleen and thymus in offspring of protein-deficient rats, 202.
KETCHESON, MARION R., GEORGE P. BARRON AND DENNIS H. COX. Relationship of material dietary zinc during gestation and lactation to development and zinc, iron and copper content of the postnatal rat, 303.
KIM, YOON SOO, AND JOHN P. LAMBOOY. Biochemical and physiological changes in the rat during riboflavin deprivation and supplementation, 467.
KIMURA, TOSHIZO. See Murata, Kiku, 427.
KING, K. W. See Bunce, G. E., 159, 168.
KING, KENDALL W. See Soliman, Abdel-Gawad M., 255.
KLATSKIN, GERALD. See Simon, Jerome B., 188.
KOBAYAKAWA, KATSUTO. See Fillios, Louis Charles, 105.
KOCH, MARY. See Cheng, Chuan Huan, 64.
KONLANDE, JAMES E., AND HANS FISHER. Evidence for a nonabsorptive antihypercholesterolemic action of phytosterols in the chicken, 435.
KUMAR, MAHENDRA, AND A. E. AXELROD. Circulating antibody formation in scorbutic guinea pigs, 41.

L

Lactalbumin, amino acid retention efficiency with, 159.
Lactate dehydrogenase in thiamine deficiency, 325.
LAMBOOY, JOHN P. See Kim, Yoon Soo, 467.
LARIN, F. See Zigmond, M. J., 71.
Leucine transaminase and vitamin B_6 deficiency, 420.
LEVEILLE, GILBERT A. In vivo fatty acid and cholesterol synthesis in fasted and fasted-refed chicks, 367.
LEVEILLE, GILBERT A. See Yeh, Yu-Yan, 356.
LEVENSON, STANLEY M. See Geever, Erving, 95.
LEVENSON, STANLEY M., CHARLES GRUBER AND DORINNE KAN. Similarity in passage rates of plasma proteins into the gut of germfree and conventionalized rats, 99.
Lipid mobilization, in rats exposed to cold or starvation, role of previous feeding, 25.
Liver vitamin A, effect of DDT on rats raised on alpha-protein rations, 319.

—, weanling rats, dietary regulation of transketolase activity, 64.
— xanthine dehydrogenase depletion and repletion in chicks, 193.
— xanthine oxidase and plasma amino acids in rats, influence of vitamin D and zinc, 351.

M

Magnesium and fluoride, dietary, effect on bone mineralization in young chicks, 271.
Man, bile salt enhancement of riboflavin and flavin mononucleotide absorption, 288.
MARTIN, Y. G. See Miller, W. J., 411.
MAYERSOHN, MICHAEL, STUART FELDMAN AND MILO GIBALDI. Bile salt enhancement of riboflavin and flavin mononucleotide absorption in man, 288.
MAYNARD, LEONARD A. Francis Gano Benedict — a biographical sketch, 1.
McCOY, KIM E., AND PAUL H. WESWIG. Some selenium responses in the rat not related to vitamin E, 383.
McGRATH, HOPE. See Sabine, John R., 312.
MEADE, ROBERT C. See Barboriak, Joseph J., 373.
MEHLMAN, M. A. See Therriault, D. G., 25.
Men, young, partial dietary replacement of milk protein by nonspecific nitrogen, 9.
MESSER, MICHAEL, EVELYN B. THOMAN, ALBERTO GALOFRE TERRASA AND PETER R. DALLMAN. Artificial feeding of infant rats by continuous gastric infusion, 404.
Metabolic alterations in "control animals" used in studies on biotin-deficient rats, effect of food restriction, 235.
— derangements in response of rats to ingestion of imbalanced amino acid mixtures, 255.
Metabolism and toxicity of heptachlor, effect of feeding rats different protein levels, 90.
—, arginine, effect of α-aminoisobutyric acid in chicks, 225.
—, glycine, in vitamin B_6-deficient and deoxypyridoxine-treated rats, 113.
—, one-cargon, in neonatal vitamin B_{12}-deficient rats, 139.
—, tryptophan, of rats fed a threonine-free amino acid diet, 427.
MILLER, W. J., Y. G. MARTIN, D. M. BLACKMON AND P. R. FOWLER. Effects of high protein diets with normal and low energy intake on wound healing, hair growth, hair and serum zinc, and serum alkaline phosphatase in dairy heifers, 411.
Minerals and enzymes in maternal zinc deficiency, 449.
— and enzymes, maternal and fetal, 459.
MISTRY, S. P. See Patel, M. S., 235.
MONGELLI, J. See Rothschild, M. A., 395.
MOORE, R. O., AND F. D. YONTZ. Effect of thiamine deficiency in rats on adipose tissue lactate dehydrogenase isozyme distribution, 325.
Mouse, dietary fat and the inhibition of hepatic lipogenesis, 312.
MURATA, KIKU, AND TOSHIZO KIMURA. Tryptophan metabolism of rats fed a threonine-free amino acid diet, 427.

N

Neuromotor development in progeny of underfed mother rats, 18.
Nitrogen, nonessential, effect of various sources in promoting growth of chicks fed carbohydrate-containing and "carbohydrate-free" diets, 297.
—, nonspecific, partial dietary replacement of milk protein in young men, 9.

O

ORATZ, M. See Rothschild, M. A., 395.
Ornithine-urea cycle studies nutritionally, 153.
Orotic acid, influence of, in inducing fatty liver in rats, 33.

P

PARKER, H. E. See Rucker, R. B., 57.
PARKER, H. E. See Spierto, Francis, 271.
PATEL, M. S., AND S. P. MISTRY. Effect of food restriction on metabolic alterations in "control animals" used in studies on biotin-deficient rats, 235.
Phenols and tannins, effect of feeding on growth in rats, 119.
Phosphorus and calcium requirements for maximal growth and mineralization of the rat, 443.
Phytosterols in the chicken, evidence for a nonabsorptive antihypercholesterolemic action, 435.
Pigs, baby, fed infant soybean formulas, growth, 279.
PRONCZUK, ANDREJ. See Fillios, Louis Charles, 105.
PROKSCH, ANN M. See Fomon, Samuel J., 241.
Protein and fat, impairment of gastrointestinal processing by ethanol in rats, 373.
— -deficient rats, development of spleen and thymus in offspring, 202.
—, dietary, effect on hepatic lipogenesis in the growing chick, 356.
—, dietary, effect on the toxicity and metabolism of heptachlor, 90.
— intake and energy, effect of restriction on atherosclerosis and associated physiological factors in cockerels, 335.
—, milk, partial replacement with nonspecific nitrogen in young men, 9.
Proteins, effects of severe alkali treatment on amino acid composition and nutritive value, 45.
—, plasma, similarity in passage rates into the gut of germfree and conventionalized rats, 99.

R

Rat, biochemical and physiological changes during riboflavin deprivation and supplementation, 467.

—, calcium and phosphorus requirements for maximal growth and mineralization, 443.
—, influence of coprophagy on experimental iron absorption, 95.
—, interaction of environmental lighting, food consumption and dietary protein content on hepatic tyrosine transaminase rhythm, 71.
—, maternal, excess dietary zinc, and zinc, iron, copper, calcium, and magnesium content and enzyme activity in maternal and fetal tissues, 459.
—, maternal, zinc deficiency during gestation, and zinc, iron, copper, and calcium content and enzyme activity in maternal and fetal tissues, 449.
—, postnatal, zinc, iron and copper in, 303.
—, selenium responses not related to vitamin E, 383.
—, young, fed varying levels of casein, amino acid retention and balance, 168.
—, young, fed varying levels of lactalbumin, amino acid retention and balance, 159.
Rats, biotin-deficient, effect of food restriction on metabolic alterations in "control animals," 235.
—, choline-deficient, hepatic ATP and triglyceride levels in, with and without dietary orotic acid supplementation, 188.
—, effect of ambient temperature and dietary amino acids on carcass fat deposition, 344.
—, effect of thiamine deficiency on adipose tissue lactate dehydrogenase isozyme distribution, 325.
—, effect of vitamin B_6 deficiency and deoxypyridoxine treatment on glycine metabolism, 113.
— fed a threonine-free amino acid diet, tryptophan metabolism, 427.
— fed diets containing different amounts of casein and 10% tyrosine, urinary simple phenols in, 217.
— fed different protein levels, toxicity and metabolism of heptachlor, 90.
— fed purified and nonpurified diets, urinary simple phenols in, 209.
— fed tannins and phenolic compounds, effect on growth, 119.
— force-fed a threonine-devoid diet, studies on blood glucose and hepatic glycogen, 477.
—, germfree and conventionalized, similarity in passage rates of plasma proteins in the gut, 99.
—, impairment of gastrointestinal processing of fat and protein by ethanol, 373.
—, induction of orotic acid fatty liver, studies of influencing factors, 33.
—, infant, artificial feeding by continuous gastric infusion, 404.
—, influence of zinc and vitamin D on plasma amino acids and liver xanthine oxidase, 351.
—, lean or obese, cholesterol synthesis and distribution and effect of diet at liver endoplasmic reticula and plasma membranes, 105.
—, neonatal vitamin B_{12}-deficient, study of one-carbon metabolism, 139.
—, protein-deficient, development of spleen and thymus in offspring, 202.
— raised on alpha-protein rations, effect of DDT: growth and storage of liver vitamin A, 319.
—, response to ingestion of imbalanced amino acid mixtures, metabolic derangements, 255.
—, starved or exposed to cold, influence of previous feeding on lipid mobilization, 25.
—, thiamine-deprived and thiamine antagonist-treated, effects of forced-feeding on blood levels of pyruvate, glucocorticoids and glucose and on adrenal weight, 147.
—, underfed mother, neuromotor development in progeny, 18.
—, weanling, dietary regulation of liver transketolase activity, 64.
Renner, Ruth. Effectiveness of various sources of nonessential nitrogen in promoting growth of chicks fed carbohydrate-containing and "carbohydrate-free" diets, 297.
Riboflavin and flavin mononucleotide absorption in man, bile salt enhancement, 288.
— deprivation and supplementation in the rat, biochemical and physiological changes, 467.
Riley, Doris Romine, Inez Harrill and Elizabeth Dyar Gifford. Influence of zinc and vitamin D on plasma amino acids and liver xanthine oxidase in rats, 351.
Rock, G. C. Nutritional evidence for the absence of the complete ornithine-urea cycle in the insect, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae), 153.
Rogers, Ronald R. See Fomon, Samuel J., 241.
Rogler, J. C. See Rucker, R. B., 57.
Rogler, J. C. See Spierto, Francis, 271.
Rose, R. J., and S. L. Balloun. Effect of restricted energy and protein intake on atherosclerosis and associated physiological factors in cockerels, 335.
Rothschild, M. A., M. Oratz, J. Mongelli, L. Fishman and S. S. Schreiber. Amino acid regulation of albumin synthesis, 395.
Rucker, R. B., H. E. Parker and J. C. Rogler. Effect of copper deficiency on chick bone collagen and selected bone enzymes, 57.
Runyan, Thora, and Stanley N. Gershoff. Glycine metabolism in vitamin B_6-deficient and deoxypyridoxine-treated rats, 113.

S

Sabine, John R., Hope McGrath and S. Abraham. Dietary fat and the inhibition of hepatic lipogenesis in the mouse, 312.
Salazar, I. See Israel, Y., 222.
Sarett, Herbert P. See Schneider, Donald L., 279.
Sarma, D. S. R., and H. Sidransky. Studies on orotic acid fatty liver in rats: factors influencing the induction of fatty liver, 33.
Satoh, Tadashi. See Fillios, Louis Charles, 105.
Savini, S. See Bernhart, F. W., 443.
Scheig, Robert. See Simon, Jerome B., 188.
Schlicker, Sandra A. See Cox, Dennis H., 449, 459.

SCHNEIDER, DONALD L., AND HERBERT P. SARETT. Growth of baby pigs fed infant soybean formulas, 279.
SCHOLZ, R. W., AND W. R. FEATHERSTON. Dietary-induced depletion and repletion of avian liver xanthine dehydrogenase, 193.
SCHREIBER, S. S. See Rothschild, M. A., 395.
SCRIMSHAW, N. S., V. R. YOUNG, P. C. HUANG, O. THANANGKUL AND B. V. CHOLAKO:. Partial dietary replacement of milk protein by nonspecific nitrogen in young men, 9.
Selenium responses in the rat not related to vitamin E, 383.
SHANK, ROBERT E. See Cheng, Chuan Huan, 64.
SHAO, TSANG-CHENG, AND D. C. HILL. Effect of α-aminoisobutyric acid on arginine metabolism in chicks, 225.
SHERWIN, R. W. See Simonson, M., 18.
SHIFLETT, J. M., AND BETTY E. HASKELL. Effect of vitamin B_6 deficiency on leucine transaminase activity in chick tissue, 420.
SHOEMAKER, W. J. See Zigmond, M. J., 71.
SIDRANSKY, H. See Sarma, D. S. R., 33.
SIDRANSKY, H., D. S. WAGLE, M. BONGIORNO AND E. VERNEY. Studies on blood glucose and hepatic glycogen in rats force-fed a threonine-devoid diet, 477.
SIMON, JEROME B., ROBERT SCHEIG AND GERALD KLATSKIN. Hepatic ATP and triglyceride levels in choline-deficient rats with and without dietary orotic acid supplementation, 188.
SIMONSON, M., R. W. SHERWIN, J. K. ANILANE, W. Y. YU AND B. F. CHOW. Neuromotor development in progeny of underfed mother rats, 18.
SLUMP, P. See de Groot, A. P., 45.
SOLIMAN, ABDEL-GAWAD M., AND KENDALL W. KING. Metabolic derangements in response of rats to ingestion of imbalanced amino acid mixtures, 255.
Soybean formulas, infant, fed to baby pigs, growth, 279.
SPIERTO, FRANCIS, J. C. ROGLER AND H. E. PARKER. Effect of dietary magnesium and fluoride on bone mineralization in young chicks, 271.
STOWE, HOWARD D. Composition of a complete purified equine diet, 330.
SUGAHARA, M., D. H. BAKER, B. G. HARMON AND A. H. JENSEN. Effect of ambient temperature and dietary amino acids on carcass fat deposition in rats, 344.

T

Temperature, amino acid balance and fat deposition in rats, 344.
TERRASA, ALBERTO GALFORE. See Messer, Michael, 404.
Test, simplified hemolysis, for vitamin E deficiency, 390.
THANANGKUL, L. See Scrimshaw, N. S., 9.
THERRIAULT, D. G., AND M. A. MEHLMAN. Effect of prior diet on lipid mobilization in rats during starvation or exposure to cold, 25.
Thiamine deficiency, effect in rats on adipose tissue lactate dehydrogenase isozyme distribution, 325.
— -deprived and thiamine antagonist-treated rats, effects of forced-feeding on blood levels of pyruvate, glucocorticoids and glucose and on adrenal weight, 147.
THOMAN, EVELYN B. See Messer, Michael, 404.
THOMAS, LORA N. See Fomon, Samuel J., 241.
TINSLEY IAN J. DDT effect on rats raised on alpha-protein rations: growth and storage of liver vitamin A, 319.
TOMARELLI, R. M. See Bernhart, F. W., 443.
Tryptophan metabolism of rats fed a threonine-free amino acid diet, 427.

U

UGARTE, G. See Israel, Y., 222.
Urinary simple phenols in rats fed diets containing different amounts of casein and 10% tyrosine, 217.
— simple phenols in rats fed purified and nonpurified diets, 209.

V

VALENZUELA, J. E. See Israel, Y., 222.
VERNEY, E. See Sidransky, H., 477.
Vitamin A in liver, effect of DDT on rats raised on alpha-protein rations, 319.
— B_6 deficiency, effect on glycine metabolism in rats, 113.
— B_6 deficiency, effect on leucine transaminase activity in chick tissue, 420.
— B_{12} and hydrocephalus, 139.
— D and zinc, influence on plasma amino acids and liver xanthine oxidase in rats, 351.
— E deficiency, a simplified hemolysis test for, 390.
— E, selenium responses not related to in the rat, 383.

W

WAGLE, D. S. See Sidransky, H., 477.
WEATHERHOLTZ, W. M., T. C. CAMPBELL AND R. E. WEBB. Effect of dietary protein levels on the toxicity and metabolism of heptachlor, 90.
WEBB, R. E. See Weatherholtz, W. M., 90.
WESTMORELAND, NELSON, AND W. G. HOEKSTRA. Histochemical studies of alkaline phosphatase in epiphyseal cartilage of normal and zinc-deficient chicks, 83.
WESTMORELAND, NELSON, AND W. G. HOEKSTRA. Pathological defects in the epiphyseal cartilage of zinc-deficient chicks, 76.
WESWIG, PAUL H. See McCoy, Kim E., 383.
WOODARD, JAMES C. Study of one-carbon metabolism in neonatal vitamin B_{12}-deficient rats, 139.
WURTMAN, R. J. See Zigmond, M. J., 71.

Y

YEH, YU-YAN, AND GILBERT A. LEVEILLE. Effect of dietary protein on hepatic lipogenesis in the growing chick, 356.
YOKONO, OSAMU. See Fillios, Louis Charles, 105.
YONTZ, F. D. See Moore, R. O., 325.
YOUNG, V. R. See Scrimshaw, N. S., 9.
YU, W. Y. See Simonson, M., 18.

Z

ZIGMOND, M. J., W. J. SHOEMAKER, F. LARIN AND R. J. WURTMAN. Hepatic tyrosine transaminase rhythm: interaction of environmental lighting, food consumption and dietary protein content, 71.

Zinc and vitamin D, influence on plasma amino acids and liver xanthine oxidase in rats, 351.
— deficiency, effect on alkaline phosphatase in epiphyseal cartilage of chicks, 83.
— deficiency in the maternal rat during gestation, and zinc, iron, copper, and calcium content and enzyme activity in maternal and fetal tissues, 449.
— deficiency, pathological defects in epiphyseal cartilage of chicks, 76.
—, excess dietary, for the maternal rat, and zinc, iron, copper and magnesium content and enzyme activity in maternal and fetal tissues, 459.
—, iron and copper in the postnatal rat, 303.