Relationship of Specific Nutrient Deficiencies to Antibody Production in Swine[1,2]

I. VITAMIN A

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The effect of deficiencies of particular vitamins upon antibody response has been pursued extensively. Axelrod ('60) has reviewed much of the work concerning the dependence of antibody production upon specific nutritional sufficiencies. Some workers, Werkman ('23) with rats and rabbits; Cramer and Kingsbury ('24) with rats; Simola and Brunius ('33) with guinea pigs; Natvig ('41) with rats, and Feller et al. ('42) with humans, have reported that deficiencies of vitamin A did not result in depressed antibody production. However, many of these studies were carried out without the benefit of purified diets. Lassen ('30) reported some reduction in serum agglutination titers in vitamin A-deficient rats when compared with positive controls. Greene ('33) reported that rabbits deficient in vitamin A produced lower hemolysin titers than control rabbits. Ludovici and Axelrod ('51) observed that rats fed a vitamin A-deficient diet for 4 weeks had lower hemagglutination titers than the control rats. Pruzansky and Axelrod ('55) reported similar findings following the injection of diphtheria toxoid into rats that received either a vitamin A-deficient diet or a control diet for 12 days. Underdahl and Young ('56) could find no difference between the hemagglutination inhibition titer of vitamin A-deficient or control mice. However, the morbidity following the infection of swine influenza virus was greater in the vitamin A-deficient mice.

The present study was initiated to investigate the effect of quantitated vitamin A deficiency upon antibody production in swine and the subsequent antibody production following vitamin A repletion.

METHODS

Two trials involving a total of 30 Yorkshire-Hampshire crossbred pigs were conducted to determine the effect of a vitamin A deficiency upon antibody production. In the first trial, 16 pigs were weaned at 5 days of age and placed in individual wire-mesh bottom metal cages. In the second trial, 14 pigs were weaned when 12 hours old and handled in the same manner as in the first trial. All pigs were given a semisynthetic milk diet containing 20% solids consisting of 30% of casein,[5] 10% of lard, 55% of glucose,[6] 5% of minerals and vitamins (Miller et al., '54) with vitamin A omitted. The diet was prepared by dispersing the solid material into warm water, mixing and homogenizing at a cylinder pressure of 3000 psi. The semisynthetic diets were fed as a liquid (20% solids) 5 times daily during the first 5 weeks and as a dry meal the remainder of the trial. Two per cent cellulose[7] replaced an equal quantity of glucose when the diet was fed in the dry form. All the pigs in each trial received the vitamin A-deficient diet until two weeks of age at which time they were uniformly allotted according to weight, sex and litter background, either to the vitamin A-deficient

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[5] Labco, Vitamin-Free Casein, The Borden Company, New York.

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

[7] Solka Floc, Brown Company, Boston.

diet or to the same basal diet to which vitamin A acetate (3960 IU vitamin A/kg of dry diet) had been added.

All pigs were weighed weekly. Four weeks after allotting to treatment and weekly thereafter, blood was drawn via the anterior vena cava and the serum vitamin A concentration was determined by the method of Sobel and Snow ('47) which was modified to utilize the Beckman DU spectrophotometer.

The antigenic challenge for subsequent measurement of antibody production was initiated within a litter when the serum vitamin A concentration of the pigs fed the deficient diet averaged less than 14 μg/100 ml. The antigen was phenolized *Salmonella pullorum*[8] which was administered in 6 daily intraperitoneal injections. The amount of antigen administered at each injection was proportional to the estimated blood volume as determined by Hansard et al. ('51, '53). One milliliter of diluted antigen with a turbidity corresponding to 1.00 on a McFarland nephelometer scale was injected per 100 ml of estimated blood volume as described by Harmon et al.[9] and Miller et al. ('62). Prior to the first injection and one week subsequent to the last injection blood was drawn via the anterior vena cava and the serum was removed and frozen for later measurement of the immunological response. Tube serial dilution agglutination titers were determined in duplicate for each sample by the method as described by Smith and Conant ('60).

Following the measurement of immunological response all the pigs were group-fed a corn-soybean oil meal diet fortified with vitamins and minerals in accordance with recommendations of the National Research Council subcommittee on swine nutrition ('59). This constituted the start of a repletion period for the pigs suffering from a vitamin A deficiency. After feeding the natural diet for 6 to 7 weeks, the serum vitamin A concentration was again determined and the pigs were challenged with type O, Rh positive, human erythrocytes. Six daily 5.0 ml intraperitoneal injections of a 20% suspension of erythrocytes were administered. Hemagglutination titers were determined previous to the antigen injection and one week following the last antigen injection. The hemagglutination titers were determined by the tube serial dilution method of Paul and Bonnell as described by Stafseth et al. ('56) except that 2% human erythrocytes were substituted as the antigen.

Total serum protein concentration and percentages of the electrophoretic components of the serum protein were determined at the time the antibody titers were measured. Total serum protein concentration was determined by the method of Waddell ('56) and the serum protein components were separated by paper electrophoresis.[10]

RESULTS

In the first trial the pigs were maintained with the respective experimental treatments for an average of 10 weeks. The pigs fed the complete diet did not gain faster than the pigs receiving the vitamin A-deficient diet (table 1) during this period. In the second trial the pigs that had been weaned at 12 hours instead of 5 days as in the first trial received the experimental dietary treatments for only 6 weeks. However the pigs fed the control diet in this trial gained significantly faster ($P < 0.01$) and required significantly less ($P < 0.05$) feed per unit of bodyweight gain than did the deficient pigs.

The serum vitamin A concentration of the pigs receiving the vitamin A-deficient diet in both trials was significantly lower ($P < 0.01$) than in the control pigs after 4 weeks on treatment (table 2). However, the average serum vitamin A of the deficient pigs in the first trial had dropped to 13.8 μg/100 ml after an average of 8 weeks on the treatment. The pigs fed the deficient diet in the second trial, which were weaned at 12 hours of age, had an average serum vitamin A value of 12.6 μg/100 ml after only 4 weeks on the treatment. Apparently the vitamin A stores previous to weaning in the first trial were

[8] Unstained pullorum tube antigen concentrate, Vineland Poultry Laboratories, Vineland, New Jersey.

[9] Harmon, B. G., E. R. Miller, D. E. Ullrey, D. A. Schmidt, J. A. Hoefer, R. W. Luecke 1959 Antibody production in the baby pig. J. Animal Sci., *18*: 1559 (abstract).

[10] Spinco instruction manual RIM 5, Beckman Instrument Company, Spinco Division, Palo Alto, California.

TABLE 1

Growth and feed utilization of pigs fed a complete or vitamin A-deficient diet

	Trial 1 Dietary treatment[1]		Trial 2 Dietary treatment[1]	
	Vitamin A-deficient	Vitamin A-fortified	Vitamin A-deficient	Vitamin A-fortified
No. of pigs	9	7	7	7
Initial wt, kg	3.36 ± 0.30[2]	3.30 ± 0.33	2.27 ± 0.16	2.27 ± 0.20
Experimental depletion				
Final wt, kg[3]	15.7 ± 2.80	17.0 ± 2.00	7.4 ± 0.45	9.63 ± 0.33[4]
Daily gain, kg	0.20 ± 0.074	0.24 ± 0.018	0.12 ± 0.013	0.18 ± 0.006[4]
Feed/gain	1.48 ± 0.15	1.44 ± 0.09	2.01 ± 0.36[5]	1.24 ± 0.04
Experimental repletion				
Final wt, kg[3]	42.1 ± 6.5[5]	42.5 ± 4.63	25.3 ± 2.73[5]	34.7 ± 2.18[6]
Daily gain, kg	0.50 ± 0.006	0.50 ± 0.077	0.43 ± 0.036	0.60 ± 0.018[6]

[1] All pigs received a complete natural diet during repletion.
[2] SE of mean.
[3] Final weights were recorded at the time the post injection immunological response was measured.
[4] Significantly greater than corresponding value for the other treatment ($P < 0.01$).
[5] Three pigs died.
[6] Significantly greater than corresponding value for the other treatment ($P < 0.05$).

TABLE 2

Serum vitamin A values of pigs fed a complete or vitamin A-deficient diet

	Trial 1 Dietary treatment[1]		Trial 2 Dietary treatment[1]	
	Vitamin A-deficient	Vitamin A-fortified	Vitamin A-deficient	Vitamin A-fortified
Experimental depletion				
4 Weeks,[2] μg/100 ml	17.3 ± 0.36[3]	24.6 ± 0.55[4]	12.6 ± 1.00	26.3 ± 0.84[4]
6 Weeks, μg/100 ml	14.6 ± 2.13	27.2 ± 0.85[4]	10.9 ± 1.11	26.6 ± 1.12[4]
8 Weeks, μg/100 ml	13.8 ± 1.76	29.3 ± 2.08[4]		
Experimental repletion				
Final, μg/100 ml	39.8 ± 1.54	35.3 ± 1.53	30.9 ± 1.28	32.4 ± 1.33

[1] All pigs received a complete natural diet during repletion.
[2] Weeks after allotment to treatment.
[3] SE of mean.
[4] Significantly greater than corresponding value for other treatment ($P < 0.01$).

sufficient to delay the appearance of severely reduced serum vitamin A concentration. Heaney[11] has reported a fivefold increase in the serum vitamin A concentration in baby pigs during the first 24 hours of life. The average serum vitamin A concentration of the control pigs at the various intervals during the experimental depletion feeding period ranged from 24.6 to 29.3 μg/100 ml. The average values for the pigs fed the complete diet agree with the observations of Hentges et al. ('52) who reported normal serum vitamin A values in swine of 15 to 32 μg/100 ml.

The deficient pigs in both trials evinced much hyperkeratinization of the epidermis, apparent xerophthalmia and faulty locomotion. A vitamin A-deficient pig together with a control pig from the second trial is shown in figure 1. Three deficient pigs in each trial failed to recover following the return to the vitamin A-adequate diet.

The total serum protein level was not affected by the removal of vitamin A from the diet (table 3). The percentages of α- and γ-globulin fractions were significantly greater ($P < 0.01$) and the percentage of albumin was significantly less ($P < 0.01$) in the vitamin A-deficient pigs (table 3). Erwin et al. ('59) reported a decreased serum albumin in vitamin A-deficient calves.

[11] Heaney, D. P. 1960 Effects of marginal vitamin A intake during gestation in swine. Ph.D. Thesis Michigan State University.

Fig. 1 Control pig (left) and a vitamin A-deficient pig (right) from the second trial 6 weeks after allotment to treatment.

TABLE 3

Serum protein values of pigs fed a complete or vitamin A-deficient diet (trials 1 and 2)

	Experimental depletion phase		Experimental repletion phase[1]	
	Vitamin A-deficient	Vitamin A-fortified	Vitamin A-deficient	Vitamin A-fortified
Total serum protein, %	4.95 ± 0.30[2]	4.92 ± 0.18	5.47 ± 0.18	5.45 ± 0.13
Albumin, %	34.36 ± 1.74	45.48 ± 1.02[3]	41.58 ± 1.93	44.23 ± 2.49
α-Globulin, %	33.55 ± 1.19[3]	27.20 ± 0.66	22.06 ± 0.73	22.90 ± 1.07
β-Globulin, %	17.94 ± 0.75	17.32 ± 0.92	15.72 ± 0.86	16.52 ± 0.95
γ-Globulin, %	15.44 ± 0.93[3]	10.09 ± 0.75	14.33 ± 0.55	12.35 ± 0.88

[1] All pigs received a complete natural diet during repletion.
[2] SE of mean.
[3] Significantly greater than corresponding value for other treatment ($P < 0.01$).

TABLE 4

Summary of antibody titers, serum vitamin A values and growth of pigs fed a complete or vitamin A-deficient diet (trials 1 and 2)

	Dietary treatment[1]	
	Vitamin A-deficient	Vitamin A-fortified
Experimental depletion phase		
Reciprocal of antibody titer[2]	24.7 ± 5.98[3]	308.6 ± 59.60[4]
Serum vitamin A, μg/100 ml	12.5	27.9[4]
Avg daily gain, kg	0.16	0.22
Experimental repletion phase		
Reciprocal of antibody titer[5]	67.0 ± 14.61	75.7 ± 8.2
Correlation coefficients		
Serum vitamin A vs. agglutination	0.70[6]	
Agglutination vs. rate of gain	0.55[7]	
Serum vitamin A vs. rate of gain	0.35	

[1] All pigs received a complete natural diet during repletion.
[2] Tube agglutination with *Salmonella pullorum* antigen.
[3] SE of mean.
[4] Significantly greater than corresponding value for other treatment ($P < 0.01$).
[5] Tube agglutination with human erythrocyte antigen.
[6] Statistically significant ($P < 0.01$).
[7] ($P < 0.05$).

The reciprocals of the net agglutination titers produced in response to the injections of *Salmonella pullorum* antigens were significantly greater ($P < 0.01$) for the control pigs than for the vitamin A-deficient pigs, 308.6 and 24.7, respectively (table 4).

Correlation analysis (Snedecor, '56) of the data showed a significant correlation coefficient of 0.70 between net antibody

titer and serum vitamin A concentration. The correlation coefficients between daily gain and net antibody titer and between daily gain and serum vitamin A concentration were 0.55 and 0.35, respectively (table 4). This suggests that the net antibody titer was more closely related to the serum vitamin A concentration than was daily gain. The low correlation between daily gain and antibody titer indicates that inanition did not account for the reduced antibody production by the vitamin A-deficient pigs. This point was further substantiated by the similar growth rates and divergent agglutination titers of the deficient and control pigs in the first trial. Axelrod et al. ('47) have reported that inanition as such did not affect the immune response of an animal.

After a 6- to 7-week repletion period the formerly vitamin A-deficient pigs had hemagglutination responses to human erythrocytes, serum protein values, and serum vitamin A values which were similar to the values of pigs that received the complete diet throughout the study. However, in the second trial during this repletion period the formerly vitamin A-deficient pigs continued to gain weight more slowly ($P < 0.05$) than the control pigs.

SUMMARY

Studies were conducted with 30 baby pigs in two trials to determine the effect of a vitamin A deficiency upon antibody production in swine. Using a semisynthetic milk diet the pigs were fed either a complete diet or a diet deficient in vitamin A. Pigs were weaned at 5 days of age and 12 hours of age in the first and second trials respectively. The average serum vitamin A level of the deficient pigs dropped to 13.8 μg/100 ml 8 weeks after allotment to treatment in the first trial and 12.6 μg/100 ml 4 weeks after allotment to treatment in the second trial. A series of antigen injections was begun when the average serum vitamin A concentration of the deficient pigs in each trial fell below 14 μg/100 ml. The net serum antibody titer of the pigs deficient in vitamin A was significantly lower ($P < 0.01$) than the titer of the control pigs. The correlation coefficient between serum vitamin A and antibody titer was 0.70 and highly significant. Only in the second trial did the pigs on the deficient diet gain less ($P < 0.01$) and require more feed per unit gain ($P < 0.05$). The correlation coefficient between serum vitamin A concentration and rate of gain was 0.35 and not statistically significant. The deficient pigs had greater percentages of serum α-globulin ($P < 0.01$) and γ-globulin ($P < 0.01$) and a lesser percentage of serum albumin ($P < 0.01$) than the control pigs. Following a repletion period of 6 to 7 weeks, the formerly vitamin A-deficient pigs had values of serum vitamin A, serum protein, and antibody titer which were similar to the control pigs. In the second trial, pigs which were formerly vitamin A-deficient continued to gain weight more slowly ($P < 0.05$) during repletion than the control pigs.

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Relationship of Specific Nutrient Deficiencies to Antibody Production in Swine[1,2]

II. PANTOTHENIC ACID, PYRIDOXINE OR RIBOFLAVIN

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In a previous study (Harmon et al., '62) this laboratory reported that pigs deficient in vitamin A experience a severe impairment of agglutinin production to *Salmonella pullorum*. Stoerk and Eisen ('46), Axelrod et al. ('47), Stoerk et al. ('47), Agnew and Cook ('49) and Axelrod and Pruzansky ('55) have demonstrated the deleterious effects of pyridoxine deficiency upon serum antibody response to various antigens in rats. Axelrod et al. ('61) have also reported severely inhibited antibody production in pyridoxine-deficient guinea pigs injected with diphtheria toxoid. Stoerk et al. ('47) reported that deficiencies of pantothenic acid or riboflavin in rats did not influence the immunological response to injections of sheep erythrocytes. However, Axelrod et al. ('47), Ludovici et al. ('49), Wertman and Sarandria ('51) and Zucker et al. ('56) reported that rats deficient in pantothenic acid produced very low antibody titers when compared with positive controls. Riboflavin deficiencies in rats have been shown by Axelrod et al. ('47), Wertman et al. ('52) and Pruzansky and Axelrod ('55) to cause moderate suppression of antibody production.

Miller et al.[5] reported that 4-week-old baby pigs, fed diets deficient in pantothenic acid, pyridoxine or riboflavin, produced extremely low serum antibody titers. However, production by the control pigs also was low. Harmon et al.[6] and Miller et al. ('62) have reported that antibody production remains low in baby pigs until about 5 to 6 weeks of age.

The present study was initiated to investigate the effects of deficiencies of pantothenic acid, pyridoxine or riboflavin upon antibody production in swine and the subsequent antibody production following repletion. In addition, observations were made of other blood cellular and serum components.

METHODS

Two trials involving a total of 48 Yorkshire-Hampshire crossbred pigs were conducted to determine the effect of deficiencies of pantothenic acid, pyridoxine or riboflavin upon antibody production. In each trial the pigs were removed from the sow at 14 days of age and placed in individual wiremesh-bottom metal cages. All pigs were given a semisynthetic milk diet containing 20% solids consisting of 30% of casein,[7] 10% of lard, 55% of glucose,[8] 5% of minerals and vitamins (Miller et al., '54) with pantothenic acid, pyridoxine and riboflavin omitted. The diet was prepared by dispersing the solid material into warm water, mixing and homogenizing at a cylinder pressure of 3000 psi. The semisynthetic diets were fed as a liquid (20% solids) 5 times daily during the first three

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[5] Miller, E. R., D. A. Schmidt, D. E. Ullrey, J. A. Hoefer and R. W. Luecke 1957 Differences in antibody production between nursing pigs and those fed synthetic milk diets. J. Animal Sci., *16:* 1038 (abstract).

[6] Harmon, B. G., E. R. Miller, D. E. Ullrey, D. A. Schmidt, J. A. Hoefer and R. W. Luecke 1959 Antibody production in the baby pig. J. Animal Sci., *18:* 1559 (abstract).

[7] Labco, Vitamin-Free Casein, The Borden Company, New York.

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

weeks and as a dry meal the remainder of the trials. Two per cent cellulose[9] replaced an equal quantity of glucose when the diet was fed in the dry form. All pigs in each trial received the diet deficient in the three B-vitamins until 4 weeks of age. At that time they were uniformly allotted into 4 treatment groups according to weight, sex and litter background. All treatments received the previously mentioned diet which had been fortified with 21.4 mg of pantothenic acid, 4.6 mg of pyridoxine and 14.3 mg of riboflavin/kg of solids or this same fortified diet with individual omissions of pantothenic acid, pyridoxine or riboflavin.

The pigs were weighed weekly. All the pigs received a series of 6 daily intraperitoneal antigen injections two weeks after allotment to treatment in the first trial and three weeks after allotment in the second trial. *Salmonella pullorum*[10] was used as the antigen following experimental depletion in the first trial and type O, Rh positive, human erythrocytes were used in the second trial. Prior to the first injection and one week subsequent to the last injection blood was drawn via the anterior vena cava and the serum was removed and frozen for later measurement of the immunological response. The amount of antigen administered at each injection was proportional to the estimated blood volume as determined by Hansard et al. ('51, '53). One milliliter of the diluted *S. pullorum* antigen solution with a turbidity corresponding to 1.00 on a McFarland Nephelometer scale was injected/100 ml of estimated blood volume. This method has been described previously by Harmon et al.[11] and Miller et al. ('62). The agglutination titers for the *S. pullorum* antigen were determined in duplicate by the tube serial dilution method as described by Smith and Conant ('60). The human erythrocytes were suspended in a 20% suspension and 1 ml was injected for each 100 ml of estimated blood volume. The hemagglutination titers were determined by the tube serial dilution method of Paul and Bonnell as described by Stafseth et al. ('56) except that 2% human erythrocytes were used as the antigens.

As an indicator of the severity of the pyridoxine deficiency, urinary xanthurenic acid concentrations were determined by the method of Wachstein and Gudaitis ('52). Urine collections were made for 24 hours before and after the feeding of 100 mg of DL-tryptophan/kg of body weight. These analyses were made in the pyridoxine-deficient pigs and the control pigs at the time antigen injections were initiated during the experimental depletion period. Hematocrit, hemoglobin, erythrocyte, leukocyte and differential determinations were also carried out at the time of antibody determination. Hematocrit values were determined by the method of McGovern et al. ('55), hemoglobin values by the method of Crosby et al. ('54), blood cell counts by the method of Ham ('56) on Neubauer counting chambers and differential counts by the manner described by Wintrobe ('56).

Following the determination of the antibody titers, all the pigs were grouped according to size and given a corn-soybean oil meal diet fortified with vitamins and minerals in accordance with recommendations of the National Research Council subcommittee on swine nutrition ('59). After 6 to 7 weeks the pigs were challenged with 6 daily intraperitoneal injections of type O, Rh positive, human erythrocytes in the first trial and *S. pullorum* in the second trial. The order of the use of the antigens was reversed in the two trials to determine whether the choice of antigens had any influence on the antibody response. The procedures for measuring the immunological response were identical with those used in the experimental depletion phase of the trials.

Total serum protein concentration and percentages of the electrophoretic components of the serum protein were determined at the time the antibody titers were measured. Total serum protein concentration was determined by the method of Waddell ('56) and the serum protein components were separated by paper electrophoresis.[12]

In the second trial, three groups of 4 pigs (one pig from each treatment comprised a group) were allotted to a paired

[9] Solka Floc, Brown Company, Boston.

[10] Unstained pullorum tube antigen concentrate, Vineland Poultry Laboratories, Vineland, New Jersey.

[11] See footnote 6.

[12] Spinco instruction manual RIM 5, Beckman Instrument Company, Spinco Division, Palo Alto, California.

feeding study for the entire experimental depletion period. The quantity of feed offered a pig within a group was limited to an amount equal to that of the pig consuming the least.

RESULTS AND DISCUSSION

The weight gain of the control pigs was significantly greater ($P < 0.01$) than the gain by any deficiency group at the time antibody production was measured, and the control pigs required less feed per unit gain than did the pigs fed the deficient diets (table 1). Miller et al. ('54, '57) and Stothers et al. ('55) have observed the rapid development of reduced growth rate in pigs deficient in pantothenic acid, pyridoxine or riboflavin. The pigs fed each of the deficient diets exhibited roughened hair coat, intermittent diarrhea and anorexia. In addition, rectal prolapse occurred in at least two pigs from each deficiency treatment. Epileptiform seizures were observed in two of the pyridoxine-deficient pigs. The seizures occurred in periods of excitement such as feeding or weighing. One pig from each of the 4 treatments in the first trial is shown in figure 1.

The pyridoxine-deficient pigs exhibited significantly lower ($P < 0.05$) hematocrit and hemoglobin values (table 2). Cartwright et al. ('44), Moustgaard ('53) and Miller et al. ('57) have all reported a decrease in the level of hemoglobin in pyridoxine-deficient pigs. The erythrocyte count of the pyridoxine-deficient pigs was significantly lower ($P < 0.05$) than the same value for the pantothenic acid or riboflavin-deficient pigs. The leukocyte counts were lower in the pyridoxine-deficient pigs than for any other treatment, although the differences were not significant. In the first trial the riboflavin-deficient pigs had a significantly greater ($P < 0.05$) percentage of neutrophils than did the pigs on the other deficient diets. However, after analyzing the combined data of the two trials, the increased percentage of neutrophils of the riboflavin-deficient pigs was not significantly greater than for

TABLE 1

Growth and feed utilization by pigs fed a control diet or diets deficient in pantothenic acid, pyridoxine or riboflavin

	Dietary treatment			
	Control	Deficient vitamin		
		Pantothenic acid	Pyridoxine	Riboflavin
Trial 1				
No. of pigs	5	5	5	5
Initial wt, kg	6.45 ± 0.26[1]	6.40 ± 0.20	5.91 ± 0.37	6.00 ± 0.35
4-Week wt, kg[2]	12.18 ± 0.90[3]	7.64 ± 0.75	9.27 ± 0.93	8.00 ± 1.03
Daily gain, kg	0.20 ± 0.04[3]	0.07 ± 0.02	0.11 ± 0.04	0.08 ± 0.04
Feed/gain	1.58 ± 0.11	3.34 ± 0.70	2.61 ± 0.23	5.25 ± 1.37[4]
Repletion[5]				
Final wt, kg[2]	39.60 ± 1.36[3]	31.91 ± 1.04	32.04 ± 2.91	29.91 ± 1.91
Daily gain, kg	0.66 ± 0.02[3]	0.56 ± 0.02	0.55 ± 0.05	0.52 ± 0.02
Trial 2				
No. of pigs	6	4	4	4
Initial wt, kg	5.50 ± 0.41	5.36 ± 0.57	5.64 ± 0.34	4.95 ± 0.44
4-Week wt, kg	10.64 ± 0.90	8.41 ± 0.88	9.41 ± 0.76	8.36 ± 0.86
5-Week wt, kg[2]	12.04 ± 0.85[3]	8.86 ± 0.28	9.95 ± 1.04	9.45 ± 0.98
Daily gain, kg	0.19 ± 0.02[3]	0.09 ± 0.01	0.13 ± 0.02	0.13 ± 0.01
Feed/gain	1.32 ± 0.06	2.67 ± 0.27[3]	1.95 ± 0.30	1.78 ± 0.08
Repletion[5]				
Final wt, kg[2]	53.2 ± 2.59[6]	38.6 ± 0.45	47.7 ± 2.36	45.0 ± 5.23
Daily gain, kg	0.72 ± 0.03[6]	0.51 ± 0.01	0.65 ± 0.04	0.61 ± 0.07

[1] SE of mean.
[2] Weights were recorded at the time the post injection immunological response was measured.
[3] Significantly greater than corresponding value for all other treatments ($P < 0.05$).
[4] Significantly more feed per unit of gain than pyridoxine-deficient or the control pigs ($P < 0.05$).
[5] All pigs received a complete natural diet during repletion.
[6] Significantly greater than corresponding value for the pantothenic acid-deficient pigs ($P < 0.05$).

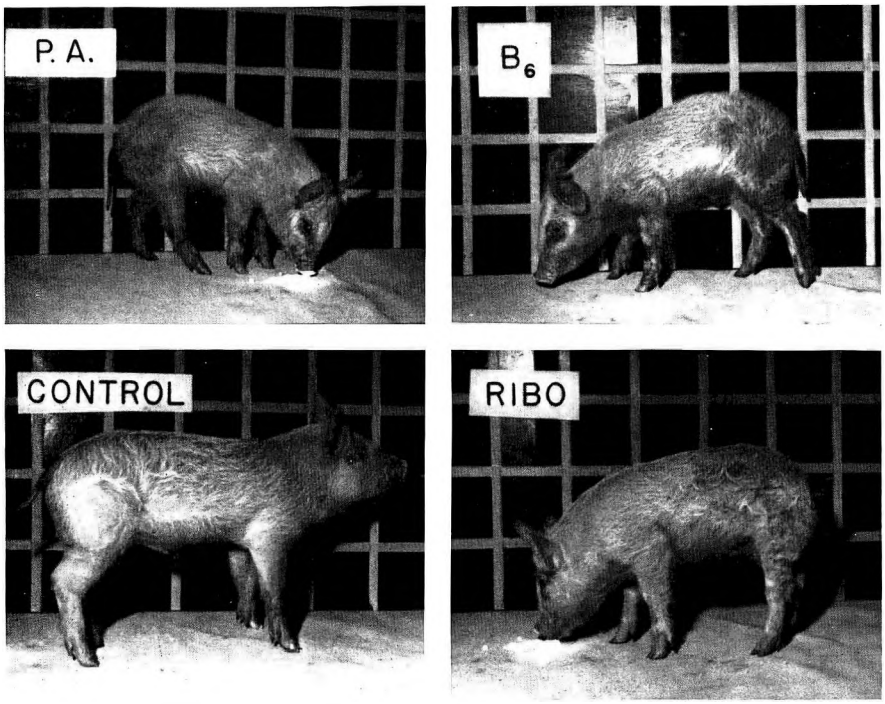

Fig. 1 Control pig and pigs deficient in pantothenic acid, pyridoxine or riboflavin from the first trial 5 weeks after allotment to treatment.

TABLE 2

Hematology of pigs fed a complete diet or diets deficient in pantothenic acid, pyridoxine or riboflavin

	Dietary treatment			
	Control	Deficient vitamin		
		Pantothenic acid	Pyridoxine	Riboflavin
Hematocrit, %	41.2 ±1.16[1]	37.4 ±1.83	33.8 ±2.09[2]	41.9 ±1.62
Hemoglobin, %	12.4 ±0.95	12.1 ±0.82	10.0 ±0.67[2]	12.7 ±0.38
Erythrocytes, 10^6	7.54 ±0.23	8.31 ±0.61	6.93 ±0.42[3]	8.48 ±0.34
Leukocytes, 10^3	20.97 ±2.00	19.95 ±3.99	18.19 ±1.71	20.97 ±2.34
Lymphocytes, %	64.6 ±4.74	60.6 ±3.03	63.5 ±4.90	55.9 ±6.20
Neutrophils, %	31.6 ±4.99	36.1 ±3.23	34.6 ±4.50	42.3 ±6.27

[1] SE of mean.
[2] Significantly less than corresponding value for all other treatments ($P < 0.05$).
[3] Significantly less than corresponding value for pantothenic acid and riboflavin-deficient group ($P < 0.05$).

the other treatments. Mitchell et al. ('50) reported an increased percentage of neutrophils in riboflavin-deficient pigs.

Urine analysis from the pyridoxine-deficient and control pigs showed that xanthurenic acid concentrations were significantly greater ($P < 0.01$) in the pyridoxine-deficient pigs both before and after the feeding of additional tryptophan (table 3). Miller et al. ('57) have suggested that urinary xanthurenic acid concentration was an excellent indicator of the severity of a pyridoxine deficiency.

In the first trial the pigs receiving a control diet produced significantly higher ($P < 0.01$) reciprocals of net antibody titers to *S. pullorum* than did the pigs fed diets deficient in pantothenic acid, pyri-

TABLE 3

Urinary xanthurenic acid values[1] of pigs fed a control diet or a diet deficient in pyridoxine

	Dietary treatment	
	Control	Pyridoxine-deficient
Pre-tryptophan	21.7 ± 3.3[2]	83.5 ± 10.2[3]
Post-tryptophan	23.4 ± 3.7	256.2 ± 57.6[3]

[1] Urinary xanthurenic acid values expressed as micrograms per milliliter of urine.
[2] SE of mean.
[3] Significantly greater than corresponding value for other treatment ($P < 0.01$).

doxine or riboflavin (table 4). After the deficient pigs had been repleted the response to injections of human erythrocytes was similar in all treatments. In the second trial the control pigs produced significantly more ($P < 0.01$) measurable antibodies to human erythrocytes than the pigs fed diets deficient in pantothenic acid, pyridoxine or riboflavin. Following repletion the pigs formerly receiving the deficient diets produced antibodies to injections of *S. pullorum* that were equal to the production by

TABLE 4

Reciprocals of agglutination titers of pigs fed a control diet or diets deficient in pantothenic acid, pyridoxine or riboflavin

	Dietary treatment[1]			
	Control	Deficient vitamin		
		Pantothenic acid	Pyridoxine	Riboflavin
Trial 1				
Experimental depletion[2]	176 ± 16.0[3,4]	15 ± 6.3	33 ± 9.5	23 ± 7.2
Experimental repletion[5]	60 ± 12.6	33 ± 4.8	65 ± 24.5	56 ± 14.4
Trial 2				
Experimental depletion[5]	67 ± 9.6[3]	6 ± 1.6	4 ± 2.5	12 ± 1.9
Experimental repletion[2]	267 ± 38.1	200 ± 40.0	200 ± 23.1	165 ± 26.3

[1] All pigs received a complete natural diet during repletion.
[2] Tube agglutination with *Salmonella pullorum* antigen.
[3] Significantly greater than corresponding value for all other treatment groups ($P < 0.01$).
[4] SE of mean.
[5] Tube hemagglutination with human erythrocyte antigen.

TABLE 5

Serum protein values of pigs on a control diet or on diets deficient in pantothenic acid, pyridoxine or riboflavin

	Dietary treatment			
	Control	Deficient vitamin		
		Pantothenic acid	Pyridoxine	Riboflavin
	Experimental depletion			
Total serum protein, %	5.62 ± 0.99[1]	5.47 ± 0.28	6.06 ± 0.28	5.52 ± 0.26
Serum protein components, %				
Albumin	49.2 ± 2.69	43.9 ± 3.89	47.2 ± 2.17	49.3 ± 1.92
α-Globulin	20.8 ± 0.84	27.0 ± 3.40[2]	23.6 ± 1.66	23.2 ± 0.53
β-Globulin	16.4 ± 0.87	15.4 ± 0.87	13.6 ± 0.56[3]	14.6 ± 0.47
γ-Globulin	13.7 ± 1.30	13.7 ± 1.95	15.7 ± 1.75	12.4 ± 1.23
	Experimental repletion[4]			
Total serum protein, %	6.82 ± 0.63	6.77 ± 0.44	6.58 ± 0.02	6.28 ± 0.17
Serum protein components, %				
Albumin	48.4 ± 0.97	48.3 ± 2.18	50.1 ± 2.17	47.7 ± 2.41
α-Globulin	18.8 ± 0.60	18.9 ± 0.81	17.8 ± 0.99	19.5 ± 0.88
β-Globulin	13.5 ± 0.55	13.0 ± 0.70	12.4 ± 0.56	13.1 ± 0.56
γ-Globulin	21.0 ± 1.79	19.8 ± 1.42	19.4 ± 1.49	19.7 ± 1.40

[1] SE of mean.
[2] Significantly greater than the corresponding value of the control pigs ($P < 0.05$).
[3] Significantly less than the corresponding value of the control pigs ($P < 0.05$).
[4] All pigs received the complete natural diet during repletion.

TABLE 6

Paired feeding study of pigs fed a control diet or diets deficient in pantothenic acid, pyridoxine or riboflavin

	Dietary treatment			
	Control	Deficient vitamin		
		Pantothenic acid	Pyridoxine	Riboflavin
No. of pigs	3	2[1]	2[1]	3
Avg total gain, kg	6.50[2]	3.59	4.05	5.05
Hematocrit, %	38.7	39.2	33.3[3]	43.7
Hemoglobin, %	11.7	11.8	9.9	12.3
Erythrocytes, 10^6	8.0	8.8	7.2[4]	9.2
Reciprocals of hemagglutination titers	63[5]	4	4	14

[1] One pig died.
[2] Significantly greater than corresponding value for all other treatments ($P < 0.05$).
[3] Significantly lower than corresponding value for riboflavin-deficient pigs ($P < 0.05$).
[4] Significantly lower than corresponding value for pantothenic acid or riboflavin-deficient pigs ($P < 0.05$).
[5] Significantly greater than corresponding value for all other treatments ($P < 0.01$).

the control pigs. However, it appears that pigs respond with higher antibody titers to *S. pullorum* than to human erythrocytes.

Deficiencies of pantothenic acid, pyridoxine or riboflavin did not bring about a change in the total serum protein values (table 5). However, the pantothenic acid-deficient pigs had a significantly higher ($P < 0.05$) percentage of α-globulin than the control pigs. The pyridoxine-deficient pigs, on the other hand, had a significantly lower ($P < 0.05$) percentage of β-globulin than the controls. The suppression of measurable reciprocals of net antibody titers by all deficient diets was not accompanied by a uniform effect upon the protein electrophoretic profile. Following repletion all pigs produced similar values for serum protein and immunological response. However, the formerly deficient pigs continued to gain weight less rapidly than the control pigs did.

The paired feeding study indicated that on equalized feed intake the pigs fed the control diet gain weight significantly faster ($P < 0.05$), more efficiently ($P < 0.05$) and produce higher reciprocals of antibody titers ($P < 0.01$) (table 6) than any deficient group.

SUMMARY

Studies were conducted with 48 pigs in two trials to determine the effect of deficiencies of pantothenic acid, pyridoxine or riboflavin upon antibody production in swine. Using a semisynthetic milk diet the pigs were fed either a complete diet or diets deficient in pantothenic acid, pyridoxine or riboflavin. The pigs were weaned at two weeks, supplied with a diet deficient in the three B-vitamins under study for two weeks and allotted to the 4 treatment diets at 4 weeks of age. Two weeks after allotment to treatment a series of *Salmonella pullorum* injections was begun in the first trial. In the second trial injections of human erythrocytes were initiated after three weeks on the respective treatment. Reciprocals of net serum antibody titers were determined one week after the last injection in each trial. Control pigs produced significantly greater ($P < 0.01$) reciprocals of antibody titers than the deficient pigs regardless of the antigen used. A paired feeding study consisting of one pig from each treatment in a group showed that the control pigs on equal feed intake gained weight significantly faster ($P < 0.05$), more efficiently ($P < 0.05$) and produced significantly greater ($P < 0.01$) reciprocals of net antibody titers than did any deficient group. The pyridoxine-deficient pigs displayed oligocythemia and oligochromemia and had significantly higher ($P < 0.01$) urinary xanthurenic acid concentrations than the control pigs did.

Following repletion, the pigs formerly deficient in B-vitamins had values of serum protein and antibody responses to either *S. pullorum* or human erythrocyte antigens which were similar to the control pigs. However, the formerly deficient pigs continued to gain weight more slowly than the control pigs.

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Nitrogen Balance Studies with Subjects Fed the Essential Amino Acids in Plasma Pattern Proportions[1]

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The determination of free amino acids in the blood plasma of healthy individuals receiving an adequate diet has shown that during post-absorption the nutritionally essential amino acids are present in rather constant proportions (Stein and Moore, '54; Frame, '58; Swendseid et al.[2]). From the values obtained, these proportions differ from the pattern of the essential amino acids in food proteins of good nutritive quality in that valine and lysine are relatively high and isoleucine and leucine are relatively low. It has been demonstrated many times that the proportionality pattern of the nutritionally essential amino acids is an important consideration in determining the metabolic response to a given protein (Allison, '55). Therefore it seemed pertinent to ascertain whether amino acids are efficiently utilized by the metabolic processes of the body when they are fed in plasma pattern proportions. For this purpose, studies were carried out with college students wherein a diet containing amino acids supplied as plasma pattern was fed during one period and an isonitrogenous diet containing amino acids supplied as egg pattern was fed during another period. The effects of these diets on nitrogen balance were measured and compared.

EXPERIMENTAL PROCEDURES

Four college students, two women and two men, in good health as ascertained by medical examination, were the subjects for this study. Three were 25 years of age and one was 26. They were given first a controlled diet of ordinary food which contained 10 gm of nitrogen/day. When nitrogen equilibrium was established, they received the isonitrogenous experimental diet. The diet containing the amino acids in egg pattern proportions was fed during one period of 7 days or longer and an isonitrogenous experimental diet containing the amino acids in plasma pattern proportions was fed during another period of similar length. The sequence of these periods was varied for the different subjects. The methods and the dietary procedures were the same as in previous studies (Swendseid et al., '61, '62). The dietary components were also the same and, in addition to purified essential amino acids and whole egg protein, consisted of: (1) the basal diet of ordinary food low in protein; (2) variable amounts of nitrogen-free food to adjust the caloric intakes for men to 50 to 55 Cal./kg of body weight a day and for women to 45 to 50 Cal.; and (3) amounts of glycine and diammonium citrate as needed to achieve a total nitrogen intake of 10 gm/day. When the amino acids proportioned to plasma pattern were fed, purified amino acids were added to the whole egg and the nitrogen-containing components of the basal diet in amounts such that the proportions of the plasma pattern would be maintained in the total diet. Likewise, when amino acids were fed in egg pattern proportions, purified amino acids were added to whole egg and the other nitrogen-containing foods in amounts such that the egg pattern ratio would prevail in the entire diet.

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[2] Swendseid, M. E., B. Friedrich and S. G. Tuttle 1961 The effect of negative nitrogen balance on plasma amino acids. Federation Proc., 20: 8 (abstract).

TABLE 1

Ratios and daily intakes of essential amino acids for plasma and egg pattern

L-Amino acids	Pattern ratios Threonine = 1		Daily intake, young women EAA[1] nitrogen = 0.86 gm		Daily intake, young men EAA[1] nitrogen = 1.30 gm	
	Plasma	Egg	Plasma pattern	Egg pattern	Plasma pattern	Egg pattern
			gm	*gm*	*gm*	*gm*
Threonine	1.0	1.0	0.70	0.74	1.07	1.10
Isoleucine	0.6	1.2	0.42	0.89	0.64	1.33
Leucine	1.2	1.7	0.84	1.30	1.28	1.93
Lysine	2.0	1.7	1.40	1.09	2.14	1.63
Methionine and cystine	1.2	1.2	0.84	0.98	1.28	1.20
Phenylalanine and tyrosine	1.4	1.5	0.98	1.13	1.50	1.68
Tryptophan	0.6	0.3	0.42	0.24	0.64	0.36
Valine	1.8	1.3	1.26	0.93	1.93	1.39

[1] Essential amino acid.

The plasma amino acid and the egg patterns were evaluated in diets that were isonitrogenous with respect to the amount of essential nitrogen as well as the amount of total nitrogen. The amounts of essential nitrogen given each day were those amounts found adequate to maintain nitrogen equilibrium in a previous study in which the amino acids were proportioned to the Food and Agriculture Organization (FAO) provisional pattern and to egg pattern (Swendseid et al., '61, '62). These amounts were 0.86 gm and 1.30 gm of essential nitrogen/day for women and for men, respectively. The ratios and daily intakes of the essential amino acids during the periods when they were fed in plasma and in egg proportions are shown in table 1.

RESULTS AND DISCUSSION

In table 2 are shown the average daily nitrogen balance values for the young women and young men fed the experimental diets with amino acids supplied as plasma pattern and as egg pattern. All 4 subjects were in positive nitrogen balance when they were fed the amino acids proportioned to the egg ratio. Three subjects were in negative nitrogen balance and one subject was in positive balance with the diet containing amino acids supplied as plasma pattern. For all 4 subjects, however, less nitrogen was retained per day when the diet which furnished amino acids as plasma pattern was fed (subject JH, − 1.83 gm; MS, − 0.30 gm; TS, − 1.06; AH, − 0.69 gm). Although the number of subjects studied was small, this consistent and pronounced decrease in nitrogen retention appears to constitute good evidence that amino acids proportioned to plasma pattern are not as efficiently utilized by the body processes as are the amino acids in egg pattern proportions.

In table 1 it is indicated that the daily intake of two of the essential amino acids is less when plasma pattern proportions rather that egg pattern proportions are supplied in the diet. These two amino acids are isoleucine and leucine. Also, it can be calculated that when the amino acids are administered as plasma pattern.

TABLE 2

Nitrogen balances of subjects fed amino acids in plasma and egg pattern proportions

Subject	Sex	Body wt	Avg daily nitrogen balance values[1]	
			Plasma pattern	Egg pattern
		kg	*gm/day*	*gm/day*
JH	female	56.4	− 1.75(1)[2]	+ 0.08(2)[2]
MS	female	62.7	+ 0.28(2)	+ 0.58(1)
TS	male	85.5	− 0.95(1)	+ 0.11(2)
AH	male	80.0	− 0.43(2)	+ 0.26(1)

[1] The dietary periods varied in length from 7 to 10 days.
[2] Figures in parentheses show the sequence of dietary periods.

the daily intakes of all of the essential amino acids for both young men and young women are in excess of the proposed minimal requirements (reviews by Rose, '57 and Leverton, '59) with the exception of isoleucine. The daily intake of young women for this amino acid is 0.42 gm as compared with the requirement value of 0.45 gm, and the daily intake of young men is 0.64 gm as compared with the requirement value of 0.70 gm. These data suggest that isoleucine, and possibly also leucine, are the limiting amino acids for the plasma pattern.

The results of this study also add to the evidence (Swendseid et al., '61, '62) that 0.86 gm and 1.30 gm of essential amino acid nitrogen are sufficient to maintain young women and young men, respectively, in nitrogen equilibrium when the essential amino acids are supplied in egg pattern proportions in a diet containing 10 gm of total nitrogen.

SUMMARY

Two young women and two young men were fed the essential amino acids proportioned to plasma pattern during one period and an isonitrogenous amount of essential amino acids proportioned to egg pattern during another period in diets that contained 10 gm of total nitrogen/day. Nitrogen balance values showed that less nitrogen was retained when the amino acids were administered in plasma pattern ratios. This observation is evidence that amino acids supplied as the plasma pattern are utilized less effectively for metabolic purposes than amino acids in egg pattern proportions.

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Evaluation of the Effect of Breed on Vitamin B_6 Requirements of Chicks[1]

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The need of chicks for vitamin B_6 has been demonstrated by several workers, and specific requirements for this vitamin have been proposed on the basis of growth and feed efficiency responses.

Briggs et al. ('42) were the first to determine the requirement by adding various levels of vitamin B_6 to a purified ration of known vitamin B_6 content. They concluded that the young chick requires 275 to 300 μg of vitamin B_6/100 gm of feed.

Fuller and Field ('58) reported that the vitamin B_6 requirement of broiler chicks is between 1 and 2 mg/454 gm of feed. A year later, Fuller and Kifer ('59) showed that, in both battery brooders and floor pens, supplemental vitamin B_6 improved weight gains of chicks fed corn-soybean meal type rations. In a purified diet, 1.5 mg of vitamin B_6/454 gm of ration gave maximal growth and feed efficiency.

Numerous reports are available concerning the relationship of vitamin B_6 to transaminase activity of mammalian tissue (Ames et al., '47; March et al., '55; Babcock, '59). However, little information is available on the relationship of this vitamin to transaminase activity of avian tissue. Brin and Olson[3] observed that, during vitamin B_6 deficiency, the activity of the glutamic-oxalacetic transaminase system in cardiac muscle of ducklings was depressed 40% and that the activity of the glutamic-alanine system was 45% below normal.

Goswami and Robblee ('58) reported that, at three weeks of age, the aspartic-glutamic transaminase activity of blood, heart and liver of deficient Leghorn chicks was 40, 53 and 50%, respectively, of that observed in one-day-old chicks. Feeding pyridoxine at a level in excess of the chick's requirement did not increase the enzyme activity over that of chicks receiving the requirement level.

In the research to be reported, two experiments were conducted to re-evaluate the vitamin B_6 requirements of the chick. Criteria used were weight gain, feed efficiency, serum glutamic-oxalacetic transaminase (SGO-T) activity, gross clinical symptoms of deficiency and examination of histological sections of livers, gizzards and adrenal glands.

Differences between breeds in nutritional requirements have been reported in relation to a number of nutrients. Since no reports on the effect of breed on vitamin B_6 requirement of chicks could be found, experiment 3 using Single Comb White (SCW) Leghorns, Rhode Island Reds, and Vantress × Arbor Acre chicks was designed to study the influence of breed on the vitamin B_6 requirement of the chick. The criteria used in this experiment were the same as those for the first two experiments.

EXPERIMENTAL PROCEDURE

Commercial Vantress × Arbor Acre male chicks were used in all experiments. In addition, for experiment 3, SCW Leghorns and Rhode Island Red chicks were obtained from the Iowa State University Poultry Farm. All experiments were conducted in electric battery brooders, and experimental rations and water were allowed ad libitum. Waterers were washed

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[3] Brin, M., and R. E. Olson 1951 Effect of pyridoxine deficiency upon respiration and transaminase activity of cardiac muscle in ducklings. Federation Proc., *10*: 166 (abstract).

daily to keep microbial growth, and hence vitamin synthesis, at a minimum. Crystalline pyridoxine hydrochloride[4] was used in all studies. Blood was obtained by cardiac puncture, using a 5-ml glass syringe. The SGO-T activity was measured by the method of Karmen ('55).

In experiment 1, 120 day-old chicks were assigned randomly to 10 pens. The semipurified diet used contained the following ingredients expressed as percentage of the total ration: dextrose 65.75; isolated soybean protein,[5] 22.00; soybean oil, 2.00; nonnutritive fiber,[6] 3.00; vitamin mixture, 1.00; mineral mixture, 5.30; DL-methionine, 0.50; glycine, 0.20; and choline chloride, 0.25. The vitamin mixture added the following per kilogram of ration: vitamin A, 10,000 IU; vitamin D_3 1500 ICU; vitamin E, 22 IU; inositol, 132 mg; folic acid, 3.3 mg; *p*-aminobenzoic acid, 66 mg; niacin, 88 mg; Ca pantothenate, 22 mg; riboflavin, 8.8 mg; thiamine·HCl, 4.4 mg; menadione, 4.4 mg; ascorbic acid, 220 mg; vitamin B_{12}, 22 μg; and biotin, 220 μg. The mineral mixture contributed the following per kilogram of complete ration: sodium chloride, 5 gm; calcium, 12 gm; phosphorous, 6.4 gm; zinc, 6.4 mg; potassium, 11.3 gm, manganese, 70 mg; iron 91 mg; cobalt 2.5 mg; copper, 6.9 mg; magnesium, 880 mg; iodine, 3.6 mg.

The practical diet used in experiment 1 contained the following ingredients expressed as percentage of the total ration; ground yellow corn, 59.0; soybean oil meal (50% protein), 28.0; meat and bone scraps (50% protein), 4.0; soybean oil, 2.0; condensed fish solubles, 2.0; dehydrated alfalfa meal (20% protein), 2.0; salt mixture, 0.6; dicalcium phosphate, 1.0; oyster shell, 0.5; vitamin mixture, 1.0. The vitamin mixture contributed the following per kilogram of complete ration: vitamin A, 6600 IU; vitamin D_3, 1100 ICU; vitamin E, 11 IU; riboflavin, 4.4 mg; niacin, 37 mg; Ca pantothenate, 8.8 mg; vitamin B_{12}, 16.5 μg; menadione, 2.2 mg; penicillin, 11 mg; and 3-nitro-4-hydroxylphenylarsonic acid, 50 mg. The salt mixture contributed the following per kilogram of complete ration: sodium chloride, 4 gm; manganese, 120 mg; iodine, 0.55 mg; iron, 40 mg; copper, 30 mg; zinc, 2.9 mg; cobalt, 0.50 mg; and sulfur, 17 mg.

Two pens of chicks were allotted to each of 5 experimental treatments under a completely randomized design. The 5 experimental diets consisted of the semipurified diet with 4 levels of vitamin B_6 (table 1), and the practical diet, containing approximately 5.5 mg of total vitamin B_6/kg of ration.

Design of experiment 2 was similar to that of experiment 1 except that the 5 experimental diets consisted of the semipurified diet with 5 levels of vitamin B_6 (table 2). At 18 days of age, 4 birds from each group, as nearly as possible the average weight of their group, were selected from groups receiving 1.1, 3.3, and 5.5 mg of vitamin B_6/kg of ration. These were bled, decapitated, and livers, gizzards and adrenal glands removed for histological

[4] Merck and Company, Rahway, New Jersey.
[5] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[6] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Effect of vitamin B_6 on growth rate, feed efficiency and serum glutamic-oxalacetic transaminase (SGO-T) activity — experiment 1

Basal ration	Vitamin B_6	Weight[1] Age in days					4-week feed conversion	SGO-T	
		(1	7	14	21	28)		14 days	28 days
	mg/kg	*gm*	*gm*	*gm*	*gm*	*gm*		*μ/ml*	*μ/ml*
Purified	1.1	42	71	98	152	188[2]	—	68 ± 8.9[3]	67 ± 3.6
Purified	3.3	42	98	202	340	483	1.86	148 ± 11.4	149 ± 10.7
Purified	5.5	43	99	202	344	505	1.68	129 ± 10.3	144 ± 14.2
Purified	9.9	43	96	204	327	484	1.72	150 ± 6.7	164 ± 6.0
Practical	5.5	43	96	200	345	502	1.84	156 ± 7.9	144 ± 10.5

[1] Two replicate pens of 12 chicks each.
[2] Mean weights of only three chicks that were injected with pyridoxine hydrochloride at 14 days of age.
[3] Mean ± SE.
[4] Approximate calculated vitamin B_6.

TABLE 2

Mortality and autopsy observations — experiment 1

Observation	Total vitamin B_6				
	Semi-purified diet				Practical-type diet
	mg/kg	*mg/kg*	*mg/kg*	*mg/kg*	*mg/kg*
	1.1	3.3	5.5	9.9	5.5
Mortality	21/24	2/24	0/24	0/24	1/24
Gizzard erosion	16/20	5/20	3/12	2/12	1/12
Wing hemorrhage	4/12	0/12	0/12	0/12	0/12

studies. The tissues were fixed and imbedded in paraffin, and sections were cut at 6 μ, mounted and stained, using two types of stains, (1) hematoxylin-eosin, and (2) Gomori's one step trichrome. Standard histological procedures were used in preparing the slides.

A total of 288 one-day-old male chicks of three breeds (SCW Leghorn, Rhode Island Red, Vantress X Arbor Acre) were used in experiment 3. Ninety-six chicks of each breed were randomly assigned to 8 pens of 12 chicks each. Two pens of each breed were allotted to each of the 4 experimental diets. The same semipurified diet used in the previous two experiments was used in this experiment and fed at 4 levels of vitamin B_6 (1.3, 1.8, 2.2 and 2.6 mg/kg of ration).

RESULTS

Experiment 1. Chicks receiving 1.1 mg vitamin B_6/kg of ration demonstrated retarded growth at one week of age (table 1). The 3.3 mg level was the lowest level that supported good growth and feed efficiency. The best feed conversion, however, was obtained when the semipurified diet contained 5.5 mg of vitamin B_6/kg. There were no statistically significant differences in either 4-week weight or feed conversion when the practical diet was compared with the semipurified diet containing 3.3 mg or more of vitamin B_6.

The SGO-T activity was significantly depressed at 14 and 28 days when chicks were fed the purified diet containing 1.1 mg of vitamin B_6/kg.

As early as 8 days of age, some of the birds fed the diet containing the lowest level of vitamin B_6 exhibited vitamin B_6 deficiency symptoms. These symptoms have been described by several workers (Jukes, '39; Fuller and Kifer, '59). Immediately after death, chicks were autopsied, and abnormalities were recorded. The most prominent pathological findings observed were hemorrhages in various areas of the body. These were particularly striking around follicles of the wing feathers as shown in figure 1.

Vitamin B_6 deficiency also significantly increased the incidence of gizzard erosion as shown in table 2. Gizzard erosion was, in most cases, accompanied by hemorrhages and varied greatly in severity among birds receiving the same diet. The number of lesions in eroded gizzards varied and usually increased with the length of time chicks were fed the low-vitamin B_6 diet.

Experiment 2. A marked difference in weight between groups fed 1.1 mg of vitamin B_6 and all other groups was observed at the end of the first week of age (table 3). Chicks fed the diet containing 1.1 mg did not gain weight after the second week. No significant differences in weight or feed conversion at 4 weeks were observed among groups fed 2.2, 3.3, 4.4 or 5.5 mg of vitamin B_6/kg of ration.

The SGO-T activity of chicks receiving 2.2 mg of total vitamin B_6 was lower (table 3) than in any of the other groups. This difference, however, was not statistically significant. Since no survivors remained at 4 weeks in groups receiving 1.1 mg vitamin B_6/kg ration, no SGO-T values are available for this treatment.

Manifestations of vitamin B_6 deficiency observed in this experiment were similar to those described in experiment 1. Results of autopsies made on chicks at the end of the experiment are presented in table 4. Spleen weights per 100 gm of body weight were a little higher in the group fed 2.2 mg of vitamin B_6, but this

Fig. 1 An excised wing from a vitamin B_6-deficient chick showing hemorrhages around follicles of the feathers. Few feathers are shown to have been broken at the point of hemorrhage and fallen off.

TABLE 3

Effect of vitamin B_6 on growth rate, feed efficiency and serum glutamic-oxalacetic transaminase (SGO-T) activity — experiment 2

Added pyridoxine·HCl	Total vitamin B_6	Average weights[1] Age in days					4-week feed conversion	SGO-T 4-weeks of age
		1	7	14	21	28		
mg/kg	*mg/kg*	*gm*	*gm*	*gm*	*gm*	*gm*		*μ/ml*
0.0	1.1	40	69	72	72[2]	—	—	—
1.1	2.2	40	94	182	315	454	1.86	104 ± 10.5[3]
2.2	3.3	40	92	186	316	460	1.84	126 ± 19.3
3.3	4.4	40	94	183	312	440	1.90	131 ± 19.8
4.4	5.5	40	93	186	310	448	1.89	129 ± 15.0

[1] Two replicate pens of 12 chicks each.
[2] Mean of only three chicks that survived out of 24 chicks started.
[3] Mean ± SE.

TABLE 4

Mortality and autopsy observations — experiment 2

Observation	Total vitamin B_6, mg/kg				
	1.1	2.2	3.3	4.4	5.5
Body wt, gm	—	461 ± 15.9[1]	456 ± 13.7	426 ± 12.8	451 ± 9.2
Liver wt, gm/100 gm body wt	—	3.3 ± 0.14	3.6 ± 0.20	3.4 ± 0.16	3.4 ± 0.12
Spleen wt, mg/100 gm body wt	—	235 ± 23.1	227 ± 27.1	244 ± 19.8	224 ± 31.6
Gizzard erosion	15/24	2/12	2/12	3/12	1/12
Wing hemorrhage	4/24	0/12	0/12	0/12	0/12
Mortality	24/24	0/24	3/24	0/24	4/24

[1] Mean of 8 chicks ± SE.

difference was not statistically significant. Since all chicks fed the basal diet died before 4 weeks of age, data are incomplete for this treatment.

Several histopathological manifestations were observed in gizzard tissue sections from chicks fed 1.1 mg of vitamin B_6/kg of ration (figs. 2 and 3).

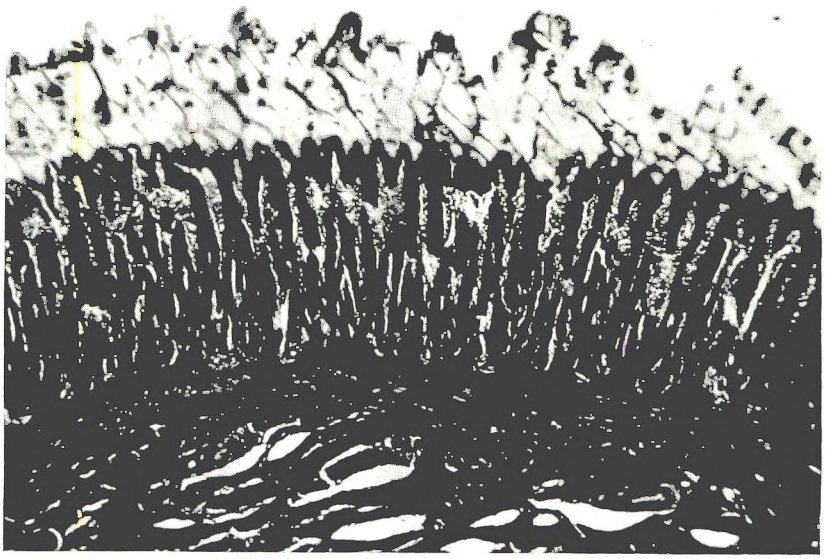

Fig. 2 A cross section of a normal gizzard stained with Gomori's one step trichrome, taken from a chick receiving adequate vitamin B_6. From top to bottom, horny latery, tunica propria with the glands in it, submucosa and part of the muscle layer. Photomicrograph 95 ×.

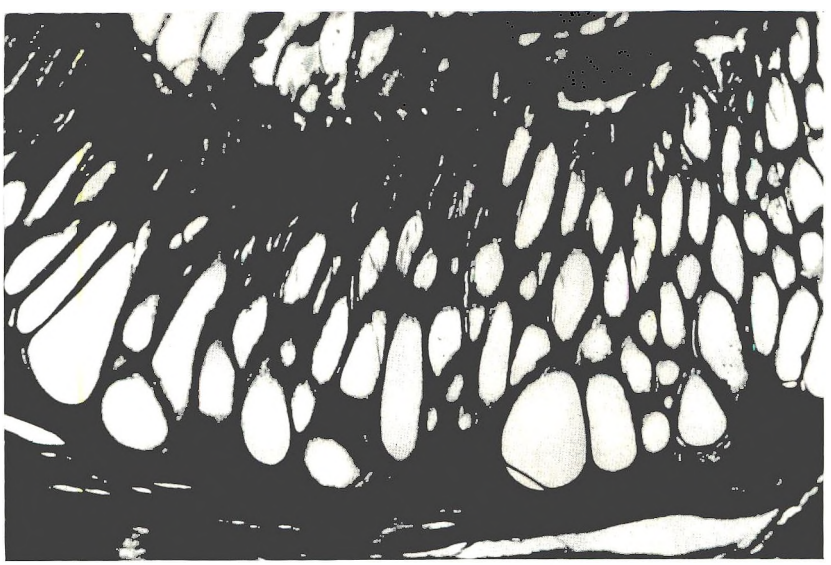

Fig. 3 A cross section of an eroded gizzard stained with Gomori's one step trichrome, taken from a chick receiving vitamin B_6-deficient diet. From top to bottom: part of the horny layer, tunica propria with extreme dilation of the glands, submucosa and a thin portion of the muscle layer. Photomicrograph 95 ×.

These consisted of the appearance of cystic glands in the tunica propria and extreme dilatation of these glands. Cells lining the cystic glands were almost squamous in nature, flattened and no longer cuboidal. The horny layer varied in thickness and was eroded at different locations. In some instances, the submucosa also varied in thickness from one area to the next.

Experiment 3. Table 5 presents weekly chick weights and 4-week feed conversion data for experiment 3. With Leghorns, it is observed that, at the end of the first, second and third weeks, there is a linear improvement in weight with increases of vitamin B_6 from 1.3 to 2.6 mg/kg of diet. At the end of the fourth week, however, the difference between groups receiving 2.2 and 2.6 mg of vitamin B_6 had largely disappeared. In Rhode Island Reds, the same trend is evident, but Rhode Island Red chicks receiving 2.6 mg vitamin B_6 gained slightly more than those receiving 2.2 mg at all ages. With Vantress X Arbor Acre cross chicks at all ages, a linear response of weight gain to vitamin B_6 in the diet is evident up to 2.2 mg vitamin B_6, but increasing the vitamin to 2.6 mg/kg ration did not further increase gains.

The SGO-T activity determinations made at 4 weeks of age are presented in table 5. In Leghorns, SGO-T activity was not significantly reduced by lowering the vitamin B_6 level in the diet. In both the Rhode Island Reds and the Vantress X Arbor Acre cross, transaminase activity was not statistically different between groups fed 2.2 and 2.6 mg of vitamin B_6/kg of diet. For both breeds, however, means from groups receiving 1.3 and 1.8 mg of vitamin B_6 were significantly different from those of groups receiving 2.2 or 2.6 mg vitamin B_6/kg diet.

The relationship of transaminase activity to growth in the different breeds studied is graphically represented in figure 4. The figure illustrates that the transaminase activity was affected more by vitamin B_6 deficiency in the faster-growing breeds. It also demonstrates that, once an optimal level of vitamin B_6 is reached, there is no further increase of transaminase activity as a result of an increase in vitamin B_6 in the diet.

Leghorns showed a much higher mortality rate than either of the other two breeds (table 5). Gizzard erosion incidence was highest in chicks receiving 1.3 mg of vitamin B_6/kg of diet, regardless of breed. This supports results reported in the previous two experiments. Results also indicate that gizzard erosion cannot be com-

TABLE 5

Effect of vitamin B_6 level as influenced by breed — experiment 3

Breed	Vitamin B_6	Weight[1] Age in days					4-week feed conversion	SGO-T	Mortality	Gizzard erosion
		1	7	14	21	28				
	mg/kg	*gm*	*gm*	*gm*	*gm*	*gm*		*μ/ml*		
SCW Leghorn	1.3	39	60	74	92	107	2.36	102 ± 22.4[2]	12/24	6/12
SCW Leghorn	1.8	39	66	101	138	188	2.41	118 ± 18.1	2/24	2/12
SCW Leghorn	2.2	39	69	116	178	259	2.00	119 ± 18.4	0/24	3/12
SCW Leghorn	2.6	39	72	129	193	268	2.10	125 ± 18.2	1/24	2/12
RI Red	1.3	41	60	75	102	123	2.02	91 ± 3.7	3/24	4/12
RI Red	1.8	41	68	103	150	208	1.88	108 ± 16.7	2/24	3/12
RI Red	2.2	41	78	140	220	306	2.12	145 ± 14.9	0/24	2/12
RI Red	2.6	42	84	152	236	332	2.10	151 ± 6.0	0/24	0/12
Vantress × Arbor Acre	1.3	41	80	107	144	180	1.77	66 ± 15.0	4/24	4/12
Vantress × Arbor Acre	1.8	41	87	155	242	340	1.72	90 ± 13.6	0/24	1/12
Vantress × Arbor Acre	2.2	41	92	180	283	429	1.66	142 ± 11.7	0/24	1/12
Vantress × Arbor Acre	2.6	41	92	181	298	433	1.76	142 ± 21.2	0/24	1/12

[1] Each figure represents the mean of 24 chicks, minus mortality.
[2] Mean ± SE.

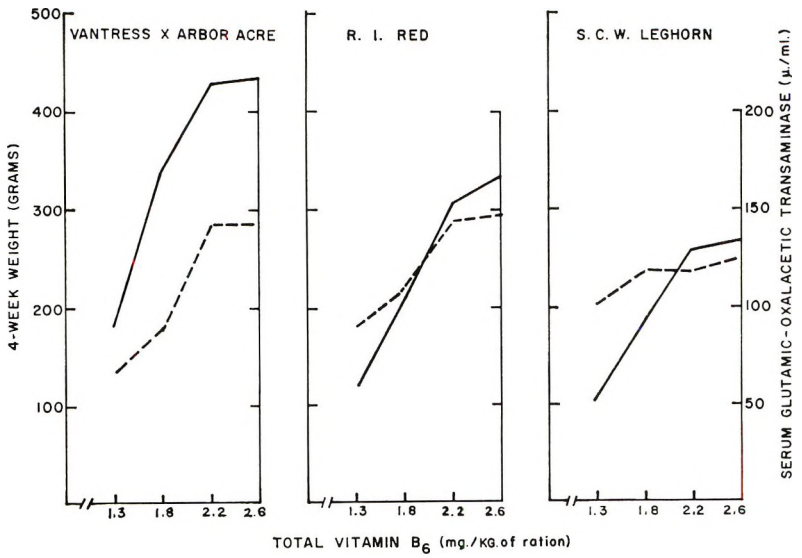

Fig. 4 Effect of vitamin B_6 level on growth rate and serum glutamic-oxalacetic transaminase (SGO-T) activity in three different breeds of growing chicks. Solid line indicates body weight: broken line, SGO-T activity.

pletely eliminated by adding adequate levels of vitamin B_6 to the diet.

DISCUSSION

The vitamin B_6 requirement of the growing chick was investigated in three experiments carried out at different times of the year but with little variation in management procedures.

Results presented from experiment 1 indicate that, among the levels of vitamin B_6 studied, 3.3 mg of vitamin B_6/kg of diet was the lowest level that gave optimal growth and feed efficiency responses. This agrees with the report of Hogan et al. ('41) who estimated the requirement to be between 3 and 5 mg/kg of diet. The experiments of these workers, however, involved only these two levels of vitamin B_6.

Fuller and Field ('58) and Fuller and Kifer ('59) reported that the vitamin B_6 requirement of broiler chicks is between 2.2 and 4.4 mg/kg of feed. In experiment 2, 5 levels of vitamin B_6 were studied and were chosen at closer-spaced intervals than in experiment 1. Results obtained in experiment 2 show no significant differences in weight or feed conversion at 4 weeks between groups fed 2.2 and 3.3 mg of vitamin B_6/kg of feed. This observation is supported by Kratzer et al. ('47) who concluded that chicks require 2 mg of vitamin B_6/kg of feed.

The purpose of experiment 3 was to study the effects of breed on the vitamin B_6 requirement of the chick. Four-week weight data showed statistical significance in breed-vitamin B_6 level interaction.

Leghorns fed the vitamin B_6-deficient diet had a much higher mortality rate than either of the other two breeds. Thornton and Shutze ('60) showed that, with thiamine-deficient diets, the percentage mortality, as well as a shorter survival time, was more evident in Leghorns than in New Hampshire × Delaware cross chicks. Since no critical work has been found in the literature on the effect of different breeds on vitamin B_6 requirement of the chick, results obtained in experiment 3 cannot be evaluated in comparison with previous work. However, Lucas et al. ('46) suggested a higher vitamin B_6 requirement for a Rhode Island Red × Barred Plymouth Rock Cross. This suggestion was based on observations of symptoms similar to vitamin B_6 deficiency symptoms in chicks receiving diets considered adequate in vitamin B_6.

The SGO-T activity was studied as a second major criterion in the evaluation of vitamin B_6 nutrition in the chick. In all

three experiments, it was shown that SGO-T activity reflected the severity of deficiency. The groups receiving the vitamin B_6-deficient diet in experiment 1 had about 45% of the transaminase activity of those receiving diets adequate in vitamin B_6. This observation is in agreement with March et al. ('55). Sass and Murphy ('58) found that administration of 25 mg of pyridoxine/day reversed a significant drop in glutamic-oxalacetic transaminase activity in whole blood of human patients. A group of other vitamins failed to give this response.

Increasing the vitamin B_6 content to 9.9 mg/kg in a semipurified diet increased SGO-T activity slightly in experiment 1, but differences were not statistically significant. Goswami and Robblee ('58) showed that feeding pyridoxine at a level in excess of the chick's requirement did not result in any increase in blood aspartic-glutamic transaminase activity over those receiving the requirement level. It was observed in experiment 2 that groups receiving 2.2 mg of vitamin B_6 had lower SGO-T activity than those receiving 3.3 mg/kg of feed. Growth rates, however, did not differ up to 4 weeks. This suggests that 2.2 mg of vitamin B_6 is sufficient for growth but may be marginal for SGO-T activity.

Experiment 3 indicates that SGO-T activity differs in different breeds, particularly when the intake of vitamin B_6 is low or marginal. The severity of decrease in SGO-T activity appears to be more pronounced in the faster-growing than in the slower-growing breeds.

The precision and sensitivity of the two major criteria used to estimate dietary adequacy was evaluated. Bliss and György ('51) suggested the use of two statistics in measuring the sensitivity and precision of a particular criterion used in biological studies. These are the steepness of the response curve and the extent to which individual observations vary about the mean response line. To measure these two parameters, the above authors made use of the statistic, λ, which is simply the standard deviation of the response divided by the linear regression coefficient of that particular response criterion.

The λ was calculated for 4-week weights and SGO-T activity (table 6). Since a low λ value indicates high sensitivity, the 4-week weight was a more precise criterion than SGO-T activity in all three experiments. This is also clearly shown in average values. It is observed that λ values decrease, and, hence, sensitivity is improved from experiment 1 to 2, and from 2 to 3, considering the same breed of chicks. In experiment 3, the λ for both 4-week weight and SGO-T activity of the Vantress × Arbor Acre Cross are lower than those for the other two breeds. Likewise, the λ values of Rhode Island Reds for both 4-week weight and SGO-T activity are lower than those for Leghorns. This demonstrates that the sensitivity of these two criteria increases with an increasing rate of growth.

The above comparison of the precision and sensitivity of 4-week weights and SGO-T activity in investigations of vitamin B_6 nutrition in the chick, using the statistic, λ, leads to the conclusion that growth remains the preferred criterion in vitamin assays. Furthermore, the fast-growing breeds, such as the modern

TABLE 6

Sensitivity of 4-week chick weights vs. serum glutamic-oxalacetic transaminase (SGO-T) activity as a measure of vitamin B_6 requirement of the chick, using the statistic λ

Exp. no. and breed used		λ (SD/regression coefficient)	
		4-week wt	SGO-T activity
1	Vantress × Arbor Acre	0.154	0.300
2	Vantress × Arbor Acre	0.095	0.581
3	Vantress × Arbor Acre	0.084	0.166
	Rhode Island Red	0.098	0.224
	SCW Leghorn	0.128	0.627
	Average	0.112	0.380

broiler crosses, are more desirable for such assays than is the Leghorn.

Vitamin B_6 deficiency significantly increased the incidence of gizzard erosion in all experiments. Gizzard erosion is a complex pathological manifestation which appears to be due to several factors, nutritional or otherwise. Our data indicate that vitamin B_6 is possibly one of the nutritional factors involved. The implication of vitamin B_6 in gizzard erosion has not been previously reported in the literature. Bird et al. ('36) observed characteristic lesions in the gizzard lining while studying a factor, called at that time, vitamin B_4. It is quite possible that vitamin B_6 could have been part of this so-called vitamin B_4 factor. In studying the essential nature of vitamin B_6 for chicks, Hegsted et al. ('40) reported severe gizzard erosion in chicks fed some of the basal diets used. No indication was given, however, as to which diet was actually responsible for the erosion.

SUMMARY

The vitamin B_6 requirement of the chick was studied, using weight, feed conversion, serum glutamic-oxalacetic transaminase (SGO-T) activity and gross clinical and histopathological symptoms as criteria. In the first two experiments, broiler-type chicks were fed diets containing 1.1, 2.2, 3.3, 4.4, 5.5 and 9.9 mg total vitamin B_6/kg of diet. In the third experiment, Single Comb White Leghorns, Rhode Island Reds and Vantress × Arbor Acre chicks were used to study breed effects on vitamin B_6 requirement.

Chicks started on a vitamin B_6-deficient diet regimen showed retarded growth at one week of age. Clinical symptoms were first observed at about 8 days of age and were characterized by hyperexcitability, decreased appetite, extreme weakness, ruffled feathers, convulsions and death. Pathological findings revealed hemorrhages in various areas, most striking around follicles of wing feathers.

Vitamin B_6 deficiency increased gizzard erosion. Histopathological manifestations of eroded gizzards were extreme dilatation of the glands of the tunica propria, with the cells lining these glands becoming almost squamous in nature.

The SGO-T activity was depressed when the diet contained 1.1 mg of vitamin B_6, but was not significantly affected by levels above 2.2 mg/kg feed. The SGO-T activity in Leghorns was significantly less affected by low vitamin B_6 than in the other two breeds.

For the three breeds studied, 2.2 to 2.6 mg vitamin B_6/kg ration was adequate for best growth and feed conversion. Growth was a more sensitive criterion than was SGO-T activity for vitamin B_6 adequacy.

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Effect of the Level of Dietary Protein with and without Added Cholesterol on Plasma Cholesterol Levels in Man[1,2]

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Several workers have reported on the basis of epidemiological studies that there are positive correlations between per capita intake of total calories, fat, and protein and mortality due to coronary heart disease (Keys, '53; Yerushalmy et al., '57; Yudkin, '58; Jolliffe et al., '59). Although the validity of some of the data on which these calculations have been made was commented upon by some of these authors (Yerushalmy et al., '57; Yudkin, '58; Jolliffe et al., '59) and criticized in some detail by Mann ('57), nonetheless these associations have indicated areas that should be investigated by the direct experimental approach. An assumption currently held by many investigators working in this field is that high serum lipid levels potentiate the development of atherosclerotic lesions, thus increasing the probability of clinical coronary disease.

The role of dietary fat in affecting serum lipids has received most attention and has been investigated intensively but comparatively little study has been made of the effect of dietary protein in man. Furthermore many of the experiments that have been performed to date can be criticized on one or another basis. For example only recently has it been clearly established that the animal sterol, cholesterol, (Beveridge et al., '59, '60; Connor et al., '61)[3] and such plant sterols as the sitosterols (Beveridge et al., '61) make significant contributions to the hyper- and hypocholesterolemic effects, respectively, of certain animal and plant fats. Again plant and animal protein foods usually have associated with them triglycerides which may themselves have variable effects (Kinsell et al., '52, '58; Ahrens et al., '54, '57; Beveridge et al., '54; Bronte-Stewart et al., '56). A consideration of these factors makes it apparent that unless purified protein sources are used in conjunction with a basal diet of constant composition, it is impossible to attribute with confidence any changes observed to alterations in dietary protein. Undoubtedly the conflicting nature of some of the reports in the literature is due to neglect of one or another of these features. Further information on this problem in man was sought by the use of homogenized formula rations in which the protein moiety was varied from 5 to 25% of total calories by equicaloric substitution at the expense of carbohydrate.

EXPERIMENTAL

The methods used in preparing and storing the diets, together with the experimental design, have been described in previous publications (Beveridge et al., '56, '57) and only a brief description will be given here. Two basal rations, differing only in that one of these contained an additional amount of cholesterol (500 mg/ 950 Cal.) were made up of the ingredients shown in table 1.[4] These were homogenized with water to produce a thick milk-like fluid. Protein was altered by making appropriate equicaloric changes in the carbohydrate component, thus maintaining constant the proportion of calories (30%) derived from the fat moiety (butter). The

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[1] This work was financed by grants from the Ontario Heart Foundation, Toronto, Ontario, and the Nutrition Foundation, Inc., New York.
[2] Presented in abbreviated form at the meeting of the Council on Arteriosclerosis of the American Heart Association, October 24–25, 1962.
[3] Bronte-Stewart, B. 1961 Lipids and atherosclerosis. Federation Proc., 20: 127 (abstract).
[4] The authors are indebted to the following firms for generous supplies of certain of the dietary ingredients: Mead Johnson of Canada, Ltd., and Mil-ko Products, Ltd., Canada.

TABLE 1

Composition of basal diet;[1] amounts required to make a 950-Cal. sample

Ingredient	Amount	Protein	Fat	CHO
	gm	*gm*	*gm*	*gm*
Skim milk powder[2]	28.1	10	0.1	14.6
Calcium caseinate[3]	29.1	25.6	0.6	—
Sucrose	20.0	—	—	20.0
Maltose and dextrins[4]	98.0	—	—	96.0
Butter	38.3	—	31.0	—
Total		35.6	31.7	130.6
Calories	950.1	142.4	285.3	522.4
		%	%	%
Calories		15.0	30.0	55.0

[1] Two grams of iodized salt were added per 950-Cal. batch. A mixture of vitamins was also added to supply the following amounts of these substances (in milligrams) per 950 Cal.: thiamine, 0.6; riboflavin, 0.6; niacin, 5.0; pyridoxine, 5.0; Ca pantothenate, 5.0; ascorbic acid, 25.
[2] Mil-ko, Mil-ko Products, Ltd., Canada.
[3] Casec, Mead Johnson of Canada, Ltd., Belleville, Ontario.
[4] Dextri Maltose. Mead Johnson of Canada, Ltd.

only other items permitted were water, tea and coffee without sugar or milk. A small number of subjects were unable to continue with the diet and dropped out for various reasons — illness or inability to adjust to the dietary regimen. The subjects were instructed to take enough of the ration to maintain their body weight constant and only a few subjects gained or lost more than one to 1.5 kg. The average change in weight between day 8 and 16 was −0.4 kg. Blood samples were taken from the subjects in the fasting state between 7:00 and 8:30 AM on days zero, 4, 8, 12 and 16. Plasma cholesterol was determined by the technique of Abel et al. ('58).

Seventy-nine male university students were assigned at random to the two basal rations which were identical except that one contained an added amount of cholesterol (500 mg/950 Cal.). Both provided 15% of calories in the form of protein and 30% of calories as butterfat. After 8 days, each of the two groups was divided into 5 sub-groups with similar plasma cholesterol distribution and for another similar period they were fed diets modified by the addition or withdrawal of the protein, calcium caseinate,[5] for equicaloric amounts of maltose-dextrins.[6] The percentage of calories supplied by protein was as follows: 5, 10, 15, 20, and 25. Sixty-five subjects completed the experimental period.

In addition to the male subjects, 39 female students consumed the basal ration without the added cholesterol and were then divided into three groups and transferred to diets providing 5, 15, or 25% of calories as protein. All but 7 of the female subjects finished the experiment.

RESULTS AND DISCUSSION

The behavior of the plasma cholesterol in terms of average percentage change at day 16 from the values found at day 8 is shown in figure 1, and the statistical data are presented in table 2 for those on the ration without added cholesterol. Similar data are presented in table 3 for those fed the ration containing added cholesterol. The only significant changes observed were the increases resulting from consumption of the diet low in protein (5% of calories). The plasma cholesterol values with this low protein diet at day 8 and 16 for the unsupplemented group were 152.0 and 171.1 mg/100 ml respectively, (+ 19.1 mg/100 ml, $P < 0.05$) and for the cholesterol-supplemented group were 168.3 mg/100 ml and 180.2, respectively (+ 11.9 mg/100 ml, $P < 0.05$). The group receiving the cholesterol supplement had as expected because of our previous work on the effect of dietary cholesterol in man (Beveridge et al., '59, '60), a higher cholesterol value at day 8 than did those not receiving the added cholesterol. Presumably this might help to account for the smaller increase due to the feeding of the low protein diet over the period day 8 to 16 observed in the groups receiving the cholesterol supplement.

[5] Casec, Mead Johnson of Canada, Ltd.
[6] Dextri-Maltose, Mead Johnson of Canada, Ltd.

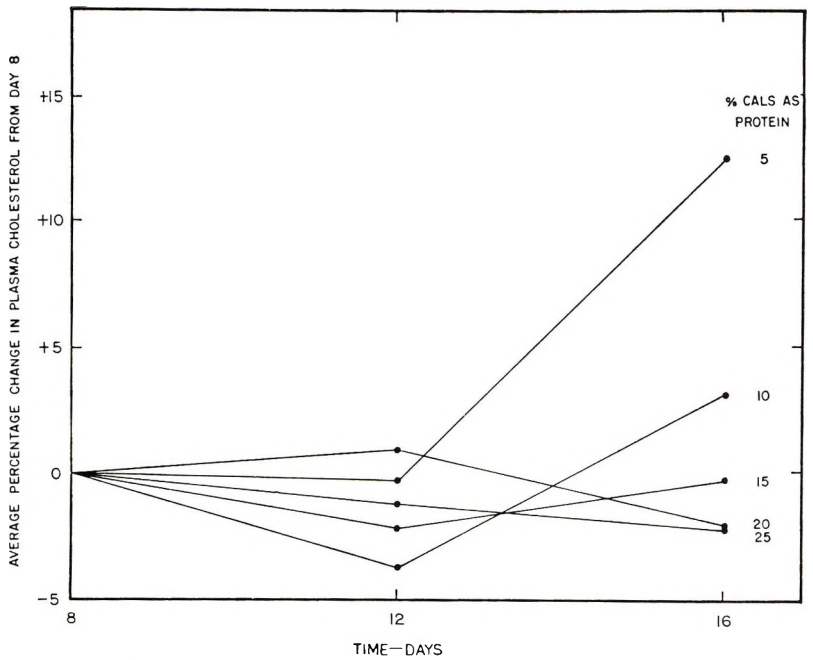

Fig. 1 The average percentage change in plasma cholesterol for groups, each comprised of 6 or 7 male university students, following transfer from a basal diet providing 15% of calories to diets in which the protein component, calcium caseinate, was varied by isocaloric substitution for the maltose-dextrins.

TABLE 2

Effects on plasma cholesterol levels in male subjects of the addition or withdrawal of calcium caseinate for equicaloric amounts of maltose-dextrins in a basal diet providing 15% of calories as protein[1]

Group no.	% Calories as protein	No. of subjects	Average plasma cholesterol		Mean difference between day 8 and day 16	*t*	P
			Day 8	Day 16			
			mg cholesterol/100 ml		*mg cholesterol/100 ml*		
1	25	6	157.3	154.0	— 3.3	1.14	0.3 –0.4
2	20	6	149.6	146.6	— 3.0	0.40	0.7 –0.8
3	15	7	162.8	162.4	— 0.4	0.09	0.9
4	10	7	165.9	171.2	+ 5.3	1.58	0.1 –0.2
5	5	7	152.0	171.1	+19.1	3.04	0.02–0.05

[1] All subjects ate the basal diet providing 15% of calories as protein for 8 days prior to the change in dietary protein.

The results from the 32 female students who consumed the ration without added cholesterol are shown in table 4. The only change observed was a small but significant decrease of 7.4 mg/100 ml (*P*, 0.01 to 0.02) in the group transferred to the diet high in protein.

These data, obtained from the first large scale experiment to be performed on normal young subjects consuming a formula ration, provide no support for the possibility that high intakes of protein per se are implicated in the development of cardiovascular disease through any elevating effect on plasma lipid levels. Indeed these studies indicate not only that high levels of dietary protein did not increase plasma cholesterol but that low protein diets were hypercholesterolemic.

Due to the variety of experimental conditions used by previous workers in this field, it is a difficult task to reconcile all

TABLE 3

Effects on plasma cholesterol levels in male subjects of the addition or withdrawal of calcium caseinate for equicaloric amounts of maltose-dextrins in a basal diet providing 15% of calories as protein[1] and a supplement of 500 mg cholesterol per 950 calories

Group no.	% Calories as protein	No. of subjects	Average plasma cholesterol		Mean difference between day 8 and day 16	t	P
			Day 8	Day 16			
			mg cholesterol/100 ml		*mg cholesterol/100 ml*		
6	25	7	172.8	172.0	− 0.8	0.22	0.8 –0.9
7	20	6	181.8	189.6	+ 7.8	1.45	0.2 –0.3
8	15	7	175.2	178.1	+ 2.9	0.46	0.6 –0.7
9	10	6	158.0	160.3	+ 2.3	0.58	0.5 –0.6
10	5	6	168.3	180.2	+11.9	3.24	0.02–0.05

[1] All subjects ate the basal diet providing 15% of calories as protein, plus 500 mg cholesterol per 950 calories for 8 days prior to the change in dietary protein.

TABLE 4

Effects on plasma cholesterol levels in female subjects of the addition or withdrawal of calcium caseinate for equicaloric amounts of maltose-dextrins in a basal diet providing 15% of calories as protein[1]

Group no.	% Calories as protein	No. of subjects	Average plasma cholesterol		Mean difference between day 8 and day 16	t	P
			Day 8	Day 16			
			mg cholesterol/100 ml		*mg cholesterol/100 ml*		
1	25	10	172.8	165.4	−7.4	3.13	0.01–0.02
3	15	11	171.4	171.5	+0.1	0.03	0.9
5	5	11	176.1	176.8	+0.7	0.37	0.7 –0.8

[1] All subjects ate the basal diet providing 15% of calories as protein for 8 days prior to the change in dietary protein.

the results without some qualification. The carefully designed experiment reported by Furman et al. ('59) deserves mention. Although data on only one subject, a 70-year-old woman with mild postmenopausal osteoporosis, were reported, these authors have obtained similar results on a number of other subjects.[7] The investigators found a highly significant decrease in serum cholesterol when skim milk powder, which supplied all of the protein (15% of calories) in the formula ration, was replaced with glucose. A salt solution was added to compensate for the minerals removed from the diet, but no other changes were made to take into account the other components eliminated from the ration by removal of the skim milk powder. These conditions differ from those used by us in that a diet free from protein was used, whereas the lowest level used in our work was 5% of calories.

Olson et al. ('58) using diets comprised of the usual variety of foodstuffs tested the effect of varying the level of dietary protein in 9 subjects, 5 of whom were hypercholesterolemic. The low protein diet, which supplied daily 25 gm of vegetable protein from cereals, rice, and legumes, led to lower serum cholesterol values than did the diet high in protein which was derived mainly from animal sources. Although it is stated that the low protein diet was similar in fat content, no indication of the cholesterol and plant sterol levels are given. In the light of our observations on the effect of dietary cholesterol in man (Beveridge et al., '59, '60) and the recent confirmation of these by others (Connor et al., '61),[8] Olson's results can be readily explained on the basis of difference in cholesterol content of the diets he used, because the high protein ration derived mainly from animal sources would presumably provide significantly more cholesterol than the low protein diet in which

[7] Personal communication.
[8] See footnote 3.

all the protein was obtained from vegetables. A further consideration, in view of our recent work on the dose-response relationship between the plant sterol, sitosterol, and plasma cholesterol levels reported at the 5th International Congress of Biochemistry ('61), is that the low protein diet would undoubtedly provide significant amounts of these substances thus contributing to the observed decrease in serum cholesterol. The report by Walker et al. ('60) that a diet in which the protein moiety was supplied mainly from vegetable foods led to lower serum cholesterol and phospholipid levels than did one in which animal products provided essentially all of this dietary component is in accord with the explanation offered here for the observations of Olson and his colleagues.

Keys and Anderson ('57) have reported that increasing the protein from 65 to 132 gm and 83 to 131 gm daily by isocaloric substitution of skim milk powder for carbohydrate in "a typical American diet" had no effect on the serum cholesterol level of schizophrenic men. Similar results were obtained by Lutz et al.[9] with 10 men given diets of the usual variety of foodstuffs providing from 60 to 160 gm of protein/day. These observations on the effect of protein at relatively high levels are entirely compatible with the results of the present investigation.

Rations containing added amounts of cholesterol (500 mg/950 Cal.) were used to test the possibility that the added sterol might accentuate any change observed due to alteration in dietary protein. No such effect was noted. However, the 32 subjects who consumed the ration with added cholesterol during the first 8 days of the experimental period had significantly higher plasma cholesterol levels than did the 33 subjects who ate the same diet without the added cholesterol. The average zero and 8 day values were 178.2 and 171.4 mg/100 ml for the group given the added cholesterol and 175.4 and 157.8 mg/100 ml for the group not receiving the added sterol. The mean 8 day values are significantly different (P, 0.01 to 0.02). These results attest further to the validity of our previous reports on the significant effect of dietary cholesterol in man.

There appears to be the possibility of a sex difference in response of plasma cholesterol to changes in dietary protein and it is proposed to examine this further in the near future.

These results on man are similar to those initially reported by Pick et al.[10] and confirmed by others (Kokatnur et al., '58; Leveille et al., '61; Marion et al., '61) for the chick and also similar to those reported for the rat (Moyer et al., '56; Jones and Huffman, '56).

Finally, although it is tempting to suggest that the effect of the low protein diet in causing an increase in plasma cholesterol may be due specifically to a deficiency of the sulfur-containing amino acids, as has been described by Mann and others for certain experimental animals (Mann et al., '53; Fillios and Mann, '54),[11] this possibility appears unlikely because of the short-term nature of the experiment. This is a point, however, that should be determined experimentally.

SUMMARY

Sixty-five male university students were divided into two groups and given a formula ration providing protein and fat (butter) at levels of 15 and 30%, respectively, with or without a supplement of 500 mg cholesterol/950 Cal. for 8 days. The two groups were each divided into 5 sub-groups and transferred for another 8 days to diets in which the protein moiety was changed to 5, 10, 15, 20 and 25% by the addition or withdrawal of the protein preparation, calcium caseinate for equicaloric amounts of maltose-dextrins. Only the diets low in protein (5% of calories) caused a significant change in plasma cholesterol and this was in the form of an increase. The plasma cholesterol values in milligrams per 100 milliliters for these two groups fed the diets low in protein at day 8 and 16 were, respec-

[9] Lutz, R. H., R. H. Barnes, E. Kwong and H. H. Williams 1959 Effect of dietary protein on blood serum cholesterol in men consuming mixed diets. Federation Proc., *18*: 534 (abstract).

[10] Pick, R., J. Stamler and L. N. Katz 1957 Effects of high protein — high vitamin supplements on cholesterolemia and atherogenesis in cholesterol-fed cockerels. Federation Proc., *16*: 101 (abstract).

[11] Mann, G. V. 1960 Decompensation of sterol metabolism in monkeys. Federation Proc., *19*: 15 (abstract).

tively, 152.0 and 171.1 mg (+ 19.1, $P < 0.05$, without added cholesterol) and 168.3 and 180.2 (+ 11.9, $P < 0.05$, with added cholesterol). Thirty-two female students consumed the unsupplemented diet for 8 days. They were then divided into three groups and continued to receive for another 8 days diets, modified as described above, providing 5, 15, or 25% of calories as protein. The only significant change observed in the plasma cholesterol was a small but significant decrease in the case of the diet high in protein (− 7.4 mg/100 ml, P, 0.01 to 0.02). These data obtained from the first large scale experiment to be performed on normal young subjects fed a formula ration provide no support for the possibility that high intakes of protein per se are implicated in the development of cardiovascular disease through any elevating effect on plasma cholesterol levels.

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Effect of the Addition of Imbalanced Amino Acid Mixtures to a Low Protein Diet, on Weight Gains and Plasma Amino Acids of Chicks[1]

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Reduced feed consumption and weight gains and histopathological changes in various tissues are known to occur when animals are fed amino acid-deficient diets. Histopathological changes occur particularly rapidly when the amino acid-deficient diet is force-fed (Sidransky and Baba, '60).[2] However, it has not been established clearly whether these deleterious effects are primarily a result of an overall protein deficiency, perhaps in combination with more than adequate energy intake, or of the intake of large amounts of amino acids that are not utilizable for growth and must be disposed of by the animal.

In the following experiment a study was made of the effect of incorporating amino acid mixtures devoid of an essential amino acid into a diet containing a low level of a well balanced protein. In this way diets were created which were markedly deficient in a single amino acid but which with respect to nitrogen content were comparable to a diet containing over 20% of a well balanced protein.

EXPERIMENTAL

White Plymouth Rock pullets, obtained from a commercial hatchery, were fed the basal diet (basal 1, described below) from one-day-old to two weeks of age. At this time, 7 or 8 birds, depending on the experiment, were alloted to each pen so that the average weights of pens of birds were about equal and the range in weights over the whole experiment was relatively small. The experimental diets were assigned to the pens of birds at random and feeding was continued ad libitum for one week with feed consumption and weight gains being recorded. At three weeks of age the birds were killed, blood samples taken from the carotid artery, and free amino acids in the blood plasma assayed microbiologically. Details of the preparation of the blood samples and the assay procedures have been given previously (Gray et al., '60; Hill et al., '61).

Basal 1 contained 9.5% of C–1 protein[3] supplemented with DL-methionine and glycine (3% and 2% of the protein preparation, respectively) and was identical to diet A described by Hill et al.[4] with the exception that 7% of non-nutritive cellulose[5] was used in the diet rather than 2%.

The amino acid mixture was formulated to contain the same relative proportions of amino acids as contained in the C–1 protein, including the supplementary methionine and glycine. The formulation was in terms of the L- isomers of the amino acids with the exception of methionine, for which the L- and DL- forms were considered to be nutritionally equivalent, and was based on microbiological assays of C–1 protein for 15 amino acids and literature values for alanine, proline and serine. Racemic mixtures of α-alanine, isoleucine, serine and valine were added at twice the levels of the corresponding L- isomers contributed by the C–1 protein.

The percentage composition of the complete amino acid mixture was as follows:[6]

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[2] Platt, B. S., K. Calder and B. H. Doell 1962 Pathology of acute experimental protein malnutrition in the force-fed rat. Proc. Nutrition Soc., *21:* VI (abstract).
[3] Isolated soybean protein, ADM C-1 Assay Protein, Archer-Daniels-Midland, Cincinnati, Ohio.
[4] We are indebted to Merck and Co. Ltd., and Pfizer Canada, both of Montreal, Quebec, and Distillation Products Industries, Rochester, New York, for several of the vitamins used in the experimental diet.
[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[6] L-Lysine·HCl was generously donated by E. I. duPont de Nemours and Company, Wilmington, Delaware. Other amino acids were purchased from Nutritional Biochemicals Corporation, Cleveland.

DL-α-alanine, 5.40; L-arginine·HCl, 7.37; L-aspartic acid, 7.42; L-cystine, 0.55; L-glutamic acid, 12.63; glycine, 5.22; L-histidine·HCl, 2.29; DL-isoleucine, 8.16; L-leucine, 6.52; L-lysine·HCl (95%), 6.31; DL-methionine, 3.96; L-phenylalanine, 3.88; L-proline, 1.88; DL-serine, 10.31; DL-threonine, 6.21; L-tryptophan, 0.89; L-tyrosine, 2.57; DL-valine, 8.52.

When this mixture was incorporated into basal 1 at a level of 16.1% it formed a diet which was considered equivalent to one containing 25% of C–1 protein (including supplemental methionine and glycine) with respect to its contribution of the L-forms of the amino acids.

To obtain amino acid-deficient diets, arginine, isoleucine, leucine, lysine and valine were omitted singly in turn, and the resulting mixtures incorporated into basal 1 to give the equivalent of a diet containing 25% of C–1 protein but deficient in one of the above essential amino acids.

The proportions of amino acids as specified above were used in all experiments except experiment 1. For this experiment somewhat higher proportions of glutamic acid, aspartic acid, proline and serine were used (10, 1, 0.5, and 1% of the diet, respectively) and alanine was omitted.

Sodium bicarbonate and anti-acid[7] were added to diets containing amino acid mixtures, each at 1% of the diet. All additions to the basal diet including the above substances and the amino acid mixtures were made at the expense of dextrose.

Tests of statistical significance were made by applying the *t* test to comparisons planned in the experiment (Snedecor, '56).

RESULTS AND DISCUSSION

Hill et al. ('61) observed that when 15% of zein was incorporated into a low protein diet, similar to the basal diet used here, weight gain, feed consumption and the plasma lysine level of chicks were reduced. In experiment 1 of the present study a comparison was made of the effects of zein and an amino acid mixture devoid of lysine. It has already been noted that the amino acid mixture used in this experiment differed somewhat from that used in all succeeding experiments in that it contained higher proportions of glutamic acid, aspartic acid, proline and serine but no alanine. Whether zein or the amino acid mixture deficient in lysine was used, a similar depression occurred in the level of plasma lysine (table 1). Weight gains and feed consumption were also depressed but much more severely in the case of the amino acid mixture. These differences in weight gains were accepted as statistically significant (zein vs. basal, $P < 0.1 > 0.05$; AA less lysine vs. basal, $P < 0.01$). Since the mixture of amino acids supplied a considerably different complement of amino acids than the zein, it may be concluded that the effects observed were not unique for zein but were most likely a reflection of the severe deficiency of lysine common to both diets.

It seemed worthwhile, therefore, to study the effect of less severe lysine deficiency. This was done by increasing the level of lysine in basal 1 from 0.51 to 0.78% by adding increments of C–1 protein. The amino acid mixture lacking lysine was then added in sufficient amount to provide in each case the equal of 25% of C–1 protein in the diet with respect to contribution of amino acids. It was found (table 2) that weight gains and plasma lysine were reduced at each level of lysine when the amino acid mixture was added, but the effect decreased as the amount of mixture added decreased. These effects were statistically significant ($P < 0.05$). Weight gains of chicks fed the three deficient diets supplemented with lysine were quite comparable and about 10% less than that of chicks fed the diet containing 25% of C–1 protein.

Further studies were made omitting in turn leucine, isoleucine, valine and arginine from the amino acid mixture, thus creating diets deficient in these amino acids (table 3, experiment 1). For each deficient diet average gains were significantly less ($P < 0.01$) than the 41.5-gm gain exhibited by chicks fed the basal diet. Again the diet containing the complete amino acid mixture produced somewhat smaller gains than the diet containing 25% of C–1 protein.

It has been shown by several workers (Reid et al., '56; Norris et al., '58; Davis et al., '62) that chicks fed diets contain-

[7] Amphojel (aluminium gel), John Wyeth and Brother, Canada, Ltd., Walkerville, Ontario.

TABLE 1

Effect of zein and an amino acid mixture devoid of lysine on weight gain, feed consumption and free amino acids in blood plasma

Diet[1,2]	Nitrogen in diet	Avg gain/ chick	Avg feed consumption/ chick	Amino acids in plasma		
				Lysine	Threonine	Trypto-phan
	%	*gm*	*gm*	*mg/ 100 ml*	*mg/ 100 ml*	*mg/ 100 ml*
Basal 1 (9.5% of C-1 protein)[3]	1.36	28.5	91.2	2.9	0.9	1.5
+15% of zein	3.37	20.8	77.5	0.8	12.0	1.4
+AA less lysine	3.82	9.5	47.0	0.8	12.9	1.8
+AA complete	3.87	94.0	114.9	5.4	6.7	1.6

[1] Each diet was fed to triplicate groups of 7 birds each for one week.
[2] AA indicates amino acid mixture without lysine, added in sufficient amounts to give the equivalent of 25% of the soybean protein for all amino acids except lysine.
[3] ADM C-1 Assay Protein, Archer-Daniels-Midland, Cincinnati, Ohio.

TABLE 2

Relationship between dietary lysine level and the effect of feeding an amino acid mixture devoid of lysine

Diet[1,2]	Lysine in diet	Plasma lysine	Avg gain/ chick	Avg feed consumption/ chick
	%	*mg/100 ml*	*gm*	*gm*
Basal 1 (9.5% of C-1 protein)	0.51	2.6(0.1)[3]	33.2	107
+16.1% of AA	0.51	0.9(0)	8.1	38
+16.1% of AA+0.83% L-lysine	1.34	10.7(1.5)	90.4	120
Basal 2 (12.0% of C-1 protein)	0.64	2.2(0.5)	56.0	118
+13.5% of AA	0.64	1.1(0.1)	23.0	61
+13.5% of AA+0.69% of L-lysine	1.33	7.7(1.4)	93.2	115
Basal 3 (14.5% of C-1 protein)	0.78	3.0(0)	76.0	139
+10.9% of AA	0.78	1.8(0.5)	36.5	74
+10.9% of AA+0.56% of L-lysine	1.34	7.1(0.5)	91.7	120
Basal 5 (25% of C-1 protein)	1.34	7.5(0.2)	103.6	133

[1] AA indicates amino acid mixture without lysine, added in sufficient amounts to give the equivalent of 25% of C-1 protein for all amino acids except lysine.
[2] Each diet was fed to duplicate groups of 8 chicks each for one week.
[3] Figures in parentheses show difference between values for duplicate lots.

TABLE 3

Effect of incomplete amino acid mixtures on weight gains and feed consumption of chicks

Diet[1,2]	Experiment 1		Experiment 2[3]	
	Avg gain/ chick	Avg feed consumption/ chick	Avg gain/ chick	Avg feed consumption/ chick
	gm	*gm*	*gm*	*gm*
Basal 1 (9.5% C-1 protein)	41.6	116	39.1	125
+ AA less leucine	7.1	34	−1.9	40
+ AA less isoleucine	11.7	51	—	—
+ AA less valine	3.2	36	1.0	44
+ AA less arginine	19.8	67	15.6	70
+ AA complete	91.0	123	97.7	136
+ 15.5% of C-1 protein	94.8	130	107.3	145

[1] Each diet was fed to duplicate groups of 8 chicks each for one week.
[2] AA is amino acid mixture which, when fed complete, was considered the equivalent of 15.5% of C-1 protein in respect to contribution of amino acids to the diet. The indicated amino acids were omitted one at a time from this mixture.
[3] In experiment 2 all diets including the pre-experimental diet were supplemented with a trace mineral mixture which contributed in mg per kg of diet: $Na_2MoO_4 \cdot 2H_2O$, 20; $AlNH_4(SO_4)_2 \cdot 12H_2O$, 133; $CoCl_2 \cdot 6H_2O$, 26; $CuSo_4 \cdot 5H_2O$, 30; $ZnCl_2$, 20.

ing isolated soybean protein have an increased requirement for certain trace minerals. Molybdenum is of particular interest in the present instance, not only because there is a decreased xanthine dehydrogenase activity in the tissues of molybdenum deficient chicks (Higgins et al., '56), but it is reasonable to believe that large excesses of dietary amino acids might increase the molybdenum requirement due to greater demands on the enzyme for the formation of uric acid.

Accordingly, a mineral supplement, which included a molybdenum salt among others, was added to the experimental diets, and also to the pre-experimental diet for 11 days prior to the assignment of the experimental diets. The supplement provided the following in mg per kg of diet: $Na_2\ MoO_4 \cdot 2H_2O$, 20; $AlNH_4\ (SO_4)_2 \cdot 12H_2O$, 133; $CoCl_2 \cdot 6H_2O$, 26; $CuSO_4 \cdot 5H_2O$, 30; $ZnCl_2$, 20.

The data obtained, using these fortified diets, (table 3, experiment 2) indicated that a deficiency of the above minerals was not an explanation for the results of the preceding experiment.

It is reasonable to believe that the depression in weight gains observed in the foregoing experiments was caused by an amino acid imbalance. Fisher and Shapiro ('61) observed a similar effect when they fed chicks diets that were deficient in tryptophan, lysine or methionine, and it has been known for some time that a similar type of imbalance can be demonstrated with rats (Harper, '58; Salmon, '58; Kumta and Harper, '60; Sauberlich, '61).

It is unlikely that the basal 1, containing 9.5% of C-1 protein supplemented with methionine and glycine presented a perfectly balanced mixture of amino acids to the chick. Consequently, the addition to this diet of an amino acid mixture, even when such was devoid of an essential amino acid, might be expected in most instances to induce a small but positive growth effect. However, it is clear that in our experiments the ability of the imbalanced amino acid mixtures to produce growth depressions was sufficient to more than offset any growth stimulation promoted by the mixtures.

From the data of table 4 it appears that modification of the diet to give deficiencies of leucine, isoleucine, valine and arginine was in each case reflected in a marked depression in the level of the dietary deficient amino acid in the blood plasma. ($P < 0.01$ except for valine in which case P was approximately 0.1). This observation extends previous findings with respect to tryptophan in rat plasma (Sauberlich and Salmon, '55) and lysine and tryptophan in chick plasma (Hill

TABLE 4

Effect of amino acid deficiencies on free amino acids in blood plasma[1]

Diet[2]	Leucine	Isoleucine	Valine	Arginine	Threonine	Lysine
	mg/100 ml plasma	*mg/100 ml plasma*	*mg/100 ml plasma*	*mg/100 ml plasma*	*mg/100 ml plasma*	*mg/100 ml plasma*
Basal 1 (9.5% of C-1 protein)	2.3(0.3)[3]	1.5(0.3)	1.9(0.3)	4.3(2.2)	1.0(0.3)	2.8(0.4)
+ AA less leucine	0.6(0.1)	6.6(0.2)	13.6(1.6)	6.2(0.6)	15.1(0.3)	11.0(2.4)
+ AA less isoleucine	3.5(0.6)	0.4(0.1)	7.9(0.3)	5.6(1.8)	17.3(1.4)	10.1(2.3)
+ AA less valine	2.8(0)	2.3(0.1)	1.0(0.1)	3.8(0.3)	15.7(1.5)	9.2(1.1)
+ AA less arginine	3.6(0.1)	3.2(0.1)	7.4(0)	1.2(0.1)	25.5(1.8)	10.9(1.4)
+ AA complete	3.3(0.6)	2.6(0.4)	6.6(0.7)	8.7(1.2)	10.6(4.6)	7.9(3.4)

[1] Plasma samples are from same birds for which growth and feed consumption data are given in table 3, experiment 1.

[2] AA is amino acid mixture which, when fed complete, was considered the equivalent of 15.5% of C-1 protein in respect to contribution of amino acids to the diet. The indicated amino acids were omitted one at a time from this mixture.

[3] Figures in parentheses show difference between values for duplicate pens.

et al., '61) and strengthens the belief that the phenomenon is a general one which will be exhibited by any amino acid when it becomes limiting for growth. Of interest are the elevated levels of plasma isoleucine and valine when the leucine-deficient diet was fed, whereas deficiencies of isoleucine or valine appeared to have little influence on the levels of the other two amino acids. This observation may be related to a report by Snyderman et al.[8] that in adult male humans there is an inverse relationship between the leucine and valine content of plasma. Also noteworthy is the elevation of threonine and lysine in the plasma when the diet was made deficient in any of the 4 amino acids. This effect was anticipated in view of previous observations with respect to the behavior of these amino acids (Gray et al. '60). The exceptionally high level of threonine resulting with the arginine-deficient diet is without obvious explanation.

The practical significance of the depression in weight gains caused by amino acid mixtures depends on whether a similar effect occurs when amino acid-deficient diets composed of proteins only are consumed. The effect appears to be given by certain protein mixtures since a reduction in weight gains was observed when zein was incorporated into the basal diet in the present experiments and also in previous experiments (Hill et al., '61). The effect was not so severe with zein as with amino acid mixtures but the rather poor digestibility of zein[9] might explain this difference, particularly if the phenomenon results from effects at the cellular level. On the other hand, when gelatin was used rather than zein, creating a tryptophan deficiency, it was not too well established that gains are depressed (Hill et al., '61). Furthermore, in an experiment not reported in detail here it was found that one-week average gains of birds fed the basal diet and the basal diet plus 15% of gelatin were 29.0 and 24.4 gm, respectively, a statistically nonsignificant difference. An explanation of this result may possibly be the considerable contribution of glycine and arginine to the diet by 15% of gelatin. Snetsinger and Scott ('61) reported that glycine and arginine could partially counteract the depression in weight gains of chicks resulting from the consumption of excess lysine. It might also be claimed that the amino acid mixtures per se, unrelated to an amino acid deficiency, produced the depression in weight gains, particularly as rather large amounts of such mixtures were added to the diets. Indeed the consistently poorer growth obtained when the complete amino acid mixture was added to the basal 1 rather than 15.5% of extra C-1 protein supports this argument. However, the observed maximal difference in weight gains given by these two diets (about 20%) was not sufficient to account completely for the effect produced by the imbalanced amino acid mixtures. In this connection Chen et al. ('62) have suggested that dietary amino acids may influence growth and feed consumption of rats by inducing a high osmotic pressure in the stomach. However, in our experimental diets the added amino acids replaced dextrose and it appears unlikely that major alterations in osmotic pressure occurred.

There was also the possibility that the DL-isomers in the amino acid mixture may have contributed to the observed effects. Accordingly, in an additional experiment, the DL-alanine, DL-isoleucine in the valine-deficient diet were replaced at one-half their amount in the diet with the corresponding L-isomers and the DL-methionine with an equal amount of L-methionine. The valine-deficient diet containing the D-isomers and the modified diet gave average weight gains per chick for the one-week experimental period of −0.6 and 3.1 gm, respectively, and the low protein control diet, 34.7 gm. Omission of the DL-serine in addition to replacement of the other DL-isomers produced a weight gain of 2.4 gm. In view of these results it does not appear likely that the DL-isomers included in the mixture were a major factor affecting the results.

Spolter and Harper ('61) and Stucki and Harper ('61) observed that rats would adapt to imbalances of amino acids with

[8] Snyderman, S. E., D. C. Cusworth, E. Roitman and L. E. Holt, Jr. 1959 Amino acid interrelationships: The effect of variation in leucine intake. Federation Proc., *18*: 546 (abstract).

[9] McIndoo, E. M., E. M. Olsen and D. C. Hill 1961 Factors affecting the excretion of dietary zein by chicks. Poultry Sci., *40*: 1430 (abstract).

TABLE 5

Effect of pre-experimental diet on weight gains given by amino acid deficient diets

Diet 0–2 weeks	Avg wt at 2 weeks[1]	Diet 2–3 weeks	Avg starting wt[2]	Avg gain 2–3 weeks	Avg % gain[3]
	gm		*gm*	*gm*	
Basal 1	79.2	Basal 1	81.5	30.6	31.6
		+ AA less isoleucine[4]	81.8	13.6	15.3
+ AA less isoleucine[4]	66.5	Basal 1	68.6	36.6	42.1
		+ AA less isoleucine[4]	69.9	19.3	24.3
Basal 1	72.7	Basal 1	84.4	34.6	34.0
		+ 15% of zein	84.1	19.5	20.8
+ 15% of zein	64.8	Basal 1	71.5	35.7	40.0
		+ 15% of zein	72.8	22.8	27.1

[1] Average of 35 chicks/group.
[2] Average of 12 birds/group selected to give approximately equal starting weight for comparable groups.
[3] $\frac{\text{Average gain}}{\text{Average weight during third week}} \times 100$.
[4] AA is amino acid mixture which when fed complete was considered the equivalent of 15.5% of C-1 protein in respect to contribution of amino acids to the diet. In this experiment isoleucine was omitted from the mixture.

respect to feed consumption and growth. This possibility was not investigated thoroughly in the experiments just described. Nevertheless, we have shown that the change from the pre-experimental diet to the experimental diets when the birds were two weeks of age, a procedure in all our experiments, was not an explanation of the observed effects (table 5). When the diets were fed from the time the birds were one day old, the average weights at two weeks were in favor of the basal diet and, regardless of the pre-experimental diet, smaller gains were recorded when the amino acid-deficient diets were fed during the third week. However, it may be of significance that average percentage gain during the third week was consistently better for birds that had received the deficient diets from hatching. This observation suggests that some adaptation to the amino acid deficient diets had taken place during the period from hatching to three weeks.

SUMMARY

Amino acid mixtures from which a single essential amino acid was omitted (arginine, isoleucine, leucine, lysine or valine) were incorporated into a diet containing 9.5% of soybean protein. Weight gain, feed consumption and the level of the dietary deficient amino acid in the plasma were reduced for chicks consuming these diets. Similar results were observed when zein was used rather than mixtures of amino acids.

The depression in weight gain appears to have resulted from the ingestion of relatively large quantities of amino acids which had to be deaminated or excreted.

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Effect of Starvation and a Nonprotein Diet on Blood Plasma Amino Acids, and Observations on the Detection of Amino Acids Limiting Growth of Chicks Fed Purified Diets[1]

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It has been reported by Charkey et al. ('53, '54), and confirmed by Gray et al. ('60) that when chicks are deprived of feed, a marked increase occurs in the levels of free lysine and threonine in the blood plasma.

As an explanation for this effect, Charkey et al. ('53) proposed that lysine and threonine are particularly resistant to deamination and accumulate in the blood when large amounts of amino acids resulting from tissue breakdown are released during starvation.

If a rapid breakdown of tissue protein during starvation results from a deficiency of available energy then birds that are not completely fasted, but receive a nonprotein diet rich in energy, should not exhibit elevated levels of plasma lysine and threonine.

This hypothesis was tested in the following experiments. In addition, some experimental results are reported from the investigation of a potentially useful method for detecting the amino acids in chick diets that are limiting growth. The method is a modification of one proposed by Longenecker and Hause ('59) who used dogs as subjects.

EXPERIMENTAL

In all experiments one-day-old White Plymouth Rock pullets, obtained from a commercial hatchery, were fed a basal diet to two weeks of age. At this time, birds, whose weights lay within a relatively narrow range, were assigned to experimental pens, 12 to 13 birds/pen, so that in each experiment the average weights of the groups were approximately equal. The various experimental treatments or diets were given during the third week.

The basal diet was similar to diet A described by Hill et al. ('61) with the exception that it contained 12 or 15% of soybean protein[2] rather than 9.5%, the extra protein replacing dextrose. The nonprotein diet (NPD) was formulated by replacing the soybean protein with dextrose and substituting pure choline chloride for a 25% commercial mixture. In two trials, glycine and DL-methionine which were added to the basal diet at levels of 2 and 3.0%, respectively, of the soybean protein, were also in the nonprotein diet.

Experiments 1 and 2 were designed primarily to compare the effects of fasting and of a nonprotein diet. In these experiments birds were fed a basal diet containing 15% of soybean protein during the first two weeks and, during the third week, the alterations in feeding (from full feeding of a complete diet to fasting, and from a complete diet to a nonprotein diet) were made at such times that all birds were killed and blood sampled when the birds were 21 days old. All treatments were applied to duplicate groups. Water was available to the birds at all times regardless of the treatment.

Experiment 3 was a further investigation of the effect of feeding a nonprotein diet on plasma amino acids, and incorporated a test of a method for detecting the amino acids limiting growth from observations on plasma amino acid levels. For

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[2] Isolated soybean protein, ADM C-1 Assay Protein, Archer-Daniels-Midland, Cincinnati, Ohio.

this experiment a basal diet containing 12% of soybean protein was fed until the chicks were two weeks of age. At this time each of 6 experimental diets were assigned to 4 groups of chicks. Two groups from each diet regimen were killed when the birds were 21 days old and the other two groups 24 hours later during which interval the birds had received a nonprotein diet.

The 6 experimental diets were derived from the basal diet by altering the protein source, and the additional ingredients, thus introduced, replaced dextrose in the diet. In place of the 12% of soybean protein the following combinations were used to obtain the 6 diets: 9.5% of soybean protein plus 15% of zein with and without the addition of 0.63% of L-lysine·HCl,[3] 9.5% of soybean protein plus 15% of gelatin with and without 0.12% of L-tryptophan and 20% of vitamin-free casein with and without 1% of L-arginine·HCl.

Blood samples were taken from the carotid artery and pooled for each group of birds, and free amino acids in the blood plasma were assayed microbiologically. Details of the preparation of the blood plasma and the assay procedures have been given previously (Gray et al., '60; Hill et al., '61; Olsen et al., '61).

RESULTS AND DISCUSSION

Effect of fasting and a nonprotein diet. Influence of the duration of fasting on the concentration of free amino acids in the plasma, and a comparison of the effect of fasting with the feeding of a nonprotein diet are recorded in table 1.

When birds were fasted the concentration of 8 amino acids, arginine, glutamic acid, glycine, histidine, methionine, phenylalanine, tryptophan and tyrosine decreased, and the concentration of 6 amino acids, glutamine, isoleucine, leucine, lysine, threonine and valine increased.

Lysine and threonine were the most affected of the 6 amino acids that increased in concentration when the birds were deprived of feed. This result was in general accord with earlier work in which less restricted diets were used (Gray et al., '60). However, in the present experiments the lysine concentration recorded for the fasted chicks was about twice the concentration found by Gray et al. ('60) who used practical-type diets and 4-week-old Barred Plymouth Rock cockerel chicks. Although this difference may be a reflection of a difference in the chicks used, the possibility cannot be ignored that a deficiency of some trace ingredients in the purified diet may have influenced the results. In particular it may be pointed out that no supplemental source of molybdenum, a component of xanthine oxidase, was added to the purified diets and, furthermore, the practical-type and purified diets may have differed sufficiently with respect to vitamin B_{12} content to influence the result (Charkey et al., '54).

In evaluating the fasting effect it is well to remember that, if there is correlation between dietary and plasma amino acid concentration during the consumption of feed, the degree of increase or decrease of an amino acid observed in any particular experiment will be influenced by previous diet. It was found in our laboratory,[4] although details have not been published, that plasma concentrations of glycine, isoleucine, leucine, lysine, methionine, threonine and valine were approximately proportional to their dietary level. For example, concentrations of dietary lysine of 0.5, 1.0, 1.4 and 1.9% (obtained by increasing the protein in the diet from 8.7 to 29.9%) produced corresponding concentrations of plasma lysine of 2.5, 5.4, 8.1 and 10.5 mg/100 ml. It was also observed that concentrations of plasma arginine, histidine, phenylalanine, tryptophan and tyrosine increased markedly when the dietary protein was increased from 8.7 to 21.1%, although these amino acids were not greatly affected by further increases in dietary protein.

However, apart from these considerations, an increase of any magnitude in the concentration of plasma amino acids when fasting is imposed is indicative of an increased contribution of these amino acids from some endogenous source, the actual concentration they reach in the plasma

[3] L-Lysine·HCl was generously donated by Merck, Sharp and Dohme, Rahway, New Jersey.

[4] Olsen, E. M., D. C. Hill, J. A. Gray and H. D. Branion 1959 Effect of dietary protein level on the concentration of free amino acids in the blood plasma of chicks. Poultry Sci., *38:* 1234 (abstract).

TABLE 1

Effect of fasting and a nonprotein diet on amino acids in blood plasma[1]

Treatment	Arginine	Glutamic acid[2]	Glutamine[2]	Glycine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Tyrosine	Valine
	mg/100 ml		*mg/100 ml*		*mg/100 ml*		*mg/100 ml*		*mg/100 ml*		*mg/100 ml*		*mg/100 ml*	
Experiment 1														
Full-fed	7.3(1.0)	3.0	15.7	7.7(0.3)	2.6(0.4)	2.2(0.1)	3.2(0.2)	4.9(0.2)	5.1(0.4)	2.8(0.2)	2.6(0)	1.8(0)	4.5(0.4)	3.0(0.2)
Feed removed 12 hr	3.9(0.2)	2.8	19.6	4.6(0.1)	2.0(0)	2.6(0.4)	4.5(1.1)	9.7(2.0)	1.6(0.3)	2.0(0.4)	3.8(0)	1.3(0.2)	3.6(0.1)	4.0(0.5)
Feed removed 24 hr	3.7(0)	3.4	19.6	5.5(0.1)	2.5(0.4)	3.8(0)	6.2(0.2)	25.9(1.2)	1.8(0.1)	2.1(0)	9.2(0)	1.6(0)	2.9(0.2)	6.0(0.2)
Feed removed 36 hr	3.6(0.2)	2.9	16.7	5.7(0.1)	2.4(0.2)	3.5(0)	5.6(0.2)	25.8(3.7)	1.6(0.1)	2.0(0)	11.1(0.1)	1.5(0.1)	2.2(0.1)	5.4(0.3)
Feed removed 48 hr	2.9(0.5)	2.8	17.2	6.1(0.5)	2.4(0.3)	3.4(0.2)	5.1(0.5)	24.6(6.8)	1.6(0.1)	2.0(0.1)	11.1(1.0)	1.4(0.1)	2.2(0.1)	5.2(0.4)
NPD[3] 24 hr	1.0(0.1)	2.3	7.8	6.2(0.5)	1.4(0.2)	1.0(0)	2.0(0.2)	5.4(0.8)	5.9(0.3)	1.6(0)	1.9(1.2)	1.4(0.1)	2.8(0.3)	1.7(0.1)
Experiment 2														
Full-fed	7.2(0.6)	4.2	14.7	7.2(0.6)	3.0(0.6)	2.3(0.2)	4.2(0.3)	6.5(2.1)	6.8(0.8)	3.0(0.3)	3.6(1.9)	2.0(0.2)	4.1(0.2)	3.3(0.5)
Feed removed 24 hr	3.5(0.1)	3.5	18.4	5.5(0.2)	2.9(0.2)	3.4(0)	7.4(0.1)	23.1(1.0)	2.3(0)	2.0(0)	9.3(2.0)	1.4(0.1)	3.1(0.2)	5.9(0.5)
NPD[3] 24 hr	1.1(0)	2.8	6.6	5.8(0.2)	1.1(0.3)	1.1(0.1)	2.7(0.4)	4.7(1.4)	6.9(1.7)	1.8(0.2)	2.4(0.1)	1.4(0.1)	2.8(0.1)	2.0(0.1)
NPD[4] 24 hr	1.0(0)	2.1	4.7	3.4(0.1)	1.5(0.2)	0.9(0)	2.4(0.1)	5.0(0.4)	0.9(0)	1.6(0)	1.9(0)	1.2(0.1)	2.2(0.1)	1.6(0)
NPD[4] 6 days	1.0(0)	2.5	11.6	5.0(0.5)	2.0(0.1)	0.7(0.1)	2.2(0)	9.4(1.2)	0.5(0)	1.0(0)	3.5(0.5)	0.8(0.1)	0.9(0.1)	1.6(0)
NPD[4] 6 days, then 24-hr fast	2.6(0.1)	3.4	20.7	7.0(0.8)	2.5(0)	2.6(0.2)	5.8(0.4)	17.6(2.2)	1.4	1.5(0.1)	7.5(1.2)	0.7(0)	1.4(0.2)	4.4(0.2)

[1] Values are averages for samples from two pens of birds, with the difference between the two values given in parentheses.

[2] Determination on pooled plasma from two replicated pens.

[3] Nonprotein diet contained supplements 0.3% of glycine and 0.45% of DL-methionine.

[4] Supplements of glycine and DL-methionine omitted.

probably being largely governed by their susceptibility to excretion or deamination.

Data from experiments 1 and 2 support the hypothesis, previously stated, that birds fed a nonprotein diet should not exhibit elevated levels of plasma amino acids. When the nonprotein diet was fed, with the exception of the concentration of lysine as recorded in experiment 1, and that for methionine and glycine when they were included in the nonprotein diet, plasma levels of all amino acids assayed were substantially below levels given by the complete diet. In experiment 1 the level of lysine remained slightly above that given by the complete diet, but was considerably less than the very high level exhibited by fasted birds in both experiments.

The available data cannot provide an explanation of these observations. However, it is reasonable to suggest that the primary cause of the increases following a 24-hour fast was an accelerated breakdown of body protein resulting from a deficiency of energy, and that this breakdown was prevented or slowed down when energy was supplied by the nonprotein diet.

As part of experiment 2 the nonprotein diet was fed for 6 days and then a 24-hour fast was imposed. This treatment was included to investigate the possibility that the response of plasma amino acid concentration to fasting, particularly of lysine, was related to the protein reserves of the birds.

With continued feeding of the nonprotein diet for the 5 additional days, certain amino acids increased in concentration, others decreased, and still others changed little. Most marked effects were increases in glutamine, glycine, lysine and threonine. The 24-hour fast following the 6-day depletion period increased the concentration of all amino acids with the exception of tryptophan which decreased slightly. The response of lysine to fasting was considerably less than that obtained with nondepleted birds, possibly a reflection of depleted protein reserves.

The observations in experiments 1 and 2 raise a question regarding the significance of fasting amino acid levels in the young chick, particularly if such levels are to be used as a base line for studying the effects of diet on amino acid concentrations in the blood. Fasting levels do not appear to represent a steady metabolic state, but possibly a condition involving a rapid breakdown of body protein.

Detection of the amino acids limiting growth. Longenecker and Hause ('59) proposed a method for relating concentration of plasma amino acids to the nutritional value of dietary protein using adult dogs as test animals. Plasma amino acid ratios were calculated by relating the levels of plasma amino acids, determined following a protein meal and corrected for levels obtained after an 18-hour fast, to the amino acid requirements of the adult dog. Such ratios when ranked in order of magnitude were found to provide a reasonably good prediction of the amino acids which were limiting in several proteins for maintenance of the dog.

The method as outlined by Longenecker and Hause ('59) cannot readily be applied to chicks, particularly because of the increase in plasma lysine and threonine which occurs consistently when birds are fasted. However, in view of the results of experiment 1 and 2 it appears that levels of plasma amino acids obtained after feeding a nonprotein diet might be a suitable substitute for fasting levels in applying the method.

Experiment 3, which was used to test this modification, comprised 6 diets with certain known or suspected amino acid deficiencies. In addition, the experiment was designed to investigate the influence of the preceding diet on the levels of plasma amino acids obtained following a 24-hour period of feeding a nonprotein diet. Data yielded by this experiment are given in table 2.

Plasma amino acid levels produced by diets 1, 2, 3 and 4 were essentially in agreement with those observed in previous experiments in which similar diets were used (Hill et al., '61). Diets 5 and 6 were not investigated previously with respect to their effect on plasma amino acids, but the low level of plasma arginine produced by diet 5 was anticipated because of the demonstrated deficiency of this diet in arginine.

Quite divergent plasma amino acid values were obtained for the various groups killed following the period of feed-

TABLE 2

Effect of diet composition on the amino acids in blood plasma[1]

Diet or treatment no.	Protein source during third week	Avg gain 2–3 weeks	Arginine	Glycine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Tyrosine	Valine
		gm	*mg/100 ml*		*mg/100 ml*		*mg/100 ml*		*mg/100 ml*		*mg/100 ml*		*mg/100 ml*	
	Complete diets													
1	9.5% of soybean protein +15% of zein	23.5(3.0)	3.7(0.3)	6.4(1.3)	2.8(0.2)	1.9(0.1)	5.9(0.1)	0.9(0.2)	2.9(0)	3.5(0.1)	12.8(1.6)	1.3(0)	6.8(0.3)	2.7(0.2)
2	As above +0.63% of L-lysine·HCl	53.5(5.1)	2.1(0)	5.4(0.2)	2.7(0)	2.1(0.1)	7.1(0.7)	8.0(1.5)	3.6(0.2)	3.7(0.2)	9.8(1.3)	0.6(0.1)	6.4(0.4)	2.7(0.1)
3	9.5% of soybean protein +15% of gelatin	31.1(2.6)	9.5(0.6)	29.4	1.4(0.3)	1.3(0.1)	2.4(0.1)	9.6(0.8)	1.9(0.3)	1.9(0.1)	5.4(0)	0.3(0.2)	1.7(0)	3.3(0.4)
4	As above +0.12% L-tryptophan	61.4(4.5)	12.3(1.2)	40.3(4.0)	0.7(0.1)	0.9(0.1)	2.6(0.3)	7.2(1.1)	2.4(0)	1.6(0)	2.8(0.3)	1.4(0.1)	1.2(0)	3.5(0.2)
5	20% of casein	25.8(2.8)	1.5(0.2)	5.1(0)	3.7(1.8)	3.0(0.3)	4.2(0.5)	23.6(9.1)	2.4(0)	2.5(0.2)	26.7(5.8)	1.8(0.5)	6.0(0.8)	6.5(0.8)
6	As above +1% of L-arginine·HCl	85.7(9.8)	5.6(1.2)	3.6(0.1)	3.6(0.4)	2.7(0.2)	4.0(0.8)	20.0(0.2)	2.7(0.1)	2.9(0.2)	16.8(0.5)	1.8(0.2)	5.0(0.2)	6.1(0.6)
	Nonprotein diets,[2] 24 hr													
7	As 1	25.8(3.1)	0.6(0.3)	2.8(0.7)	0.9(0.3)	0.5(0)	1.2(0.2)	2.0(0.2)	0.5(0)	1.3(0.2)	1.9(0.2)	0.9(0.1)	2.2(0.2)	0.9(0.2)
8	As 2	61.4(5.4)	0.9(0.1)	4.0(0.4)	1.2(0.1)	0.9(0)	1.9(0.1)	4.0(1.1)	0.9(0.1)	1.7(0)	3.9(0.3)	1.4(0.2)	3.3(0.3)	1.7(0)
9	As 3	31.5(4.8)	0.8(0.4)	2.5(0.2)	0.8(0.1)	0.6(0)	1.3(0.1)	3.3(0.7)	0.4(0)	1.1(0.1)	1.4(0.1)	0.9(0.1)	1.4(0.1)	1.0(0.1)
10	As 4	66.0(11.1)	1.5(0.4)	2.4(0.1)	0.6(0)	0.7(0.1)	1.4(0.3)	3.8(0.3)	0.7(0.1)	1.3(0)	1.5(0.2)	1.1(0)	2.2(0)	1.2(0.2)
11	As 5	30.2(5.9)	0.8(0.2)	3.5(0.1)	1.6(0)	0.8(0.1)	1.7(0.2)	4.9(1.7)	0.6(0.1)	1.5(0.1)	7.2(0.1)	1.1(0.1)	2.0(0.2)	1.8(0)
12	As 6	88.4(4.7)	1.1(0.1)	4.8(0)	1.3(0.1)	1.2(0.1)	2.1(0)	9.2(0.3)	1.0(0)	1.7(0.1)	7.9(0.3)	1.6(0.2)	2.7(0.7)	2.3(0.1)

[1] Values are averages for samples from two pens of birds with the difference between the two values given in parentheses.
[2] See Experimental section in text.

ing the nonprotein diet. Presumably these differences were related to the composition of the preceding diet. Statistical analysis of the data for each amino acid (Snedecor, '56) showed that the effects of the preceding diet were highly significant ($P < 0.01$) for all amino acids. Data on hand do not permit an explanation of these differences but possibly further experiments in which the nonprotein diet was fed for both shorter and longer periods than 24 hours might clarify the problem.

Sets of plasma amino acid ratios were calculated for each of the 6 diets and recorded in table 3. The ratios were obtained for each amino acid by subtracting the plasma concentration following the feeding of the nonprotein diet from that following the feeding of the test diet, and dividing the difference by the amino acid requirement. The requirements used were in the percentages of diet as given by the National Research Council ('60). For example, the plasma amino acid ratio for arginine in diet 1 was calculated as

$$\frac{3.7 - 0.6}{1.2} = 2.58.$$

It should be emphasized that only the relative magnitude of the ratios was considered, the largest negative ratio or the smallest positive ratio being taken to represent the first-limiting amino acid. No significance was attached to the absolute magnitude of any one ratio.

On the last two lines of table 3 are shown the predicted first and second limiting amino acids based on the relative magnitude of the ratios. Prediction of the first limiting amino acid was apparently quite successful for each of the 6 diets. Predictions were unequivocable in the case of diets 1, 3 and 5, in view of the effects on weight gains which resulted from supplementation of the diets with lysine, tryptophan or arginine. In the case of diet 2 it was found in previous studies (Hill et al., '61) that tryptophan was the limiting amino acid. Direct confirmation has not been obtained that the phenylalanine-tyrosine complex is limiting with diet 4. However, the amino acid composition of this diet, based on published data for the amino acid composition of soybean protein and gelatin, supports the view that such

TABLE 3

Plasma amino acid ratios and the prediction of limiting amino acids[1]

Diet no.	1	2	3	4	5	6
Protein source	Soybean + zein	Soybean + zein + lysine	Soybean + gelatin	Soybean + gelatin + tryptophan	Casein	Casein + arginine
Amino acid						
Arginine	2.58	1.00	7.25	9.00	0.58	3.75
Glycine	3.60	1.40	26.90	37.90	1.60	−1.20
Histidine	6.33	5.00	2.00	0.33	7.00	7.67
Isoleucine	2.33	2.00	1.17	0.33	3.67	2.50
Leucine	3.36	3.71	0.79	0.86	1.79	1.36
Lysine	−1.10	4.00	6.30	3.40	18.70	10.80
Methionine[2]	5.11	3.38	1.88	2.13	2.25	2.13
Phenylalanine[2]	3.14	1.43	0.57	0.21	0.71	0.86
Threonine	18.20	9.83	6.67	2.17	32.50	14.80
Tryptophan	2.00	−4.00	−3.00	1.50	3.50	1.00
Tyrosine	6.57	4.43	0.43	−1.43	5.71	3.29
Valine	2.25	1.25	2.88	2.88	5.88	4.75
Phenylalanine + tyrosine	4.86	3.64	0.78	−0.50	3.57	2.50
Limiting order						
First	lysine	tryptophan	tryptophan	phenylalanine-tyrosine	arginine	glycine
Second	tryptophan	arginine	phenylalanine-tyrosine	histidine-isoleucine	glycine	tryptophan

[1] Ratios calculated from data of table 2.

[2] Calculation on basis that no cystine or tyrosine in the diet.

is the case, chiefly due to a deficiency of tyrosine. With respect to diet 6, glycine is most likely the limiting amino acid (Waterhouse and Scott, '61; Jenkins et al., '62).

Prediction of the second limiting amino acid was successful for diets 1 and 2 (Hill et al., '61) and probably also for diets 3 and 5. However, in the case of diets 4 and 6 the prediction is suspect. The calculated amino acid composition of diet 4, in fact, bears out that the concentration of histidine and isoleucine are low, but suggest the methionine-cystine complex as second limiting. Also investigations with a diet basically similar to diet 6 in our laboratories (Jenkins et al., '62) support the view that the sulfur amino acids were second limiting rather than tryptophan as indicated by the plasma amino acid ratios. Possibly the sulfur amino acids behave exceptionally with respect to the diet-blood plasma relationship. On the other hand, the calculated amino acid ratios for methionine may be in error due to an over estimation by the assay method of the available methionine or underestimation of the requirements or, possibly, an unusual value obtained following the feeding of the nonprotein diet. Of course, the possibility cannot be dismissed that under the experimental conditions peculiar to experiment 3 the sulfur amino acids were not second limiting in diet 6. Unfortunately plasma values were not available for cystine.

If one attempts to predict the third limiting amino acid, difficulties are immediately apparent. For diet 1, although valine is predicted by the ratios, the third limiting amino acid is, as explained above, undoubtedly arginine.

It is perhaps not unexpected that, when the birds are actively growing and consuming feed up to the time of sacrifice as in this experiment, the prediction becomes uncertain beyond the first or second limiting amino acid. In addition to the demands for growth, many factors are certainly operating which modify the amino acid concentrations in the plasma. All amino acids, other than the most limiting, are to a varying degree in excess for growth purposes, and the more in excess they are the more predominant becomes the influence of these factors in relation to the demand for amino acids for tissue synthesis in governing the plasma amino acid levels.

The diets used in experiment 3 are more or less severely deficient in essential amino acids for the chick, and it has not been established that the procedure as described above would be sensitive enough to detect borderline deficiencies. Also open to question is whether lysine and threonine, in view of their tendency to exhibit elevated levels in the plasma when they are in excess, would be readily detected as second limiting amino acids in diets where they had this status. Nevertheless, despite these and other possible weaknesses in the procedure as applied in the present study, further investigation of its potentialities seems warranted.

SUMMARY

Chicks were fed a diet containing 15% of isolated soybean protein and then deprived of feed for periods of 12 to 48 hours. The removal of feed resulted in a marked elevation in the concentrations of lysine and threonine in the blood plasma, with maximal levels being recorded in 24 to 36 hours. Concentrations of glutamine, isoleucine, leucine and valine were also elevated, but to a considerably lesser degree, and concentrations of arginine, histidine, phenylalanine, tryptophan and tyrosine were depressed.

When chicks received the nonprotein portion of the diet for 24 hours, rather than being completely deprived of feed, the concentrations of all amino acids investigated, including lysine and threonine were below values obtained when the complete diet was fed.

Six diets with known or suspected amino acid deficiencies were fed, and plasma amino acid ratios were calculated for each amino acid by relating the plasma amino acid concentration, corrected for the concentration obtained when a nonprotein diet was fed, to the amino acid requirement. It was found that the ratios, on the basis of their relative magnitude, revealed the first and possibly the second limiting amino acids for the diets used.

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Studies on Ornithine Synthesis in Relation to Benzoic Acid Excretion in the Domestic Fowl

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Jaffe (1877) reported that the domestic fowl excreted benzoic acid conjugated with ornithine. Williams ('59) cites several additional studies where other aromatic acids such as pyromucic, phenylacetic, *p*-nitrophenylacetic, picolinic acid, and nicotinic acid were shown to be conjugated with ornithine by the hen. Benzoic acid is also excreted by hens as the glucuronide (Baldwin et al., '59).

To our knowledge, the source of the ornithine for conjugation with benzoic acid has never been investigated extensively in the chicken. Crowdle and Sherwin ('23) studied the end products of nitrogen metabolism of hens given doses of benzoic acid and concluded that ornithine for this reaction was synthesized at the expense of "waste" nitrogen. The proportion of total nitrogen excreted as uric acid decreased to the extent that ornithine nitrogen increased in hens given benzoic acid.

If the chicken could use "waste" nitrogen to synthesize ornithine, the question arises as to what biochemical pathways may be utilized. Chickens apparently cannot synthesize arginine and the absence of this amino acid from the diet will result in death.[1] Dietary citrulline can replace arginine but ornithine will not substitute for arginine (Klose and Almquist, '40). Pathways for the synthesis of ornithine from glutamic acid or proline have been described in the rat (Sallach, '51) but these interconversions have not been investigated in the fowl.

The studies described in this paper were intended to determine the source of ornithine for conjugation with benzoic acid in the chicken and to study the possible nutritional importance of this reaction.

To approach the problem as outlined, arginine was considered a possible source of ornithine for conjugation with benzoic acid. If this hypothesis were true, feeding benzoic acid could result in an arginine deficiency, which could be corrected by dietary sources of either arginine or ornithine. Other possible precursors of ornithine could also be studied in this way. This was the initial approach to the problem, followed by further studies of conjugated ornithine excretion and the conversion of radioactive precursors into ornithuric acid.

EXPERIMENTAL

White Plymouth Rock male chicks from a commercial hatchery were placed on experiment when they were two days old and fed the experimental diets for either 14 or 21 days. The chicks were housed in electrically heated, thermostatically controlled battery brooders with raised wire floors, and given feed and water ad libitum. The chicks were group-weighed at weekly intervals. The body weight data were subjected to analysis of variance and differences among treatment means were detected by Duncan's multiple range test.

The basal diets for use in the chick and hen studies are given in table 1.

Colostomized hens were prepared by the method of Ariyoshi and Morimoto ('56). Hens prepared in this manner in our laboratory have lived up to a year after the operation and have continued to lay eggs after a short pause following the operation. The hens used in this study were approximately 9 months old and were colostomized three weeks prior to beginning these experiments. Benzoic acid additions to the diet were made at the expense

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[1] Ousterhout, L. E. 1959 Effect of chronic amino acid deficiencies on the survival time of chicks. Federation Proc., *18*: 540 (abstract).

TABLE 1

Diets used in chick and hen studies

	Chick diet	Hen diet
	%	%
Isolated soybean protein[1]	25.00	18.82
Cornstarch	—	62.53
Glucose	61.15	—
Cellulose	3.00	3.00
Soybean oil	—	5.00
Corn oil	3.00	—
DL-Methionine	0.60	0.16
Glycine	0.40	0.16
Mineral mixture	5.63[2]	5.63[2]
Vitamin mixture	1.22[2]	1.22[2]
$CaCO_3$	—	2.62
L-Arginine·HCl	—	0.86

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[2] Vitamin mix and mineral mix are the same as described by Nesheim et al. ('62).

of glucose. Feed consumption of the hens was slightly restricted so that all consumed nearly the same quantity of feed each day. Total urine collections were made from each hen for two or three consecutive two-day periods. Urine samples were removed from the collection apparatus twice daily and frozen. A few drops of formaldehyde were added to prevent bacterial decomposition of urine samples. All analyses were made on two-day urine collections from each hen.

For analysis, the urine collections were thawed, diluted to a convenient volume (usually 700 ml) and homogenized in a Waring Blendor. The solids were allowed to settle and aliquots of the suspension were taken for analysis for urea, creatine-creatinine, free and conjugated ornithine. Urea was determined by the urease method of Van Slyke and Cullen ('14), and creatine by a modification of the method of Taussky ('54). Free ornithine was determined by application of the acid ninhydrin method of Chinard ('52) to an appropriate dilution of urine. Conjugated ornithine was determined by measuring ornithine liberated following treatment of the urine sample with 6 N H_2SO_4 for 9 hours in an autoclave at 25 pounds pressure.

Radioactivity measurements were made by dissolving 3.5 mg of the isolated dibenzoyl ornithine in 0.1 cc of 1.0 N ammonium hydroxide in a planchet. Even distribution was achieved by placing a disc of lens paper in the planchet and drying in an oven at 70 to 80°C. The counting was done in a Nuclear-Chicago gas-flow counter with a micromil window in place. All samples were counted at the same mass and no attempts were made to relate radioactivity recovered to dose since the isolation was not quantitative.

The dibenzoyl ornithine was isolated from filtered urine by acidifying the filtrate to pH 1 to 2 with HCl. After 18 hours, the supernatant was decanted and the crystals were washed three times with hot water on a sintered glass filter. The crystals were dissolved in dilute NH_4OH in a volume of 200 cc, decolorized with charcoal, filtered and reprecipitated by acidifying the solution. The crystals were collected, washed with hot water and recrystallized several times from ethanol until the melting point was constant and agreed with literature values for dibenzoyl ornithine (182 to 184°C).

RESULTS

A preliminary experiment indicated that dietary levels of 1, 2 and 3% of benzoic acid depressed growth rate of chicks in a manner linearly related to benzoic acid concentration. On the basis of these results a dietary level of 1.5% of benzoic acid was chosen for future studies since this level depressed growth but did not appear to cause the chick undue distress.

The results of a series of experiments conducted to determine whether various amino acids would reverse the growth depression caused by benzoic acid are summarized in table 2.

In experiment 1, 1.5% of benzoic acid markedly depressed growth rate of chicks. The depression in growth caused by benzoic acid was considerably less when arginine or ornithine was added to the diet. Glutamic acid did not influence the growth depression caused by benzoic acid feeding. Arginine and glutamic acid did not improve growth of chicks fed diets without benzoic acid. The arginine and ornithine levels used were equal molar and were calculated to supply sufficient ornithine to conjugate with the benzoic acid used. The glutamic acid levels used were equal molar and two times the molar levels of arginine and ornithine used.

The results of experiment 2 are also shown in table 2. Levels of ornithine and arginine fed were the same as in experiment 1. The glycine level was equal molar with the added levels of arginine and ornithine. Glycine had no effect on the growth depression caused by benzoic acid feeding, whereas ornithine and arginine again partially overcame the detrimental effects of benzoic acid.

The first two experiments indicated that additional arginine or ornithine reversed part, but not all, of the growth depression caused by benzoic acid feeding. An additional experiment was conducted to determine whether all of the growth-depressing effects of a slightly lower level of benzoic acid could be reversed by arginine. The plan and results of this experiment are shown in table 3.

Growth of chicks fed 1% of benzoic acid was less depressed than by the 1.5% used in the first experiments. Chicks fed 0.88% of arginine plus benzoic acid grew nearly as rapidly as those receiving the basal diet. Higher levels of arginine were not more effective. In the absence of benzoic acid, the 1.76% of L-arginine·HCl was slightly growth-depressing, possibly because the chloride level of the diet was

TABLE 2

Effect of various amino acids on growth depression caused by benzoic acid

Treatments	Avg wt, 2 weeks[1]	Multiple range test[2]
	gm	
Experiment 1		
Basal	189	a
+1.5% benzoic acid	125	c
+1.5% benzoic acid+1.36% L-arginine·HCl	158	b
+1.5% benzoic acid+1.1% L-ornithine·HCl	158	b
+1.5% benzoic acid+0.87% glutamic acid	126	c
+1.5% benzoic acid+1.74% glutamic acid	132	c
+1.36% L-arginine·HCl	177	ab
+1.74% L-glutamic acid	187	a
Experiment 2		
Basal	195	ab
+1.5% benzoic acid	127	e
+1.5% benzoic acid+1.36% L-arginine·HCl	153	cd
+1.5% benzoic acid+1.1% L-ornithine·HCl	169	c
+1.5% benzoic acid+0.48% glycine	136	de
+1.36% L-arginine·HCl	192	b
+1.1% L-ornithine·HCl	198	ab
+0.48% glycine	214	a

[1] Weights represent an average of 3 replicate groups of 8 chicks each/treatment.
[2] Means not followed by same letter are significantly different at the 1% level of probability — Duncan's multiple range test (Federer, '55).

TABLE 3

Effect of levels of arginine and L-proline on growth depression caused by benzoic acid

Treatment	Avg wt, 2 weeks[1]	Multiple range test[2]
	gm	
Basal	198	a
+1% benzoic acid	169	b
+1% benzoic acid+0.88% L-arginine·HCl	192	a
+1% benzoic acid+1.36% L-arginine·HCl	186	ab
+1% benzoic acid+1.76% L-arginine·HCl	183	ab
+1% benzoic acid+0.70% L-proline	171	b
+1.76% L-arginine·HCl	180	ab
+0.70% L-proline	194	a

[1] Weights represent an average of 3 replicate groups of 8 chicks each/treatment.
[2] Means not followed by same letter are significantly different at the 5% level of probability — Duncan's multiple range test (Federer, '55).

too high. The L-proline was ineffective in overcoming the growth depression caused by feeding 1% of benzoic acid.

An additional experiment was conducted to determine the effect of arginine on the growth depression caused by feeding high levels of phenylalanine and tyrosine. It was considered possible that feeding large excesses of these amino acids would result in the urinary excretion of metabolites that may be conjugated with ornithine; also that arginine might have an effect on the growth depression caused by these amino acids. The results of an experiment to test this hypothesis are shown in table 4. DL-Phenylalanine depressed growth more than L-tyrosine at the levels fed. Chicks receiving the basal diet supplemented with phenylalanine and arginine grew significantly faster than those fed the diet containing phenylalanine alone. However, this improvement in growth rate was relatively small compared with the effect of arginine on benzoic acid toxicity observed in the previous experiments. There was no significant effect of arginine on the growth depression observed in chicks fed the basal diet supplemented with L-tyrosine.

The experiments described above indicated that only arginine or ornithine could partially overcome the growth depression caused by feeding benzoic acid to chicks. This suggested that arginine was the source of ornithine for conjugation with benzoic acid and that ornithine was not synthesized from glutamic acid or proline.

To establish further the source of ornithine for conjugation with benzoic acid and to supplement the nutritional data obtained, an experiment was conducted with C^{14}-labeled glucose and arginine. Two colostomized hens were each injected intraperitoneally with 46 μc of L-arginine-1-C^{14} and two colostomized hens were each injected intraperitoneally with 138 μc of uniformly labeled C^{14} glucose. The hens were fed the basal diet shown in table 1 for 10 days prior to the injection of radioactive compounds. Shortly after the radioisotopes were given, 1 gm of benzoic acid was given to each hen orally in a gelatin capsule. Twenty-four-hour urine collections were made and the dibenzoyl ornithine isolated and radioactivity determined. The two samples of dibenzoyl ornithine from the hens receiving the labeled arginine had a specific activity of 28,600 and 23,400 count/min/mg, respectively. Essentially no activity was observed in the dibenzoyl ornithine isolated from hens given the radioactive glucose. This indicates that the hen does not possess a metabolic pathway to synthesize ornithine from glucose.

Additional studies were conducted with colostomized hens to obtain data on the quantitative aspects of the effect of benzoic acid feeding on arginine metabolism. Measurements were made on urinary excretion of "bound" ornithine of hens fed the basal diet and then on the same hens fed the basal diet supplemented with 2% of benzoic acid or 2% of DL-phenylalanine. Three hens were used in the series in which benzoic acid was studied and three were used in the series where phenylalanine was studied.

The data for these experiments are included in table 5. Except where indicated, the data represent averages of analyses on three separate two-day urine collections. The data indicate that conjugated ornithine excretion of hens fed the basal diet

TABLE 4

Effect of arginine on the toxicity of phenylalanine and tyrosine

Treatment	Avg wt, 2 weeks[1]	Multiple range test[2]
	gm	
Basal	358	a
+3% DL-phenylalanine	216	b
+3% L-tyrosine	260	c
+3% DL-phenylalanine + 1.65% L-arginine·HCl	244	c
+3% L-tyrosine+1.65% L-arginine·HCl	273	c

[1] Weights represent an average of 3 replicate groups of 8 chicks each/treatment.
[2] Means not followed by same letter are significantly different at the 5% level of probability — Duncan's multiple range test (Federer, '55).

TABLE 5

Effect of benzoic acid and DL-*phenylalanine feeding on excretion of conjugated ornithine*

Hen no.	Conjugated ornithine excreted/ 48 hr	Urea excreted/ 48 hr	Creatine — creatinine/ 48 hr	Conjugated ornithine excreted/ 48 hr	Urea excreted/ 48 hr	Creatine — creatinine/ 48 hr
	mmole	*mmole*	*mmole*	*mmole*	*mmole*	*mmole*
	Basal			Basal + 2% benzoic acid		
1	0.40	6.51	0.36	11.70	11.54	1.10
2	0.76	12.01	0.87	11.76	11.43	1.34
3	0.56	9.00	0.80	9.14	9.83	0.92
Average	0.57	9.17	0.68	10.87	10.93	1.12
				Basal + 2% DL-phenylalanine		
4	0.61[1]	10.84[1]	0.66[1]	0.88	10.60	1.07
5	0.68	8.62	0.61	0.93	9.75	0.70
6	0.96	5.71	0.69	1.18	6.83	1.08
Average	0.75	8.39	0.65	1.00	9.06	0.95

[1] Average of determination on 2 consecutive 48-hour urine collections. All other values in table are averages of determinations on 3 consecutive 48-hour urine collections.

was very low. Based on calculated arginine intake over the collection period, only about 2% of the ingested arginine could be accounted for in this way, assuming the only source of the ornithine was arginine. When benzoic acid was fed, however, conjugated ornithine increased markedly, and based on calculated arginine intake, about 40% of the ingested arginine would be required to provide the urinary ornithine found.

There was a slight increase in conjugated ornithine excretion following phenylalanine feeding. This increase is of doubtful significance and the data indicate that little ornithine conjugation of metabolites occurs following ingestion of excess phenylalanine.

In the acid ninhydrin method used for ornithine determination, lysine will also react to give some interfering color. Urine samples from hens given benzoic acid were acidified, and the resulting precipitate treated with 6 N HCl for 9 hours at 25 pounds pressure in an autoclave. After removing the hydrochloric acid, the hydrolyzed samples were chromatographed in a tertiary butanol, formic acid and water (70:15:15) system on Whatman no. 1 filter paper. Ornithine was the only amino acid observed on the chromatograms. Lysine could not be detected in the hydrolysate even though the system was shown to separate lysine and ornithine.

Data obtained in studies of urea and creatine-creatinine excretion of colostomized hens receiving benzoic acid are summarized in table 5. The excretion of these compounds was measured because ornithine can be derived from arginine as a result of breakdown of arginine to urea and ornithine or by transfer of the guanidino group to glycine to form guanidino acetic acid. Although the creatine-creatinine and urea excretion of hens receiving benzoic acid increased compared with the same hens fed the control diet, this increase is not equivalent to the increase in ornithine excretion. The urea excretion by these hens was quite high even in the absence of dietary benzoic acid. Since the arginine level of the hen diet was quite high, sufficient arginine may have been broken down to urea and ornithine to supply sufficient ornithine to conjugate with benzoic acid without a further increase in urea excretion.

DISCUSSION

The data presented above indicate that dietary arginine is probably the source of ornithine in the fowl for conjugation with aromatic acids such as benzoic acid. Apparently pathways for ornithine synthesis from glutamic acid, proline or glucose precursors do not exist in the domestic fowl.

The studies on ornithine excretion by colostomized hens fed benzoic acid indicates that a sizeable amount of the ingested arginine may be used for conjugation with ornithine. Based on feed

consumption records, percentage of benzoic acid fed, calculated arginine intake, and ornithine excretion, our data indicate that about 60% of the benzoic acid dose was excreted as the ornithine conjugate. This calculation assumes that only dibenzoyl ornithine was excreted and no monobenzoyl ornithines. Baldwin et al. ('59) found by isotopic procedures that 25 to 43% of an ingested benzoic acid dose was excreted as the ornithine conjugate by hens. Possibly the higher values were obtained in these studies because of the higher arginine content of the diets used.

Although arginine requirements can be increased by feeding benzoic acid, normally arginine needs should be affected to a negligible extent by ornithine synthesis. In the absence of dietary benzoic acid, conjugated ornithine excretion was very small.

McGilvery and Cohen ('50) showed that ornithuric acid synthesis occurred in hen kidney but not in the liver. Baldwin ('36) suggested many years ago that the main function of arginase in the hen kidney was to supply ornithine for detoxication.

Lysine has been shown to be converted to the 4,4′-diaminolysuric acid on reaction with *p*-aminobenzoic acid in chicken kidney slices by Efimochkina ('51). In the same studies, DL-proline or L-glutamic acid would not form benzoic acid conjugates, but arginine, citrulline and N^5-benzoyl-DL-ornithine did give rise to ornithuric acid in kidney slices. This data would be in agreement with evidence obtained in the present report. Although Efimochkina produced a lysine conjugate in *in vitro* experiments, no conjugated lysine was demonstrated in urine samples studied in our experiments.

Although feeding benzoic acid to chicks apparently caused a slight arginine deficiency, none of the outward manifestations of amino acid deficiency were present. Characteristic poor feathering is generally observed with arginine deficiency in young chicks, but in the experiments reported here feathering was not affected by benzoic acid feeding, and except for poor growth the appearance of the chicks was normal.

SUMMARY

The source of ornithine for conjugation with benzoic acid in the chicken was investigated. The growth depression caused by feeding benzoic acid to chicks could be partially reversed by dietary additions of ornithine and arginine but not by glycine, proline, or glutamic acid. In studies with colostomized hens, dibenzoyl ornithine isolated from urine of hens that received intraperitoneal injections of L-arginine-1-C^{14} was highly radioactive, whereas no radioactivity was observed in dibenzoyl ornithine isolated from hens receiving uniformly labeled glucose-C^{14}. Excretion of ornithine from hens receiving 2% of dietary benzoic acid was high, amounting to nearly 40% of dietary arginine equivalent.

These studies show that the chicken is apparently unable to synthesize ornithine and that dietary arginine is the source of ornithine for conjugation with benzoic acid.

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Vitamin Stability in Diets Sterilized for Germfree Animals[1]

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Diets used for germfree animals must be autoclaved rigorously to insure complete absence of viable bacteria and bacterial spores. To this date, the most practical method of sterilization has been to autoclave with steam. In the Lobund Laboratory diets are routinely autoclaved at 17 pounds steam pressure (123°C) for 25 minutes. Under these conditions a number of changes may take place in the chemical composition of the diets. Luckey et al. ('55) reported vitamin retention data subsequent to sterilization of diets for germfree animals which demonstrated considerable loss of thiamine and lesser losses of other selected B vitamins.

The purpose of the work reported herein was to reinvestigate the retention, after autoclaving, of a number of vitamins in three stock diets used at the Lobund Laboratory. Preliminary investigations had demonstrated improved thiamine retention when water had been added to the diet prior to autoclaving. This observation was verified by the present studies and in principle extended to other water soluble vitamins and vitamin A.

EXPERIMENTAL

Thiamine, riboflavin, vitamin B_6 and calcium pantothenate stabilities were determined in L-356, L-462 and L-473E5 — three formulas routinely used at the Lobund Laboratory. L-356 (Larner and Gillespie, '57) is a semisynthetic, L-462 (Wostmann, '59) a more practical-type diet for rats and mice. L-473E5, a diet fed to rabbits and guinea pigs, is an adjusted modification of diet L-462. All diets are described in detail in table 1.

Portions of each of the above formulas were mixed with distilled water to obtain samples containing 100, 80, 60, 40, and 20% of air-dried feed (solids). Samples containing 60, 40 and 20% solids were placed in refrigerator trays to a depth of approximately 0.75 inch, whereas the 100 and 80% solids samples were made into packs 0.75 × 5 × 5 inch by wrapping in cheese cloth following by Kraft paper. Three sterilization runs, each containing a replication of the above samples, were then conducted under the following conditions: 20 minutes of vacuum (approximately 22 inches) without heat (in the jacket), followed by a 25-minute period with 17 pounds steam pressure (123°C). Subsequent to completion of the three sterilization runs, the comparable samples from each run were combined and stored at −20°C until assayed.

For thiamine, vitamin B_6 and riboflavin analysis, duplicate extracts were prepared by refluxing samples with 0.1 N hydrochloric acid for one hour, whereas distilled water was used in place of hydrochloric acid to prepare duplicate pantothenic acid extracts. The above conditions were knowingly inadequate for complete extraction of bound vitamins, but they were considered adequate to remove the added crystalline vitamins. These added vitamins supplied the majority of the total vitamins. The relative loss of the added vitamins after sterilization was of primary interest. The extracts were adjusted to approximately a pH of 5, filtered through Whatman no. 40 paper, and stored in brown bottles at −20°C until assayed. Thiamine was determined fluorometrically as thiochrome

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TABLE 1

Composition of diets

Constituent	Diet L-356	Diet L-462	Diet L-473E5
	amount/ 100 gm	*amount/ 100 gm*	*amount/ 100 gm*
Casein, gm	20[1]	5[2]	5
Lactalbumin, gm	—	10.5	10.5
Rice flour, gm	58.5	—	—
Whole wheat flour, gm	—	32	32
Yellow corn meal, gm	—	34	13
Rolled oat groats, gm	—	—	21
Whole milk powder, gm	—	10.5	10.5
Liver powder, gm	2	2	2
Yeast extract, gm	2	—	—
Alfalfa meal, gm	—	2	2
Corn oil, gm	5	—	—
Powdered cellulose,[3] gm	5	—	—
Salts, gm	5	1.8	3.8
Ascorbic acid, mg	200	—	—
Thiamine·HCl, mg	6	3	3
Riboflavin, mg	6	1.5	1.5
Nicontinic acid, mg	5	2.5	2.5
Nicotinamide, mg	5	2.5	2.5
Ca pantothenate, mg	30	15	15
Choline chloride, mg	200	100	100
Pyridoxine·HCl, mg	2	1	1
Pyridoxamine dihydrochloride, mg	0.4	0.2	0.2
Biotin, mg	0.1	0.05	0.05
Folic acid, mg	1	0.5	0.5
p-Aminobenzoic acid, mg	5	2.5	2.5
i-Inositol, mg	100	—	50
Vitamin B_{12} (0.1% trituration) in manitol, mg	—	12.5	12.5
Cornstarch, mg	217.5	108.75	58.75
Fat-soluble vitamins			
Vitamin A conc., natural ester form, IU	800	800	800
Vitamin D,[4] IU	100	100	100
Vitamin E, mixed tocopherols, mg	150	37.5	37.5
d,l α-tocopheryl acetate, mg	—	10	10
Vitamin K_3, menadione, mg	10	10	10
Corn oil to make a total in gm	2	2	2
$CaCO_3$, gm	1.500	500[5]	500
$CaHPO_4$, mg	275	—	—
K_2HPO_4, gm	1.125	—	—
Na_2HPO_4, mg	1000	—	—
NaCl, mg	250	300	550
K acetate, mg	—	—	900
Mg acetate, mg	—	—	950
KI, mg	3.75	4.5	4.5
$MgSO_4 \cdot 7H_2O$, mg	375	450	450
$MnSO_4 \cdot H_2O$, mg	62.5	57.7	57.7
$Fe(C_6H_5O_7)_2$, mg	375	450	450
$CuSO_4 \cdot 5H_2O$, mg	19.2	22.4	22.4
$CoCl_2 \cdot 6H_2O$, mg	2.5	3.0	3.0
$ZnSO_4 \cdot H_2O$, mg	5	3.8	3.8
NaF, mg	—	1.5	1.5
$Na_2B_4O_7 \cdot 10H_2O$, mg	2.5	3.0	3.0
MoO_3, mg	3.7	3.0	3.0
$AlK(SO_4)_2 \cdot 12H_2O$, mg	—	4.5	4.5
pH Before sterilization[6]	6.1	5.5	5.8
pH After sterilization[6]	6.0	5.6	5.8

[1] Labco, The Borden Company, New York.
[2] Technical — 30-mesh, National Casein Sales, Chicago 20.
[3] Cellophane Spangles, Microfiber, Inc., Pawtucket, Rhode Island.
[4] Delsterol, E. I. Du Pont de Nemours and Company, Wilmington, Delaware.
[5] Added separately.
[6] Mixture of one part air-dried diet to 4 parts of water.

TABLE 2

Effect of steam sterilization on B vitamin stability in Lobund diets at graded moisture levels

Treatment		Diet L-356		Diet L-462		Diet L-473E5	
	% solids	% retention	μg/gm	% retention	μg/gm	% retention	μg/gm
				Riboflavin			
Raw[1]		100.0	33.7 ± 0.8[2]	100.0	20.8 ± 0.1	100.0	22.6 ± 0.3
SS[3]	100[4]	94.4	31.8	95.2	19.8	88.1	19.9
SS	80	95.5	32.2	96.2	20.0	93.8	21.2
SS	60	95.3	32.1	96.6	20.1	96.5	21.8
SS	40	92.0	31.0	96.2	20.0	94.7	21.4
SS	20	93.8	31.6	93.3	19.4	92.0	20.8
				Pyridoxine			
Raw		100.0	21.2 ± 1.3	100.0	12.0 ± 0.3	100.0	14.0 ± 0.4
SS	100	83.0	17.6	68.3	8.2	64.3	9.0
SS	80	90.6	19.2	69.2	8.3	78.6	11.0
SS	60	90.6	19.2	76.7	9.2	80.7	11.3
SS	40	91.5	19.4	79.2	9.5	92.8	13.0
SS	20	97.2	20.6	85.0	10.2	98.6	13.8
				Thiamine			
Raw		100.0	52.4 ± 1.6	100.0	27.4 ± 0.5	100.0	34.2 ± 1.9
SS	100	11.8	6.2	24.7	6.7	9.4	3.2
SS	80	31.5	16.5	39.8	10.9	42.1	14.4
SS	60	37.0	19.4	58.4	16.0	52.6	18.0
SS	40	42.4	22.2	56.6	15.5	59.1	20.2
SS	20	65.5	34.3	61.3	16.8	62.6	21.4
				Calcium pantothenate			
Raw		100.0	385 ± 3	100.0	194 ± 6	100.0	218 ± 6
SS	100	72.7	280	59.8	116	53.2	116
SS	80	81.0	312	65.5	127	76.6	167
SS	60	88.6	341	77.3	150	81.7	178
SS	40	85.7	330	79.4	154	90.4	197
SS	20	92.7	357	81.4	158	95.0	207

[1] Raw indicates fresh nonsterilized diet.
[2] SD.
[3] SS designates steam-sterilized.
[4] Indicates percentage of solids in diet.

according to a modification of the method by Bouman ('48), described by Wostmann and Knight ('60). Vitamin B_6 was assayed microbiologically with *Saccharomyces carlsbergenisis* (ATCC 9080) (Hurley, '60), riboflavin fluorometrically (AOAC, '60) and calcium pantothenate microbiologically with *Lactobacillus plantarum* (ATCC 8014) (AOAC, '60). In two separate series with diet L-462, vitamin A and carotene were determined colorimetrically by the AOAC ('60) methods for mixed feeds. Dry matter was determined by drying feed samples at 100 to 105°C for 24 hours.

The data were treated by analysis of variance. In this way variances due to experimental treatments and analysis technique were removed from the error variances. Standard deviations were calculated from the error variances and are presented in the tables.

RESULTS AND DISCUSSION

The experimental data concerning the B vitamins are summarized in table 2. Vitamin content is expressed as micrograms per gram of air-dried feed and as percentage recovery or retention.

In the air-dried diets (100% solids) thiamine destruction was extensive (75 to 90% of initial level), followed in order of increasing stability by calcium pantothenate, vitamin B_6 and riboflavin. This order of stability was the same in each of the three formulas. Furthermore, in air-dried diets the destruction of each of the above vitamins was greatest in L-473E5. This diet is very similar to L-462, except for the addition of potassium and magnesium as the acetate salts.

Water additions to the air-dried diets prior to autoclaving improved the stability of the above B vitamins, with the exception of riboflavin which was not affected. In general, the greatest improvement in stability was obtained from the smallest addition of water. However, each increasing moisture level further improved vitamin retention. Diet L-473E5, which showed the greatest losses of thiamine, calcium pantothenate and vitamin B_6 in sterilized air-dried diet, also demonstrated the greatest benefit in improved vitamin stability with water additions.

The effect of autoclaving diet L-462 at graded moisture levels on the retention of added vitamin A and naturally occurring carotene was investigated in a second experiment. These data are summarized in table 3. There was only moderate destruction of vitamin A and carotene in the air-dried autoclaved diet. Graded water additions appeared not to change the vitamin A retention materially. However, in the case of carotene, there was a definite trend of decreasing retention with increasing moisture content — an effect directly opposite to that noted with the B vitamins.

The mechanism by which water additions to the diets protect some B vitamins is at this time unknown. That 3 out of 4 of the B vitamins studied demonstrated this response indicates some common, general mechanism. The results presented here suggest agreement with the observations of Waibel et al. ('54). These workers observed improved retention of thiamine in a purified diet when stored in an area of high relative humidity as opposed to a lower humidity. They had demonstrated that the strongly hygroscopic dibasic potassium phosphate contributed greatly to the thiamine instability in their diets. Kandutsch and Baumann ('53) had earlier postulated that thiamine destruction takes place in the aqueous film on food particles and that oxygen is the destructive agent. The high salt concentration in these water layers, in which the value of the pH does not necessarily parallel that measured in a dilute diet suspension (table 1), might be instrumental in this process. Waibel et al. ('54) also claimed that destruction took place in the aqueous film and suggested that the protective effect of diet storage at high humidity may be due to a dilution of the aqueous phase surrounding the food particles. Protection of other B vitamins by increasing moisture content might possibly be explained by similar mechanisms.

The retention of vitamin A in sterilized L-462 is in agreement with the data of Reyniers et al. ('50), who recovered approximately 80% of the original vitamin A from a semisynthetic diet after similar sterilization procedures. Water additions to the diet had no observable effect on vitamin A stability. An explanation for the apparent detrimental effect of water in the diet on carotene stability is not known by the authors.

On many occasions, steam sterilization of air-dried diets has given rise to problems, as anticipated earlier (Wostmann, '59). The margin between absolute sterility and over-sterilization characterized by impaired performance of the animal appears small with these diets. Experience at this laboratory has shown that reproduction in

TABLE 3

Effect of steam sterilization on vitamin A and carotene stability in Lobund diet L-462 containing graded moisture levels

Treatment		Vitamin A		Carotene	
	% solids	*% retention*	*IU/gm*	*% retention*	*μg/gm*
Raw[1]		100	16.8 ± 1.3[2]	100	2.26 ± 0.14
SS[3]	100[4]	84.4	14.2	93.8	2.12
SS	80	86.1	14.5	88.0	1.99
SS	60	88.9	15.0	83.2	1.88
SS	40	86.2	14.5	79.6	1.80
SS	20	81.3	13.7	69.0	1.56

[1] Raw indicates fresh nonsterilized diet.
[2] SD.
[3] SS designates steam-sterilized.
[4] Indicates percentage of solids in diet.

germfree rats and mice decreases sharply at thiamine levels of 4 to 8 μg/gm diet (after sterilization) — levels considered to be more than adequate for all normal requirements (Cuthbertson, '57). Additional thiamine fed with the drinking water repairs this situation. The observation appears to indicate the formation of one or more strongly inhibiting anti-metabolites during sterilization. The danger might be not so much in the loss of B vitamins during sterilization, as in their transformation to structurally closely related anti-metabolites. Increases in moisture content of the diet, therefore, would not only increase recovery of these vitamins after sterilization, but would also reduce the formation of interfering products.

SUMMARY

Three diets used routinely in the Lobund Laboratory, containing graded additions of water were steam-sterilized and then analized for thiamine, vitamin B_6, calcium pantothenate and riboflavin. In a separate, similar experiment diet L-462 was sterilized and analyzed for vitamin A and carotene.

Vitamins, rated in the order of increasing stability in autoclaved air-dried diets, were as follows: thiamine, calcium pantothenate, pyridoxine, vitamin A, carotene and riboflavin. Graded additions of water to the air-dried diets progressively improved the apparent stability of thiamine, pyridoxine and calcium pantothenate, had little effect on vitamin A and riboflavin stability, and decreased the stability of carotene.

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Nutritive Value of Marine Oils

I. MENHADEN OIL AT VARYING OXIDATION LEVELS, WITH AND WITHOUT ANTIOXIDANTS IN RAT DIETS[1,2]

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Certain marine or vegetable oils when fed to experimental animals have caused various types and degrees of abnormalities. Restricted growth (Anglemier and Oldfield, '57); reduced efficiency of feed utilization (Oldfield and Anglemeir, '57); deposition of soft fat susceptible to oxidation (Barnes et al., '43; Lea, '53); and incidence of steatitis or "yellow fat disease" in mink (Lalor et al., '51), pigs (Davis and Gorham, '54), cats (Coffin and Holzworth, '54) and rats (Mason et al., '46) are among symptoms reported.

Although marine oils have been used as dietary sources of some essential nutrients (especially vitamins A and D) for humans, farm animals and fowl, they have seldom been used as the sole or major lipid component of diets. However, certain animals, notably mink, consume high fish diets and thus may derive considerable of their energy supply from fish oils. Menhaden and perhaps other marine oils, are commercially available in quantities which would allow such use and constitute a significant potential source of energy. Due to their high degree of unsaturation, fish oils pose problems of instability which affect both palatability of the diet and quality of the animal product (Brown, '31). There is evidence that many of these problems are oxidative in nature (Barnes et al., '43; Johnson, '56; Matsuo, '61) and that antioxidants may be useful means of protection.

The studies reported herein were concerned with the preparation of marine oils for feeding and the consequent effects on laboratory rats. Three separate experiments were conducted. The first utilized menhaden triglyceride fraction (MTG)[3] separated from the oil by molecular distillation. The triglyceride was used at three widely different levels of oxidation. The second experiment tested the effectiveness of antioxidants added to previously oxidized clay-bleached[4] menhaden oil, and the third followed a similar pattern using a fresh oil. Evaluation of the quality of both diet and animal body lipids was made by the thiobarbituric acid (TBA) procedure of Sinnhuber and Yu ('58). This procedure determines malonaldehyde, which is formed in lipid peroxidation (Zalkin and Tappel, '60).

To obtain a clear definition of the problems involved, oxidative levels considerably higher than those generally encountered in edible marine oils were used.

EXPERIMENTAL

Experiment 1. Four groups of 10 male albino rats each were used. The rats were caged individually and fed for a 21-day period on diets composed as shown in table 1. The various B-vitamins were weighed out in quantities listed and made up to one gram with starch. The MTG was oxidized in closed glass containers under a slight positive oxygen pressure in the light at room temperature and in this way, successive aliquots having TBA numbers[5] of 9, 519, and 1082 and with peroxide

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[2] Carried out under Contract no. 14-17-0001-352, U.S.D.I. Bureau of Commercial Fisheries. Appreciation is tendered M. E. Stansby and staff, Fisheries Technological Laboratory, Seattle, for counsel and assistance in conducting this project.

[3] Prepared at the Fisheries Technological Laboratory, U.S.D.I., Seattle, Washington. The product may be referred to as being free from nonsaponifiable materials and polymers.

[4] Clay bleaching is a process utilized for removal of odors, free fatty acids and pigments from the oil. Charcoal is also used with the clay in this process.

[5] TBA number = mg malonaldehyde/1000-gm sample.

TABLE 1
Composition of basal diet

	gm
Cornstarch	644
Fibrin, beef, washed	180
Menhaden triglyceride (MTG)	100
Glucose	6
Salt mix[1]	40
B-vitamin mix	10
Cellulose, wood, purified	20
Total	1000

B-vitamin mix	*mg*
Thiamine	0.6
Riboflavin	1.2
Pyridoxine	0.4
Niacin	5.0
Ca pantothenate	4.0
Inositol	100.0
Choline Cl	200.0
p-Aminobenzoic acid	2.5
	μg
Biotin	1.0
Folic acid	1.0
Cyanocobalamin	1.0

[1] Jones and Foster ('42).

values (POV) of 2, 125 and 310 were obtained. These were used in diets for groups 1–2, 1–3 and 1–4 respectively. Diet 1–1 contained 10% of non-antioxidant treated lard replacing the MTG.

The prepared oils were measured out in appropriate daily aliquots and stored in vials at − 10°C to prevent changes in their oxidation levels. Each day the MTG or lard was mixed with other dietary ingredients and immediately placed before the animals. Refused feed was recorded daily and the feed dishes were thoroughly cleaned prior to refeeding. Animal weights and feed consumption were determined weekly. After 21 days on experiment the animals were killed with ether and samples of blood, fat, liver and kidney tissues were removed for further study.

Experiment 2. Six groups of 4 each male and female rats were established, and the animals individually fed rations formulated as shown in table 1 but having clay-bleached menhaden oil substituted at a 15% level. This oil replaced the MTG used in experiment 1, plus 5% of the cornstarch in the basal ration. The clay-bleached menhaden oil was oxidized in a manner analogous to that used with the MTG. Samples having the following characteristics were prepared: (a) low oxidation level; POV, 2.6, TBA, 35.3; (b) medium oxidation level; POV, 15.5, TBA, 48.8; (c) high oxidation level; POV, 61.0, TBA, 162.0. A lard-supplemented diet was fed as a control to lot 2–1. Lots 2–2, 2–3 and 2–4 received diets containing the low, medium and high oxidation levels of oils, respectively. Lots 2–5 and 2–6 received the highly oxidized oil plus the *in vivo* antioxidants, *dl*-α-tocopheryl acetate or 1,2-dihydro-6-ethoxy, 2,2,4-trimethylquinoline (ethoxyquin),[6] respectively. The α-tocopheryl acetate was added at the level of 0.0044%, and the ethoxyquin was added at the level of 0.0125% in the diet. Storage and incorporation of the oil in the diet and general feeding practices were similar to those in experiment 1. After 100 days on experiment the rats were killed with ether and samples of blood, brain, fat, heart, kidney and liver tissues were removed for further study.

Experiment 3. Four groups of 10 male rats each were used. The animals were individually fed ration formulas similar to those described in experiment 2, but using clay-bleached menhaden oil (POV, 3.0; TBA, 86.0) from the stock previously used in experiment 2, and substituting casein for beef blood fibrin. Lots 3–1 served as the control group and received 15% lard instead of the oil. Lot 3–2 received 15% of unprotected menhaden oil while lots 3–3 and 3–4 received the same amount of oil with either α-tocopherol or ethoxyquin added. Further experimental oxidation of the oil was not carried out in this experiment. The antioxidants were premixed with the daily aliquots of oil prior to freezing and storage at levels of 0.005% and 0.0125% of the total diet, respectively. Care of the animals and assembly of data were carried out as previously described. The experiment lasted 6 weeks.

RESULTS

Experiment 1. Body weights and feed consumption data are presented in table 2. Growth performance was similar in groups 1–1 and 1–2 which were fed lard and fresh MTG, respectively. Groups 1–3 and 1–4, fed medium and highly oxidized MTG

[6] Ethoxyquin (Santoquin) is a product of the Monsanto Chemical Co., St. Louis, Missouri.

TABLE 2

Performance of rats fed menhaden triglyceride (MTG) ration

Group diet description	Body weights					Feed consumption			
	Start	Week			Mean	Week			Mean
		1	2	3	Gain	1	2	3	Intake
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
1–1 Lard	73	91	108	115	42	64	69	68	201
1–2 Fresh MTG	70	87	108	121	50	60	65	69	194
1–3 Medium oxidation MTG	73	80	99	108	35	54*	64	64	183
1–4 High oxidation MTG	73	71**	80**	94*	21*	46**	48**	53*	146**

* Indicates significant difference from control (1–1) group ($P < 0.05$).
** Indicates significant difference from control (1–1) group ($P < 0.01$).

TABLE 3

Effect of menhaden triglyceride on liver and kidney weights, hemoblogin and hematocrit levels

Group	Average				Average	
	Liver wt		Kidney wt		Hemoglobin	Hematocrit % RBC
	gm	%[1]	*gm*	%[1]	*gm/100 ml*	
1–1	4.11	3.57	1.04	0.90	15.4	32.6
1–2	5.35**	4.44	1.13	0.94	13.0**	33.2
1–3	4.88*	4.51	1.09	1.01	13.3**	33.6
1–4	4.40	4.40	0.97	1.03	13.6**	34.3

[1] % of total body weight.
* Indicates significant difference from control (1–1) group ($P < 0.05$).
** Indicates significant difference from control (1–1) group ($P < 0.01$).

showed correspondingly lower gains as the level of oxidation was increased. Statistical significance was reached in comparison with the control (lard) diet only in the case of the highly oxidized MTG ration (group 1–4); however, gains with the fresh MTG-supplemented diet (group 1–2) were consistently greater ($P < 0.01$) than those on the highly oxidized MTG and overall mean gains with the same diet (1–2) were significantly ($P < 0.05$) greater than those fed the medium oxidized MTG mix. Feed consumption was generally directly related to gains except that the lard-fed group ate slightly more than the group fed fresh MTG. Again, there was no significant difference in feed consumption between the lard-fed and fresh MTG-fed groups. Both of these groups (1–1 and 1–2) ate significantly more than group 1–4 which received the highly oxidized MTG.

The weights of certain organs are recorded, along with blood characteristics, in table 3. Livers tended to be larger in all groups fed MTG than in the lard-fed group, regardless of state of oxidation of the triglyceride. This is further emphasized when percentages that liver weights make up of total body weights are calculated. Similar observations apply to kidney weight:body weight percentages. Blood from rats fed MTG was significantly lower in hemoglobin than that from lard-fed rats. Differences among the 4 groups with respect to hematocrit were not significant.

Lipid peroxidation using the TBA procedure to measure malonaldehyde, determined on liver and kidney tissues taken from all animals at necropsy as well as on 24-hour collections of urine and feces from single randomly chosen animals from each group, are presented in table 4. The TBA numbers of liver and kidney tissues reflect the oxidation level of the dietary fat. Values were lowest in rats fed fresh

TABLE 4

Thiobarbituric acid (TBA) number[1] of certain tissues and excreta of rats fed menhaden triglyceride (MTG)

Group	Tissues		Excreta	
	Liver	Kidney	Urine	Feces
1–1	1.97	3.42	0.0	0.0
1–2	1.73	5.61	14.0	61.6
1–3	4.40	8.24	115.0	232.0
1–4	6.83	11.60	201.0	589.7

[1] TBA number = mg malonaldehyde/1000-gm sample.

MTG and lard and increased as triglycerides of increasing rancidity were fed.

The TBA numbers for 24-hour collections of feces and urine also reflect the condition of the oils fed. Based on the amounts of urine and feces voided, the following total excretions of malonaldehyde have been calculated: 0.0 for group 1–1; 0.1063 mg for group 1–2; 0.5214 mg for group 1–3; and 0.4010 mg for group 1–4. The higher excretion from group 1–3 than from group 1–4 (highly rancid MTG) probably reflects the considerably higher food intake by the former (table 2).

Experiment 2. Average body weights of male and female rats at 4-week intervals are presented in table 5. Values recorded are average weights of not less than three rats per group. Groups were dropped from experiment when only two rats survived. Mortality was higher among the males than the females.

From the first interval (table 5) body weights of all groups fed fish oil, except group 2–6, tended to be lower than those in the lard-fed control group (2–1). Protection was afforded by ethoxyquin, but not by *dl*-α-tocopheryl acetate, under the conditions of the experiment. Differences were statistically significant ($P < 0.05$) between groups fed the most highly oxidized oil (group 2–4) and the control or ethoxyquin-treated groups. The *dl*-α-tocopheryl acetate added to the already highly oxidized oil had little protective effect among the females at the level used. Group 2–5 females were significantly lighter than the controls.

The growth-depressant effects of the less-highly oxidized oils became more apparent during the 5- to 8-week period when weights of all rats receiving unprotected fish oils were significantly lower than those of the controls. Mortalities commenced during the 5- to 8-week period also and were apparently influenced by the oxidation level of the oil; occurring first in the highly oxidized oil lot (2–4), next on the medium oxidation level (group 2–3) and so on (table 6). A sex difference in mortality rates is evident, the males being more frequently affected. The growth and mortality patterns established during the first two intervals were extended through the third. Feed consumption data are presented in table 7.

TABLE 6

Mortalities and abnormal fat coloration[1] of rats fed menhaden oil at various oxidation levels

Groups	Experimental interval			Total mortality
	1–4 weeks	5–8 weeks	9 weeks – end of trial	
2–1	—	—	—	0
2–2	w	—	y y y	4
2–3	—	y y y	y y y y	7
2–4	y y	—	y y y y y	7
2–5	—	w	y	2
2–6	—	—	w	1

[1] Each letter represents one mortality; w = white fat, y = yellow fat.

TABLE 5

Average body weights at 4-week intervals for rats fed menhaden oil at various oxidation levels

Groups		Experimental interval					
		1–4 weeks		5–8 weeks		9–12 weeks	
		Males	Females	Males	Females	Males	Females
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
2–1	Lard	186	149	257	195	283	215
2–2	Low oxidation oil	171	133	170*	143**	—	135**
2–3	Medium oxidation oil	159	136	163*	152*	—	139**
2–4	High oxidation oil	122*	108*	—	134**	—	—
2–5	High oxidation oil + vitamin E	164	97*	227	150**	231*	154**
2–6	High oxidation oil + ethoxyquin	192	143	253	180	248	202

* Indicates significant difference from control (2–1) group ($P < 0.05$).
** Indicates significant difference from control (2–1) group ($P < 0.01$).

TABLE 7

Average weekly feed intake for male and female rats fed menhaden oil at various oxidation levels

Groups	1–4 weeks		5–8 weeks		9–12 weeks	
	Males	Females	Males	Females	Males	Females
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
2–1	276	265	316	300	332	271
2–2	260	237	233*	204**	190*	193*
2–3	247	238	227*	216**	—	204*
2–4	200*	187*	—	204**	—	168**
2–5	251	190*	310	248	285	223*
2–6	304	258	326	281	324	265

* Indicates significant difference from control group 2–1 ($P < 0.05$).
** Indicates significant difference from control group 2–1 ($P < 0.01$).

TABLE 8

Effects of menhaden oil and antioxidants on rat weight gains

Group		Initial wt	Wt gain at weekly intervals					
			1	2	3	4	5	6
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
3–1	Lard	58.4	86.7	115.1	138.7	175.6	219.6	289.4
3–2	Fresh oil	58.8	80.4**	89.8**	102.5**	125.3**	140.4**	169.0**
3–3	Oil + vitamin E	59.8	89.1	110.1	133.1	171.5	211.0	250.6
3–4	Oil + ethoxyquin	58.4	87.8	107.3	131.7	167.2	203.3*	236.2*

* Average weights significantly different from those of lard group ($P < 0.05$).
** Average weights significantly different from those of lard group ($P < 0.01$).

Feed consumption by the control (2–1) and the ethoxyquin-treated oil (2–6) groups was equal but higher than that of the other groups. The three groups fed the unprotected oils showed progressive and significantly lower feed intakes, the depression occurring first in the case of the most highly oxidized oil (group 2–4). The *dl*-α-tocopheryl acetate did not improve feed intake, particularly among the female rats, at the level used.

Before experiment 2 terminated, 21 out of the 48 rats involved had died. Observations at necropsy showed that yellowish coloration of depot fat appeared about two weeks after feeding of the most highly oxidized oil commenced and slightly later with the oils of lower oxidation levels. Mortality rate was higher among groups fed medium and highly oxidized oils. Addition of the antioxidants to diets of groups 2–5 and 2–6 retarded pigmentation of the depot fat and protected the animals from early death. Data in table 6 represent number of mortalities from each treatment group as well as depot fat color noted at necropsy.

Experiment 3. Data concerning body weights of the experimental animals at weekly intervals are listed in table 8.

Among the oil-fed groups, improved gains were afforded by the antioxidants, more so by the *dl*-α-tocopherol than by ethoxyquin. Better performance was obtained with α-tocopherol in this study over that in experiment 2 (where the acetate form was used) possibly because of higher levels and because the chemical form in this latter case permitted some *in vitro* antioxidant activity.

Feed intake and efficiencies of feed conversion were calculated for the entire trial, and are included in table 9. Differences noted were consistent throughout the trial, and did not change from week to week. Net gains are listed for comparison. The average absolute weights of livers, left kidneys, spleens and testes are listed in table 10 together with the percentages that these organs make up of total body weight. Even though total body weights were less, the liver weights of rats fed menhaden oil protected by antioxidants were significantly greater ($P < 0.01$) than those of the con-

TABLE 9

Relationship of weight gains and feed consumption by rats fed menhaden oil and antioxidants

Group	Net gain	Total feed intake	Feed consumed/ gm gain
	gm	*gm*	*gm*
3–1	201.8	606.0	3.02
3–2	108.7**	425.3**	3.95**
3–3	190.8	574.6	3.03
3–4	177.8**	577.7	3.26*

* Indicates significant difference from lard group 3–1 at ($P < 0.05$).
** Indicates significant difference from lard group 3–1 at ($P < 0.01$).

trol group. In group 3–2 which received fresh oil, although the absolute liver weight was low, the percentage that liver represented of total body weight was again elevated. Kidney weights were significantly affected only by the fresh oil diet (group 3–2) in which case they made up a larger percentage of body weight. Spleen weights tended to follow the same pattern as livers, being heavier in all three oil-fed groups than those of controls. Testes weights indicated retardation of development in the fresh oil-fed group (3–2). Lack of normal pigmentation of the upper incisors was also noted in this group.

Blood hemoglobin and hematocrit were determined and results are listed in table 11. Values for the rats fed the fresh-oil diet were significantly lower than those for the control or ethoxyquin-protected oil diet groups ($P < 0.01$). The *dl*-α-tocopherol was effective against lowering of hemoglobin level, but not against depression of hematocrit.

Liver characteristics, in terms of dry matter and ether extract contents and TBA values, are also included in table 11. There were no statistically significant differences among any of these items. Although considerable variation occurred in TBA values, all these were extremely low as compared with oxidized oils in the diets and were considered indicative of a low level of oxidation. There was no evidence of steatitis in any animals on this experiment.

DISCUSSION

Growth. Evidence has been presented to indicate a difference in the growth response of rats to type, level and condition of lipid fed. Fresh menhaden triglyceride supported growth similar to that of lard when both were given at a 10% level in the diet for three weeks (table 2). Clay-

TABLE 10

Effects of menhaden oil and antioxidants on organ weights

Group	Body wt	Liver		Left kidney		Spleen		Testes	
	gm	*gm*	%	*gm*	%	*gm*	%	*gm*	%
3–1	260.4	10.12	3.88	1.010	0.389	0.60	0.23	2.66	1.02
3–2	169.0	9.66	5.76**	0.740**	0.439*	0.56	0.39**	1.82**	1.05
3–3	250.6	12.95**	5.19**	0.979	0.391	0.75*	0.30*	2.73	1.09
3–4	236.2	12.16**	5.17**	0.969	0.411	0.72	0.31**	2.61	1.11

* Indicates significant difference from lard group 3–1 ($P < 0.05$).
** Indicates significant difference from lard group 3–1 ($P < 0.01$).

TABLE 11

Effects of menhaden oil and antioxidants on blood and liver

Group		Blood characteristics		Liver characteristics		
		Hemoglobin	Hematocrit	D.M.[1]	E.E.[2]	TBA no.[3]
		%	%	%	%	
3–1	Lard	13.88	38.9	28.51	11.73	4.6
3–2	Fresh oil	10.78**	29.8**	27.10	8.25	9.2
3–3	Oil + vitamin E	12.62	32.2**	27.96	9.28	4.04
3–4	Oil + ethoxyquin	13.33	35.3	27.48	10.42	5.1

[1] D.M. indicates dry matter in liver homogenates.
[2] E.E. indicates ether extract of dry liver samples.
[3] TBA number = mg malonaldehyde/1000-gm sample.
** Indicates significant difference from control group or those fed ethoxyquin-protected oil diet ($P < 0.01$).

bleached oil (POV, 2.6; TBA, 35.3) at a 15% dietary level also supported growth similar to lard but only for 4 weeks following which growth depression resulted (table 5). Slightly more oxidized clay-bleached oil (POV, 3.0; TBA, 86.0) failed to support growth equivalent to lard from the second week (table 8). These results are in agreement with previous observations, at this station, of growth depression in pigs following feeding of 8.25% sardine oil in the diet (Anglemier and Oldfield, '57). However, normal growth was obtained with 5% menhaden oil (Oldfield and Anglemier, '57). This difference of effect shown in experiments 1, 2 and 3 may be due to the composition of the lipid used (pure triglyceride vs. clay-bleached oil) as well as to the degree of oxdiation and the level of the latter lipid. Clay-bleaching of the oil may have rendered it free from all natural tocopherols and could have contributed pro-oxidants (i.e. metals), therefore increasing its susceptibility to rapid oxidation when exposed to room temperature for the 24-hour feeding periods. It has been noted elsewhere (Buxton, '42) that fish liver oils treated with activated carbon had a shortened induction period and an increased rate of peroxide formation.

As the degree of MTG oxidation increased, a corresponding decrease in growth rate became evident (table 2). Similarly, growth depression was obtained as the level of oxidation of the clay-bleached oil was elevated (table 5). There was also an increase in mortality rate, more so among the males than the females, which corresponded to the increased level of oxidation (table 6). Sex difference in rate of mortality may be due to the lower growth stress in females or to the higher content of vitamin E in the female rat as shown by Edwin et al., ('61).

Ethoxyquin was effective in preventing growth depression caused by highly oxidized oil (experiment 2; table 5). It was less effective, however, when added to the oil in experiment 3 where a beneficial response occurred during the first 4 weeks only (table 8). The level used was the same in both trials. This situation suggests that the rapidly oxidizing oil used in experiment 3 may have created a deficiency of an essential metabolite, such as vitamin E, whose role was not filled by the synthetic antioxidant. Alternatively, it may mean that a higher antioxidant level was necessary to protect oils in earlier stages of oxidation than in later ones.

The α-tocopheryl acetate used in experiment 2 at a dietary level of 0.0044% was ineffective against growth depression caused by highly oxidized clay-bleached oil (table 5). This was partly due to its unavailability as an antioxidant *in vitro* prior to ingestion with the diet. According to Ames[7] tocopherol esters have no antioxidant activity until hydrolyzed to free the tocopherol, thus it could be assumed that oxidation levels above those established initially (POV, 61.0; TBA, 162) may have been reached during the period when the diet was before the rats. On the other hand, the *dl*-α-tocopherol used in experiment 3 acted immediately as an antioxidant when mixed with the oil thus giving both *in vitro* and *in vivo* antioxidant action. The higher level used (0.005%) may have been an added advantage.

Feed intake. No difference in the feed intake due to the feeding of fresh MTG or lard was noted in the first short-term (3-week) experiment (table 2). When fresh clay-bleached oil was fed, however, feed intake was reduced suggesting the susceptibility of this oil to oxidative rancidity during the period of exposure in the feeding pans. Sinnhuber[8] has demonstrated the extent of oxidative changes in menhaden oil mixed with a synthetic diet which changed from an initial TBA value of 32.5 to a value of 1220 over a 24-hour period at room temperature. Reductions in feed intake by pigs fed oxidized sardine oil have been reported (Anglemier and Oldfield, '57).

Addition of antioxidants to the oil (experiment 3; table 9) improved feed intake to a level only slightly below that of the lard-fed group of rats, and as shown in table 9, total feed intake in both protected groups was not significantly lowered from the control group. The α-tocopherol was more effective in improving efficiency of

[7] Ames, S. R. 1957 Vitamin E (alpha tocopherol) in Poultry Nutrition: Recent Developments. Tech. Presentation no. 57-1. Distillation Products Industries, Rochester, New York, March.

[8] Unpublished material, Department of Food Science and Technology, Oregon State University, 1961.

feed utilization than ethoxyquin at the levels used. As the level of oxidation was increased in both MTG and clay- bleached oil there was a corresponding decrease in feed intake (tables 2 and 7).

Organ size. Evidence is presented in both experiments 1 and 3 indicating that feeding menhaden oil, either as a pure triglyceride or as a clay-bleached oil, led to liver hypertrophy (tables 3 and 10). The extent of this condition is evident when the percentages that liver weights make up of total body weights are compared (table 10). The degree of oxidation of the oil, and the type and amount of antioxidant used appear to influence the degree of hypertrophy. As the level of oxidation of MTG increased, there was a corresponding decrease in absolute liver weights (table 3).

Hypertrophy of the liver in rats fed menhaden oil was not due to alterations in the ratio of tissue components dry matter content as well as ether extractable materials in liver homogenates showed only slight differences among the various groups (table 11). Kaunitz et al.[9] however, have indicated that an increase in liver lipid content occurred as a result of feeding oxidized fat.

Hypertrophy of the kidneys was demonstrated when rats were fed clay-bleached oil but not MTG, and when the percentages that kidney weights make up of total body weights were compared. When comparisons were on an absolute weight basis kidneys from rats fed clay-bleached oil were smaller compared to those from lard-fed or protected-oil groups (table 10). In this study, the effect of antioxidants in overcoming the hypertrophic effect of menhaden oil was demonstrated. It was reported by Kaunitz ('60) that larger livers and kidneys were obtained from rats fed polymers of oxidized lard or cottonseed oil. An increase in kidney weights was also shown in swine fed polymerized menhaden oil (Oldfield and Anglemier, '57).

Results of experiment 3 also demonstrated the hypertrophic effect of fresh oil on the spleen (table 10). The absolute weight of this organ was higher in both protected oil groups than in the control group, whereas this was not evident in the unprotected group because of the relatively smaller size of animals. The hypertrophic effect was further demonstrated in all three oil-fed groups when the percentages that spleen weights make up of total body weight were compared with the unprotected group showing the highest values. This study also demonstrates some slight reduction by both antioxidants of the hypertrophic effect of the oil.

Development of the testes (normally late developing organs) was evidently retarded by feeding unprotected fish oil (table 10). This specific growth retardation may have its cause in an induced vitamin E deficiency. Retarded growth of testes in mice was demonstrated by Horikawa ('58) as a result of vitamin E deficiency. Damage to rat testes as a result of feeding rancid fat was also reported (Kaunitz, '60).

Blood characteristics. Feeding menhaden oil or its triglyceride to rats led to a small but significant reduction in blood hemoglobin irrespective of the oxidation level of the oil (tables 3 and 11). Both antioxidants used in experiment 3 were effective, ethoxyquin more so than alpha tocopherol in preventing reduction of hemoglobin.

Rats fed MTG showed no significant changes in their blood hematocrit. Those fed clay-bleached oil, however, showed a significant reduction in hematocrit values, a condition which was effectively overcome by the use of ethoxyquin but not by α-tocopherol. Hematocrit values closely followed those of hemoglobin with respect to the effects of both the oil and the antioxidants (table 11).

It has been shown that low blood hemoglobin may result from decreased formation of the pigment due to protein malnutrition or iron deficiency (Follis, '58), vitamin E deficiency (Indovina, '51) or alteration in the rate of transfer of ferric to ferrous iron (Anonymous, '58). Rats receiving the unprotected menhaden oil mixed with a vitamin E-free synthetic diet may have been exposed to some if not all of the above circumstances.

Incisor depigmentation. Depigmentation of the upper incisors was observed in rats

[9] Kaunitz, H., C. A. Slanetz, R. E. Johnson, B. Puetzer, G. B. Levy and E. Windholz 1955 Influence of heated and aerated cottonseed oil on fat content of liver and kidney. Federation Proc., 14: 408 (abstract).

fed the unprotected menhaden oil in experiment 3. A similar symptom has been observed by other workers who have related it to iron deficiency (Follis, '58), low dietary protein (Irving, '58; Anonymous, '59), and to deficiencies of vitamins A and E (Irving and Richards, '39; Irving, '42; Garton et al., '52; Moore and Mitchell, '55; Golberg and Smith, '60). In this work, the simultaneous lowering of hemoglobin and hematocrit levels is suggestive of lowered availability of iron induced by a deficiency of vitamin E. Lewis et al. ('51), among others, demonstrated that the latter may be occasioned by the presence of highly unsaturated fish oil in the diet.

Steatitis and malonaldehyde level. Malonaldehyde, which is measured by the TBA reaction, is indicative of the extent of lipid peroxidation. Malonaldehyde is only one carbonyl that results when polyunsaturated fats undergo autoxidation. This very reactive substance probably does not exist in the free state, but results from the action of TBA on lipid hydroperoxides or malonaldehyde derivatives.

Results obtained from these experiments indicate the effect of the oxidized lipid on the condition of liver lipids, depot fats and TBA values of excreta. Malonaldehyde concentrations of certain tissues and excreta of the animals in experiment 1 were directly related to the levels in the diet lipids (table 4). Although the animals were apparently able to excrete most of the malonaldehyde, measurable levels accumulated in the liver and kidneys. These levels were not, however, indicative of any extensive oxidation. Subsequent experience has shown that this compound also accumulates in the blood, in which connection it may be a useful diagnostic criterion for oxidized oil effects. It seems probable that a causal relationship exists between malonaldehyde retained in the animal body and toxicity symptoms which developed. In experiment 3, TBA values for livers were similar to those obtained for rats fed medium and highly oxidized MTG in experiment 1 (tables 4 and 11).

An important observation in both experiments 2 and 3 was the effectiveness of the antioxidants at the levels used in preventing steatitis or "yellow fat" disease. In experiment 2, both *dl*-α-tocopheryl acetate and ethoxyquin performed well in this respect. Since the oil was considerably oxidized initially in experiment 2 (POV, 60) and since the acetate form is inactive *in vitro*, it is obvious that the effect took place after ingestion by the animal. The consistent observation of yellow to brown fat deposits in the animals which died on experiment 2 (table 6) indicates the importance of steatitis in the syndrome of toxic effects due to ingesting oxidized oils. Steatitis has been shown to occur in many species of animals fed different types of oils, and has been effectively prevented by α-tocopherol and its esters, (Davis and Gorham, '54; Gorham, et al., '51; Lalor et al., '51; Robinson and Coey, '51).

SUMMARY

1. Pure triglyceride separated from fresh menhaden oil proved the equivalent of lard in supporting growth of laboratory rats when added to a semisynthetic basal diet at a 10% level. When experimentally oxidized in glass containers to high peroxide levels (POV = 125 to 310) the triglyceride caused steatitis, enlarged livers and caused high levels of malonaldehyde in the blood and excreta of the animals.

2. Clay-bleached menhaden oil was shown to undergo extensive oxidation changes over a brief period (24 hours). Such "fresh" oil caused toxic symptoms in rats when fed at a 15% dietary level. Symptoms included anorexia, steatitis, lowered hemoglobin levels and death, and they became more extensive or severe as the level of oxidation of the dietary fat increased.

3. Antioxidants, ethoxyquin or α-tocopherol were effective in causing remission of most of the symptoms attributed to presence of the oxidized fats in the diet. Considerable protection was obtained *in vivo* even after the oil had been allowed to oxidize (POV = 60;) however, most effective protection was achieved when the antioxidants were added to the fresh oil. Differences were shown in antioxidant activity relating to the form of tocopherol and level used.

4. The nature of the metabolic changes resulting from feeding oxidized menhaden oil was discussed, and possible nutrient relationships involved are postulated.

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Supplementation of Cereal Proteins with Amino Acids

IV. LYSINE SUPPLEMENTATION OF WHEAT FLOUR FED TO YOUNG CHILDREN AT DIFFERENT LEVELS OF PROTEIN INTAKE IN THE PRESENCE AND ABSENCE OF OTHER AMINO ACIDS[1,2]

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In a previous report (Bressani et al., '60) the effect of supplementing wheat flour protein with its limiting amino acids according to the FAO reference protein was studied in children using the nitrogen balance technique. Compared with the essential amino acid pattern of the FAO reference protein (FAO, '57), the order of the limiting amino acids in the wheat basal diet was: lysine, tryptophan, methionine, isoleucine, valine, and threonine.

The results obtained with these children (Bressani et al., '60; Barness et al., '61) indicated clearly that lysine is the most limiting amino acid in wheat protein. The nitrogen retention values when lysine alone was added were considerably above those found when the basal diet was fed, although below those obtained with isoproteic amounts of milk (Bressani et al., '60; Rosenberg et al., '54). The addition of both tryptophan and lysine to the basal diet gave a slightly greater and more constant response.

Several reports (Bressani et al., '58; Almquist et al., '50; Becker et al., '57; Grau, '48; Brinegar et al., '50) from experiments with animals have shown that the relative amounts of the essential amino acids required for maximal response increase with the percentage of protein in the diet. Similar studies in humans have not been reported. It seemed pertinent, therefore, to determine whether a basal diet of wheat, supplemented with both lysine and tryptophan in appropriate amounts, would give consistent nitrogen retention values as high as those obtained with milk, and also whether the effect of a given amount of lysine per gram of nitrogen added to wheat flour is altered by the level of protein intake.

MATERIALS AND METHODS

The first experiment compared nitrogen retention observed in two children fed isoproteic levels of milk, of wheat flour supplemented with lysine, and of wheat flour supplemented with other limiting amino acids. The second experiment investigated the effect of adding different amounts of lysine to wheat flour at two levels of protein intake in 5 children. The age and weight of the children at the start of each experiment are shown in table 1.

The basal diet (in gm/100 gm) consisted of: wheat flour, 85; wheat gluten, 7; glycine, 3; and cornstarch, 5. The test diets supplied the specified level of protein and a part of the calories which were adjusted to the desired level by the addition of sugar and vegetable fat. The various amino acids were substituted for an equal amount of cornstarch, their nitrogen replacing glycine nitrogen so that all the diets remained isonitrogenous, as well as isocaloric. Corrections for the D- form of the amino acids were made by doubling the amount added, except for DL-methionine which was assumed to be fully utilized. The amount of lysine was corrected

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[2] INCAP Publication I-260.

TABLE 1

Age and weight of children at start of each experiment

Case no.	Exp. no.	Age	Weight	Protein intake	Calorie intake
			kg	*gm/kg/day*	*kg/day*
97	1	3 years, 8 months	14.7	2	90
98	1	2 years, 9 months	10.6	2	90
92	2	3 years, 6 months	13.7	2	90
109	2	6 years, 4 months	18.4	2	90
103	2	3 years, 8 months	12.8	2	90
105	2	4 years, 6 months	15.3	3	90
115	2	2 years, 11 months	9.0	3	100

for the hydrochloride molecule present. Amino acid contents and supplements are expressed as milligrams of amino acids per gram of nitrogen to facilitate comparison with the amino acid pattern of the FAO reference protein.

In most instances, the diet was prepared for each three-day balance period and refrigerated until all was used. The two food preparations were a gruel and a pudding of a thicker consistency. The children were fed both preparations three times a day, and a multivitamin and mineral mixture[3] was given daily. The basal diet contained 101 mg of lysine/gm of nitrogen.

In experiment 1, the quantities of amino acids added to the basal diet were those needed to bring its essential amino acid pattern to that of the FAO reference protein. In experiment 2, different levels of L-lysine·HCl were added to wheat flour so that the ratios of lysine-to-trytophan in the diet were 2/1, 3/1, 4/1, 5/1 and 6/1. The 6/1 lysine supplement gave a total of 270 mg lysine/gm N, the amount contained in the essential amino acid pattern of the FAO reference protein.

In all trials weight either remained constant or increased slightly. Balance studies were usually carried out with a two-day adaptation period followed by three 3-day balance periods on each diet. At the end of each three-day balance period, the entire collections of urine and feces and samples of the diet were prepared for nitrogen analysis by the Kjedahl method.

Results for successive three-day periods on the same regimen are given as averages; variation among periods was similar to that described previously for identical procedures (Bressani et al., '60). Values for individual three-day periods were used in the analysis of variance.

RESULTS

Experiment 1

Table 2 presents the results with child PC-97, who was fed the basal wheat diet with additions of lysine, of lysine and tryptophan, and of a mixture of lysine, tryptophan, methionine, isoleucine, valine, and threonine. Nitrogen intake was relatively constant, but the absorbed nitrogen tended to be slightly lower during the milk feeding period than when the wheat protein was fed. Nitrogen retention with the wheat basal diet supplemented with lysine alone was only slightly lower than with any of the amino acid mixtures or with milk.

Table 2 also shows the nitrogen balance results obtained with child PC-98, who received the wheat basal diet with different amino acid supplements. Nitrogen intake remained relatively constant throughout the study, whereas absorbed nitrogen was slightly higher with the wheat flour diets than with milk. Nitrogen retention was similar with both milk and wheat flour supplemented simultaneously with lysine, tryptophan, methionine, valine, isoleucine, and threonine at the specified levels.

Nitrogen retention was greatest with wheat protein supplemented with lysine and tryptophan followed by lysine alone, by lysine and valine, and by the basal diet in order of decreasing retention.

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

TABLE 2

Comparison of nitrogen balance results with milk and a wheat flour basal diet supplemented with amino acids

Diet	No. of balance periods	Nitrogen			Absorption	Retention
		Intake	Fecal	Urine		
		mg/kg/day			*% of intake*	*% of intake*
		Child PC — 97				
Milk	3	288	51	185	82.3	18.0
Basal + L-lysine·HCl[1]	4	302	58	221	87.4	14.2
Basal + amino acid mixture[2]	4	290	30	205	89.6	19.0
Basal + L-lysine·HCl + DL-tryptophan[3]	3	295	44	194	85.1	19.3
		Child PC — 98				
Milk	2	329	51	209	84.5	21.0
Basal	3	338	49	260	85.5	8.6
Basal + L-lysine·HCl[1]	8	328	42	245	87.2	12.5
Basal + amino acid mixture[2]	4	317	48	183	84.8	27.1
Basal + L-lysine·HCl + DL-tryptophan[3]	4	328	37	224	88.7	20.4
Basal	3	320	43	236	86.6	12.8
Basal + L-lysine·HCl + DL-valine[4]	3	304	41	210	86.5	17.4
Milk	2	321	71	165	77.9	26.5

[1] To give 270 mg lysine/gm N in diet.
[2] To give 270 mg lysine, 90 mg tryptophan, 270 mg methionine, 270 mg isoleucine, 270 mg valine and 180 mg threonine/gm N in diet.
[3] To give 90 mg tryptophan/gm N in diet.
[4] To give 270 mg valine/gm N in diet.

Experiment 2

Protein intake: 2 gm/kg/day. Table 3 shows the nitrogen balance results obtained with each of the three children fed the wheat basal diet at a protein intake of 2 gm/kg/day, supplemented with increasing levels of L-lysine·HCl. The supplementation to 162 mg lysine/gm N in the diet increased nitrogen retention above that observed with the basal diet alone. Higher levels of supplemental lysine failed to improve nitrogen balance significantly. Child PC-109 had a preliminary feeding of milk, which gave slightly higher balance results than those observed with the lysine supplemented wheat protein diet.

Protein intake: 3 gm/kg/day. Table 4 summarizes the nitrogen balance results obtained from feeding each of three children the wheat basal diet at a protein intake of 3 gm/kg/day, supplemented with increasing levels of lysine. The basal diet gave low nitrogen balance values, but the supplementation to 162 mg of lysine/gm N again improved nitrogen balance. It was further increased in one child by the addition to 208 mg lysine/gm N. In child PC-105 this level of lysine gave nitrogen values similar to those observed during the initial treatment with milk.

Statistical analysis of lysine supplementation results at the two levels. The analysis of variance of the nitrogen retention data, expressed as percentage of nitrogen intake, shows a significant difference in the percentage of retention among the various lysine levels of supplementation studied ($P = 0.01$). This difference can be ascribed to the increment in percentage of retention compared with that obtained with 101 mg lysine/gm N in the diet and was observed in all cases when lysine was added at the rate of 162 mg lysine/gm N in the total diet. Higher levels of lysine failed to produce significant additional increases in nitrogen retention. Since there is no statistical indication of either a lysine level × protein level interaction or a lysine level × child interaction, it can be concluded that within the range of protein intake studied a uniformly high level of percentage of nitrogen retention was attained starting with lysine additions to the level of 162 mg of lysine/gm N.

TABLE 3

Effect of increasing levels of supplemental lysine on nitrogen retention of children fed a wheat basal diet at a protein intake of 2 gm/kg/day (average of three 3-day balance periods)

Diet	Nitrogen			Absorption	Retention
	Intake	Fecal	Urine		
	mg/kg/day			*% of intake*	*% of intake*
	Child PC — 92				
Basal (101 mg lysine/gm N)[1]	325	47	274	85.5	1.2
Basal+lysine (162 mg lysine/gm N)	322	45	227	86.0	15.5
Basal+lysine (208 mg lysine/gm N)	313	49	211	84.3	16.9
Basal+lysine (260 mg lysine/gm N)	301	54	191	82.1	18.6
	Child PC — 103				
Basal (101 mg lysine/gm N)	330	44	269	86.7	5.2
Basal+lysine (162 mg lysine/gm N)	335	44	244	86.9	14.0
Basal+lysine (208 mg lysine/gm N)	322	52	239	83.8	9.6
Basal+lysine (260 mg lysine/gm N)	320	54	222	83.1	13.7
Basal+lysine (282 mg lysine/gm N)	317	36	224	88.6	17.9
	Child PC — 109				
Milk	312	68	201	78.2	13.8
Basal (101 mg lysine/gm N)	325	34	270	89.5	6.5
Basal+lysine (162 mg lysine/gm N)	328	27	264	91.8	11.3
Basal+lysine (194 mg lysine/gm N)	313	29	241	90.7	13.7
Basal+lysine (208 mg lysine/gm N)	318	27	258	91.5	10.4
Basal+lysine (282 mg lysine/gm N)	331	29	272	91.2	9.1

[1] Total lysine in diet.

TABLE 4

Effect of increasing levels of supplemental lysine on nitrogen retention in children fed a wheat basal diet at a protein intake of 3 gm/kg/day (average of three 3-day balance periods)

Diet	Nitrogen			Absorption	Retention
	Intake	Fecal	Urine		
	mg/kg/day			*% of intake*	*% of intake*
	Child PC — 103				
Milk	468	79	345	83.1	9.4
Basal (101 mg lysine/gm N)[1]	473	59	455	87.5	−8.7
Basal+lysine (162 mg lysine/gm N)	475	53	422	88.8	10.5
Basal+lysine (208 mg lysine/gm N)	495	65	379	86.9	10.3
Basal+lysine (260 mg lysine/gm N)	505	59	446	88.3	9.5
Basal+lysine (282 mg lysine/gm N)	493	52	384	89.5	11.6
	Child PC — 105				
Milk	482	77	341	84.0	13.3
Basal (101 mg lysine/gm N)	495	66	400	86.7	5.9
Basal+lysine (162 mg lysine/gm N)	479	48	382	90.0	10.2
Basal+lysine (208 mg lysine/gm N)	488	47	385	90.4	11.5
Basal+lysine (260 mg lysine/gm N)	508	40	390	92.1	16.3
Basal+lysine (282 mg lysine/gm N)	504	41	385	91.9	15.5
	Child PC — 115				
Basal (101 mg lysine/gm N)	481	61	398	87.3	4.6
Basal+lysine (162 mg lysine/gm N)	509	64	345	87.4	19.6
Basal+lysine (194 mg lysine/gm N)	530	70	379	86.8	15.3
Basal+lysine (208 mg lysine/gm N)	533	59	400	88.9	13.9
Basal+lysine (282 mg lysine/gm N)	509	60	363	88.2	16.9

[1] Total lysine in diet.

DISCUSSION

The nitrogen retention results of the first experiment confirm the previous report of Bressani et al. ('60) that in young children the addition of lysine to a basal wheat diet brings about a marked increase in nitrogen retention. Nevertheless, the increase is lower than that observed from feeding isonitrogenous amounts of milk. The present results indicate that the addition of lysine, tryptophan, methionine, isoleucine, valine and threonine to the wheat basal diet in amounts called for by the pattern of the FAO reference protein (FAO, '57) results in nitrogen retention values as high as those obtained with milk, or higher. They further show that, of the 6 amino acids added in these amounts, lysine and tryptophan together, added to wheat protein fed at intakes between 2 and 3 gm of protein/kg of body weight, produce about 90% of the nitrogen retentions obtained with the same amount of milk protein.

Contrary to the results with a previous child (Bressani et al., '60) valine did not improve nitrogen retention beyond that obtained with lysine alone in the child given the tryptophan and valine combination in the present study. The deficiencies of methionine, isoleucine, and threonine in wheat flour are also minimal. As in the previous study by Bressani et al. ('60), the addition of the 4 amino acids to wheat flour supplemented with lysine and tryptophan produced only a small response; nevertheless, the present experiments and those of investigators working with rats (Hutchinson et al., '58; Bender, '58; Rosenberg et al., '54; Ericson, '60) suggest the need of these amino acids to obtain maximal response with wheat flour protein.

One of the 6 children appeared to require 194 mg of lysine/gm N in the diet for maximal retention rather than 162 mg. The higher figure is equivalent to 102 mg of lysine/kg of body weight, very close to the 103 mg of lysine suggested for children by Snyderman et al. ('59) on the basis of nitrogen balance studies in children one to two months of age, and higher than the figures reported by Nakagawa et al. ('61) for children 11 years old.

The results suggest that when wheat is the only source and is fed at a level of 2.00 to 3.00 gm of protein/kg, the optimal quantity of lysine to be added to the diet is between 162 and 194 mg of lysine/gm N. Adding these quantities of lysine to those in the basal diet results in a lysine-to-tryptophan ratio between 3/1 and 4/1, as proposed in the FAO reference pattern. In a recent review on lysine in human nutrition, Jansen ('62) expressed in several ways the lysine requirements for man and rats observed by a number of investigators. Expressed as the relationship of lysine-to-tryptophan, most of the values were between 3/1 and 4/1.

That the maximal retention of nitrogen was obtained at a much lower level in the present studies helps to explain why the addition of L-lysine at a level of 270 mg lysine/gm N in the studies of Bressani et al. ('60) did not result in nitrogen retention values as high as those obtained with the lower level. It also suggests the reason that the addition of tryptophan to the higher levels of lysine supplementation improved balance; the higher additions of lysine probably caused tryptophan to become the first limiting amino acid for children. The addition of only 162 mg lysine/gm N to the diet was sufficient to balance the available tryptophan in the wheat flour. If larger amounts of lysine are supplied, other amino acids must be added to maintain favorable proportions among the essential amino acids. This appears to be a fundamental limitation in the use of the FAO or any other amino acid reference pattern.

In using any amino acid pattern for the evaluation of proteins, it is necessary to consider not only the adequacy of the amount of each amino acid per gram of nitrogen when others are present in optimal quantities, but also the effect of excesses. This agrees with Rosenberg's conclusion ('59) that the amino acid supplementation of a deficient protein gives an improved response only when it corrects the most limiting amino acid in such a way as to improve the balance or proportion among the other essential amino acids. Some of the amino acids may have little or no adverse effect on nitrogen utili-

zation when they exceed the quantity per gram of nitrogen recommended in the FAO reference pattern; others, such as methionine, valine, and leucine, when present in excess, may under some conditions produce imbalances. An improvement in the present amino acid pattern concept would be to include upper, as well as lower, limits for each amino acid concentration per gram of nitrogen so that when used to evaluate foods or diets the pattern would warn of possible imbalances due to amino acid excesses, as well as adverse effects of deficiencies.

SUMMARY

In the first of two experiments using the nitrogen balance technique in children, it was shown that the addition of lysine to a basal wheat diet brings about a marked increase in nitrogen retention. Nitrogen retention values as high as or higher than those obtained with isonitrogenous feeding of milk were obtained by feeding a wheat basal diet supplemented with a mixture containing the FAO levels of lysine, tryptophan, methionine, isoleucine, valine, and threonine. About 90% of the response, however, was found to be due to the combined lysine and tryptophan supplement.

In the second experiment, the effect of protein level of intake on the minimal lysine supplement to be added to wheat flour was studied in 6 children. Maximal retention of nitrogen was obtained with the addition of 162 to 194 mg lysine/gm of nitrogen to a basal diet fed at a rate of 2 and 3 gm of protein/kg of body weight per day, respectively. Within the range of the protein fed, the amount of lysine per gram of nitrogen that must be added did not vary significantly with protein intake.

The results demonstrate once again that it is necessary in amino acid supplementation studies to consider not only the adequacy of the amount of each amino acid, when others are present in optimal quantities, but also the effect of excesses of some amino acids. Maximal response is obtained only when a correction is made in the amount of the most limiting amino acid in such a way as to improve the balance or proportion among the other essential amino acids. This is a fundamental characteristic of the FAO or any other amino acid reference pattern.

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Further Studies of the Influence of Diet on Radiosensitivity of Guinea Pigs, with Special Reference to Broccoli and Alfalfa[1]

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Confirmation of the observation of Lourau and Lartigue ('50) that supplementation of a bran and oats diet with cabbage decreases the radiosensitivity of guinea pigs, was reported by Duplan ('53) and Spector and Calloway ('59). The latter workers showed that the protective effect was not due to ascorbic acid and that supplementation with broccoli was also effective. Duplan stated that if the basal bran and oats diet was replaced by a balanced synthetic diet, identical results were obtained. However, examination of guinea pigs fed the bran-oats diet led Calloway and Munson ('61) to conclude that at least part of the observed response was due to improved nutritional status. Accordingly, a series of tests were conducted in which various improvements of the diet were investigated. The data to be presented indicate that the radioprotective quality is shared by a number of plant materials; that this quality cannot be demonstrated in the presence of a purified casein diet; and that the protective agent in alfalfa is a water-soluble substance.

METHODS AND MATERIALS

The standardized assay procedure, described below, used by the two collaborating laboratories, was followed except as otherwise noted. Young guinea pigs weighing 250 to 350 gm were maintained for 10 to 14 days with a basal diet consisting of 50% of field oats and 50% of wheat bran, supplemented with vitamin C by addition of 0.24% of sodium ascorbate to the dry diet or in the drinking water as 1.5 gm ascorbic acid plus 1.0 gm sodium bicarbonate/liter.[2] Animals received in poor physical condition were discarded during this standardization period. After standardization, the animals were divided at random into experimental groups of approximately 20 each and given various supplements for two weeks prior to, and for 30 days after, irradiation.

The purified diet used was patterned after the Reid and Briggs ('53) diet no. 13 and was composed of: (gm/100 gm) vitamin-free casein, 30; corn oil, 6.3; sucrose, 9.3; non-nutritive cellulose,[3] 15.0; glucose,[4] 7.8; potassium acetate, 2.5; magnesium oxide, 0.5; USP Salts XIV, 5.998; zinc carbonate, 0.002; cornstarch, 19.4; glycerine, 1.0; B vitamins in cornstarch,[5] 1.0; fat-soluble vitamins in corn oil,[6] 1.0, and ascorbic acid, 0.2. The analyzed composition of this diet, together with that of the bran-oats diet and of broccoli, is shown in table 1. Formulation of other supplements was based upon these compositional data.

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[2] Groups were composed of either all males or equal numbers of males and females, as experience indicated there was no apparent difference in response due to sex. The adequacy of vitamin C supplementation was established by measurement of whole blood levels. Values obtained in 24 animals ranged from 0.88 to 2.68 mg/100 ml, average 1.6 mg/100 ml.

[3] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

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

[5] Vitamins added/100 gm diet: (in mg) thiamine·HCl, 1.6; riboflavin, 1.6; pyridoxine·HCl, 1.6; Ca pantothenate, 4.0; niacin, 20.0; folic acid, 1.0; inositol, 200.0; choline chloride, 200.0; α-tocopherol, 1.82; 2 methyl-1-,4-naphthoquinone, 0.20; (in μg) biotin, 60.0; vitamin B_{12}, 4.0; (in IU) vitamin A palmitate, 1776; and vitamin D_3, 160.

[6] See footnote 5.

TABLE 1

Composition of basal diets and raw broccoli

Ingredient/100 gm of diet	Purified diet	Bran-oats	Broccoli, raw	Ingredient/100 gm of diet[1]	Purified diet	Broccoli, raw
Moisture, gm	8.85	10.55	91.8	Calcium, gm	0.86	0.11
Nitrogen, gm	4.02	2.08	0.37	Phosphorus, gm	0.73	0.069
Amino acids, gm				Sodium, gm	0.172	0.062
Arginine	1.10	1.03	0.23	Potassium, gm	0.450	0.255
Cystine	0.082	0.208	0.030	Magnesium, gm	0.330	0.046
Methionine	0.732	0.192	0.037	Chlorine, gm	0.640	0.046
Cysteine	0.264	0.176	0.067	Fluorine, mg	2.1	0.1
Histidine	0.820	0.320	0.078	Bromine, mg	15.7	2.4
Isoleucine	1.59	0.52	0.11	Iodine, mg	0.57	0.24
Leucine	2.76	0.94	0.19	Zinc, mg	1.2	0.3
Lysine	2.46	0.65	0.20	Aluminum, mg	3.3	0.4
Phenylalanine	1.40	0.54	0.11	Manganese, mg	0.8	0.3
Tyrosine	1.39	0.42	0.08	Iron, mg	22.2	1.5
Threonine	1.26	0.79	0.13	Copper, mg	0.4	0.3
Tryptophan	0.369	0.275	0.045	Selenium, μg	<2	5
Valine	2.12	0.79	0.20	Molybdenum, μg	<10	25
Crude fat, gm	7.61	2.74	0.113	α-Tocopherol, mg	<2	2.0
Fiber, gm	15	12.6	1.3	β-Carotene, mg	—	0.820
Ash, gm	6.95	4.90	0.766	Thiamine, mg	0.733	0.098
Silicon, mg	5.15	347.0	1.71	Riboflavin, mg	1.54	0.180
Boron, mg	<0.5	0.5	2.0	Niacin, mg	21.5	0.738
Sulfur, mg				Pyridoxine, mg	1.59	0.18
Total	206	156	111	Ca pantothenate,		
Organic	179	156	70	mg	6.52	1.33
Arsenic				Folic acid, μg	590	46
(as AS_2O_3), μg	6	36	12	Biotin, μg	76	8
Nickel, mg	1.7	1.7	8.3	Vitamin B_{12}, μg	0.87	NF
				Choline chloride, mg	212	44
				Inositol, mg	207	40
Para-aminobenzoic acid, mg	1.0	NF[2]	NF			

[1] Data on the composition of bran-oats with respect to these constituents were presented previously, (Calloway and Munson, '61).
[2] NF = None found.

Fresh vegetables and fruits were usually purchased locally and held under conditions appropriate for the product, for not more than 5 days. All alfalfa was from a single lot of high-protein, high-vitamin A (approximately 65,000 μg β-carotene/100 gm), dehydrated leaf meal, held under inert gas by the supplier.[7] Fifty grams of raw materials were provided in a separate food cup. Percentage supplementation of the bran-oats diet was at the expense of the total diet. All mixed diets, basal or supplemented, were fed ad libitum.

The X-radiation was administered at the Argonne National Laboratories or at the USAF Radiation Laboratory, University of Chicago, using a General Electric Maximar X-ray machine.[8] Radiation factors were: 180 kv; 15 ma; no filtration added; 21 to 22 r/min in air; 105-cm target distance. Six animals were irradiated simultaneously in a horizontal beam and representatives from each treatment were included in each exposure. Except where noted, animals were exposed to 400 r, half of the dose being applied to each side of the whole body.

At Stanford Research Institute, gamma radiation was administered from a 1600 curie Co^{60} source, with filtration through 0.95 cm of aluminum. Exposure rate was 16 rad/min (ferrous sulfate dosimetry); total-body exposure dose was 350 rad, half of the dose being applied to each side of the body. Twenty animals, including representatives of each dietary treatment, were irradiated simultaneously.

[7] Consolidated Blendors, Fremont, Nebraska.

[8] We are indebted to Dr. D. E. Smith and Joseph Trier of Argonne National Laboratories and to Dr. K. P. DuBois of the USAF Radiation Laboratory for arranging radiation services.

Animals were housed individually in suspended, screen-bottom cages in a controlled environment (26 ± 2°C). Body weight and food intake were recorded intermittently throughout the experimental periods. The criterion from which the efficacy of any treatment was determined was percentage of mortality subsequent to irradiation.

PROCEDURE AND RESULTS

Series 1. Influence of vitamin A on radiosensitivity of guinea pigs fed bran and oats. The basal bran-oats diet is devoid of vitamin A and low in content of protein, riboflavin, calcium, sodium, and the halogens. Earlier studies (Calloway and Munson, '61) revealed reduced hepatic levels of vitamin A in guinea pigs fed the deficient diet for as little as 2 to 4 weeks, and gross pathologic changes associated in this species with deficiency of vitamin A. Therefore, experiments were carried out in which crude and refined sources of vitamin A were evaluated for ability to decrease the radiosensitivity of guinea pigs fed the deficient cereal diet. Several raw plant foods, providing nearly equal amounts of water and fiber but varying in content of β-carotene, were tested as were dehydrated alfalfa leaf meal and certain dairy products. The vitamin A-free diet was fed to all animals during a two-week standardization period before introduction of the indicated supplements.

Data in table 2 relative to unsupplemented, cabbage- and broccoli-supplemented treatments which have been replicated many times in the course of these and earlier experiments, serve to indicate the extent of variance of the assay. Percentage mortality 20 days after 400-r X-irradiation was 97 ± 5, 54 ± 17 and 42 ± 16 for these respective groups. Both supplemented groups differ significantly from the unsupplemented ($P < 0.001$), but not from each other. A few deaths occurred between post-irradiation days 20 and 30; 30-day survivors have been maintained for an additional 6-week period without additional mortality. As is frequently the case with this species, some mortality was experienced among control non-irradiated animals: 5 to 20% of guinea pigs fed the deficient diet and zero to 5% of these given vitamin A-contributing supplements. Radiation mortality values have not been corrected for this factor as there is no valid basis for determining whether a given death would have occurred in the absence of the superimposed stress.

All β-carotene-containing vegetables and fruits exerted some beneficial effect. Mortality sustained 20 days after irradiation was about 30 to 50% of groups fed raw green vegetables or carrots.[9] Dietary supplementation with 20% of dehydrated alfalfa reduced mortality to a very low level; no deaths occurred by the 20th day and only 19% of the group died thereafter. Tomatoes and oranges supply less β-carotene and were less beneficial; the percentage mortalities were 62 and 78, respectively. Beets, apples, and white potatoes were essentially without effect.

Supplements providing pre-formed vitamin A showed no consistent effect on radiosensitivity. Although only 65% of a group given dry whole milk died by post-irradiation day 30, none of the groups fed fluid whole milk or butter survived. Percentage mortality was reduced to 72 at 20 days and 83 at 30 by daily peroral administration of vitamin A together with all vitamins incorporated into the Reid-Briggs diet 13, in the amount that would be contained in an average daily intake of 20 gm of that diet.

To determine whether these responses were attributable to variation in amount or in type of vitamin A source used, guinea pigs were depleted of vitamin A reserves, by preliminary feeding of the deficient cereal diet to induce a 20% reduction of body weight; then they were provided graded amounts of vitamin A palmitate or of crystalline β-carotene during the pre-irradiation period of two weeks and the post-irradiation period of 30 days. All animals continuously given the deficient diet following depletion succumbed within the period of observation, whether irradiated or not. However, exposure to

[9] The low mortality (44% at 20 days) experienced when a supplement of carrots was given requires comment in view of the report of Duplan ('53) that cabbage promotes superior radioresistance in comparison with carrots. In our single experiment with carrots, the vegetable of the cabbage family tested was mustard greens, supplementation with which resulted in only 12% mortality. Thus, our data indicate a similar trend.

TABLE 2

Radiosensitivity of vitamin A-depleted guinea pigs fed a basal diet of bran and oats plus ascorbic acid without vitamin A or with supplements varying in vitamin A content (Series 1)

Supplement[1]	Mortality following irradiation	
	Day 20	Day 30
	%	%
400 r X-radiation		
None	97 ± 5 (10)[2]	99 ± 2
Cabbage	54 ± 17(9)	56 ± 17
Broccoli	42 ± 16(7)	42 ± 10
Alfalfa leaf meal, 20%	0	19
Mustard greens	12, 50	
Green beans	31	
Lettuce	44	
Carrots (root only)	44	
Tomatoes	62	
Oranges	78	
Beets (roots only)	90	90
Apples	94	
White potatoes	100	100
Dry whole milk, 20%	60	65
Fluid whole milk, vitamin D-fortified[3]	94	100
Butter, 10%	100	100
All vitamins[4]	72	83
350 rad Co^{60}-gamma radiations[5]		
None[6]	100	100
Vitamin A palmitate, IU/100 gm[6]		
1775	100	100
4775	95	95
14775	95	95
β-Carotene, μg/100 gm[6]		
2960	90	100
7960	90	90
24625	75	75
Alfalfa leaf meal, 20%	33	33

[1] 50 gm/day unless otherwise noted.
[2] Figures in parentheses indicate number of separate tests included in mean and standard deviation.
[3] 400 IU/quart of pasteurized, homogenized milk.
[4] Given once daily by mouth in amount supplied by 20 gm of Reid-Briggs Diet 13.
[5] All animals included in this group were standardized by feeding the vitamin A-free diet until 20% of body weight was lost. See text.
[6] 3% of corn oil added as vehicle for supplements.

350 rad of gamma radiation shortened average survival time by 8 days. Not more than two non-irradiated animals per group died when given vitamin A or β-carotene after depletion.

Radiation-induced mortality was 100% of those given 1775 IU of vitamin A/100 gm of diet, the amount included in Reid-Briggs diet 13, and 95% of animals that received 4775 and 14,775 IU/100 gm (see table 2). Provision of 2960 or 7960 μg of β-carotene/100 gm of diet was equally ineffective. Mortality was 75% of the group given the extremely high level of β-carotene, 24625 μg/100 gm of diet (12 times the average content of broccoli-supplemented diets and double that of the diet containing 20% of alfalfa). In the presence of alfalfa, only 33% of the animals died. Average survival time was equal in all supplemented groups and was extended about 5 days beyond that of the unsupplemented, irradiated group (12 to 15 days vs. 8 days).

For brevity, neither food intake nor body weight of the large number of groups included in table 2 is given. It was observed that irradiation induced in all groups the same alteration in patterns of food intake. Food consumption decreased for a few days immediately following exposure, returned to near normal levels during the latent period of radiation sickness, and decreased again coincident with the major occurrence of death between the 11th and 15th days. Recovery was

accompanied by steadily increasing food intake, attaining pre-irradiation values by the end of the third week.

Total caloric intake (basal plus supplement) was usually greater in groups given supplements. Consumption of the basal diet was approximately equal among all groups; that is, there was little compensatory decrease in basal food intake in the presence of supplementary food. Supplements contributed about 20% of the total caloric intake. However, variations in food intake bore no consistent relationship to survival. For example, intakes of groups fed beets or cabbage were approximately equal but mortalities were 90 and 54%, respectively.

Marked differences in growth were observed within and between experiments. Neither growth nor body weight were reliable indicators of radiation resistance, although all effective supplements supported better weight gains than were achieved in the controls.

Series 2. Radiosensitivity of guinea pigs fed broccoli or alfalfa in conjunction with a purified casein diet. To determine whether improved nutritional status was the only factor responsible for enhanced radioresistance of guinea pigs fed the bran-oats diet with broccoli or alfalfa, these supplements were fed in conjunction with an adequate purified diet. The modified Reid-Briggs diet 13 served as the basal diet in this series, according to the same test plan used in series 1: two-week standardization with the basal diet followed by two-week pre-irradiation and 30-day post-irradiation periods with or without supplements.

Some difficulty was encountered in acceptance of the synthetic diet despite gradual introduction by admixture with ground commercial feed. In two such experiments, mortality subsequent to 400-r X-irradiation was 62 to 67% of animals fed the basal diet alone and 56% of those given, in addition, 50 gm of raw broccoli daily. Of animals fed ground commercial guinea pig chow,[10] supplemented with 0.24% of sodium ascorbate, 50% died after irradiation.

Satisfactory food acceptance was achieved in a third experiment. After standardization, groups of 40 guinea pigs were assigned the basal, unsupplemented diet or received, in addition, 50 gm of raw broccoli daily or 20% of alfalfa leaf meal incorporated into the basal diet at the expense of cornstarch. All broccoli was procured from a single producer in California and was shipped by air twice weekly with ice to assure freshness. Eighteen animals from each diet group were exposed to either 400 or 500 r of X-radiation.

Percentage mortality of groups fed the basal diet alone, with broccoli and with alfalfa was, respectively: after 400 r, 22, 39 and 11; after 500 r, 44, 61 and 28. Analysis of variance of proportions of animals killed during the 30-day test (using arc sine transformation) showed both supplemented groups to differ significantly from the basal: i.e., higher mortality with broccoli and lower with alfalfa.

Body weight data, graphed in figure 1, show that animals given the basal diet alone or with alfalfa grew at an equal, slow rate of about 15 gm/week prior to irradiation, whereas the weight increment of the group fed broccoli was about double that. Following irradiation at either dose level, growth of both of the supplemented groups was superior to that of the control group. Food intake followed roughly the same pattern. Again, approximately the same amount of basal diet was consumed in the presence of the raw broccoli supplement as in its absence. Growth was not necessarily associated with resistance to radiation, as noted earlier, since the highest mortality was sustained by the broccoli group at both radiation levels.

Series 3. Radiosensitivity of cereal-fed guinea pigs given a mixture of pure materials with the same composition as broccoli, or given raw broccoli with alfalfa meal. The next experiment was designed to determine whether the radioprotective agent was a recognized substance, by providing in chemically-pure form all of the constituents of broccoli shown in table 1. Forty-eight ingredients made up the "synthetic broccoli," which matched the natural composition, except that only one-third of the sulfur was derived from organic sources and chloride was in excess due to unavoidable additions

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

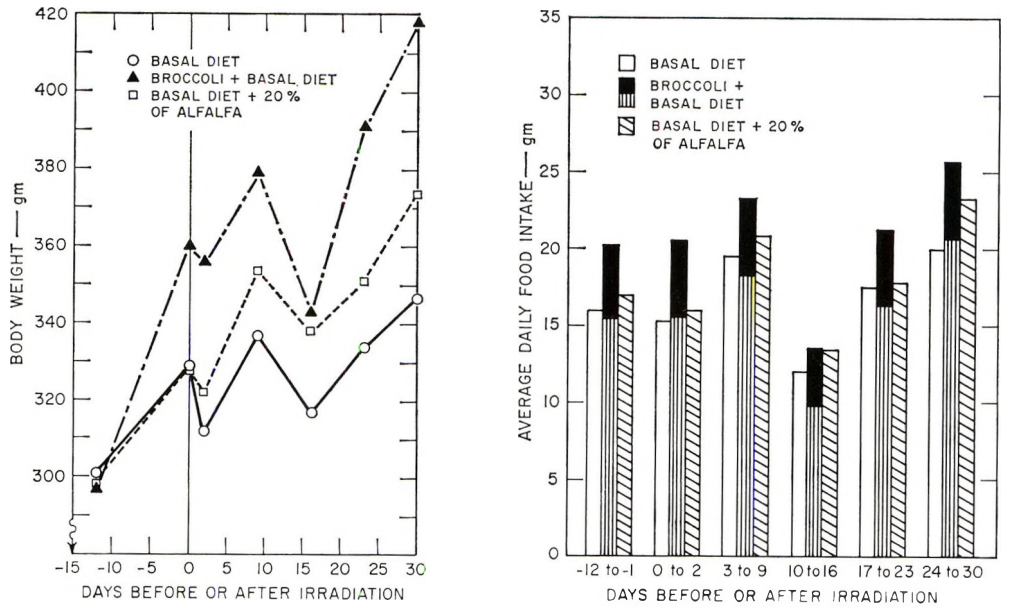

Fig. 1 Growth curves and ad libitum food intake of guinea pigs fed a purified casein diet, alone or with supplements of raw broccoli or dehydrated alfalfa, and exposed to 400 r of X-radiation. To aid comparison, amount of broccoli consumed is shown on a dry solids basis, as part of the total food intake (series 2).

from vitamins and amino acids (1.788% of dry solids instead of 0.56%). The L-form of amino acids was used except for isoleucine, phenylalanine, threonine and valine where racemic mixtures were used. Total nitrogen content was made up with glutamic acid and monosodium glutamate. Since accurate data on the composition of broccoli lipids and carbohydrates were not available, another plant oil, cottonseed, was selected as the source of the small amount of crude fat, and glucose[11] and cellulose[12] as carbohydrates. The basal bran-oats diet was supplemented with 1% of vitamin A-fortified corn oil (1776 IU/100 gm as palmitate) and 0.24% of sodium ascorbate. This diet was fed to all animals during the standardization period and to the dietary control group thereafter.

Although dietary supplementation with either broccoli or alfalfa effectively decreases the radiosensitivity of cereal-fed guinea pigs, there is no valid reason to assume that these protect by the same mechanism. If the mechanisms differ, combination of the supplements might prove more effective than either one alone. Therefore, crude supplements included in this experiment were: raw broccoli, 50 gm/day; 20% of alfalfa leaf meal; or 10% of alfalfa plus raw broccoli, 25 gm/day.

Mortality statistics are summated with others of this series in table 3. The group given only vitamin A gained the least weight before irradiation and sustained the most severe losses afterward. Radiation death occurred in 75% of this group, an improvement over that usually experienced in the absence of vitamin A. The diet containing synthetic broccoli was accepted well and weight gain was improved over that of the control group, but mortality (95%) was higher than in the control group. Of animals given alfalfa, 15% died after irradiation and 50% of those receiving broccoli. Only 10% of the group given the combined supplements succumbed, indicating a possble synergism.

The combined influences of broccoli and alfalfa were then evaluated in animals subjected to 350 rad gamma irradiation. In 14 separate experiments, the 30-day radiation-induced mortality was 86 ± 10%

[11] Cerelose.
[12] Alphacel.

TABLE 3

Radiosensitivity of guinea pigs fed a basal diet of bran and oats plus ascorbic acid and vitamin A, supplemented with pure compounds, broccoli and alfalfa, or alfalfa fractions

Supplement[1]	Mortality 30 days following irradiation	
	400 r X ray	350 rad Co^{60}-gamma
	%	%
Vitamin A palmitate, 1776 IU/100 gm	75, 67	86 ± 10(14)[2]
Synthetic broccoli, 20%[3]	95	
Broccoli, 50 gm/day	50	56, 30
Alfalfa leaf meal, 20%	15, 28	51 ± 16(13)
Alfalfa leaf meal, 10%		63
Alfalfa leaf meal, 5%		89
Broccoli, 50 gm/day + alfalfa leaf meal, 20%		20
Broccoli, 25 gm/day + alfalfa leaf meal, 10%	10	28, 25
Vitamin A, 1776 IU/100 gm +		
d-α-tocopheryl acetate, 110 mg/100 gm		89
Hesperidin, 50 mg %		94
Coumesterol, 2.0 mg %		94
Water-soluble fraction of alfalfa I[4]	28	39, 44
Water-insoluble residue of alfalfa II[4]	67	80, 75
Alfalfa fractions I + II[4]	24	15
Alfalfa ash, 2.4%		72
Alfalfa ash, 4.8%		76

[1] Supplements fed two weeks before and 30 days after irradiation. All diets included 0.24% of sodium ascorbate and 1% of corn oil. SRI diets (Co^{60}-series) also contained 11 mg/100 gm *d*-α-tocopheryl acetate. All animals received vitamin A during the two-week standardization period.
[2] Figures in parentheses indicate numer of separate tests included in mean and SD.
[3] Mixture of 48 ingredients matching broccoli composition given in table 1.
[4] Weights of fractions equal to 20 gm of dry alfalfa were added to 80 gm of basal bran-oats diet.

of animals given the bran-oats diet supplemented with sodium ascorbate and vitamin A palmitate (see table 3). With inclusion of 20% of alfalfa leaf meal, mortality was 51 ± 16% of 13 groups of guinea pigs. A single experiment showed 63% mortality of a group given 10% of alfalfa meal and 89% of those given 5%, indicating dose-dependency.

In the first trial of the conjoint supplements, using 25 gm of broccoli and 10% alfalfa, synergism was again suggested. Mortality was 56 and 45% of the usual raw broccoli (50 gm), and alfalfa (20%) groups, respectively, and 28% of the combined-supplement group. Essentially no such effect was noted in a second experiment, where 20 to 30% of mortality occurred in all groups.

Other substances found in plant materials, that have been suggested to have relevance to radiation sensitivity, were also tested. Supplements to bran-oats diet in addition to vitamin A and sodium ascorbate were: (mg/100 gm) *d*-α-tocopheryl acetate, 110; the flavonoid hesperidin, 50; and coumesterol, the major estrogenic compound found in alfalfa,[13] 2.0. As shown in table 3, none of these reduced the mortality of animals exposed to 350 rad of gamma radiation.

Series 4. Radioprotective effect of alfalfa fractions in the cereal-fed guinea pig. Since the protective effect of broccoli could not be matched by dietary supplementation with a comparable mixture of pure amino acids, vitamins and minerals, isolation of the factor has been undertaken.[14] Alfalfa leaf meal was selected as the starting material because it is reliably effective and is easier to handle and store than is fresh produce.

Alfalfa leaf meal was extracted with water for 4 hours and filtered, yielding a water-soluble fraction (I) and a water-insoluble pulp residue (II). Fraction I

[13] Dr. G. O. Kohler, Western Utilization Research and Development Division, USDA, kindly provided the crystalline coumesterol and assayed the alfalfa for its potency, found to be equal to 92 ppm of coumesterol.

[14] Fractionation was carried out at the Quartermaster Research and Engineering Center Laboratories, Natick, Massachusetts, under the direction of Drs. Louis Long and Torsten Hasselstrom of the Pioneering Research Division, and at the Stanford Research Institute under Dr. W. E. Skinner.

contained 20% of the original solids. However, due to the addition of water in the extraction process, the weights of fractions equal to 100 gm of the dehydrated leaf meal were 55 gm (I) and 190 gm (II). Therefore, supplementation representing 20 gm of alfalfa meal required the addition of 11 gm of fraction I and 38 gm of fraction II to 80 gm of the basal diet (bran and oats with sodium ascorbate and vitamin A palmitate).

Ash of alfalfa leaf meal, prepared by incineration in a muffle furnace, was tested at two levels of supplementations, 2.4 and 4.8% of the diet.

The data, assembled from both laboratories in table 3, show clearly that the agent which promotes survival following irradiation was completely retained in the water-soluble portions of alfalfa, and was absent from the insoluble residue and the ash.

Neither body weight nor weight of spleen, liver, kidneys, adrenals or gonads revealed any differences which appeared to correlate with survival potential. Growth prior to irradiation was superior in the alfalfa-fed group, but was essentially equal in all others.

DISCUSSION

Two hypotheses can be offered to account for the responses observed. First, the bran-oats diet may impose a requirement which does not exist in conjunction with the purified diet, either due to the presence of some substance detrimental to absorption or to a specific nutrient imbalance. Alternatively, an unrecognized essential substance may be provided by both green plant materials and some ingredient of the purified diet.

Failure to provide protection against radiation injury by supplementation of a vitamin A-deficient diet with pure vitamin A and natural sources of the preformed vitamin was most unexpected. Prolongation of survival time until death under these conditions suggests that the animals were in improved condition at the time of irradiation. Since damage to the intestinal tract is a cardinal effect of irradiation, it is possible that faulty absorption of vitamin A may be a post-irradiation complication. To accept this hypothesis, one must assume that less damage to the intestinal tract occurs in animals pre-fed the alfalfa supplement; or that the provitamin A of alfalfa is more readily absorbable or utilizable than is the pure vitamin; or both possibilities. Ershoff and Hernandez ('60) have, in fact, reported that alfalfa contains an unidentified water-soluble factor which improves utilization of endogenous vitamin A in the rat, as well as promoting absorption of this nutrient.

Deficiency of vitamin A, either prolonged or transient, has been shown to decrease the radioresistance of rats (Ershoff, '52; Ershoff and Greenberg, '53). However, mortality among animals fed alfalfa following vitamin A depletion was as low as that noted in nondepleted animals, so the transient deficiency of vitamin A in this series appears to have introduced no significant confounding.

There is no indication that the radioprotective mechanism differs from the increased resistance to Salmonellosis resulting from cabbage-feeding in the guinea pig.[15] Certainly, an agent that promotes resistance to bacterial infection might be expected to increase survival following irradiation where bacterial invasion plays a prominent role. However, preliminary studies by O'Dell suggest that supplementation with alfalfa does not influence resistance to Salmonella infection.[16] Either the disease - resistance and radiation - resistance agents are different or alfalfa and the Brassicaceae protect against radiation injury by different mechanisms.

Our observations suggest that the radioprotective agent may not be identical with the guinea pig growth-stimulating factors described by others (Kohler et al., '38; Cannon and Emerson, '39; Ershoff, '57)[17,18] as the radioprotective activity is retained in a fraction that does not stimulate growth, and growth stimulation occurs in the absence of a radioprotective effect with the purified diet. The distribution of the radioprotective agent appears to parallel that of the factor from lettuce or grass juice which is also water-soluble.

[15] O'Dell, B. L., D. P. Nabb and W. O. Regan 1960 Effect of cabbage on growth and Salmonellosis in guinea pigs. Abstracts, Fifth International Congress on Nutrition, Washington, D. C., p. 26.

[16] O'Dell, B. L., personal communication ('61).

[17] Briggs, G. M. 1961 An unidentified growth factor(s) from plant sources for guinea pigs — an improved assay. Federation Proc., 20: 454 (abstract).

[18] See footnote 15.

SUMMARY

1. Whole-body exposure to X-radiation or Co^{60}-gamma radiation resulted in almost total mortality within 10 to 15 days among young guinea pigs fed a basal diet of bran and oats plus ascorbic acid. In confirmation and extension of previous observations, it was shown that supplementation with alfalfa, broccoli, and other plant materials high in content of β-carotene, for two weeks before irradiation and during 20 to 30 days after irradiation, consistently reduced mortality. Other categories of foods showed marginal or no beneficial effects.

2. Supplementation of the bran-oats diet with pure vitamin A or β-carotene extended survival time but did not appreciably influence radiation-induced mortality.

3. Guinea pigs fed an adequate purified diet were more resistant to radiation injury than those given the bran-oats basal diet. Supplementation with broccoli or alfalfa did not consistently affect radiosensitivity under these conditions.

4. The beneficial effect of supplementation with raw broccoli could not be duplicated by feeding a mixture of 48 chemically-pure ingredients patterned upon the composition of broccoli, in conjunction with the bran-oats diet.

5. The radioprotective agent in alfalfa is water-soluble and is destroyed upon ashing.

LITERATURE CITED

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Glucose, Sucrose and Lactose in the Diet and Blood Lipids in Man[1]

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Previous studies from this laboratory (Keys et al., '60) showed that changes in the nature of the dietary carbohydrates produce significant changes in the concentration of blood lipids in man. Diets containing carbohydrates of fruits, leafy vegetables and legumes produced lower serum cholesterol values than those containing equal calories supplied by sucrose and milk sugar. Addition of 15 gm of pectin to the daily diet produced a small but significant decrease of serum cholesterol concentration but an equal amount of cellulose had no such effect (Keys et al., '61).

The present paper reports the results of two experiments designed to obtain information about the effects of glucose, sucrose and lactose on the serum lipid levels in man.

In the first experiment (experiment AB) the blood lipids were studied under three different dietary conditions: (1) with a standard American diet; (2) with a similar diet in which part of the fat was replaced by sucrose; and (3) with a diet containing about two-thirds of the fat in the form of corn oil.

The second experiment (experiment AC) was a comparison of the effects of sucrose, glucose and lactose on the blood lipids, as well as a comparison of these high sugar diets with a standard American diet, similar to that used in experiment AB.

GENERAL PROCEDURE

The subjects of experiment AB were 23 male schizophrenic patients, long-time residents of Hastings State Hospital and clinically normal apart from their mental illness. They ranged from 37 to 59 years of age. They were divided into 4 groups matched with respect to serum cholesterol level in a common diet, age, relative body weight and psychiatric diagnosis. Each diet was given for three weeks. The diets given to each group in each period are shown in table 1. All the men ate the "controlled house diet" in periods 1 and 6. In periods 2 and 3 two of the groups were given the sucrose and corn oil diets alternately while the other groups were eating other experimental diets. Later in periods 4 and 5 the rest of the men were given the sucrose and corn oil diets in the same switchback plan.

The same subjects in the same groups were used in experiment AC except for one replacement in group W and one addition to group X to bring the number in each group up to 6. Period 6 of experiment AB was also considered to be period 1 of experiment AC. The diets given in the various periods to each group are listed in table 1.

The controlled house diet was a standard American diet providing about 35% of total calories from mixed fats. Seven menus were used in repeated cycles. All the raw foods were weighed before cooking and each batch of cooked food was divided into the required number of approximately equal portions and served. Meat, fish or eggs were served twice daily. Milk, bread and butter and coffee or tea were in every meal. Every day there were one serving of potatoes, two other vegetables, two fruits including usually one citrus fruit and one serving of hot or cold breakfast

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TABLE 1

Diets used in experiments AB and AC

Experiment and period	Group W	Group X	Group Y	Group Z
AB-1	CHD[1]	CHD	CHD	CHD
AB-2	12 C[2]	18 C[2]	sucrose[3]	corn oil[4]
AB-3	18 C[2]	12 C[2]	corn oil	sucrose
AB-4	corn oil	sucrose	18 C[2]	12 C[2]
AB-5	sucrose	corn oil	12 C[2]	18 C[2]
AB-6; AC-1	CHD	CHD	CHD	CHD
AC-2	glucose[5]	sucrose	glucose	lactose[6]
AC-3	sucrose	glucose	lactose	glucose
AC-4	lactose	glucose	sucrose	glucose
AC-5	CHD	CHD	CHD	CHD

[1] CHD indicates controlled house diet.
[2] 12 C and 18 C: These diets contained the same basic part as the others fed in the same periods but the experimental supplement was a fat mixture in which the saturated fatty acids were either predominantly of 12 to 14 carbon atoms or of 16 to 18 carbon atoms (Grande et al., '61). The data from these diets are not considered in this report.
[3] Sucrose indicates low fat diet plus 233 gm sucrose.
[4] Corn oil indicates low fat diet plus 100 gm corn oil.
[5] Glucose indicates low fat diet plus 233 gm glucose.
[6] Lactose indicates low fat diet plus 104 gm lactose plus 129 gm glucose.

cereal. Cakes or puddings were served once or twice a day.

The low-fat diet was a mixed diet prepared from low fat foods and differing from the controlled house diet mainly by having 900 Cal. less of fat. Butter was replaced by jelly, whole milk by skim milk and ordinary bread by a fat-free bread. The visible fat was trimmed from the meat. Servings of meat were reduced in size and a stew or casserole dish was served at the noon and evening meal. Puddings and cookies without fat were used. Salads were served with sweet-sour type dressings. Fruits and vegetables were used generously.

To the basic low-fat diet, supplements of approximately 900 Cal. were added. In the case of the carbohydrates these supplements were either 233 gm of sucrose, 255 gm of glucose monhydrate[3] containing 233 gm of anhydrous glucose or a mixture of 109 gm of milk sugar and 141 gm of glucose monohydrate.[4] This last supplement was equivalent to 104 gm lactose plus 129 gm of anhydrous glucose. The corn oil[5] supplement was 100 gm. These various supplements were incorporated into the food just before serving. The sugar supplements were added to drinks like fruit juice, coffee and skim milk or used on bread as a fruit flavored spread. The oil supplement was incorporated into the cooked cereal, mashed potatoes and casserole dishes.

The subjects were weighed twice weekly in night clothing after arising and urinating, but before breakfast. If a man was gaining body weight, certain items of the standard serving were withdrawn from his diet. The most common withdrawal was bread followed in order by jelly, potatoes and fruit. In spite of these adjustments in experiment AB the men gained rather steadily, the average being a gain of 1.4 kg in the 6 periods. The man who gained most, 7.2 kg, had serum lipid changes parallel to those of the other men; hence his data were not discarded. The second highest gain was 3.5 kg. In experiment AC the average body weight change was a loss of 0.1 kg in 5 periods and the maximal change was a loss of 4.0 kg. This man's blood lipid changes were similar to those of the others and his data were retained.

Blood samples were taken before breakfast on two of the last three days of each three-week diet period and the serum was analyzed for total cholesterol. Equal volumes of serum from the two days were pooled for each man and the mixture was analyzed for lipid phosphorus and for "total fatty acids and cholesterol" by methods previously described (Keys et al., '60; Grande et al., '61). Using these

[3] Donated by Corn Products Company, Argo, Illinois, courtesy of Dr. D. M. Rathmann. The glucose was Cerelose 2001, a glucose monohydrate.
[4] See footnote 3.
[5] See footnote 3.

methods serum triglycerides were calculated from the values for total fatty acids, lipid phosphorous and total cholesterol on the assumption that 73% of the serum cholesterol was esterified.

FOOD ANALYSIS

The diets were sampled for analysis by collecting 7-day composites of one standard serving. In this way all of the 7 menus were included. The composite was stored in a deep freeze. At the end of the week the mass of about 14,000 gm was allowed to thaw, mixed and completely homogenized in a gallon-size Waring Blendor. The weight of the total homogenate was measured and samples were taken for analysis.

Water was determined by heating to constant weight in a vacuum oven at 60°C with an absolute pressure of less than 0.1 atmosphere and a stream of dry nitrogen of about 50 ml/minute. Ash was determined by heating in air at 550°C to constant weight. Protein was determined by digesting a 4-gm sample with H_2SO_4 and a catalyst in a 500-ml Kjeldahl flask, followed by distillation of the ammonia from an aliquot in a micro-Kjeldahl still, collection of the ammonia in a measured portion of standard H_2SO_4 and titration of the excess H_2SO_4 with standard NaOH solution.

The fat was extracted by a modification (called S-2M) of the method described for feces by Soderhjelm and Soderhjelm ('49) which in turn is derived from a method described by Röse (1888) for dairy products. A glass-stoppered Mojonnier extraction flask[6] made with a 25-ml lower bulb and 100-ml upper bulb was the most convenient apparatus for this method. Alternatively a Röhrig ('05) tube can be used. The 4-gm sample of homogenate was placed in the Mojonnier flask, 0.2 ml of concentrated hydrochloric acid, 13 ml of ethanol (absolute or 95%), 20 ml of ethyl ether and 20 ml of petroleum ether (any boiling point from 30 to 70°C) were added. After each addition the air was flushed out of the flask by nitrogen and the contents mixed gently. Finally the flask was inverted repeatedly for 60 seconds and then allowed to stand 10 minutes or longer until the upper layer was completely clear. Most of the upper layer was decanted into a 250-ml Erlenmeyer flask. A mixture of 5 ml of ethanol, 15 ml of ethyl ether and 15 ml of petroleum ether was added and the extraction repeated. After a third such extraction the combined lipid extracts were evaporated on a water bath at a temperature a little below the maximal boiling point of the petroleum ether with the assistance of a stream of nitrogen passing through the flask. The drying was finished by placing the flask in the vacuum oven, adjusted as described for the water determination, for 5 minutes. A vial or flask of about 20-ml capacity was prepared by heating for one hour or more at 100 to 110°C, cooling in a desiccator and weighing (including two tare vials for each set of samples). The extracted fat was dissolved in 10 ml of petroleum ether. This fat solution was heated to boiling for a moment and poured through a filter made by forcing a small wad of absorbent cotton into the top of the stem of a small funnel. The filtrate was collected in the weighed vial. Most of the petroleum ether was evaporated from this vial by placing it in a water bath with a stream of nitrogen as described above. The flask in which the extracted fat was contained was washed with petroleum ether twice more and the washings were passed through the filter in the same way. The solvent was evaporated completely and the vial and tares were carefully cleaned on the outside and heated for 15 hours in the vacuum oven adjusted as before. They were cooled in a desiccator and weighed. In general the weights of the tares were different on the two occasions, presumably due to differences in moisture absorbed on the glass surface from the room air during the weighing process. The final weight was therefore corrected by subtracting from it the mean gain of the tares. The weight of fat was taken as the difference of weights after correction.

The phosphorus of the extracted lipid (fat) was determined by dissolving it in petroleum ether (b.p. 60 to 70°C) removing an aliquot containing 1 to 10 mg of lipid, evaporating this to dryness in a hard glass test tube, adding 1 ml of 60% per-

[6] Made in the University of Minnesota glassblowing shop following the ilustration published by Soderhjelm and Soderhjelm ('49).

chloric acid, oxidizing as described by Keys et al. ('60) and developing the color due to phosphorus as described by Grande et al. ('61). Phosphorus of the lipid was reported as "phosphorus third of phospholipid" by multiplying the weight of P by 6.3. This is based on the assumption that the phospholipid calculation can be made as though it were lecithin with a molecular weight of 774 of which 196 is in the part of the molecule (the "phosphorus third") which contains the choline, phosphoric acid and one-third of the glycerol. The remainder of the lecithin molecule has practically the same composition as triglyceride and this remainder was combined with triglycerides and reported as total glycerides.

For determination of total unsaponifiable lipid, a sample of about 200 mg of lipid extract was used, usually the remainder of the portion from which an aliquot had been removed for phosphorus determination. This was transferred to a 120-ml carbonated beverage bottle. The solvent was evaporated by use of a hot water bath and a stream of nitrogen. To the bottle were added 2 ml of 33% KOH and 8 ml of ethanol. The bottle was sealed with a Kel-F[7] elastomer gasket under a metal cap and heated in an autoclave at 2 atmospheres (15 p. s. i. g.) for three hours. The solution was transferred to a Mojonnier extraction flask, water was added to make the ethanol concentration 50% and it was extracted three times with 15-ml portions of petroleum ether. After each petroleum ether portion was decanted it was washed in another Mojonnier flask by the same 25-ml portion of 0.5% NaCl in H_2O containing 1 ml of ethanol. The washed petroleum ether extracts were combined in a 50-ml Erlenmeyer flask and evaporated to dryness on a water bath using a stream of nitrogen. The flask was dried in the vacuum oven as described above for 30 minutes and weighed together with two tare flasks. A 9-ml portion of petroleum ether was added and swirled in the flask, then poured out into a volumetric flask with care to leave any visible particles behind. This washing was repeated twice with 6-ml portions. The flask and tares were dried and weighed as before. The difference in weight after correction for change in weight of the tare flasks was taken as unsaponifiable lipid. The total glycerides were calculated by subtracting the total unsaponifiable lipid and the phosphorous third of phospholipids from the fat (total lipid) of the food.

The determination of individual fatty acids by gas liquid chromotography (GLC) was done on a different sample of extracted fat from that saponified in the autoclave because this treatment caused alkali isomerization of the diene fatty acids. A sample of 200 to 500 mg was sealed in a 120-ml carbonated beverage bottle with 10 ml of methanol and 0.5 ml of concentrated sulfuric acid and was heated at 60 to 65°C overnight. After adding an equal volume of water the solution was extracted with petroleum ether. The solvent was evaporated from the extract by heating it on a water bath and passing nitrogen through the container.

The residue contained the fatty acids as methyl esters and the unsaponifiable lipids and was used for GLC. The GLC column contained 30% butanediol succinate (Craig) polyester coated on acid-washed Celite, 60 to 80 mesh. The column was 6 mm in diameter and 150 cm long and was operated at 220 to 230°C with helium. A Beckman GC - 2 apparatus was used. The areas under the peaks, measured with an integrator, were found to be proportional to the concentration of fatty acids with a maximal error of about 1% of the total.

Before adoption of the present method (S-2M) of fat extraction from homogenized mixed diet samples, a procedure was tried which included vacuum drying (first in the frozen state and later at 60°C), grinding with sand and extracting with ethyl ether in the Soxhlet apparatus. By this method more than 10% of the lipid failed to be extracted from the samples used. A few homogenized lyophilized samples were analyzed by the method described for lipid extraction from tissues by Folch et al. ('57) which starts with extraction of the aqueous slurry by a mixture of chloroform and methanol. By this

[7] Obtained as 0.040 inch sheets as Material VL-1101 M 4-849 from Vernay Laboratories, Inc., Yellow Springs, Ohio, or as 0.080 inch sheets as no. 458 black Viton from the West Company, Phoenixville, Pennsylvania.

method the weight of lipid found depended on the procedure used to purify the extracted lipid. The weights of unsaponifiable lipid and of fatty acids found by the two methods were in good agreement (within 0.02 and 0.2% of total dry matter, respectively). The results indicate that the extraction of lipids is equal by both the S-2M and the Folch methods.

A modification of the S-2M method intended to hydrolyze the starches and proteins was tried. The sample in the extraction tube was heated for 60 minutes in a boiling water bath in 7N HCl. Celite filter aid, 1.5 gm, was added, the solution was cooled in an ice bath and the liquid was drawn off through a filter stick and discarded. The solids were washed with three portions of ice water, 5 ml each. Ethanol was used to wash the filter stick, thus returning all the lipids to the extraction tube. The lipid extraction was continued as described for method S2-M. The preliminary hydrolysis with HCl did not increase the amount of lipid extracted. On the other hand, the total lipid was low (17.8 as compared to 20%) and the fatty acids extracted were correspondingly low.

To test whether all the lipid had been extracted in method S-2M the aqueous slurry remaining at the end was adjusted to contain 12 ml of water and 22 ml of ethanol. One milliliter of 50% KOH was added and the mixture was boiled under reflux for 30 minutes. It was acidified by adding 2 ml of 12N HCl, diluted with 8 ml of water and extracted three times with ethyl ether. The extracted lipids were weighed and found, in three trials, to be 0, 1, and 2% of the previously extracted lipid.

Finally, analyses were made of 54 lyophilized mixed diet samples which had been independently analyzed in the Unilever Company laboratories, Rotterdam, at the request of the Netherlands Nutrition Committee.

In this independent method the lyophilized sample was boiled for 20 minutes in 4N HCl presumably to hydrolyze and dissolve carbohydrate and protein components. It was then diluted and filtered. The insoluble matter was dried at 105°C and extracted with petroleum ether in a Soxhlet apparatus. The extracted lipids were dried and weighed. The lipid content of the 54 lyophilized diet samples ranged from 12 to 30%. The mean difference between methods in lipid found was 0.03% and the two greatest differences were 1.5 and 1.0%. Since the S-2M method gave lipid yields as high as any known method its results were accepted as correct.

RESULTS

The analyses of the various diets expressed as the average daily consumption of nutrients are shown in tables 2 and 3.

TABLE 2

Average daily consumption of calories and nutrients per man in experiment AB

	Diet[1]		
	CHD	Sucrose	Corn oil
Total Calories[2]	2624	3127	2932
Carbohydrate, gm	332	593	321
Protein, gm	94	80	82
Total lipid, gm	102	48	146
P third of phospholipid,[3] gm	1.2	0.5	0.6
Unsaponifiable lipid, gm	1.5	1.1	2.2
Total glyceride, gm	99	47	143
Calories from glycerides as % of total calories:			
Total glycerides	34	13	44
Saturated	17	6	11
Monoene	14	6	14
Polyene	3	1	19

[1] For description of diets, see footnotes to table 1.
[2] See discussion for comment on the difference in caloric intake.
[3] Phosphorus of the lipid reported as "phosphorus third of phospholipid" by multiplying the weight of P by 6.3; see text under Food Analysis section.

TABLE 3

Average daily consumption of calories and nutrients per man in experiment AC

	Diet[1]	
	CHD	Sugar diets
Total Calories	2563	2994[2]
Carbohydrate, gm	323	571
Protein, gm	93	81
Total lipid, gm	100	43
P third of phospholipid,[3] gm	1.2	1.5
Unsaponifiable lipid, gm	1.5	0.7
Total glycerides, gm	97	41
Calories from glycerides as % of total calories:		
Total glycerides	35	12
Saturated	17	5
Monoene	15	6
Polyene	3	1

[1] For description of diets, see footnotes to table 1.
[2] This figure applies to the sucrose diet. The caloric value of the glucose diet was 2949 Cal. and that of the diet containing lactose and glucose was 2974 Cal. These differences exist because anhydrous glucose has a caloric value 5% less than that of the disaccharides.
[3] See footnote 3, table 2.

The mean serum lipid values corresponding to each of the three experimental diets of experiment AB, are presented in table 4. The values for the controlled house diet were computed from the mean of the values for each subject in periods 1 and 6. The serum cholesterol level was highest with the controlled house diet, and lowest with the corn oil diet, with an intermediate value for the sucrose diet. All the cholesterol differences between the diets were highly significant. The serum phospholipid level followed the cholesterol level, except that it was relatively elevated with the sucrose diet. This is shown by the cholesterol:phospholipid ratios given in the table. The most striking difference was observed in the serum triglyceride level which was highest with the sucrose diet. All the triglyceride differences between any two of the experimental diets were highly significant.

The results of experiment AC are summarized in table 5. The serum lipid values are shown in chronological order for the different experimental periods. No distinction is made between the different sugars in this table. The mean serum cholesterol values were remarkably constant. There was a mean decrease of 33 mg/100 ml in the serum cholesterol level when the sugar diets were substituted for the con-

TABLE 4

Serum lipids in Experiment AB and the differences due to exchanging diets[1]

	No. of subjects	Diet[2]			Difference		
		CHD	Sucrose	Corn	CHD *minus* sucrose	Sucrose *minus* corn	CHD *minus* corn
Cholesterol, mg/100 ml	23	214 ± 6.4[3]	185 ± 5.7	158 ± 5.4	+29 ± 3.4 $P < 0.0001$	+27 ± 4.8 $P < 0.0001$	+56 ± 4.4 $P < 0.0001$
Phospholipids, mg/100 ml	16[4]	230 ± 7.4	219 ± 7.1	179 ± 8.3	+11 ± 5.6 $P = 0.06$	+40 ± 6.1 $P < 0.0001$	+51 ± 5.6 $P < 0.0001$
C:P ratio[5]	16[4]	0.91 ± 0.022	0.82 ± 0.020	0.87 ± 0.023	0.09 ± 0.020 $P = 0.0003$	−0.05 ± 0.018 $P = 0.008$	0.04 ± 0.018 $P = 0.04$
Triglycerides, mg/100 ml	16[4]	123 ± 13.3	173 ± 22.6	86 ± 12.2	−50 ± 15.4 $P = 0.006$	+87 ± 14.4 $P < 0.0001$	+37 ± 6.8 $P < 0.0001$

[1] The probability of chance occurrence of a mean difference as large as that observed is indicated by *P*.
[2] For description of diets see footnotes to table 1.
[3] Mean ± SE of mean.
[4] The 16 subjects chosen for these analyses were the two highest and two lowest in serum cholesterol in each of the 4 matched groups.
[5] Mean of the individual ratios of cholesterol-to-phospholipids.

TABLE 5

Serum lipids in experiment AC and the differences due to exchanging diets[1,2]

Diet[3]	Period					Mean for CHD	Mean for sugars	Difference CHD *minus* sugars	P
	1 CHD	2 Sugars	3 Sugars	4 Sugars	5 CHD				
Cholesterol,[4] mg/100 ml	213 ± 7.0	180 ± 6.0	180 ± 6.5	180 ± 5.8	213 ± 6.9	213 ± 6.4	180 ± 5.9	+33 ± 3.08	< 0.0001
Phospholipids,[5] mg/100 ml	218 ± 7.6	214 ± 9.1	211 ± 8.6	199 ± 8.1	218 ± 8.1	218 ± 7.4	208 ± 8.2	+10 ± 4.4	0.04
C:P ratio[5,6]	0.91 ± 0.025	0.81 ± 0.015	0.83 ± 0.021	0.88 ± 0.019	0.94 ± 0.026	0.93 ± 0.024	0.84 ± 0.017	0.09 ± 0.018	0.0001
Triglycerides,[4] mg/100 ml	124 ± 14.4	207 ± 21.1	200 ± 20.9	196 ± 18.7	136 ± 15.6	130 ± 14.5	201 ± 18.8	−71 ± 10.2	< 0.0001

[1] Mean ± SE.
[2] The probability of chance occurrence of a mean difference as large as that observed is given by *P*.
[3] For description of diets see footnotes to table 1.
[4] For all 24 men.
[5] Only 16 subjects were chosen for these analyses including the two highest and two lowest in serum cholesterol in each of the 4 matched groups.
[6] Mean of the individual ratios of cholesterol to phospholipids for 16 men.

trolled house diet. The cholesterol level showed no further changes for the following two sugar diet periods, and returned to the original value when the men were again fed the controlled house diet. In each sugar diet period 12 men ate the glucose diet, 6 the sucrose, and 6 the lactose diet. The average serum cholesterol difference of 33 mg/100 ml between the controlled house diet and the sugar diets was highly significant.

The serum phospholipid level showed only a small decrease of doubtful statistical significance when the men changed from the controlled house diet to the sugar diets. The lack of parallelism between the cholesterol and the phospholipid responses is shown by the highly significant decrease of the cholesterol to phospholipid ratio.

Changing from the controlled house diet to the sugar diets produced a striking increase of the serum triglycerides. A corresponding decrease was observed upon return to the house diet. The mean triglyceride difference between the house diet and the sugar diets was 71 mg/100 ml or an increase of 55% above the level with the controlled house diet.

A comparison of the effects on the blood lipids of the various sugar diets used in experiment AC is presented in table 6. There are no significant differences between the three sugar diets with respect to their effects on the serum cholesterol and phospholipids. Only the triglyceride difference between the sucrose and glucose diets (32 mg/100 ml) approaches statistical significance with a *P* value of 0.04.

DISCUSSION

Grant and Fahrenbach ('59) have reported higher serum cholesterol levels in chickens and in rabbits fed diets containing sucrose than in those fed diets containing glucose. This difference was very marked in the chicks fed a diet high in cholesterol (3%) but no difference was observed when cholesterol was not added to the diet.

Wells and Anderson ('59) reported higher cholesterol levels in rabbits fed diets containing 29% of lactose and 0.35% of cholesterol than in those fed similar diets containing sucrose. The ef-

fect of dietary lactose in the rabbit was observed only at a given level of cholesterol in the diet (0.35%) and at this level a similar cholesterol-raising effect was produced by an antibiotic or by restricting the availability of feed to two or three 1-hour periods daily (Wells et al., '62). Combinations of these treatments did not produce additive effects on serum cholesterol.

In the rat Portman and co-workers ('56) observed that sucrose, glucose and fructose as dietary carbohydrate have equal effects on the hypercholesterolemia produced by cholesterol-cholic acid-containing diets.

The results of the present experiments conform to expectations from previous experiments in this laboratory and as reviewed elsewhere (Portman and Stare, '59; Olson, '59; Olson and Vester, '60) in that serum cholesterol and phospholipids were extremely low with the corn oil diet, moderate with the sugar diets and high with the diets rich in saturated fatty acids.

No significant differences in cholesterol level were observed between the three sugar diets used in experiment AC. This indicates that the cholesterol differences reported previously (Keys et al., '60) between diets rich in starch and other complex carbohydrates and diets containing sucrose and lactose are not related to the digestion products of the complex carbohydrates because starch is eventually broken down and absorbed as glucose, and no difference was found here between glucose and the other two sugars.

Recently Wells and Anderson[8] reported a decrease of serum cholesterol in 4 young men when they changed from a lactose diet to a sucrose diet. Their lactose diet provided 160 gm of lactose/day and no switchback of the diets was made.

The most striking blood lipid change observed in these experiments was the marked increase of serum triglycerides observed with the sugar diets. In experiment AB the introduction into the diet of 261 gm of carbohydrate, of which about 233 gm was sucrose, with removal of 54 gm of dietary lipid, produced a mean increase in serum triglycerides of 50 mg/100 ml (SE $\pm$ 15.4). No clear differences between the individual sugars were observed, but the observation reported in table 6 suggests that sucrose tends to produce higher triglyceride levels than glucose.

An increase in the concentration of triglycerides in the serum has been reported by others in experiments in which the amount of fat in the diet was sharply reduced (and replaced by carbohydrate) (Hatch et al., '55; Ahrens, '57; Nichols et al., '57). Antonis and Bersohn ('61) observed a similar effect in both Bantu and European men but the triglyceride level decreased as the high carbohydrate diet was continued. After 3 to 6 months the triglyceride level was as low or lower than with the high diet fat.

Apparently there is a slow adaptation to the diet which explains the fact that men habitually consuming low-fat, high-carbohydrate diets tend to have usually low serum triglyceride levels (Antonis and Bersohn, '60). The present experiments, as well as those reported by all others except the Johannesburg group, were of too short duration to exhibit the phenomenon found by Antonis and Bersohn. But even in the present experiment AC the mean serum triglyceride level made a suggestive but not statistically significant decline from a high of 207 in the first measurements after dietary fat reduction to 200 and 196 in successive three-week periods. The calorie intake was higher with the sugar diets than with the controlled house diets, the average difference being 431 and 503 Cal./day in experiment AB and AC, respectively. Such a difference in calorie intake alone might be expected to cause an increase in the serum cholesterol level unless there was a corresponding change in calorie expenditure. In experiment AB there was an average gain of 1.4 kg in body weight over a period of 12 weeks of eating the experimental diets which suggests that some but not all of the extra calorie intake was balanced by more energy expenditure. In experiment AC there was substantially no net change in body weight, indicating that calorie equilibrium was maintained.

[8] Wells, W. W., and S. C. Anderson 1962 The effect of dietary lactose on the serum cholesterol level of human subjects. Federation Proc., 21: 100 (abstract).

TABLE 6

Serum lipids of the subjects on the various sugar diets of experiment AC and the differences due to exchanging sugars[1]

Group	No. of subjects	Diet[2]			Difference		
		Glucose	Sucrose	Lactose	Sucrose *minus* glucose	Glucose *minus* lactose	Sucrose *minus* lactose
				Cholesterol, mg/100 ml			
YZ	12	177 ± 9.7		178 ± 11.2		−1 ± 3.4	
WY	12		179 ± 11.1	174 ± 10.1			+5 ± 4.5
WX	12	180 ± 6.5	185 ± 7.9		+5 ± 3.5		
				Phospholipids, mg/100 ml			
YZ	8	206 ± 11.5		199 ± 14.2		+7 ± 8.2	
WY	8		206 ± 15.7	201 ± 13.7			+5 ± 8.5
WX	8	218 ± 12.0	227 ± 11.3		+9 ± 6.3		
				Cholesterol : phospholipid ratio			
YZ	8	0.80 ± 0.024		0.83 ± 0.031		−0.03 ± 0.025	
WY	8		0.86 ± 0.032	0.84 ± 0.035			+0.02 ± 0.024
WX	8	0.83 ± 0.024	0.81 ± 0.028		−0.02 ± 0.022		
				Triglycerides, mg/100 ml			
YZ	8	143 ± 35.5		190 ± 26.3		−47 ± 52.2	
WY	8		208 ± 27.2	212 ± 30.5			−4 ± 15.8
WX	8	206 ± 31.3	238 ± 28.9		+32 ± 12.3		

[1] Mean ± SE.
[2] For description of diets see footnotes to table 1.

Under conditions of constant calorie intake and equal expenditure it is possible to predict the average serum cholesterol response to change in the proportions of total calories provided from fatty acid glycerides in the diet (Keys et al., '57, '59). These conditions of constant calories did not prevail in the present experiments but it is interesting to calculate the serum cholesterol changes that would be predicted for calorie equilibrium. The results are given in table 7. The prediction equation agrees well with observation in the comparisons between the controlled house diet and the sugar diets, but for the corn oil diet the prediction overestimates the serum cholesterol level by 6 to 12 mg/100 ml.

SUMMARY

In dietary experiments with 24 middle-aged men, sucrose, glucose and lactose were interchanged isocalorically with each other and with corn oil in a series of three-week periods and the changes in serum lipid concentrations were observed. Replacing 233 gm daily of glucose by sucrose gave no change in serum cholesterol or phospholipids and a mean increase of triglycerides of 32 mg/100 ml which was statistically of doubtful significance ($P = 0.04$). Interchanging 104 gm of glucose and lactose gave no changes in the serum lipids. When 100 gm of corn oil was replaced by 233 gm of sucrose, serum cholesterol, phospholipids and triglycerides increased by 27, 40, and 87 mg/100 ml, respectively. The increase in serum triglycerides confirms the reports of others who have made observations in the first few weeks after beginning a low fat diet but disagrees with the observation of low serum triglyceride levels in men eating low fat diets for many months or years. The differences in serum cholesterol levels of the men between sugar diet periods and periods of eating a typical American type diet with a high content of mixed fats, were predictable from the amounts of saturated and polyene fatty acids in the diets using the regression equation of Keys et al. ('59). Relative to the other diets the corn oil diet gave a mean serum cholesterol level lower by 6 to 12 mg/100 ml than that predicted from the equation. A comprehensive method of analysis of homogenized mixed diets was described which includes modified methods for determination of total lipids and unsaponifiable lipids and yields values for individual fatty acids in the diet.

ACKNOWLEDGMENT

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TABLE 7

Comparison of observed mean serum cholesterol changes with those predicted from the equation: $\Delta Chol = 2.68\ \Delta S - 1.23\ \Delta P$[1]

Exp.	Comparison	Δ Cholesterol	
		Observed	Predicted
		mg/100 ml	*mg/100 ml*
AB	CHD *minus* sucrose	29	27
AB	Corn *minus* sucrose	−27	−9(−15 to −21)[2]
AC	CHD *minus* sugars	33	30

[1] Where Chol = cholesterol, S = saturated fatty acids, and P = polyunsaturated fatty acids (Keys et al., '59).

[2] Allowing an average cholesterol depression of 6 to 12 mg/100 ml for unsaponifiable lipid in corn oil when this oil comprises about 28% of total calories (cf. Grande et al., '58; Keys et al., '60).

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Effects of Linoleate and Dietary Fat Level on Plasma and Liver Cholesterol and Vascular Lesions of the Cholesterol-fed Rat[1]

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Linoleic esters of high purity have been shown to reduce the blood cholesterol levels of rats fed cholesterol with a diet containing a "saturated" fat such as hydrogenated coconut oil (Hauge and Nicolaysen, '59; Quackenbush and Pawlowski, '60)[3] or tallow (Peifer et al., '60). However, in each case the experiments were terminated too early to observe whether or to what extent the linoleate might influence the development of vascular lesions.

In the present study we have observed the effects of 46 to 71 weeks of linoleate feeding at different levels, on vascular condition as well as on plasma and liver cholesterol. Also included are some experiments with natural fats and high dosages of vitamins A and E. A preliminary report has been presented.[4]

MATERIALS AND METHODS

The ethyl linoleate used in these experiments was 98% pure and essentially free from trans-components. Its detailed properties have been described elsewhere (Quackenbush and Pawlowski, '60). Two basal diets were used, a high-fat (20%) diet and a low-fat (2%) diet in which the ratio of all other nutrients to calories supplied as fat and carbohydrate was constant. The high-fat diet contained the following: (in per cent) casein (ether-extracted), 18; cellulose,[5] 2.0; salts, 4.0; cholesterol (USP), 1.0;[6] sodium glycocholate, 0.5;[7] glucose,[8] 54.5; and fat, 20.0. Vitamins were added to meet the dietary needs, as described previously (Quackenbush and Pawlowski, '60). For the low-fat diet, glucose was increased to 76.5%, and all other dietary components except the steroids were divided by the factor 1.2. Additions were made to the basal diets at the expense of glucose.

Weanling rats of an inbred Wistar strain were fed the diets ad libitum. In experiments 1 and 2, the rats were individually housed in wire cages. In experiment 3 they were group-housed, 5 rats/cage. Blood was obtained in a heparinized syringe from ether-anesthetized rats by heart puncture in experiments 1 and 2 and from the tail vein in experiment 3.

The methods used in extracting liver lipids (Thompson et al., '49) and determining cholesterol (Sperry and Webb, '50) have been described previously. Total liver lipids were determined gravimetrically.

The heart and aorta were removed and fixed in 10% formalin. Frozen cross sections (20 μ) were prepared of the top 5 mm of the heart and at various levels of the aorta. Sections were stained with Sudan IV and counterstained in alum hematoxylin before examination. The term sudanophilia (S) was used to indicate the appearance of stainable lipid in the intima or media, (or both) without evidence of proliferative changes. An atheromatous change (A) was recorded for an increase in the number of endothelial or subintimal cells associated with a high content of sudanophilic material, some-

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[3] Quackenbush, F. W., and M. D. Pawlowski 1959 Linoleic ester effect on hypercholesteremia in rats. Federation Proc., *18:* 542 (abstract).

[4] Beeler, D. A., and F. W. Quackenbush 1961 Linoleate, plasma cholesterol and atheroma in the rat. Federation Proc., *20:* 96 (abstract).

[5] Cellu Flour, Chicago Dietetic Supply House, Chicago.

[6] Nutritional Biochemicals Corporation, Cleveland.

[7] See footnote 6.

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

times with a definite fibrosis in the subintimal area.

RESULTS

In a preliminary experiment, 4 groups of 5 male rats were fed the basal high fat diet (20% hydrogenated coconut oil, HCO)[9] containing 0.1, 0.2, 0.4, or 0.8% USP cholesterol and 0.5% sodium glycocholate. At the end of 4 weeks, average plasma cholesterol levels were 116, 213, 422 and 674 mg/100 ml for the respective dietary cholesterol levels. On the basis of these results a diet containing 20% HCO, 1% cholesterol and 0.5% sodium glycocholate was selected as the basal diet.

Experiment 1. Two groups, each consisting of 20 male rats and 20 or more female rats, were fed the diets indicated in table 1. A third group of 15 male and 15 female rats were fed a diet containing soybean oil (SBO)[10] as the fat. Between the tenth and thirtieth weeks of treatment a few of the rats were bled and then killed to observe progress in plasma and vascular developments. The remaining rats were then bled and killed at 31 and 40 weeks as shown in table 1. The hearts and aortas were prepared for histological examination.

Plasma cholesterol levels (table 1) were higher in females than males at all periods regardless of dietary treatment. Animals receiving thiouracil had the highest levels; those receiving SBO, the lowest levels.

The most severe vascular changes occurred in those animals receiving the thiouracil supplement. On the other hand, very little vascular change was observed in those animals receiving SBO. After 40 weeks, sudanophilia was observed in a few of the arteries examined.

Experiment 2. Nine groups of 10 male rats were fed the diets as indicated in table 2. After 46 weeks on the various regimens, the rats which remained were bled and killed. The aorta was opened longitudinally and stained with a saturated solution of Sudan IV in acetone-alcohol. The intimal surface was observed through a dissecting microscope and the percentage of the intimal surface involved in sudanophilic areas estimated. Portions of the heart and aorta were prepared for histological examinations.

High plasma cholesterol levels and substantial vascular changes were observed in rats receiving the polyunsaturated fatty acid-free basal diet (table 2). Plasma cholesterol and severity of lesions were markedly lower in rats fed either of the two levels of linoleate. High dietary levels of the vitamins A and E, fed separately or in combination, had no apparent effect on

[9] Iodine value 4.5; contributed by Procter and Gamble Company, Cincinnati, Ohio.
[10] Iodine value 125; contributed by Ralston Purina Company, Lafayette, Indiana.

TABLE 1

Effect of dietary fat, cholesterol and thiouracil on plasma cholesterol and atherosclerosis in male and female rats

Dietary treatment[1]			Sex	Weeks	Avg TPC[2]	No. of rats	% with lesions[3]	
HCO	SBO	TU					S	A
%	%	%			*mg/100 ml*			
20	0	0	M	31	557	4	75	50
			F	31	917	4	100	100
			M	40	579	7	100	100
			F	40	736	10	100	100
20	0	0.05	M	31	1158	3	100	100
			F	31	1605	5	100	100
			M	40	596	11	100	100
			F	40	957	17	100	100
0	20	0	M	31	148	2	0	0
			F	31	365	2	0	0
			M	40	196	6	33	0
			F	40	401	8	62	12

[1] HCO, hydrogenated coconut oil, I.V. 4.5; SBO, crude soybean oil, I.V. 125; TU, thiouracil. All diets contained 1% USP cholesterol and 0.5% sodium glycocholate.
[2] Average total plasma cholesterol.
[3] S, vascular sudanophilia; A, atheroma.

plasma cholesterol or atherosclerosis in this experiment.

During the experimental period, dietary consumption averaged approximately 13 and 17 gm a day/rat of the basal diet and the linoleate-supplemented diet, respectively.

In another experiment lasting 7 weeks, male rats were fed basal diets containing 20% HCO and 20% SBO, each containing 1% cholesterol and 0.5% sodium glycocholate. Vitamin A (3300 IU/rat/day) was administered orally by dropper, intraperitoneally or mixed in the diet. No effect of vitamin A on plasma cholesterol levels was observed on any of the diets or routes of administration. In our experiments with the chick, in which no glycocholate was fed, similar massive doses of vitamin A had shown a hypocholesterolemic effect (Beeler et al., '62). This effect of vitamin A was eliminated when glycocholate was fed to the chick. Perhaps vitamin A exerts its effect on cholesterol absorption, an effect which could be overcome by the powerful surface and complexing action of glycocholate.

Experiment 3. Caged male rats were fed the diets as indicated in table 3. Initially, the lower level of linoleate was 0.09 to 0.10% of the diets. At the 7th week these were increased to 0.14 to 0.15% because of poor growth in rats receiving these diets. This increased level produced satisfactory growth.

Blood was obtained from 5 animals fed each diet beginning after 4 weeks and from 5 different animals each week, until all had been bled. All animals were bled again during the 5-week period following their first bleeding. Additional blood samples were obtained at the time of sacrifice (table 3).

Plasma cholesterol reached peak values within 4 to 5 weeks in animals fed all diets. After a compensatory decline a relatively steady state was attained in about 12 weeks. The peak values observed were a function of dietary fat and linoleate levels. With high fat diets the peak values were 880 mg/100 ml and 360 mg/100 ml for dietary levels of 0.15 and 2% linoleate, respectively. With low fat diets the peak values were 490 mg/100 ml and 325 mg/100 ml for dietary levels of 0.14 and 2% linoleate, respectively. Rats receiving high fat diets tended to attain steady states at higher levels of plasma cholesterol than those receiving low fat diets and a corresponding level of linoleate. The data in table 3 indicate that a small amount of linoleate had a pronounced effect in lowering plasma cholesterol and inhibiting sudanophilic deposition over a long period in the life of the rat. Linoleate at the 2% level afforded almost complete protection from vascular degeneration during the 71-week period.

Total liver lipids and cholesterol (table 3) were significantly reduced by 2% levels

TABLE 2

Effects of linoleate, vitamin A and vitamin E fed in a 20% HCO diet on plasma cholesterol and atherosclerosis in male rats at 46 weeks

Dietary treatment[1]			Avg TPC[2]	No. of rats	% with lesions[3]		S-grade[4] of aorta
EL	V-A	V-E			S	A	
			mg/100 ml				
0	0	0	535	6	100	100	22
0	330	0	538	5	100	80	29
0	0	1.0	535	5	100	100	24
0	330	1.0	705	5	100	80	21
0.5	0	0	168	9	33	0	1
0.5	330	0	176	9	67	22	1
0.5	0	1.0	136	9	67	0	2
0.5	330	1.0	163	10	60	0	1
10.0[5]	0	0	171	10	80	0	2

[1] EL, ethyl linoleate; V-A, crystalline gelatine coated vitamin A in IU/gm of diet; V-E, *dl*-α-tocopherol in mg/gm of diet. All diets contained 1% USP cholesterol + 0.5% sodium glycocholate.
[2] Average total plasma cholesterol.
[3] S, vascular sudanophilia; A, atheroma.
[4] Sudanophilic grade expressed as average percentage of intimal surface involved.
[5] HCO was reduced to 10% to maintain the dietary fat level at 20%.

TABLE 3

Effects of linoleate level in low- and high-fat diets upon vascular lesion formation and plasma and liver cholesterol

Weeks	Avg TPC[1]	No. of rats	% with lesions[2]		Liver lipids[3]		
			S	A	TL	FC	TC
		0.14% linoleate in low-fat diet[4]					
26	260	5	60	0	9.1	2.1	28
49	191	4	50	25	12.6	2.9	43
57	165	3	100	33	10.1	4.2	39
71	149	8	100	38	12.8	4.5	46
		0.15% linoleate in high-fat diet[4]					
26	166	7	71	29	16.3	5.4	61
49	276	5	100	40	15.9	3.8	60
57	529	3	100	67	15.0	4.5	60
71	233	5	100	60	18.2	5.8	70
		2.0% linoleate in low-fat diets[4]					
26	77	5	20	0	5.4	2.1	11
49	102	4	0	0	7.2	2.5	19
57	70	3	0	0	3.3	2.1	5
71	96	10	0	0	8.1	3.8	21
		2.0% linoleate in high-fat diets[4]					
26	80	5	0	0	7.3	2.5	12
49	177	5	0	0	7.0	2.4	17
57	185	4	25	0	6.0	2.4	11
71	119	3	33	0	6.8	2.6	14

[1] Average total plasma cholesterol.
[2] S, vascular sudanophilia; A, atheroma.
[3] TL, total lipid as percentage of fresh liver; FC, free cholesterol in mg/gm; TC, total cholesterol in mg/gm.
[4] HCO was added to linoleate for a total of 2% in the low-fat diet and 20% in the high-fat diet.

of linoleate in low and high fat diets. These effects were most dramatic in animals receiving the high fat diets. Liver lipid levels changed relatively little from the 26th through the 71st week.

DISCUSSION

Although data are presented in the tables only as a summary of the percentage of animals with lesions, separate counts were made in each case for coronary arteries, ascending aorta, arch, and abdominal aorta. In almost every case the most extensive vascular changes were observed in the ascending aorta. The arch sometimes showed greater involvement than the abdominal aorta. The coronary arteries were least involved, in some cases showing comparative freedom from lesions even though the ascending aorta had undergone substantial degeneration.

A high incidence of sudanophilic lesions and other vascular changes was consistently associated with high plasma cholesterol levels. Hegsted et al. ('57) have observed that vascular sudanophilia was proportional to the plasma cholesterol level in the rat. The data in table 3 suggest that liver lipids and particularly total cholesterol in the liver may parallel vascular lesions even more closely than does plasma cholesterol. The liver accumulates large amounts of cholesterol when dietary linoleate is lacking (Quackenbush and Pawlowski, '60). Using liver cholesterol as an index, one would deduce that 0.15% linoleate is clearly a marginal level (table 3). The groups that received no dietary linoleate also showed, as early as the third week of the basal diet regimen, the typical EFA deficiency symptoms of scaliness of paws and tail along with growth retardation.

The present experiments indicate that a deficiency of polyunsaturated fat predisposes the rat to plasma cholesterol accumulation and subsequent vascular degeneration. Other workers, using diets which were not so lacking in polyunsaturation, have generally found it necessary

to include a goitrogen in the diet to produce vascular lesions (Fillios et al., '56; Wilgram, '59; Wissler et al., '54). Thomas et al. ('59) have reported the goitrogen was not essential, but its omission from the diet lowered the incidence of infarcts in rats. The deficient rats under our conditions usually develop sudanophilia within 24 weeks after cholesterol feeding is begun. During this period there is a substantial accumulation of plasma cholesterol, the maximum occurring at 4 to 5 weeks, with a subsequent falling off at a slow rate. This accumulation of plasma cholesterol to peak values at the fourth or fifth week is of more than passing interest since experimental work is often done during the first 10 weeks of cholesterol feeding.

SUMMARY

Linoleate at 0.5 and 10% levels in a 20% saturated fat diet gave good protection against the formation of atheroma in male rats over a 46-week period but did not prevent the development of some sudanophilia. A 2% level of linoleate in low and high fat diets gave similar protection for periods as long as 71 weeks. Linoleate at 0.14 and 0.15% dietary levels afforded partial protection. Soybean oil also evidenced partial protection against lesion formation. Neither vitamin A nor vitamin E at high levels showed any effect on plasma cholesterol or the development of vascular lesions.

Protective levels of linoleate were associated with low plasma cholesterol and liver lipid levels. In one experiment the level of total liver cholesterol appeared to be a better index of protection than total plasma cholesterol.

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Influence of Varying Levels of Different Dietary Proteins on Growth Rate, Liver Xanthine Oxidase and Succinic Dehydrogenase of Young Rats

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The protein and amino acid requirements of growing animals usually have been determined by the estimation of weight gain. This method is considered the most valid one, because normal growth can, in general, be correlated with increase of tissue protein, the primary constituent of body. It has been also shown, however, that the body composition differs with dietary alterations; in some cases weight gain was more dependent on deposition of body fat than on increase of body protein (Mitchell, '44; Allison, '49, '55; Beadles et al., '33). It seemed worthwhile to make an approach to this problem not only by measuring weight gain, but also by following aspects of protein metabolism in the body. For this purpose, the present authors investigated the relationship between the growth rates and several liver enzyme activities of rats receiving various amounts and different types of protein.

A correlation was demonstrated previously between the level of liver nitrogen, the rate of growth and the activities of certain liver enzymes such as xanthine oxidase and succinic dehydrogenase when the dietary casein level was varied. Both maximal growth and maximal activities of these enzymes were obtained in rats receiving a 25% casein diet (Muramatsu and Ashida, '62a). Therefore it seemed pertinent to determine whether a similar relationship between them may exist when other different kinds of dietary proteins are used. A number of reports have appeared in the literature on the lability of liver xanthine oxidase (MacQuarrie and Venosa, '45; Miller, '48, '50; Westerfeld and Richert, '49; Williams and Elvehjem, '49; Litwack et al., '50; Wainio et al., '53; see Allison, '55; Knox et al., '56) and succinic dehydrogenase or succinic oxidase (Elson, '47; Potter and Klug, '47; Benditt et al., '49; Bargoni, '51; Millman, '51; Wainio et al., '53; Prigmore et al., '55; Williams, '61) in protein depletion. Zigman and Allison ('59) and Allison et al. ('61) have reported that the growth responses of rats fed different dietary proteins of varying nitrogen intakes were correlated with the responses of ribonuclease activity of liver and some other tissues.

In the present study, the influence of various levels of different dietary protein upon the weight gain and the activities of liver xanthine oxidase and succinic dehydrogenase was investigated. The protein sources used were egg albumen, fish meal, soybean protein, wheat gluten, and casein supplemented with or without DL-methionine. It was found that when the activities of liver xanthine oxidase and succinic dehydrogenase of rats fed different dietary proteins were plotted against the dietary level of protein, there was a general correlation with the growth curves plotted in a similar way. However, the correlation was greater with xanthine oxidase than with succinic dehydrogenase.

EXPERIMENTAL

Animals and diets. Male weanling rats of the Wistar strain were fed a basal diet containing 25% of casein until they attained a body weight of 55 to 60 gm. At

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that time, the animals were divided into groups each containing 5 animals. They were housed in individual cages in an air conditioned room maintained at approximately 25°C, and received the experimental diets and water ad libitum. The experimental diets consisted of a protein-free ration, and various dietary proteins, each of which was fed at several levels. Following are the levels of the protein sources studied: (in per cent) egg albumen,[2] 5 (4.0),[3] 10 (7.8), 15 (10), 20 (14) and 30 (20); fish meal,[4] 10 (8.2), 20 (16), 25 (20), 30 (24), and 40 (31); soybean protein,[5] 10 (9.2), 20 (17), 30 (27), 40 (35), and 50 (42); wheat gluten,[6] 10 (7.4), 20 (15), 30 (21), 35 (23), and 60 (40); and casein at the following levels: 10 (8.4), 15 (12), 20 (17), and 25 (21), supplemented with 0.1, 0.2, 0.25 and zero per cent of DL-methionine, respectively. All diets contained 5% of sesame oil, 5% of cellulose powder, 4% of salts mixture, 1% of vitamin mixture (Muramatsu and Ashida, '61), and α-potato starch to make 100% each. Each rat received two drops of cod liver oil per week as a source of fat-soluble vitamins. Animals were weighed daily and were killed 15 days after being given the experimental diets.

Assay procedure. The livers were removed rapidly, weighed, and homogenized with 5 volumes of cold distilled water for one minute in a Waring Blendor at low speeds and filtered through two layers of surgical gauze. An aliquot of the filtrate was used for the enzyme assays and for

[2] Commercial product (Kewpie Company), containing 10.6% of nitrogen.
[3] The value in parentheses represents the content of protein on the basis of Kjeldahl N × 6.25(%).
[4] Defatted commercial Katsuobushi (slices of dried bonito meat), containing 12.3% of nitrogen.
[5] Defatted Koritofu (food prepared from soybean protein), containing 12.6% of nitrogen.
[6] Commercial product (Kurachi Starch Chemical Industry Company), containing 10.6% of nitrogen.

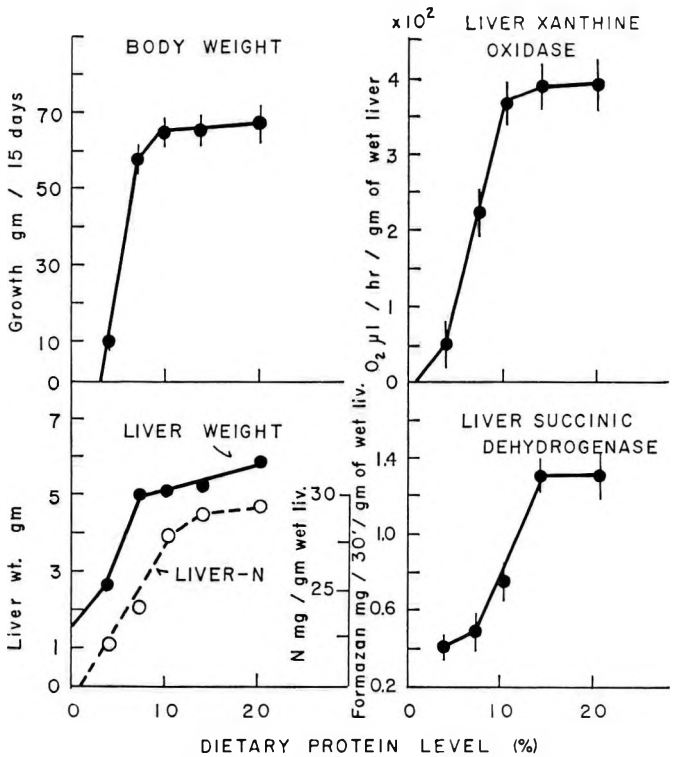

Fig. 1 Effect of various levels of egg albumen on body weight, liver weight, liver xanthine oxidase activity and liver succinic dehydrogenase activity of young rats. The protein levels are expressed on the basis of Kjeldahl N × 6.25. The vertical lines through each point represent standard errors of means.

the determination of nitrogen. The activities of liver xanthine oxidase and succinic dehydrogenase were determined as described previously (Muramatsu and Ashida, '61, '62a). The activity of xanthine oxidase was expressed as microliters of oxygen consumed per 60 minutes per gram of wet liver, and that of succinic dehydrogenase was expressed as milligrams of formazan per 30 minutes per gram of wet liver. Liver nitrogen was measured by the semimicro-Kjeldahl method and expressed as milligrams of nitrogen per gram of wet liver. The protein level in each diet was expressed on the basis of Kjeldahl N × 6.25(%).

RESULTS AND DISCUSSION

The response curves of body weight, liver weight, liver nitrogen, the activity of liver xanthine oxidase and that of liver succinic dehydrogenase in rats that received diets containing graded levels of egg albumen, fish meal, soybean protein and wheat gluten over a period of 15 days are shown in figures 1 to 4, and the protein levels required to reach each maximal value are summarized in table 1.

In the case of egg albumen (fig. 1), the maximal activity of liver xanthine oxidase was attained by feeding the 10% (based on N content)[7] protein diet, but that of succinic dehydrogenase was attained by feeding the 14% protein diet. On the other hand, the maximal levels in growth, in liver weight, and in liver nitrogen were obtained in the group fed 8 to 10% egg albumen diet. Accordingly, the inflection point of the response curve of weight gain plotted against the protein level in the diet, i. e., the protein level at which the weight gain reached the maximal value, is in agreement with that of the value of the activity of liver xanthine oxidase plotted in a similar way. These results are

[7] All values of the protein levels referred to below represent protein content on the basis of Kjeldahl N × 6.25(%).

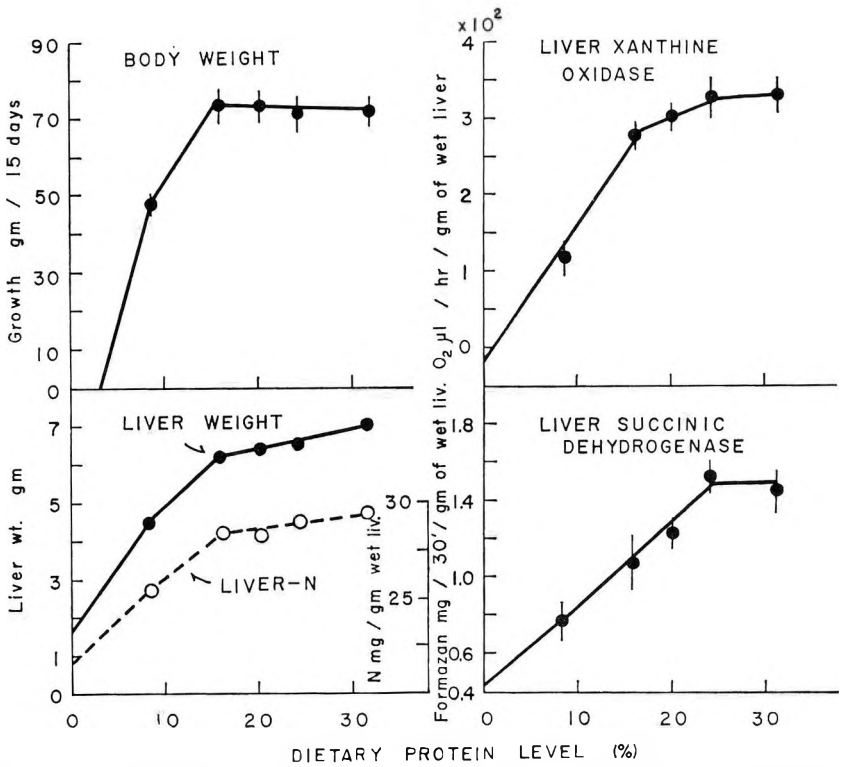

Fig. 2 Effect of various levels of fish meal on body weight, liver weight, liver xanthine oxidase activity and liver succinic dehydrogenase activity of young rats.

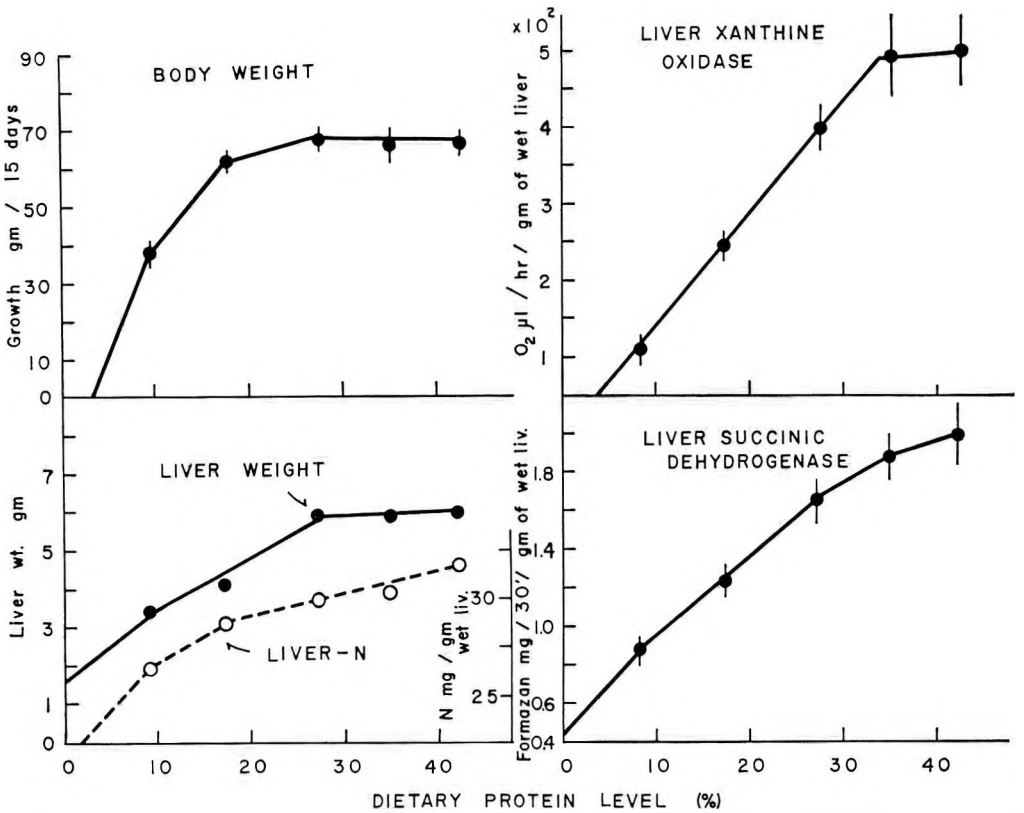

Fig. 3 Effect of various levels of soybean protein on body weight, liver weight, liver xanthine oxidase activity and liver succinic dehydrogenase activity of young rats.

TABLE 1

Minimal protein level in the diet needed for each maximal value of body weight, liver weight, liver xanthine oxidase activity and liver succinic dehydrogenase activity[1]

	Body wt	Liver wt	Liver nitrogen	Liver xanthine oxidase activity	Liver succinic dehydrogenase activity
Egg albumen	8–10	8–10	8–10	10	14
Fish meal	16	16	16	16	24
Soybean protein	17–27	27	27	35	35
Wheat gluten	—	—	—	—	—
Casein[2]	20	20	20	20	20

[1] Protein level (N × 6.25) required to reach each maximal value (%).
[2] From the data of Muramatsu and Ashida ('62a).

similar to those observed in the rats fed the casein diet reported previously (Muramatsu and Ashida, '62a).

When rats were fed diets containing varying levels of fish meal protein (fig. 2), the inflection points of the response curves of body weight, liver weight, liver nitrogen and activity of liver xanthine oxidase were observed at the 16% protein level, whereas the inflection point of the response curve of succinic dehydrogenase activity was obtained at about the 24% protein level.

In rats that received diets containing varying levels of soybean protein (fig. 3), the body weight increased as the level of soybean protein increased and the maxi-

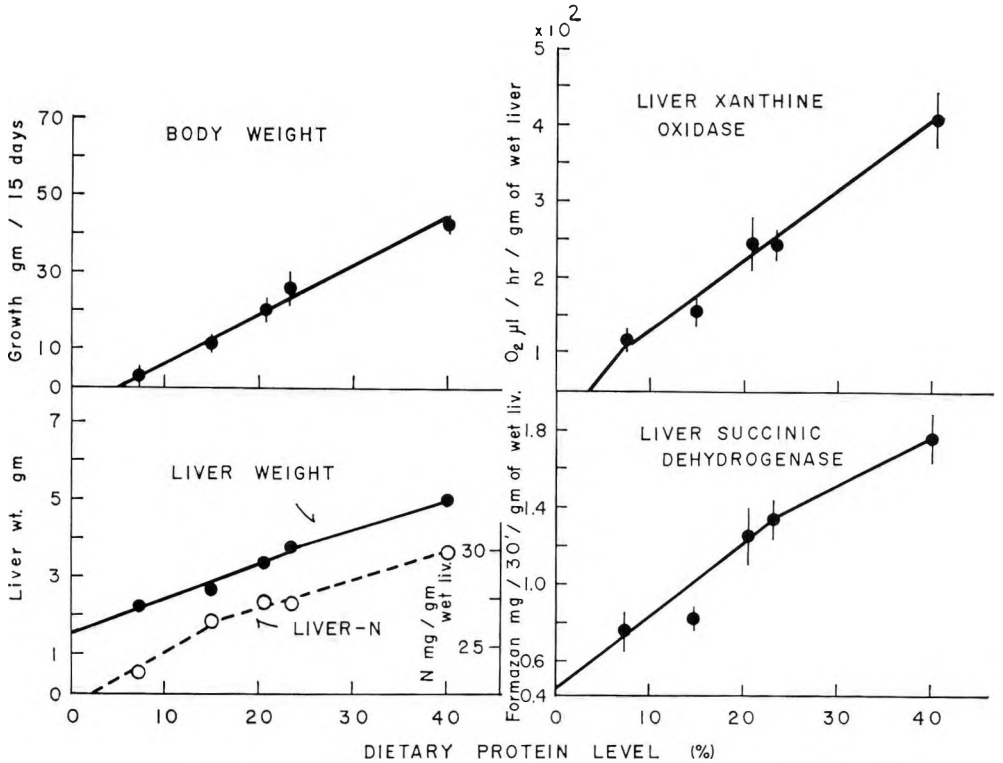

Fig. 4 Effect of various levels of wheat gluten on body weight, liver weight, liver xanthine oxidase activity and liver succinic dehydrogenase activity of young rats.

mal growth was obtained with the 17% or more soybean protein diets. The activity of liver xanthine oxidase or liver succinic dehydrogenase increased with each increase in soybean protein from zero to 35% and then showed a plateau with a further increase in level of the dietary protein. With soybean protein, however, there was no such correlation between the growth rate and liver xanthine oxidase similar to that observed with other proteins, such as casein, egg albumen or fish meal. Furthermore, the maximal activity of liver xanthine oxidase or succinic dehydrogenase obtained with soybean protein was approximately 500 or 2.0 units, respectively, which was the highest among the values obtained with all dietary proteins used in this study. The maximal activities of liver xanthine oxidase obtained with egg albumen, casein, and fish meal were approximately 400, 440 and 330 units, respectively. The maximal activities of liver succinic dehydrogenase obtained with egg albumen, casein, and fish meal were approximately 1.3, 1.3 and 1.5 units, respectively.

In rats fed increasing levels of wheat gluten (fig. 4), body weight, liver nitrogen, the activities of liver xanthine oxidase and liver succinic dehydrogenase increased in the order of the increasing protein content of the diet; accordingly the inflection points of these response curves could never be obtained even with the 40% protein diet. The activity of xanthine oxidase or succinic dehydrogenase obtained with the 40% wheat gluten diet was 400 or 1.8 units, respectively, which was equal to or above the maximal value of each enzyme in rats fed the egg albumen or casein diet, even though the maximal body weight in rats fed the former diet was still lower than in rats fed the latter diet. Whether the activity of these two enzymes observed in rats fed the wheat gluten diet represents the highest value is questionable. It is not unreasonable to assume that the

maximal values of these enzymes of rats that received a diet containing more than 40% of wheat gluten may be higher than the maximal values of these enzymes from rats receiving diets of egg albumen or casein, as observed in the case of soybean protein. Why the maximal levels of liver xanthine oxidase or succinic dehydrogenase of rats fed different dietary proteins differ from each other, what kind of physiological meaning these differences have, or whether these differences may be due to the effect of unknown substances contained in these protein preparations, are not known. Barrows ('58) and Barrows and Chow ('59) have demonstrated that the synthesis of specific tissue protein such as plasma cholinesterase in rats was stimulated by some unknown factors involved in the dietary protein preparations. Further information is needed to clarify the mechanism by which vegetable protein, such as soybean protein or wheat gluten, enhances the activities of liver xanthine oxidase and succinic dehydrogenase.

The results of this study described above lead to the suggestion that there appears to be a correlation between the growth rate and the activity of liver xanthine oxidase or succinic dehydrogenase in rats fed diets containing varying levels of different dietary proteins, except in the case of soybean protein. However, as shown in the results obtained in rats fed diets containing egg albumen or fish meal (fig. 1 and 2), the correlations of the enzyme activity with the growth rate were higher in xanthine oxidase than in succinic dehydrogenase. Namely, the minimal protein level in the diet needed for the maximal growth of rats may be correlated with that for the maximal value of liver xanthine oxidase, although not to as great a degree as for liver succinic dehydrogenase. The inflection point of the response curve of the activity of liver succinic dehydrogenase plotted against the level of dietary protein was generally shifted to a higher protein level, as compared with that of weight gain or the activity of liver xanthine oxidase. The reason for the difference in behavior of those two enzymes is obscure. The inflection points of the response curve of weight gain, liver weight, liver nitrogen and the activity of liver xanthine oxidase of rats fed diets containing various protein levels were correlated with the quality of dietary proteins examined; as the quality of protein improved, the inflection point occurred at a lower level of dietary protein.

From these results, it would be expected that if rats received casein supplemented with methionine, which is the first limiting amino acid in this protein, the inflection point of the response curve of the activity of liver xanthine oxidase, as well as that of the growth rate in rats fed this diet, may be shifted to the lower protein level as compared with that observed in rats fed diets containing casein without methionine.

The results presented in table 2 indicate that the supplementation of casein with DL-methionine shifted the inflection point of this response curve to the lower protein level, 12%; namely, in the group that received the diet containing 12% of casein with DL-methionine, the maximal values of the growth rate and the activity of xanthine oxidase were already obtained (which were equal to those of rats fed diet containing 20% casein without methionine). In this case, it was also found that the minimal protein level required for the maximal growth was in agreement

TABLE 2

Effect of supplementation of DL-*methionine to casein diets on growth rate and liver xanthine oxidase activity of young rats*

Casein level[1]	Without DL-methionine	With DL-methionine[2]
	Avg weight gain	
%	*gm/15 days*	*gm/15 days*
8	46.5 ± 1.6[3]	50.8 ± 2.9[3]
12	57.2 ± 1.8	66.8 ± 3.5
16	63.4 ± 6.0	66.4 ± 0.4
20	66.2 ± 6.3	
	Liver xanthine oxidase activity	
	O_2 µliter/ gm wet liver	*O_2 µliter/ gm wet liver*
8	208 ±33.2	196 ±30.7
12	295 ±22.2	425 ±51.6
16	355 ±34.5	383 ±26.4
20	387 ±27.4	

[1] Protein level based on Kjeldahl N × 6.25.
[2] Percentage of DL-methionine supplementation at various levels of casein: 8% casein, 0.1% DL-methionine; 12% casein, 0.2% DL-methionine; 16% casein, 0.25% DL-methionine.
[3] SE of mean.

with that for the maximal activity of liver xanthine oxidase.

Litwack et al. ('52, '53, '54) demonstrated that a correlation exists between the nutritive value of dietary protein and activity of liver xanthine oxidase in rats. The present authors have also obtained the similar results (Muramatsu and Ashida, '62b). It was considered therefore that the activity of liver xanthine oxidase could possibly be utilized as a criteria not only for the quality of dietary protein, but also for the quantity of each dietary protein required for the maximal growth of young rats. However, this procedure has a limitation, because other factors besides protein level and protein adequacy can influence for the enzyme activity, as in the case of soybean protein.

Williams ('62) carried out a time study of changes in the liver succinic oxidase system of rats during a prolonged deficiency of dietary protein. It was observed, in this study, that succinic dehydrogenase activity decreased after 10 days followed by an increase to above normal after 30 days. Accordingly, further study would be needed to determine what kind of changes in this enzyme activity may occur with time in rats fed diets containing varying levels of protein.

Westerfeld et al. ('62) reported that the activity of liver xanthine dehydrogenase of chicks increased with each increase in the dietary protein level. The results resembled those obtained with liver arginase of rats (Muramatsu and Ashida, '62a). That birds are uricotelic and rats are ureotelic animals may be an explanation for the similar results between species.

SUMMARY

Growing rats were fed diets containing varying levels of egg albumen, fish meal, soybean protein, wheat gluten and casein supplemented with or without methionine for two weeks.

The influence of these variations of dietary protein on growth rate, liver weight, liver nitrogen, the activity of liver xanthine oxidase and that of liver succinic dehydrogenase was observed.

When rats received the different dietary proteins studied, except soybean protein, it was shown that the response curve of liver xanthine oxidase or succinic dehydrogenase plotted against the protein levels in the diet was similar to those of growth rates, liver weight and liver nitrogen plotted in a similar way. The correlations of the enzyme activity with the growth rate were higher in xanthine oxidase than in succinic dehydrogenase. With soybean protein, there was no such correlation between the growth rate and the activities of these enzymes as compared with that observed with other proteins examined.

When rats were fed casein diets supplemented with methionine, the minimal protein level for the maximal growth and the maximal activity of liver xanthine oxidase was shifted from the 20% casein level to the 12% one.

Liver xanthine oxidase could be utilized as a criteria for the quantity of dietary protein required for the maximal growth of young rats. This procedure, however, has a limitation, because other factors besides protein level and protein adequacy can influence the enzyme activity, as in the case of soybean protein.

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A Study of Some of the Conditions Affecting the Rate of Excretion and Stability of Creatinine in Sheep Urine[1]

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The effects of such factors as diet, exercise, and sex upon urinary creatinine have been controversial for more than 55 years. Folin ('05) stimulated considerable interest in this subject when he pointed out that the amount of creatinine excreted in the urine by a given individual receiving a meat-free diet is a constant quantity, but that it may be different for other individuals and wholly independent of the quantitative changes in the total amount of nitrogen eliminated.

Most of the research on this subject has been conducted on man or laboratory animals and little specific information has been obtained with ruminants. Aside from marked dietary differences, there is also a considerable difference between the composition of the urine of simple-gut animals and that of ruminants which influences the stability of creatinine. Dinning et al. ('49) pointed out that the loss of creatinine from steer urine could be prevented by reducing the pH to less than 6 and by subsequent refrigeration.

Block and Schoenheimer ('39) and Block et al. ('41) using N^{15}-labeled creatine were able to demonstrate conclusively that dietary creatine can be converted into creatinine. Despite this, however, other investigations have demonstrated that the rate of urinary creatinine excretion is relatively independent of the intake of dietary creatine, as massive doses of creatine were required to increase significantly the urinary creatinine excretion of man (Folin, '05; Plimmer et al., '09; Rose and Dimmitt, '16; Chanutin, '26), dogs (Benedict and Osterberg, '23), and rabbit (Meyers and Fine, '13). The results presented by Borsook and Dubnoff ('47) which demonstrate that phosphocreatine is the immediate precursor of creatinine and those of Walker ('60) suggesting that the regulation of *in vivo* creatine synthesis is governed by the level of creatine in the muscles, help to explain these observations. On the other hand, absorbed dietary creatinine can be fully accounted for in the urine according to Maw ('48) and the experiments of other workers prove that at least 80% of the dietary dose is recovered in the urine (Meyers and Fine, '13; Rose and Dimmitt, '16; Block and Schoenheimer, '39).

The work of Dinning et al. ('49) with cattle has indicated that dietary creatinine is independent of the nitrogen content of the diet. Beard ('43) in a critical review of the metabolism of creatine and creatinine reported that urinary creatinine is highly variable and is influenced by numerous dietary factors. Such claims have often arisen when workers have included the highly variable urinary creatine excretion together with actual creatinine excretion in their estimation of creatinine outputs. Failure to consider fully the possible changes in the lean-body mass may also be involved. Thus, changes in creatinine excretion which actually reflect changes in body composition may be attributed to the effect of diet per se.

This report summarizes a series of investigations conducted to evaluate some of the conditions which may influence the rate of creatinine excretion and which would be of practical significance in experiments in which creatinine is to be used for the prediction of body composition in animals.

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[2] The data presented here constitute a part of those in the Ph.D. degree thesis presented by B. D. H. Van Niekerk to the Graduate School, Cornell University, 1962.

EXPERIMENTAL PROCEDURE

Experiments conducted. Three series of experiments were conducted: (1) In one series, the effects of the length of the storage period, pH and temperature on the rate of decomposition of creatinine in sheep urine was studied; (2) in the second series, an investigation was made of the degree of recovery from the urine of exogenous creatinine administered (a) orally, (b) via an abomasal fistula, and (c) intravenously, to sheep; and (3) in the third series, the effects of dietary level of protein and energy on the urinary creatinine excretion rate of sheep were examined.

To study the influence of length of storage period, pH, and temperature on the rate of decomposition of creatinine, a pooled sample of freshly voided urine representing 18 sheep was obtained. The creatinine concentration was determined immediately in a sub-sample of pooled urine and aliquots of the remaining urine were subjected to the following treatments: (1) Of 5 aliquots of unacidified urine kept at 27 to 30°C, toluene was added to one sample, one sample was stored in the dark, and three samples were kept under ordinary room conditions; (2) five unacidified aliquots were stored at various temperatures ranging from 4 to 39°C; and (3) six aliquots of the pooled sample were acidified to reduce the pH to 2.5 to 3.5 and stored at various temperatures ranging from zero to 39°C.

At intervals during a 5-month period of storage, the creatinine concentrations of these samples were determined to study the effects of the treatments imposed on the stability of urinary creatinine.

In the second series of experiments, the extent of recovery from the urine of exogenous creatinine administered orally to 4 sheep, via abomasal fistula to three sheep, and by intravenous (jugular vein) injection to three sheep, was determined. This was done by comparing the average daily basal creatinine outputs during a pre-treatment and a post-treatment period with the average daily excretion of creatinine during the treatment period (i.e., the period when creatinine was administered). Daily doses of approximately 1000 mg of creatinine were administered orally or abomasally in gelatin capsules. For purposes of intravenous injection creatinine was dissolved in 0.1 *N* HCl at the rate of 1000 mg/25 ml of solution. A total of 7 doses were administered to each animal during the first 7 days of 7- or 8-day urine-collection periods. The dose was administered at the beginning of each day. The last capsule (i.e., oral or abomasal administration) was introduced 48 hours before the termination of the urine collection for the treatment period, and the last injected dose was administered 24 hours before the end of urine collection for the treatment period. Each treatment period was preceded by a pre-treatment, control period generally of 7-day duration and, in all except two cases, was followed by a post-treatment period usually of 7-day duration. The urine voided by each animal during each of the pre-treatment, treatment, and post-treatment periods was collected totally, and its volume and creatinine content determined. The data obtained during the post-treatment period served to detect any possible carry-over effects attributable to the treatments and to correct for long-term changes in the basal level of creatinine excretion.

In the third series of experiments the effects on the urinary creatinine output of changing abruptly and drastically the amounts of dietary protein and energy ingested were determined. During a series of 7-day periods the following treatments were imposed: (1) A control diet of pelleted, finely ground timothy hay was fed to two sheep (observations 1 and 2, table 1) at a level estimated to provide two times the amount of energy required for maintenance, (2) a high-protein diet consisting of a mixture of pelleted alfalfa-leaf meal and linseed meal was fed to two sheep at a level computed to provide one times (observations 3 and 4, table 1) or two times (observations 5 and 6, table 1) the amount of energy required for maintenance, and (3) starvation of 5 sheep (observations 7 to 11, table 1). Each treatment period was preceded, and followed, by a 7-day control period during which the pelleted timothy-hay diet was fed at the maintenance level. Five sheep were used in this series. Observations 1, 5, and 10 were made with one animal; 2, 3, and 11, with a second animal; 4 and 8,

TABLE 1

Effect of level of intake of protein and energy and of starvation on the rate of creatinine excretion in the urine of sheep

Item of interest	Control diet		High-protein diet				Starvation				
	2 × Maintenance		Maintenance		2 × Maintenance						
Observation no.	1	2	3	4	5	6	7	8	9	10	11
Urinary creatinine, mg/day											
Pre-treatment period[1]	744	690	652	1105	711	714	1012	1100	698	698	621
Treatment period[2]	813	659	690	1183	782	830	900	1092	632	595	618
Post-treatment period[1]	711	652	640	1175	747	746	975	1067	639	667	601

[1] Control diet of pelleted timothy hay fed at the maintenance level of intake during 7-day period.
[2] Experimental treatments imposed during 7-day periods.

with a third animal; 6 and 9, with a fourth animal; and observation 7, with a fifth animal.

On the dry basis, the control diet of timothy hay contained 13% and the linseed meal-alfalfa leaf meal diet contained 22%, of protein. For a given animal, the daily intake of protein ranged from 91 gm in the pre- and post-treatment periods to 286 gm in the periods during which the high-protein diet was fed at the twice-maintenance level.

The urine voided by each animal was collected daily, compounded for each 7-day period, and the composite sample representing the entire period was analyzed for creatinine.

Collection and sampling of urine. All sheep used in the experiments described above were confined in metabolism units permitting the separate and quantitative collection of urine. The collection funnels and pans were rinsed with approximately 200 to 300 ml of water prior to collecting the urine once every 24 hours. In the second and third series of experiments, a sufficient amount of 4 *N* H_2SO_4 was added to reduce the pH to 2.5 to 3.5. A 10% aliquot of the daily urine output was compounded for each animal and period. The composite samples were stored under refrigeration at zero to 4°C. In certain instances in which it was desirable to study the day-to-day variation in the excretion of creatinine, separate daily samples were taken during 3- or 6-day periods and treated in the same way.

The treatment of the urine obtained from 18 sheep and used in the first series of experiments is detailed above.

Determination of creatinine. Creatinine was determined by means of a colorimetric method, using alkaline picrate and Lloyds reagent, as modified by Owen et al. ('54). The urine was diluted so that a final concentration of 150 to 200 μg of creatinine/100 ml were contained in the solution read. Care was taken to adjust the temperature of the alkaline creatinine picrate to 19.5°C just prior to the measurement of its optical density. The optical density of the supernatant fluid was measured with a Beckman DU model spectrophotometer at a slit width of 0.04 mm and a wavelength of 520 mμ.

RESULTS AND DISCUSSION

Stability of urinary creatinine. In the first series of experiments the concentration of creatinine in sheep urine as affected by the length of the storage period, temperature, and pH, was expressed as a percentage of the creatinine content of the urine determined immediately after collection.

The rate of loss of creatinine from urine stored at 27 to 30°C and at its normal pH (8.4 to 8.7) is shown in figure 1. These results demonstrate that the rate of decomposition of creatinine in normal sheep urine is rapid at usual summer room temperatures; within 24 hours only 86% of the original creatinine content remained in the urine, and within three weeks all traces of creatinine had disappeared. The addition to urine of toluene as a preservative or the storage of urine in the dark, both imposed at temperatures of 27 to 30°C, had no detectable effect on the rate of creatinine loss.

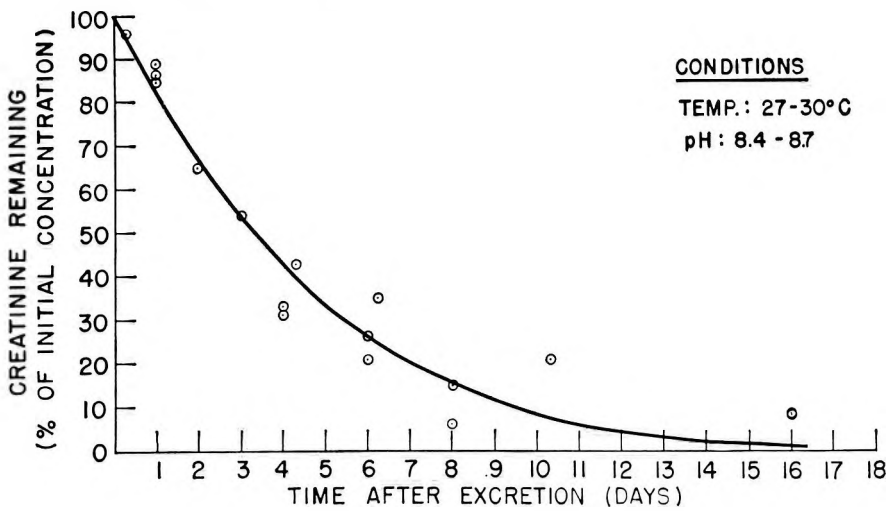

Fig. 1 The rate of loss of creatinine from sheep urine stored at 27 to 30° C at its normal pH (8.4 to 8.7).

The rate at which creatinine decays in sheep urine increases with increasing temperatures. This is demonstrated in figure 2 by the data obtained with normal urine stored up to 11 days at 4°, 15°, 28° and 39°C. The concentration of creatinine was maintained without change over 10 days in unacidified urine stored at 4°C.

Acidification of sheep urine to a pH of 2.5 to 3.5 had a marked influence on its creatinine content. The results of storing urine at this pH under temperatures of 4°, 28°, and 39°C during a 5-month period are illustrated by figure 3. No change in the creatinine content of urine stored at 4°C was detected over the 5-month period. However, at 28° and 39° C, the apparent creatinine concentration increased steadily with increasing time of storage, reaching a value within 11 days at 39° C that was 170% of the original creatinine concentration. A similar result was obtained by autoclaving acidified urine samples at 15 pounds of pressure for 30 min. This appears to be the result of the conversion of creatine to creatinine and possibly of the conversion of even other substances into Jaffé-positive chromogens.

These results are of considerable practical significance since they demonstrate that the loss of creatinine, even at usual room temperatures, can introduce a serious source of variation in experiments conducted under varying environmental temperatures. Because of the conversion of creatine into creatinine under conditions of low pH and higher ambient temperatures, it is impractical to add acid to urine collection utensils before or during the collection process as is sometimes recommended. Therefore, a study was made of the extent of the loss of creatinine from unacidified urine after 24 hours of exposure to 4 temperatures ranging from 4° to 39° C. The results of this study expressed as a percentage of the pre-treatment creatinine concentration are shown in figure 4. These data provided the basis for estimating the percentage of creatinine remaining in the urine after 24 hours by use of the following equation:

$$Y = 100 - 0.01866X^2;$$ where,

Y = percentage of creatinine remaining in unacidified sheep urine 24 hours after excretion, and

X = mean temperature in °C between 0° and 39°C.

Inter-day variation in urinary creatinine excretion. Before and during the second and third series of experiments a study was made of the day-to-day variation in the excretion of creatinine in the urine. In 9 trials of 3- or 6-day duration, the standard deviations of the daily mean outputs ranging from 674 to 1487 mg of

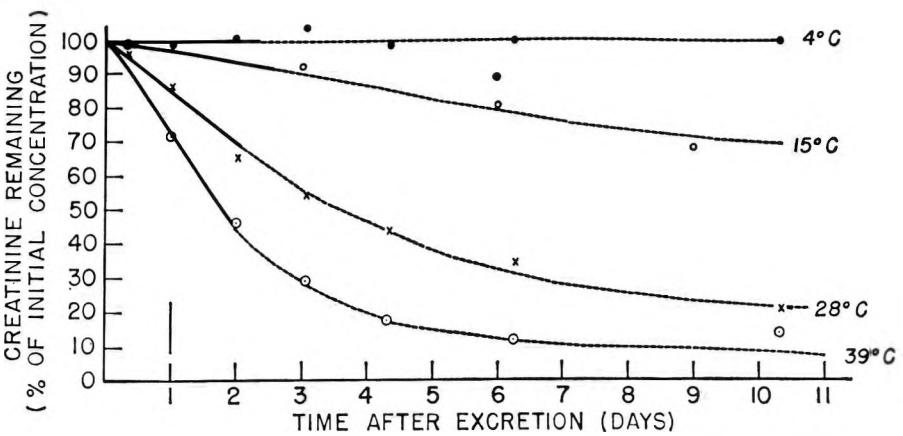

Fig. 2 The effect of temperature and storage time on the rate of creatinine loss from sheep urine stored at its normal pH (8.4 to 8.7).

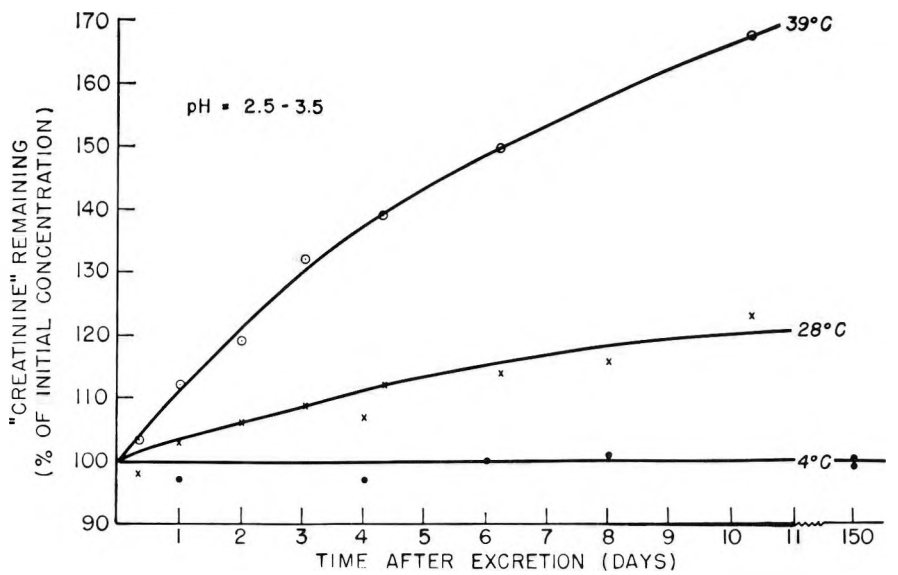

Fig. 3 The effect of temperature and storage time on the creatinine concentration of urine stored at pH 2.5 to 3.5.

creatinine ranged from 35 to 85 mg. For these data the mean inter-day coefficient of variation in urinary creatinine output was 6.3%.

Effect of administered exogenous creatinine on the urinary creatinine excretion rate. The results of administering creatinine to sheep orally, via abomasal fistula and by intravenous (jugular vein) injection are summarized in table 2. By using the average of the creatinine outputs by each sheep during the pre- and post-treatment periods as the baseline, it was found that the average recoveries of the creatinine administered by mouth, via abomasal fistula and intravenous injection were 3.5, 14.3, and 83.3%, respectively. (For sheep 3 and 4, the pre-treatment creatinine output served as the baseline.)

The apparent increase in creatinine excretion in response to oral administration lacked mathematical significance, whereas the increase effected by abomasal administration was significant ($0.02 > P >$

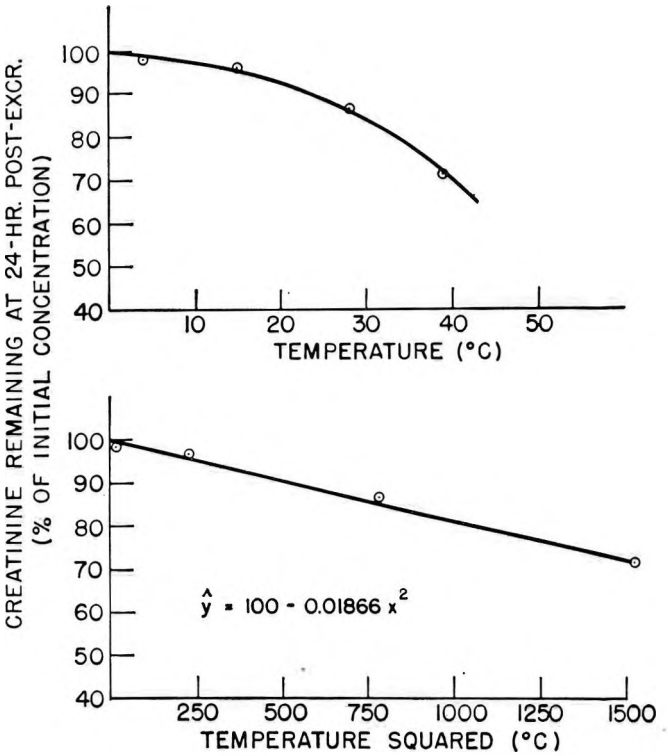

Fig. 4 Effect of temperature on the rate of decay of creatinine in sheep urine at its normal pH (8.4 to 8.7).

TABLE 2

Effect of exogenous creatinine administered orally, via abomasal fistula, and intravenously on the rate of creatinine excretion in the urine of sheep

Item of interest	Creatinine administered by:									
	Oral ingestion[1]				Abomasal fistula[2]			Intravenous injection[3]		
Observation no.	1	2	3	4	5	6	7	8	9	10
Daily dose, avg, mg/day	992	1003	1020	1009	990	1013	996	1000	1000	1000
Urinary creatinine,[4] mg/day										
Pre-treatment period	1052	747	1138	1106	818	746	1086	640	1175	675
Treatment period	1090	820	1130	1162	941	896	1186	1479	2049	1487
Post-treatment period	1105	729	—	—	714	753	1071	665	1179	700
Creatinine recovered,[5] %	1.2	8.2	−0.8	5.5	17.7	14.5	10.8	82.7	87.2	80.0

[1] Increase in creatinine excretion not significant ($P > 0.1$).
[2] Increase in creatinine excretion significant ($0.02 > P > 0.01$).
[3] Increase in creatinine excretion highly significant ($P < 0.01$).
[4] Creatinine output per day determined on composite urine samples representing 7- or 8-day periods.
[5] The creatinine recovery (%) was computed as follows:

$$\frac{UC_t - \left(\frac{UC_{p1t} + UC_{p2t}}{2}\right)}{CD} \times 100; \text{ where,}$$

UC_t = urinary creatinine (mg/day) during treatment period
UC_{p1t} = urinary creatinine (mg/day) during pre-treatment period.
UC_{p2t} = urinary creatinine (mg/day) during post-treatment period.
CD = creatinine dose (mg/day) administered during treatment period.

0.01). The creatinine output effected by intravenous administration was highly significantly ($P < 0.01$) greater than that during the control periods. No carry-over effects were detected as the result of imposing any of the treatments.

In contrast to the observations made in these studies indicating that creatinine is not absorbed to any marked degree from the gut of sheep, absorption of creatinine to the extent of 80% in nonruminants was reported by Rose and Dimmitt ('16), Block and Schoenheimer ('39) and Maw ('48). The failure to recover orally administered creatinine in the present studies appears to be attributable much less to the utilization and destruction of creatinine by microorganisms in the rumen, reticulum and omasum than to poor absorption per se. This is suggested by the observation that only 14.3% of the creatinine infused into the abomasum was recovered in the urine. In addition, the incubation of urine and creatinine solutions with washed suspensions of ruminal microorganisms at 39° C in a phosphate-buffered solution at a pH of 6.8 to 7.0 for 60 hours did not result in greater losses of creatinine than the incubation of control samples not containing microorganisms. On the other hand, creatinine incubated with crude rumen liquor was lost somewhat more rapidly. Although the limited studies made of the degradation of creatinine by ruminal microorganisms were inconclusive, the evidence obtained also indicated that bacterial action is a less important cause of the low recovery of orally administered creatinine from the urine of sheep than is poor absorption from the gut.

It is also interesting that only 83.3% of the creatinine injected intravenously was recovered in the urine. Since creatinine is not stored in the body and not excreted in any other form (Block and Schoenheimer, '39; Block et al., '41), it is probable that the creatinine not accounted for may have been excreted from the body by some other route such as the saliva, the biliary system or at some other site in the digestive tract. It is known that the salivary and sweat glands normally excrete creatinine (Spector, '56).

Effect of dietary level of protein and energy on the urinary creatinine excretion rate. During the course of the third series of experiments the abrupt increase in the intake of protein with or without an increase in energy intake imposed during 7-day periods resulted in an average increase in creatinine excretion of 9.8% more than the average daily output for the pre-treatment control period during which a pelleted, ground timothy-hay diet was ingested at the maintenance level of intake (table 1). The increased creatinine output in response to increased intake of protein was mathematically significant ($0.02 > P > 0.01$). It was found that the urinary creatinine output during the post-treatment control period immediately following the periods of high-protein intake was somewhat higher than that during the pre-treatment control period for 3 of the 4 sheep, but the difference lacked statistical significance ($0.2 > P > 0.1$).

The feeding of the control diet at the twice maintenance level did not have a consistent effect on the creatinine output of two sheep (observations 1 and 2, table 1). Also, as shown in table 1, the degree of increase in the output of creatinine by the sheep receiving the high-protein diet at the twice-maintenance level (observations 5 and 6) was not different from that of the sheep receiving the same diet at the maintenance level (observations 3 and 4). These observations indicate that the intake of energy between the maintenance and twice maintenance levels for short periods of time has very little, if any, effect on the urinary creatinine output by sheep.

The fasting of 5 sheep resulted in an output of creatinine which was 7.3% lower on the average than that during the pre-treatment period when the pelleted, ground timothy hay diet was ingested at the maintenance level (observations 7-11, table 1). This difference approached significance at the 5% level of probability. The average output during the post-treatment period immediately following the 7-day period of fasting was only 4.5% lower than that during the pre-treatment control period (table 1), but the difference was highly significant ($P < 0.01$). Fasting therefore resulted in a significant

carry-over effect which was of similar degree, but of opposite direction, to that effected by the high-protein diet.

These results demonstrate that the basal excretion of creatinine can be modified at least to a small degree by abrupt, large changes in diet imposed during brief periods of time. The carry-over effects produced by these treatments suggest that the changes in the amount of creatinine excreted are in part the reflection of changes in the composition of the lean-body mass, although this might not account fully for the changes in creatinine output. Also, the possibility exists that the results obtained in short-term experiments may be of a transient nature.

SUMMARY

Some of the factors affecting the stability and rate of excretion of urinary creatinine of sheep were studied with special reference to those conditions expected to have practical significance in investigations in which creatinine is used in the prediction of body composition in ruminants.

Creatinine was found to be unstable and to decay rapidly at the normal pH (8.4 to 8.7) of sheep urine under temperatures ranging from 15 to 39°C. However, no loss of creatinine occurred during a 5-month period when urine was stored at its normal pH but at 4°C. The storage of urine acidified to a pH of 2.5 to 3.5 and held at a temperature of 28 or 39°C resulted in a progressive increase in creatinine concentration with increasing time of storage.

The average rates of recovery from urine of creatinine administered orally, via abomasal fistula and by intravenous injection were 3.5, 14.3, and 83.3%, respectively. In contrast to nonruminants for which urinary recovery of orally administered creatinine is as high as 80%, the sheep was not found to absorb exogenous dietary creatinine to any appreciable degree. Microbial utilization or destruction of creatinine in the stomach compartments of the sheep is thus only partly responsible for the poor absorption of creatinine. Since 16.7% of the intravenously administered creatinine was not recovered in the urine, it is indicated that a significant fraction of creatinine may be voided from the body via such routes as the sweat, saliva and the digestive tract.

Abrupt, large increases in the protein intake resulted in increased outputs of urinary creatinine. Starvation resulted in a reduction in urinary creatinine. The carry-over of these effects following the return of the animals to the control diet was sufficient to indicate that the changes in creatinine output were at least partly attributable to concomitant changes in body composition.

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Effects of Rat Strain, Stilbestrol and Testosterone on the Occurrence of Hemorrhagic Diathesis in Rats Fed a Ration Containing Irradiated Beef[1,2]

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Metta et al. ('59) reported that male but not female rats died of hemorrhage when fed a ration containing irradiated beef. The difference between the sexes, the authors thought, might be due to the effect of sex hormones. A preliminary experiment in this laboratory to reproduce hemorrhagic diathesis in rats of the Holtzman strain showed their relative lack of susceptibility to hemorrhage.

In the present report the results of experiments designed to investigate the difference between two strains of rats (Holtzman and Sprague-Dawley), and the effects of stilbestrol and testosterone on the occurrence of hemorrhagic diathesis are reported.

MATERIALS AND METHODS

The diet consisted in per cent of ground nonirradiated or irradiated (5.58 megarads) beef,[3] (total solids), 35; starch 35; sucrose, 19; cod liver oil, 1.5; wheat germ oil, 0.5; salt mix, 4; and vitamin mix, 5. The salt mixture used was either salt mix 446 or USP XIV. The composition of the vitamin mixture was: (in milligrams) thiamine·HCl, 50; riboflavin, 50; pyridoxine·HCl, 50; Ca pantothenate (dextrorotatory), 400; nicotinic acid, 200; and (in grams) *p*-aminobenzoic acid, 1; inositol, 2; choline chloride, 20; and glucose,[4] 976.25. The rats were fed ad libitum fresh feed being provided three times per week in jars that had been thoroughly cleaned with detergent. Water was available at all times.

The rats were kept singly in cages with raised wire-screen bottoms at 26 to 27°C. Males were castrated at 4 weeks of age. After a recovery period of one week, the rats were allotted to treatments at random.

Two studies were undertaken. In the first, 30 weanling male Holtzman and 32 Sprague-Dawley rats were used to investigate the relative resistance to hemorrhage. The experiment was designed to compare the effect of salt mix USP 14 with that of salt mix 446, used previously by Metta et al. ('59), and to investigate variation resulting from allotting the rats to the experiment on the day of their arrival, feeding a commercial laboratory ration[5] for two days before putting them on experiment. Whether the difference was due to the use of a different strain of rat, or whether it resulted from adding vitamin K to the vitamin mix by mistake was also investigated. To find out the latter, a new batch of vitamin mix was prepared to compare with the old vitamin mixture.

Since mortality rate was not significantly affected by salt or vitamin mixes, or by the rats whether they were put on experiment on the day of arrival or later, the data of each strain were pooled and compared. Blood samples from 12 Holtz-

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[1] These studies were supported in part by contract DA-49-007-MD 72 800 with the Office of Surgeon General, Department of the Army. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of Defense. Part of this study was presented at the 44th Annual Meeting of Federation of American Societies for Experimental Biology held at Atlantic City, 1960.

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

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

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

[5] Purina Laboratory Chow, Ralston Company, St. Louis, Missouri.

man and 16 Sprague-Dawley rats were obtained by cardiac puncture for the determination of prothrombin time (Quick, '38). The rats were anesthetized with ether before taking the blood samples.

In the second study, two experiments were performed. In experiment A, 7 castrated and 7 intact male weanling Holtzman rats were allotted to each of three groups. The first group received no hormone treatment. A diethylstilbestrol dipropionate pellet[6] weighing not less than 13.6 mg and not more than 14.6 mg containing 10% binder was implanted under the skin of each rat. Each rat in the third group was implanted with a testosterone propionate pellet[7] weighing 10.3 to 16.2 mg and containing 10% binder. The skin over the pellet was massaged frequently to break any connective tissue which might have formed around the pellet, interfering with absorption of the hormone. A fourth group consisted of 7 intact Holtzman rats fed a commercial laboratory ration. The experimental period was 75 days. The feed eaten by each rat was recorded for a period of 64 days. At the end of the experiment, the rats were killed and the weights of the testes and of the seminal vesicles and prostates were recorded. Three rats from each group were bled each week to determine prothrombin times. In the beginning, pentobarbital sodium[8] only or in combination with ether were used as an anesthetic. Later, only ether was used for this purpose.

In experiment B of the second study, 8 intact and 8 castrated male weanling Sprague-Dawley rats were allotted to each of two groups. The first group, which received no hormone treatment, also contained 7 intact Holtzman rats. Each rat in the second group had been implanted subcutaneously with a diethylstilbestrol propionate pellet (13.5 to 14.8 mg). Each rat in the third group received 1 mg of testosterone[9] in 0.1 ml of corn oil subcutaneously once a week. A third group consisted of 8 intact and 10 castrated Sprague-Dawley rats and 8 intact Holtzman rats. All rats in the first three groups were fed the irradiated beef ration. A fourth group consisted of 8 Sprague-Dawley rats fed a ration containing nonirradiated beef. The experimental period was 42 days. Three rats from each group were bled for prothrombin-time determinations each week. Ether was used to anesthetize the animals before the blood samples were taken. Rats were weighed individually every week. In no case was coprophagy prevented. At the end of each experiment, the rats were killed with pentobarbital sodium and examined for signs of internal hemorrhage.

Prothrombin times longer than 20 seconds were considered to be abnormally long and suggestive of hemorrhage. Any rat dying of hemorrhage, or that died after bleeding, whose prothrombin time was longer than 20 seconds was recorded under "death due to hemorrhage." Rats that died of the anesthetic or died after bleeding, with prothrombin times less than 20 seconds, were considered to have succumbed accidentally; thus, when the mortality data were calculated, these rats were excluded from consideration. The data were subjected to arcsine (angular) transformation before statistical analysis. The mortality rate, whenever stated, pertains to the analysis after angular transformation. The statistical method was the method of least squares.

In calculating the percentages of prolonged prothrombin times, only abnormally long times (longer than 20 seconds) were considered. If a clot was not formed within two minutes, the test was stopped and prothrombin times of these samples were classified as longer than two minutes. Because the prothrombin times do not follow the normal distribution curve, the data were transformed into prothrombin rates (reciprocal of prothrombin times, sec^{-1}) for statistical analysis. In the text, the prothrombin rates are expressed as 1000/second.

Rats that died before the time of recording their final body weights were excluded in comparing the growth and feed efficiency.

RESULTS

In study one, the mortality was 58% for Sprague-Dawley rats significantly ($P <$

[6] Wick and Fry, Indianapolis, Indiana.
[7] The Upjohn Company, Kalamazoo, Michigan.
[8] Nembutal, Abbott Laboratories, North Chicago, Illinois.
[9] Delatestryl, Squibb, New York.

0.01) higher than the 22% for Holtzman rats. Four of 77 blood samples from Holtzman rats showed prothrombin times longer than 20 seconds as compared with 23 of 91 from Sprague-Dawley rats, a highly significant ($P < 0.01$) difference. The average prothrombin rates of Holtzman and Sprague-Dawley rats were 80 and 64, respectively.

To observe the effect of strain with time (fig. 1), prothrombin rates were compared only in those rats (7 from each strain) that survived and whose prothrombin rate data were complete for 6 consecutive weeks. The rates of the two strains at the third week were significantly ($P < 0.05$) different. This was also true of the averages of the prothrombin rates of approximately 12 and 16 plasma samples per week from Holtzman and Sprague-Dawley rats, respectively. A decrease in prothrombin rate at three weeks was also observed by Barnes and Fiala ('59) in rats fed a vitamin-K-free diet, but there was an additional decrease at 6 weeks.

During the first bleeding of experiment A (study 2) 5 rats (one intact, two castrated, one intact plus stilbestrol, and one castrated plus stilbestrol) died as a result of the pentobarbital sodium. During the second bleeding, one rat (castrated plus testosterone), and at the time of the third bleeding 4 rats (one castrated and three castrated plus stilbestrol) died following administration of pentobarbital sodium. The high rate of death from pentobarbital sodium was due perhaps to the decreased amount of the anesthetic required to produce the proper plane of anesthesia in the stilbestrol-treated rats.

Because of the high rate of deaths (10 of 63), a lower dose of pentobarbital sodium was used and the anesthesia was completed with ether. Later only ether was used. Only 9 rats (2%) died from anesthesia or from cardiac-puncture when ether was used. A 25% loss due to bleeding accidents in control animals has been reported by other workers (Mogenson and Jaques, '57).

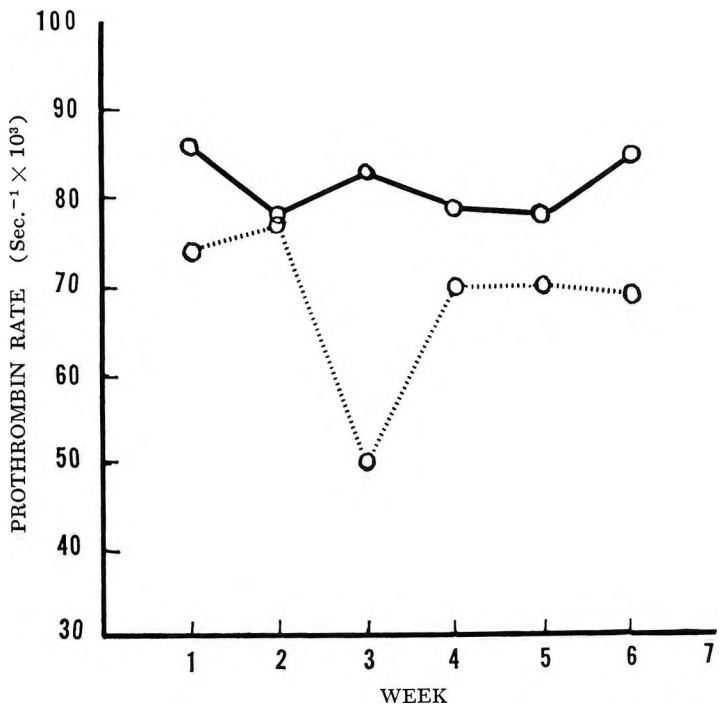

Fig. 1 Changes in the average prothrombin rates of those Holtzman and Sprague-Dawley rats whose prothrombin rates were available for the first 6 consecutive bleedings. Solid line indicates Holtzman strain rats; broken line, Sprague-Dawley strain.

The mortality due to hemorrhage in Holtzman rats was 15% and zero; of Sprague-Dawley rats, 75 and 38%, among intact and castrated rats, respectively (table 1). The decreased mortality resulting from castration was masked by treating the rats with hormones. Stilbestrol completely prevented death due to hemorrhage in both strains and the effect was highly significant ($P < 0.01$). Testosterone significantly increased ($P < 0.05$) the number of deaths in both strains of rats. Analysis of the data from all groups indicated that the Sprague-Dawley rats were significantly ($P < 0.01$) more susceptible than the Holtzman rats to death from hemorrhage. No death occurred among rats fed a commercial laboratory ration or a ration containing nonirradiated beef.

Castration increased the prothrombin rates from 83 to 89 and 75 to 79 in Holtzman and Sprague-Dawley rats, respectively (table 1), but this was not statistically significant. Castration also decreased the percentage of plasma samples with prolonged prothrombin times. There was a suggestion that stilbestrol lowered ($P < 0.05$) the prothrombin rate. Only 3% of plasma samples from stilbestrol-treated rats had prolonged prothrombin times. Testosterone reduced the prothrombin rates very significantly ($P < 0.01$) in the two strains. Testosterone caused an increase in the number of prolonged prothrombin times in the plasma samples of Holtzman rats. The Sprague-Dawley rats had consistent and significantly lower ($P < 0.05$) prothrombin rates than the Holtzman rats. The percentages of plasma samples with prolonged prothrombin times were higher in Sprague-Dawley than in Holtzman rats. The average prothrombin rate of 25 plasma samples from rats fed a commercial laboratory ration was 88 (range 70 to 104) and of 29 samples from rats fed a ration containing nonirradiated beef was 86 (range 56 to 104).

The Holtzman rats gained significantly more ($P < 0.01$) body weight in 33 days than Sprague-Dawley rats (table 2). Stilbestrol depressed the normal growth of the rats very significantly ($P < 0.01$). This effect of stilbestrol interacted significantly ($P < 0.05$) with strain, the depression being 4.45 ± 0.205 gm body weight gained/day in Holtzman as compared with 3.78 ± 0.205 gm in Sprague-Dawley rats. Castration and testosterone had no effect except that testosterone increased the body weight gained ($P < 0.05$) in the orchiectomized rats. The effect of testosterone on the occurrence of hemorrhage appeared independent of growth, there being no difference in body weight gained between the castrated group and the intact or intact-plus-testosterone groups, but the mortality rate was lower in the castrated than the other two groups.

There was no significant difference between the weight gained or feed efficiency of castrated and intact rats (table 3). Stilbestrol significantly depressed growth

TABLE 1

Effect of stilbestrol and testosterone on mortality, prothrombin rate (1000/sec) and the percentage of plasma samples with prolonged prothrombin times

Treatment		Mortality		Prothrombin time					
				Holtzman[1]			Sprague-Dawley[1]		
Rat	Hormone	Holtzman[1]	Sprague-Dawley[1]	No. samples	% Prolonged	Rate	No. samples	% Prolonged	Rate
Intact	none	2/13[2]	6/8	44	5	83±3[3]	17	24	75±4
Castrated	none	0/4	3/8	27	0	89±4	18	11	79±4
Intact	stilbestrol[4]	0/6	0/7	26	4	76±4	15	7	73±5
Castrated	stilbestrol[4]	0/2	0/8	23	0	75±4	14	0	74±5
Intact	testosterone[5]	4/14	7/8	42	21	71±3	12	17	68±5
Castrated	testosterone[5]	2/7	6/9	27	11	85±4	18	22	62±4

[1] The mortality and rate differences between Holtzman and Sprague-Dawley rats are significant at the 1 and 5% levels, respectively.
[2] No. rats died/no. rats per group, after excluding those which died of accidents.
[3] SE of mean.
[4] Stilbestrol effects on mortality and rate were significant at 1 and 5% level, respectively.
[5] Testosterone effects on mortality and rate were significant at 5 and 1% levels, respectively.

TABLE 2

Average initial body weight and the effect of hormones on body weight gained by two strains of rats

Treatment		Holtzman strain[1]			Sprague-Dawley strain[1]		
Rat	Hormone	No. rats[2]	Initial wt	Wt gain[3]	No. rats	Initial wt	Wt gain[4]
			gm	*gm*		*gm*	*gm*
Intact	none	12	109	160±4.5[5]	4	101	126± 7.8
Castrated	none	4	109	168±7.8	5	96	137± 7.0
Intact	stilbestrol[6]	6	122	18±6.4[7]	8	98	15± 5.5[7]
Castrated	stilbestrol[6]	3	114	9±9.0	8	98	15± 5.5
Intact	testosterone	10	108	167±4.9	1	102	120±15.6
Castrated	testosterone[8]	4	112	196±7.8	3	96	141± 9.0

[1] Strain effect significant ($P < 0.01$).
[2] Rats which died of accidents excluded.
[3] Weight gained for first 33 experimental days.
[4] Weight gained for first 31 experimental days.
[5] SE of mean.
[6] Stilbestrol effect significant ($P < 0.01$).
[7] Interaction between strain and stilbestrol significant ($P < 0.05$).
[8] Testosterone effect on castrated rats significant ($P < 0.05$).

TABLE 3

Effect of stilbestrol and testosterone on feed efficiency,[1] average weights of testes and of seminal vesicles plus prostate of Holtzman rats fed irradiated-beef ration for 75 days

Treatment		No. rats[2]	Avg wt gained[2]	Feed efficiency, gain/intake[2]	Testes	Seminal vesicles and prostate
Rat	Hormone					
			gm		*gm*	*gm*
Intact	none	3	287	0.181	3.37	2.16
Castrated	none	4	244	0.168	—	0.04
Intact	stilbestrol[3]	6	54	0.059	0.42[4]	0.17
Castrated	stilbestrol[3]	2	32	0.043	—	0.19
Intact	testosterone[5]	4	288	0.180	1.16[4]	3.76
Castrated	testosterone[5]	4	306[6]	0.194	—	3.90

[1] Weight gained calculated from 0 to 75 days, feed consumption from 11 to 75 days.
[2] Rats that died of accidents excluded.
[3] Stilbestrol effects on body weight gained, on feed intake, on weight of testes and of seminal vesicles plus prostate significant ($P < 0.01$).
[4] Difference between the effects of stilbestrol and testosterone on testes weights significant ($P < 0.01$).
[5] Testosterone effects on the weight of testes and of seminal vesicles plus prostate significant ($P < 0.01$).
[6] Testosterone effect on weight gained by castrated rats significant ($P < 0.05$).

($P < 0.01$). Stilbestrol reduced feed intake and significantly depressed ($P < 0.01$) feed efficiency. Whether stilbestrol reduced feed intake and affected growth indirectly or vice versa cannot be determined from these data. The effect of estrogenic substance is perhaps due to inhibition of the anterior pituitary gland (Reece et al., '39). Growth depression was induced in female rats with natural estrogens but not with stilbestrol without lowering the feed intake was reported by Meites ('49). In this study, however, the growth rate of paired control animals paralleled those of diethylstilbestrol-treated animals. Sullivan and Smith ('57) also reported that the growth rate was identical in control rats whose feed intake was restricted to the amount eaten ad libitum by male rats receiving estradiol benzoate. The data of these workers showed that voluntary restriction of feed intake was chiefly responsible for negative nitrogen balance and reduced growth in rats receiving estradiol or stilbestrol.

Testosterone significantly increased ($P < 0.05$) body weight gained by castrated rats but had no effect on feed consumption. The administration of testosterone to intact male rats had no effect on body weight gained or feed efficiency.

Stilbestrol significantly decreased ($P < 0.01$) organ weights (table 3), which indicates an interference with the development of the testes, seminal vesicles and prostate. Since the testes did not develop properly, it may be assumed that the effect of stilbestrol on seminal vesicles and

prostates was mediated by the pituitary gland. Testosterone increased ($P < 0.01$) the weights of the seminal vesicles and prostates, but decreased the weights of the testes significantly ($P < 0.01$). The average weights of testes of intact rats treated with testosterone was 1.16 gm, as compared with 0.42 gm for rats treated with stilbestrol. The difference is highly significant ($P < 0.01$). Since the stilbestrol pellet, on the average, contained 334 μmoles of diethylstilbestrol dipropionate as compared with 347 μmoles of testosterone propionate in a testosterone propionate pellet, stilbestrol was more active than testosterone in inhibiting the secretion of gonadotrophins by the pituitary. These conclusions also apply to secretion of the growth hormone by the anterior pituitary because stilbestrol, but not testosterone, had a detrimental effect on growth.

Castration interfered with the development of seminal vesicles and prostates, but its effect was masked by stilbestrol and testosterone. The effect of these hormones was significantly greater ($P < 0.01$) in castrated than in intact rats. Analysis of data correlating organ weights with body weight gains revealed that the effects of the hormones on seminal vesicles and prostates were not related to the growth of the animals, in accord with the basic concepts of endocrinology.

DISCUSSION

The results presented here indicate significantly lower resistance of Sprague-Dawley than of Holtzman rats to the factors that cause prolonged prothrombin time, hemorrhage and subsequent death. A factor might be the amount of vitamin K or other nutrients in the diets fed the mothers, thus influencing the vitamin K reserves of the pups. Richardson ('60) did not observe a significant susceptibility to hemorrhage in pups from mothers that received a diet low in vitamin K. When vitamin A was restricted in the diets of lactating females, internal hemorrhages occurred frequently in the nursing pups (Doisy et al., '60). Information obtained from the Sprague-Dawley and Holtzman companies precluded a relative lower level of vitamin A in the Sprague-Dawley diet as a factor in the lower resistance of Sprague-Dawley rats.

Holtzman rats were more susceptible to hemorrhagic diathesis than were Texas A & M rats (Richardson, '59) and Sprague-Dawley rats were more susceptible than St. Louis University strain of rat (Doisy, '61) when fed rations containing irradiated beef. The mode of action of stilbestrol in preventing hemorrhagic diathesis is not clear. Paolucci et al.[10] reported shortened prothrombin times in male rats fed a vitamin-K-deficient diet 6 hours after the injection of estrogen.

An increase of fibrinogen, prothrombin, and factor V with the increase of gestational estrogen was observed by Alexander et al. ('56). Johnson ('57) observed an increase of prothrombin and factor V, and a decrease of antithrombin following estrogen administration. Reduction of the prolonged clotting time in dogs, after exposure to radiation, was observed following estrogen administration (Michaelson, cited by Roberts, '61). No change in the various blood coagulation factors was observed in man by Borchgrevink et al., ('60), Kudish et al. ('60) or McGovern et al. ('61). Roberts ('61) cited the unpublished data of Helen Glueck who observed an increase in factor V with estrogen.

No change in prothrombin and factor V was observed up to one hour after intracardiac administration of estrogen[11] to orchiectomized rats fed commercial laboratory chow by Malhotra and Reber.[12] These data conform with the results of Mellette and Leone ('60a) in intact and castrated female rats fed a ration containing nonirradiated beef. Malhotra[13] reported that estrogen administration did not affect the prothrombin rate of rats fed a ration containing 2.79-megarad irradiated beef, but increased in those that received the 5.58-megarad irradiated beef. In the former, the rates were normal even without estrogen treatment, but were lower in

[10] Paolucci, A. M., P. B. Rama Rao and B. C. Johnson 1961 Relationship of estrogen and vitamin K in the rat. Federation Proc., 20: 452 (abstract).

[11] Premarin, Ayerst Laboratories Incorporated, New York, New York.

[12] Malhotra, O. P., and E. F. Reber (unpublished) Effect of estrogen "premarin" on prothrombin and factor V of rat.

[13] Malhotra, O. P. 1962 Hemorrhagic diathesis in rats: Effect of strain, estrogen, testosterone, vitamin K and methionine. Ph.D. thesis. University of Illinois, Urbana.

the corresponding groups of the latter, suggesting that estrogen overcame defective coagulation, but had no effect when the prothrombin rate was normal. Mellette and Leone ('60b) did not observe any appreciable effect on fibrinogen level of rats fed an irradiated-beef ration and reported that fibrinogen was not implicated in hemorrhagic diathesis.

Since testosterone did not influence body weight gain but increased mortality and prolonged the prothrombin times, it may be assumed that the effect of testosterone on the occurrence of hemorrhagic diathesis was independent of growth. Malhotra and Reber's[14] work substantiate this conclusion. Mellette and Leone ('60a) reported that testosterone decreased the factor V level in rats fed either control or irradiated-beef ration. No effect of testosterone on prothrombin levels was observed in rats receiving control diets, but there was an indication that testosterone further lowered the prothrombin level in rats fed irradiated beef.

The protective effect of estrogen and the detrimental effect of testosterone might partly be explained by the effect of these hormones on the adrenal cortex. Because the mechanism of hemostasis is complex, it may be possible that the sex hormone effects were mediated directly as well as indirectly through the adrenals.[15]

SUMMARY

Thirty Holtzman and 32 Sprague-Dawley weanling male rats were used to determine differences between strain susceptibility to hemorrhagic diathesis. Mortality was 58% for Sprague-Dawley rats, significantly higher ($P < 0.01$) than 22% for Holtzman rats. The average prothrombin rate, 50 for Sprague-Dawley rats at the end of the third week was significantly lower ($P < 0.05$) than 83 for Holtzman rats. Prothrombin rates are expressed as 1000/second. Four of 77 Holtzman and 23 of 91 Sprague-Dawley plasma samples had prolonged prothrombin times, a highly significant difference ($P < 0.01$).

Sixty-four Holtzman and 58 Sprague-Dawley rats were used to evaluate the effects of castration, stilbestrol and testosterone on the incidence of hemorrhagic diathesis. Sprague-Dawley rats were significantly less resistant ($P < 0.01$) to hemorrhagic disease than Holtzman rats. Stilbestrol completely prevented hemorrhages and subsequent deaths ($P < 0.01$), but testosterone increased both ($P < 0.05$). The effect of testosterone was independent of growth.

Sprague-Dawley rats had lower prothrombin rates ($P < 0.05$) than Holtzman rats. There was a suggestion that stilbestrol decreased the prothrombin rates ($P < 0.05$), and testosterone decreased them significantly ($P < 0.01$).

Stilbestrol decreased body weight gains ($P < 0.01$), its effect being greater on Holtzman than on Sprague-Dawley rats ($P < 0.05$). Holtzman rats grew more rapidly than Sprague-Dawley ($P < 0.01$). Stilbestrol significantly depressed ($P < 0.01$) body weight gain and feed consumption of Holtzman rats. Testosterone increased growth ($P < 0.05$) in orchiectomized rats but had no effect on feed intake.

Stilbestrol decreased the weight of seminal vesicles, prostates and testes ($P < 0.01$). Testosterone increased the weight of seminal vesicles and prostates ($P < 0.01$) but decreased the weight of testes ($P < 0.01$). The effects of sex hormones were significantly ($P < 0.01$) greater in castrated than in intact rats, and were independent of growth.

Stilbestrol was more potent than testosterone in inhibiting the secretion of gonadotrophins and somatotrophic hormones by the pituitary.

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Effect of Amino Acid Imbalance on Nitrogen Retention

II. INTERRELATIONSHIPS BETWEEN METHIONINE, VALINE, ISOLEUCINE AND THREONINE AS SUPPLEMENTS TO CORN PROTEIN FOR DOGS[1,2]

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Several investigations carried out in man (Truswell et al., '61; Scrimshaw et al., '58; Bressani et al., '58c) and animals (Mitchell et al., '32, '52; Sauberlich et al., '53; Hogan et al., '55) have indicated that corn proteins are partially deficient in both lysine and tryptophan. Reports by Sauberlich et al. ('53), Benton et al. ('55) working with rats, by Truswell et al. ('61) experimenting with adult humans and by Scrimshaw et al. ('58) and Bressani et al. ('58c) using children have indicated that corn proteins are also limited to a minor degree in isoleucine. Gillespie et al. ('58) and Sure ('53) carried out studies of threonine as a supplement to corn proteins, and, although no improvement in the nutritive value of corn of high protein content was obtained, it was suggested that threonine might be on the border line of deficiency in corn proteins.

In the studies of Scrimshaw et al. ('58) and of Bressani et al. ('58c), the amino acid pattern of the basal lime-treated corn diet was compared with that of the Food and Agricultural Organization reference pattern ('57). This comparison suggested that the basal diet was limited in the following amino acids: tryptophan, lysine, methionine, valine, isoleucine and threonine. The results indicated that the addition of sufficient amounts of lysine and tryptophan required to bring the amino acid intake up to the level called for by the reference pattern resulted in marked improvement in nitrogen retention. The addition of the indicated amount of methionine, however, brought about a decrease in nitrogen balance in every child tested. This trend apparently could be reversed by supplementation with isoleucine as a result of which retention became strongly positive. The addition of valine also appeared to have a negative effect on nitrogen retention, whereas the addition of threonine gave doubtful results; in one balance period it was negative, and in another a positive effect was apparently produced.

This report presents results of experiments made to study the above described effects of supplementation of corn proteins with isoleucine, valine, methionine, and threonine so that they would conform to the FAO amino acid reference pattern.

MATERIAL AND METHODS

Young growing mongrel dogs were used in 4 series of experiments. Three males and 4 females two months of age were used, respectively, in the first and fourth series, and 3 males and 2 female dogs four months of age were used in the second and third series, respectively.

A corn-protein basal diet contained: (in per cent) lime-treated corn (Bressani et al., '58a) 60; corn gluten,[3] 20; mineral mixture (Hegsted et al., '41) 2; hydrogenated vegetable fat, 10; L-lysine·HCl, 0.56;[4] DL-tryptophan 0.20;[5] sucrose 4.24; cod liver oil, 1.0; and glycine, 2. The diet was further supplemented with 3 ml/100 gm of a vitamin solution (Manna et al., '53). The essential amino acid content of the lime-treated corn and of the corn gluten were determined by microbiological methods

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[3] Kindly supplied by Dr. E. L. Powell, American Maize Products Company, Roby, Indiana.
[4] Kindly supplied by DuPont de Nemours, Wilmington, Delaware.
[5] See footnote 4.

TABLE 1

Amino acid composition of lime-treated corn, corn gluten and of basal diet[1]

Amino acid	Lime-treated corn	In 60 gm lime-treated corn	Corn gluten	In 20 gm corn gluten	Amino acid total	Amino acid basal diet	FAO pattern
	%	*mg*	%	*mg*	*mg*	*mg/gm N*	*mg/gm N*
Isoleucine	0.40	240	2.15	430	670	223[2]	270
Leucine	0.87	522	10.09	2018	2570	847	306
Lysine	0.26	156	1.01	202	358	119[2]	270
Methionine	0.17	150	1.08	336	486	162[2]	270
Cystine	0.08		0.60				
Phenylalanine	0.34	414	4.01	1402	1816	605	360
Tyrosine	0.35		3.01				
Tryptophan	0.048	28	0.21	42	70	23[2]	90
Valine	0.43	258	2.50	500	758	253	270
Threonine	0.28	168	2.03	406	574	191[2]	180[3]

[1] To calculate the level of amino acid to add, the difference between the level from the basal diet and that from the FAO pattern was multiplied by 3, which is the nitrogen content of the basal diet.
[2] Deficient amino acids in basal diet.
[3] Level for threonine in the FAO reference pattern was increased to 270 mg/gm N in this study.

(Bressani et al., '58b). Table 1 shows the essential amino acid content of the lime-treated corn, of the corn gluten and of the basal diet, indicating also the deficient amino acids in the basal diet used for the study. The levels of lysine and tryptophan to be used in this diet were determined by comparing the content of these amino acids in the lime-treated corn and the corn gluten with those of the FAO amino acid reference pattern. The same procedure was used in calculating the valine, methionine and isoleucine to be added to the basal diet. The amount of threonine to be added was calculated on the basis of 270 mg threonine/gm of nitrogen, rather than on the 180 mg/gm N basis of the FAO pattern. All the amino acids were added to the basal diet to replace an equal weight of sugar and the nitrogen of the amino acids replaced glycine nitrogen, making all the diets isonitrogenous and isocaloric. Corrections were made for the D- enantiomers, isoleucine, valine, and threonine, by doubling the quantity found to be limiting when compared with the FAO reference pattern. Dogs appear to utilize the DL- form of methionine as well as the L- form (Stekol, '35). The basal diet contained 18.8% crude protein.

The nitrogen balance technique was used to measure the effect of the addition of all 4 amino acids. In the first, third and fourth series of experiments, the animals were fed around 6.5 gm of protein/kg a day; in the second, the protein level was about 4.5 gm/kg a day. Each diet was tested for three consecutive 4-day periods. When the dietary treatment was changed, a two-day adjustment period was allowed.

The animals were fed at 8.00 AM and 4.00 PM throughout the experiment and were weighed daily before the first feeding. The diet, divided into two equal parts, was made into a thick gruel by adding hot water. Feces and urine were collected twice a day and stored at 4°C. Urine was collected with 1 cm^3 of concentrated acetic acid. At the end of each 4-day balance period, the feces were weighed and homogenized and the urine volume measured. Samples of both were taken for nitrogen analysis by the Kjeldahl method. A sufficient quantity of the diet was prepared for the entire dietary treatment, and three samples were analyzed for their nitrogen content, the values to serve as a basis for the nitrogen balance calculations.

RESULTS

Table 2 summarizes the nitrogen balance results observed in the first experiment consisting of 5 successive dietary treatments. At the beginning of the experiment, in which the relationships among methionine and threonine, and methionine and isoleucine were studied, the dogs weighed 3.10, 3.12 and 5.19 kg. When the dogs were fed the basal diet, the average nitrogen retention, calculated as percentage of the nitrogen intake, was 28.6%. The addition of 0.32% DL-methionine (270 mg methionine/gm N in diet) decreased

TABLE 2

Interrelationship between methionine and threonine and methionine and isoleucine as measured by nitrogen balance of dogs[1]

Diet	Order in which fed	Nitrogen				
		Intake	Fecal	Urine	Absorbed	Retained
		mg/kg/day	*mg/kg/day*	*mg/kg/day*	*% of intake*	*% of intake*
Basal	1	1111 ± 17[2]	265 ± 43	526 ± 25	76.0 ± 4.0	28.6 ± 2.6
Basal + DL-methionine	2	975 ± 10	261 ± 44	493 ± 57	73.5 ± 4.3	22.8 ± 3.5
Basal + DL-methionine + DL-threonine	3	969 ± 9	197 ± 40	463 ± 16	79.8 ± 4.0	32.0 ± 3.4
Basal + DL-methionine	4	928 ± 10	157 ± 13	541 ± 13	83.0 ± 1.4	24.7 ± 1.4
Basal + DL-methionine + DL-isoleucine	5	927 ± 4	186 ± 25	434 ± 20	79.9 ± 2.7	32.9 ± 1.9
Basal	6	787 ± 14	131 ± 12	442 ± 12	83.3 ± 1.4	27.3 ± 1.3

[1] Average, 3 balance periods of 4 days each (3 dogs).
[2] SE of mean.

TABLE 3

Interrelationship between valine and isoleucine and valine and threonine as measured by nitrogen balance in dogs[1]

Diet	Order in which fed	Nitrogen				
		Intake	Fecal	Urine	Absorbed	Retained
		mg/kg/day	*mg/kg/day*	*mg/kg/day*	*% of intake*	*% of intake*
Basal	1	713 ± 16[2]	115 ± 9	358 ± 12	83.7 ± 1.3	33.6 ± 1.7
Basal + DL-valine	2	711 ± 19	100 ± 6	423 ± 19	85.7 ± 1.0	26.3 ± 1.7
Basal + DL-valine + DL-isoleucine	3	740 ± 18	93 ± 6	384 ± 8	87.4 ± 0.6	35.5 ± 2.0
Basal + DL-valine	4	689 ± 14	98 ± 6	413 ± 11	85.7 ± 0.9	25.8 ± 0.9
Basal + DL-valine + DL-threonine	5	628 ± 43	77 ± 6	354 ± 19	87.6 ± 0.6	30.2 ± 2.4
Basal	6	534 ± 57	74 ± 7	288 ± 28	85.8 ± 0.7	31.1 ± 2.4

[1] Average, 3 balance periods of 4 days each (3 dogs).
[2] SE of mean.

the retention of nitrogen, but when 0.48% DL-threonine (270 mg threonine/gm N in diet) was added, in the presence of methionine, retention increased above the basal value.

The addition of DL-methionine alone again decreased the nitrogen retention while the addition of both DL-methionine and 0.28% DL-isoleucine (270 mg isoleucine/gm N in diet) increased the nitrogen balance to 32.9% of the nitrogen intake. A return to the basal diet fed at the end of the series resulted in a nitrogen balance of 27.3%. The changes in weight followed closely the changes and variations in nitrogen retention. The average weight gain for the three animals during this experimental period was 3.56 kg.

Table 3 shows the results of the second experiment in which the effect of valine supplementation was studied. The initial weights of the three dogs were 6.44, 7.11 and 4.14 kg. Using the basal diet, the average nitrogen retention for these animals was 33.6% of the nitrogen intake. The addition of 0.10% DL-valine (270 mg valine/gm N in diet) decreased nitrogen balance to 26.3%, and adding isoleucine in the presence of valine returned retention to approximately the basal value. Valine addition alone then decreased nitrogen balance as before, but retention was increased to 30.2% by adding threonine to valine. During the last period the basal diet gave a nitrogen balance of 31.1%. Two dogs did not consume the diet completely, and therefore, the intake of nitrogen was decreased. As in the first series, weight changes followed closely the retention of nitrogen. The animals gained an average of 2.0 kg during the entire experimental period.

Table 4 shows the results of the third series of dietary treatments. The two dogs

TABLE 4

Effect of adding threonine and isoleucine to a corn protein diet supplemented with lysine and tryptophan on nitrogen balance in dogs[1]

Diet	Order in which fed	Nitrogen				
		Intake	Fecal	Urine	Absorbed	Retained
		mg/kg/day	*mg/kg/day*	*mg/kg/day*	*% of intake*	*% of intake*
Basal	1	1011 ± 6[2]	261 ± 29	497 ± 32	74.2 ± 2.8	25.0 ± 3.6
Basal + DL-threonine	2	975 ± 13	261 ± 21	504 ± 12	73.2 ± 2.1	21.5 ± 1.7
Basal	3	947 ± 5	199 ± 20	448 ± 12	79.0 ± 2.1	31.7 ± 2.6
Basal + DL-isoleucine	4	956 ± 6	215 ± 17	489 ± 18	77.5 ± 1.8	26.3 ± 2.1

[1] Average 3 balance periods of 4 days each (2 dogs).
[2] SE of mean.

TABLE 5

Interrelationship between methionine, valine, isoleucine and threonine as measured by nitrogen balance in dogs[1]

Diet	Order in which fed	Nitrogen				
		Intake	Fecal	Urine	Absorbed	Retained
		mg/kg/day	*mg/kg/day*	*mg/kg/day*	*% of intake*	*% of intake*
Basal	1	1196 ± 47[2]	230 ± 23	669 ± 31	80.9 ± 1.7	24.9 ± 2.3
Basal + DL-methionine	2	1184 ± 48	300 ± 34	643 ± 22	75.4 ± 1.7	20.6 ± 1.6
Basal + DL-methionine + DL-valine	3	1073 ± 100	296 ± 33	578 ± 28	73.2 ± 1.8	19.1 ± 2.1
Basal + DL-methionine + DL-valine + DL-threonine	4	1022 ± 46	299 ± 41	531 ± 15	71.9 ± 2.4	18.7 ± 2.0
Basal + DL-methionine + DL-valine	5	1000 ± 33	271 ± 34	541 ± 30	73.8 ± 2.0	19.6 ± 2.7
Basal + DL-methionine + DL-valine + DL-isoleucine	6	1052 ± 40	223 ± 25	527 ± 20	79.4 ± 1.5	29.1 ± 1.4
Basal + DL-methionine + DL-valine	7	963 ± 33	229 ± 24	496 ± 14	76.7 ± 1.6	24.9 ± 1.6
Basal + DL-methionine + DL-valine + DL-isoleucine + DL-threonine	8	988 ± 30	213 ± 20	455 ± 10	78.6 ± 1.4	32.3 ± 1.0

[1] Average, three balance periods of 4 days each (4 dogs).
[2] SE of mean.

weighed 5.42 and 4.64 kg, respectively, at the start of the experiment. The addition of threonine or of isoleucine to the basal corn protein diet supplemented with lysine and tryptophan, decreased nitrogen balance. The animals gained an average of 1.73 kg in 48 days.

The results from the fourth experiment shown in table 5 combined the procedures of previous series. The intial weights of the animals were 4.17, 3.57, 3.06 and 2.05 kg. With the basal diet the animals retained an average of 24.9% of the nitrogen intake. When DL-methionine was added, nitrogen retention decreased and the addition of DL-valine in the presence of methionine further decreased nitrogen retention to 19.1%. Threonine addition or omission in the presence of methionine and valine had no effect. The addition of isoleucine in the presence of valine and methionine increased nitrogen retention to 29.1% of the nitrogen intake. Methionine and valine addition again decreased nitrogen retention, although not to a marked degree. When all 4 amino acids were added, nitrogen retention reached the highest value of the series. The 4 animals gained an average of 6 kg during the experimental period. The weight gains with each dietary treatment followed closely the changes in nitrogen balance.

DISCUSSION

The results presented in this paper agree with those obtained by Scrimshaw et al. ('58) and Bressani et al. ('58c) in experiments with children fed lime-treated corn protein diets. As with the children, methionine addition to the basal diet, which contained the levels for lysine and tryptophan in the FAO reference pattern, decreased nitrogen retention. Valine, which lowered nitrogen balance in the present experiments with dogs, gave similar results in the previous studies in children. The decrease in nitrogen retention resulting from the single addition of both methionine and valine indicates that other amino acids are more limiting in the corn basal diet. Apparently, the protein in the diet used in this study already contained adequate amounts of both methionine and valine, making supplementation with them unnecessary. Their addition may have caused an amino acid imbalance (Harper, '57–'58). The same appears to be true also for isoleucine and threonine, although the decreasing effect of these two amino acids was not as large, as with methionine and valine. Similar observations for isoleucine and threonine using growth tests with rats were reported by Rosenberg et al. ('60).

The results indicate that both isoleucine and threonine are capable of counteracting the adverse effects of excessive amounts of either methionine or valine. Isoleucine was the more effective, since it also corrected the imbalance caused by the addition of methionine and valine together, whereas threonine increased nitrogen retention of only one of these amino acids at a time. In general, threonine appeared to be more effective in correcting the effect of excess methionine than that of added valine; isoleucine was equally effective with either.

Although no direct metabolic relationship between threonine and methionine has been identified, Harper ('57–'58) has reported amino acid imbalances involving threonine when using low casein diets which were consequently low in methionine. Benton et al. ('56) reported amino acid imbalances due to excessive leucine or valine. Corn protein is known to have an unusually high ratio of leucine to the structurally related isoleucine; and it has been suggested that this increases the isoleucine required for the optimal growth of rats (Sauberlich et al., '53; Benton et al., '56; Harper et al., '55). The present results may explain the reason that corn protein has sometimes been reported to be limiting in threonine (Sure, '53; Rosenberg et al., '60). Although corn protein is not deficient in either of these two amino acids, some samples of corn may contain larger amounts of leucine, methionine and valine, and require more isoleucine and threonine to improve their nutritive value.

When the levels of amino acids in the protein of the basal diet are compared with those of the same amino acids in the FAO pattern, the order of limitation after lysine and tryptophan is: methionine, isoleucine, valine and threonine. The results of this study indicate, however, that isoleucine is the first limiting amino acid once the lysine and tryptophan needs are supplied, followed by threonine, methione and valine. This suggests that the proportions among the 4 amino acids are probably satisfactory as an increase in nitrogen retention occurred when all 4 were added simultaneously in the amounts of 270 mg of each amino acid/gm N in the diet.

SUMMARY

Comparison of the amino acid pattern of a corn protein diet with that of the amino acid pattern of the FAO reference protein indicated that, after lysine and tryptophan, methionine, isoleucine, valine and threonine were limiting in this order. The results of the present study show that the addition of methionine and valine to give corn protein the proportions of these amino acids contained in the FAO reference protein decreases nitrogen retention, in dogs when lysine and tryptophan have already been added. The adverse effect of methionine and valine, added singly or in combination, can be corrected by using the FAO indicated levels of isoleucine, whereas 270 mg of threonine/gm of nitrogen in the diet is capable of counteracting only either one of these amino acids added singly.

These results indicate that contrary to the conclusion from comparison with the

FAO reference protein, the order of the limiting amino acids in a corn protein diet, after lysine and tryptophan, is: isoleucine, threonine, methionine, and valine. Nevertheless, the simultaneous addition of all 4 amino acids to the diet supplemented with lysine and tryptophan gave higher nitrogen retention values than those observed with lysine and tryptophan supplementation alone, suggesting that the proportions of the 4 amino acids are satisfactory, when added in the amounts of 270 mg of each amino acid/gm of nitrogen in the diet.

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