INHERITED METABOLIC PATTERNS IN MICE

CALORIC REQUIREMENTS FOR PROTEIN UTILIZATION AND DETERMINATION OF PROTEIN MINIMA [1]

PAUL F. FENTON AND JOHN M. MARSH
Department of Biology, Brown University, Providence, R. I.

(Received for publication July 8, 1956)

The feeding of a synthetic ration containing 50% fat results in marked obesity in two of 4 strains of mice studied in this laboratory, A/Fn and C3H/Fn (Fenton and Dowling, '53). Another strain, C57BL/Fn, becomes moderately obese, while the I/Fn strain remains lean. The deposition of excess fat is accompanied by an increase in the fat-free component — protein, ash and water (Fenton, '56). This observation suggests that the obesity-susceptible strains may be characterized not only by excessive fat deposition but also by greater rates of protein synthesis or lower rates of protein degradation. With these possibilities in mind we undertook some studies of the nitrogen metabolism of our 4 strains of mice. Fenton and Carr ('51) showed differences in growth rates when rations of low protein content were fed. The present study represents a more detailed analysis of some genetically determined differences in protein metabolism. Making use of a 5% casein diet we have measured the growth of 4 strains under conditions of ad libitum feeding and of restricted food intake. Furthermore, we have undertaken nitrogen balance studies in which the nitrogen intake was rigidly controlled.

EXPERIMENTAL

Weanling mice of our 4 strains (C57BL/Fn, C3H/Fn, A/Fn and I/Fn) received diet 287 (table 1) containing 5% casein.

[1] Supported by grant C-1995 from the National Cancer Institute and by a grant from the Anna Fuller Fund.

Copyright 1956
The Wistar Institute of Anatomy and Biology
All rights reserved

Food intake and weight gain were measured for a period of 20 days. As the experimental situation demanded, this ration was supplied either ad libitum or in restricted quantities. In one restriction experiment a nitrogen-free supplement (298, table 1) was offered ad libitum. All the animals were housed individually in screen-bottom metal cages maintained in an air-conditioned animal room.

TABLE 1

Percentage composition of diets

INGREDIENT	DIET			
	287 [1]	292 [1]	298	311 [1]
Casein [2]	5	5	..	..
Sucrose	..	..	45	..
Dextrin	85	..	..	70
Starch	..	85	..	..
Salts A [1]	5	5	5	..
Salts HMW [3]	..	..	..	5
Corn oil	5	5	5	5
Hydrogenated vegetable oil [4]	..	..	45	20

[1] Vitamin supplement and composition of Salts A as reported by Fenton and Carr ('51).

[2] Labco, vitamin-free.

[3] Hubbell, Mendel and Wakeman ('37).

[4] Crisco.

The protein minima studies followed the principles outlined by Melnick and Cowgill ('37). Adult mice of the A/Fn and I/Fn strains were maintained in individual metabolism cages. These animals had been reared to age 6 months on a commercial stock ration.[2] The mice were fed a nitrogen-free diet (311, table 1) ad libitum. Urine collections were made during the third and 4th days on this diet. During the following three days 5 mg of nitrogen were injected intraperitoneally daily in the form of an aqueous solution of enzymatic casein hydrolysate. During a second and third three-day period the amount of nitrogen was increased to 10 and 15 mg per day respectively.

[2] Purina Laboratory Chow.

The amount of nitrogen administered as well as the urinary nitrogen were determined by the micro-Kjeldahl procedure of Ballentine and Gregg ('47). Since the nitrogen was administered intraperitoneally, fecal nitrogen was determined on a separate small group of mice and the average, 3.5 mg/day, was used in the calculation of nitrogen balance.

RESULTS

Under conditions of ad libitum feeding the C57BL and I strains consumed the smallest quantity of food, 43 and 42 gm respectively during a 20-day period. The A strain consumed

TABLE 2

Protein efficiency of 4 strains of mice

STRAIN	NO. OF ANIMALS	FOOD INTAKE 287	FOOD INTAKE 298	WEANING WEIGHT	WEIGHT GAIN	PROTEIN EFFICIENCY
Diet 287 ad libitum						
		gm	*gm*	*gm*	*gm*	
C57BL	12	43.0 ± 1.0 [1]	..	10.0	1.2	0.54
I	8	42.0 ± 1.8	..	9.7	0.8	0.35
C3H	8	61.5 ± 1.4	..	10.5	3.5	1.13
A	12	51.9 ± 1.4	..	9.8	1.2	0.46
Diet 287 restricted						
C3H	9	43.2 ± 0.4	..	10.2	— 0.7	...
A	8	42.8 ± 0.4	..	10.2	— 0.9	...
Diet 287 restricted; diet 298 ad libitum						
C3H	5	43.0 ± 0.1	5.3	10.7	0.6	0.28
A	12	42.2 ± 0.5	4.4	10.2	0.3	0.14

[1] Standard error.

over 20% more during this same interval while the C3H consumed almost 50% more. The efficiency of protein utilization for growth (grams weight gained/grams protein consumed) was more than twice as great with C3H mice as it was with the next most efficient strain, the C57BL (table 2).

When mice of the C3H and A strains were restricted in food intake to the level voluntarily consumed by C57BL mice

(43 gm/20 days), they actually lost weight. When other mice of the C3H and A strains were similarly restricted but were allowed in addition unlimited quantities of the nitrogen-free ration 298, they again showed positive weight gains. We have omitted data of those animals which did not completely consume the 43 gm of diet 287. The sum of diet 287 and diet 298 consumed did not equal the quantity of diet 287 consumed under conditions of ad libitum feeding (table 2).

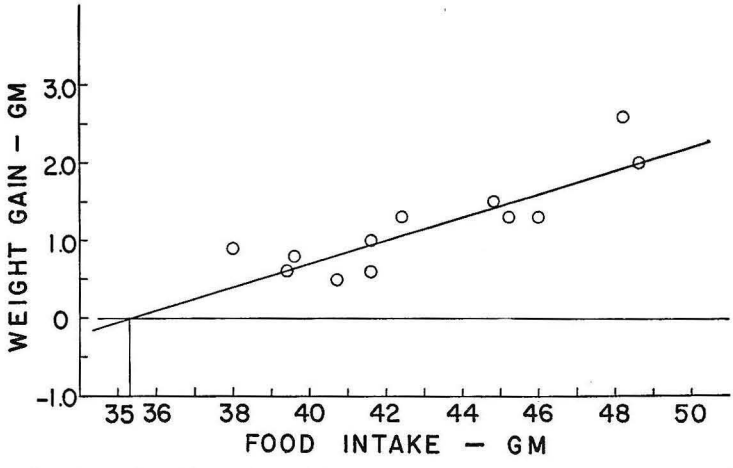

Fig. 1 Regression line of weight gain/20 days on voluntary intake of 5% protein diet of weanling C57BL male mice. Zero weight gain theoretically at food intake of 35.3 gm.

In figure 1 are plotted the body weight gains of individual C57BL animals against food intake under conditions of ad libitum feeding. The calculated regression line shows that weanling mice of this strain should show no weight change if consuming 35.3 gm of diet 287/20 days. As a check 15 weanling male mice of the C57BL strain were fed this amount of diet 287 over a period of 20 days. The average weight gain observed for the entire group was 0.1 gm. On the other hand, as was shown in table 2, mice of the A strain lost weight even when fed as much as 43 gm of diet 287. In a further experiment mice of the A strain were each fed 36 gm of diet 287 mixed with 18 gm of dextrin. The resulting mixture supplied approx-

imately as many calories as this strain consumed voluntarily under conditions of ad libitum feeding. Average food consumption for the 20-day period was 49.5 gm, corresponding to 33.0 gm of diet 287 plus 16.5 gm of dextrin. Despite this low protein intake the average weight gain for the group was 0.4 gm (table 3).

TABLE 3

Weight gains on low protein diets

STRAIN	NO. OF ANIMALS	FOOD INTAKE	WEANING WEIGHT	WEIGHT GAIN
			gm	*gm*
C57BL	15	Restricted, 35.0 gm diet 287	9.9	0.1
A	8	Restricted, 42.8 gm diet 287	10.2	—0.9
A	6	33.0 gm diet 287; 16.5 gm dextrin	9.2	0.4
C57BL	7	ad lib. 32.4 gm diet 292	9.6	—0.4

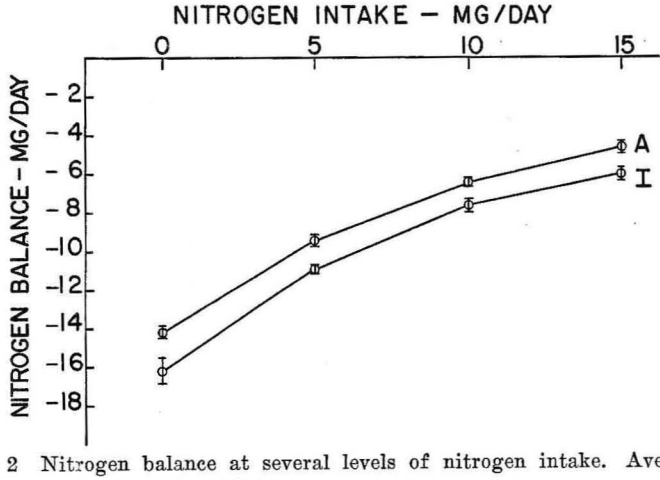

Fig. 2 Nitrogen balance at several levels of nitrogen intake. Averages for 9 A strain and 9 I strain males.

When diet 292 was fed to weanling C57BL males, food intake amounted to only 32.4 gm, and a weight loss of 0.4 gm was observed over the 20-day period. This is precisely the weight loss predicted from the regression line in figure 1. Diet 292 is identical with diet 287 except that starch was substituted for dextrin.

The results of the protein minima studies are shown in figure 2. The endogenous urinary nitrogen excretion of I strain animals was significantly greater than that of A strain mice. At all levels of nitrogen intake the I strain mice showed more negative nitrogen balances.

DISCUSSION

The large strain differences in food intake under conditions of ad libitum feeding are in line with observations we have made with other diets. Under most feeding conditions the voluntary food intake of A and C3H mice has been found to exceed that of C57BL and I strain animals. It is the A and C3H strain which can most readily be made obese by nutritional means. The observation that mice of these two strains lose weight when restricted to the quantity of food voluntarily consumed by C57BL and I strain animals indicates that they not only voluntarily consume more food, but that they also require more food in order to show gains in body weight on low protein diets. With the diets fed in these experiments the question is raised whether the A and C3H mice require more protein in order to grow or whether the need is for additional calories. The positive weight gains observed when the low protein diet was fed in restricted amounts but the nitrogen-free ration supplied in unlimited quantities indicates that the need is for calories alone. This is further supported by the experiment in which A strain mice were fed 36 gm of the low protein diet mixed with 18 gm of dextrin. It may be concluded then that A and C3H mice have a greater need for calories than do C57BL and I strain animals for the utilization of low protein diets.

The relation of caloric intake to protein metabolism in one strain of mice has been studied by Bosshardt et al. ('46, '48). Holding the protein intake constant, they found that restriction of the caloric intake decreased the rate of growth, the efficiency of protein utilization and the total energy expenditure. Extra calories in the form of protein were much more effective in stimulating growth than calorically equivalent amounts of fat and carbohydrate.

The experiments on nitrogen minima by the method of Melnick and Cowgill ('37) showed that A strain animals excrete less nitrogen per day than do I strain mice. This is true when no nitrogen is administered and one measures only the endogenous urinary nitrogen. It is equally true at each of the three levels of nitrogen administered intraperitoneally. Under the conditions used in these experiments the relation between nitrogen intake and nitrogen balance was not rectilinear as might have been expected. We attribute this to the fact that the entire daily supply of nitrogen was administered in a single dose. Presumably much of the incoming supply of amino acids was rapidly deaminated and the nitrogen excreted. This effect could be expected to be more pronounced at the higher levels of nitrogen administered, thus accounting for the curvilinear relationship between nitrogen intake and nitrogen balance. The curves indicate strongly that the A strain mouse requires less nitrogen to attain nitrogen balance than does the I strain animal.

The experiments reported here constitute further evidence of the existence of distinct metabolic patterns characterizing each of our several strains of mice. The A and C3H strains possess the greatest ability to deposit excessive quantities of fat, the greatest need for calories for the utilization of low protein diets, and, in the case of the A strain, the lowest endogenous nitrogen excretion. Whether the low nitrogen excretion of A strain mice fed nitrogen-free diets indicates a greater ability to accumulate tissue proteins and whether this ability can be related to excessive fat deposition cannot be decided as yet. The fact is that we have never seen, under

the conditions of our experiments, accumulation of excess fat without the deposition of additional protein.

SUMMARY

Two strains of mice highly susceptible to nutritionally induced obesity were found to have a higher caloric requirement for the utilization of a low protein diet than a strain which is moderately susceptible and one that is completely resistant One obesity-susceptible strain required less nitrogen than the resistant strain.

LITERATURE CITED

BALLENTINE, R., AND J. GREGG 1947 Micro-Kjeldahl determination of nitrogen. Anal. Chem., *19:* 281.

BOSSHARDT, D. K., W. PAUL, K. O'DOHERTY AND R. H. BARNES 1946 The influence of caloric intake on the growth utilization of dietary protein. J. Nutrition, *32:* 641.

———— 1948 Caloric restriction and protein metabolism in the growing mouse. Ibid., *36:* 773.

FENTON, P. F. 1956 Growth and fat deposition in the mouse. A definition of obesity. Am. J. Physiol., *184:* 52.

FENTON, P. F., AND C. J. CARR 1951 The nutrition of the mouse. X. Studies on the utilization of high and moderately low protein diets for growth in 4 strains of mice. J. Nutrition, *43:* 441.

FENTON, P. F., AND M. T. DOWLING 1953 Studies on obesity. I. Nutritional obesity in mice. Ibid., *49:* 319.

HUBBELL, R. B., L. B. MENDEL, AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. Ibid., *14:* 273.

MELNICK, D., AND G. R. COWGILL 1937 The protein minima for nitrogen equilibrium with different proteins. Ibid., *13:* 401.

THE EFFECTS OF VITAMIN DEFICIENCY ON SOME PHYSIOLOGICAL FACTORS OF IMPORTANCE IN RESISTANCE TO INFECTION

III. VITAMIN B_{12} AND FOLIC ACID DEFICIENCIES

KENNETH WERTMAN,[1] RAYMOND J. LYNN, DONALD T. DISQUE, GERALD W. KOHR AND MARY ELLEN CARROLL

The Division of Bacteriology, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania

(Received for publication June 25, 1956)

The influence of nutritional deficiencies upon the susceptibility of animals to infectious disease has been studied and reported by numerous investigators. Animals maintained on diets deficient in members of the vitamin B complex group have demonstrated a marked increase in susceptibility to certain bacterial and rickettsial infections. An increased resistance to certain viruses has been reported in animals maintained on vitamin-deficient diets.

The mechanism of this altered susceptibility to infection has not been adequately investigated. However, the ability of vitamin-deficient animals to produce circulating antibodies of various types has been rather extensively studied. The work in these fields has been recently reviewed elsewhere (Wertman, Smith and O'Leary, '54).

In three recently published papers (Wertman et al., '53, '54, '55), several observations on the effect of thiamine, niacin-tryptophan and pyridoxine deficiencies in the white rat were presented. The nonspecific physiological factors studied, which were believed to be of importance in resistance to infection, were as follows: (1) complete blood count; (2) complement activity; (3) quantitative and qualitative

[1] Present address: Department of Bacteriology, University of Arizona, Tucson, Arizona.

cellular migration in inflammation; (4) capillary permeability, as measured by the Menkin dye accumulation technique; and (5) cellular composition of bone marrow. A considerable decrease of complement activity and a substantial reduction in cellular migration to an inflamed area were observed in the white rats maintained on the niacin tryptophan-deficient diet and the pyridoxine-deficient diet. Although a significant reduction in cellular migration occurred in deficient animals, no alteration in capillary permeability could be measured by the dye accumulation technique (Menkin, '40).

Although much work has been done with vitamin B_{12} and its hematopoietic activity, very little research has been reported concerning its influence on resistance to disease. Neither have studies of this nature been performed on white rats maintained on a folic acid-deficient diet. The purpose of the previous investigations (Wertman et al., '53, '54, '55) was to study the various nonspecific physiological factors of resistance to infection concurrently in animals maintained on a well-defined diet that was deficient in thiamine, niacin-tryptophan and pyridoxine. The purpose of this investigation was to perform identical studies with a group of white rats maintained on a well-defined diet that was deficient in vitaman B_{12} as well as a similar group maintained on a well-defined diet deficient in folic acid.

EXPERIMENTAL

Male, weanling albino rats of the Sprague-Dawley strain, approximately 21 days old, were employed in the investigation. All rats were housed individually in wide-mesh, screen bottom metal cages. The animals for the vitamin B_{12} experiments were arranged into three groups, i.e., vitamin B_{12} deficient, inanition controls and ad libitum controls. The deficient group contained 80 animals and the two control groups each contained 40 animals. All animals were observed and weighed daily.

The basal diet and vitamin pill were similar to those employed by Wertman et al. ('53, '54, '55) in their previous studies. The vitamin B_{12}-deficient diet consisted of the following in percentage: defatted, whole ground yellow corn, 45.76; soy flour (low fat), 45.75; salt mixture no. 2,[2] 2.0; corn oil,[3] 5.00; L-cystine, 0.30; choline chloride, 0.10; i-inositol, 0.02; *p*-aminobenzoic acid, 0.01; iodinated casein,[4] 0.05; 2-methyl-1 4-naphthoquinone, 0.001; *d*-alpha-tocopherol acetate, 0.001; and sulfasuxidine, 1.00.

All rats in the vitamin B_{12} experiments were fed a vitamin pill daily, which contained the following vitamins in micrograms (Griffith and Farris, '49): thiamine, 80; riboflavin, 120; pyridoxine, 100; pantothenic acid, 600, nicotinic acid, 200; folic acid, 10; and biotin, 10.

All animals were stabilized for a period of one week before being placed on their respective diets. Thereafter, all animals received the basal diet plus a vitamin pill daily. The control animals were injected intraperitoneally every other day with 1 ml of vitamin B_{12} [5] in saline (0.4 μg vitamin B_{12} per ml). Ad libitum controls and vitamin B_{12}-deficient animals received diets ad libitum, while inanition control animals were fed only enough to maintain their respective weights equal to the deficient animals with which they were paired. In addition, each rat received 3000 USP units of vitamin A and 24 USP units of vitamin D per week.[6]

The animals were maintained on their diets until the deficient animals began to plateau or lose weight which was approximately 6 weeks. Initial and final mean weights for each of these groups appear in table 1.

The basal diet for the folic acid experiments had the following percentage composition: "vitamin-free" casein, 25; sucrose, 57.76; hydrogenated vegetable oil, 10.00; U.S.P. salt

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[3] Mazola.
[4] Protomine.
[5] William H. Rorer, Inc., Philadelphia, Pa.
[6] Two drops of Abbott's Haliver oil per week.

mixture no. 2,[2] 4.00; corn oil,[3] 2.00; sulfasuxadine, 1.00; choline chloride, 0.20; i-inositol, 0.03; *dl*-alpha-tocopherol acetate, 0.01; and 2-methyl-1,4-naphthoquinone, 0.001.

Each animal in the folic acid studies received a vitamin pill daily. The pills prepared for the control animals contained the following vitamins in micrograms: thiamine, 40; riboflavin, 60; pyridoxine, 50; calcium pantothenate, 300; nicotinic acid, 150; biotin, 3; and folic acid, 20. Lactose was used as the binder in the preparation of the pills. Folic acid was omitted from the pills prepared for the vitamin-dencient group of animals.

TABLE 1

Distribution and initial and final mean weights of rats

GROUP	NUMBER OF RATS	VITAMIN B_{12} DEFICIENCY		NUMBER OF RATS	FOLIC ACID DEFICIENCY	
		Mean body weights			Mean body weights	
		Initial	Final		Initial	Final
		gm	*gm*		*gm*	*gm*
Ad libitum controls	40	58.2	176.8	10	43.1	203.9
Inanition controls	40	59.4	115.5	14	43.6	126.2
Deficient	80	56.7	112.7	39	43.5	125.5

The addition of sulfasuxidine to a diet has been reported to have an adverse effect on the bacterial synthesis of biotin in the rat intestine (Martin, '42; Welch, '42). A biotin deficiency is produced as well as interference with the utilization of pantothenic acid (Wright and Welch, '44). It was for these reasons that a supplement of biotin, 2 μg, and calcium pantothenate, 150 μg, was added beyond the accepted normal requirement of the rat.

In addition to the vitamins supplied in the basal diet and the supplementary pills, each animal received 3000 USP units of vitamin A and 24 USP units of vitamin D each week.[7] The animals were maintained on the basal diet and vitamin preparations for a period of 36 days. In the last few days of this period, a high death rate became apparent. Initial and

[7] See footnote 6, page 475.

TABLE 2

Cell counts and differential enumeration of the peripheral blood in vitamin B_{12}- and folic acid-deficient and control rats

		AD LIBITUM CONTROL	INANITION CONTROL	VITAMIN B_{12} DEFICIENT	AD LIBITUM CONTROL	INANITION CONTROL	FOLIC ACID DEFICIENT
		Total count					
R.B.C., 1×10^4 cells/mm³	Median	805	867	804	850	870	710
	M.D.[1]	92.5	114	34.5	70.6	93.2	35.3
	Range	610–1090	619–1101	501–1046	720–1070	520–1140	440–1490
W.B.C., 1×10^3 cells/mm³	Median	19,000	12,900	8,750	13,600	6,700	4,200
	M.D.	2,240	4,720	2,480	2,623	2,078	1,370
	Range	11,900–34,300	5,050–31,000	3,700–17,200	10,200–19,700	3,900–10,800	1,700–6,900
		Differential count in per cent[2]					
Neutrophiles, segmented	Median	13.0	12.8	11.7	8	11	3
	M.D.	3.8	8.7	9.5	2.2	2.6	1.8
	Range	6–20	1–53	1–48	7–15	9–20	1–7
Eosinophiles	Median	1	1.1	1	1	1	1
	M.D.	..	..	..	..	..	..
	Range	0–3	1–3	0–3	0–1	1–2	0–1
Lymphocytes, large and small	Median	85	83.8	87.3	86	81	88
	M.D.	3.2	8.8	6.5	2.5	4.7	2.2
	Range	45–92	45–97	45–98	80–88	72–86	82–91
Monocytes	Median	1.9	1.3	1.5	3	4	8
	M.D.	..	0.2	0.9	1.1	2.4	1.9
	Range	0–9	0–4	0–8	2–4	2–10	4–12

[1] Mean deviation.

[2] Band, basophile and blast cell enumerations were also conducted but did not exceed 1% in any instance and are not included in the table.

final mean weights for each group in the folic acid experiments appear in table 1.

The day before the rats were to be sacrificed, blood samples for complete blood counts were obtained from all animals by tail bleeding. Standard hematological techniques were employed for these counts. The results are recorded in table 2. Following the tail bleeding, each rat was injected intraperi-

TABLE 3

Complement activity of vitamin B_{12}- and folic acid-deficient and control rats

GROUP	SERUM POOL [2]	VITAMIN B_{12} COMPLEMENT ACTIVITY E. U.[1]	SERUM POOL	FOLIC ACID COMPLEMENT ACTIVITY E. U.[2]
Ad libitum controls	1	0.08 ml of 1:6 dilution	1	0.12 ml of 1:6 dilution
	2–5	0.12 ml of 1:6 dilution	2–5	0.15 ml of 1:6 dilution
	6	0.10 ml of 1:6 dilution		
	7–8	0.14 ml of 1:6 dilution		
Inanition controls	1–2	0.12 ml of 1:6 dilution	1–3	0.21 ml of 1:6 dilution
	3–4	0.20 ml of 1:6 dilution	4	0.24 ml of 1:6 dilution
	5–8	0.14 ml of 1:6 dilution		
Deficient animals	1	0.16 ml of 1:6 dilution	1	0.24 ml of 1:6 dilution
	2–3	0.14 ml of 1:6 dilution	2–3	0.21 ml of 1:6 dilution
	4	0.18 ml of 1:6 dilution	4	0.24 ml of 1:6 dilution
	5–6	0.14 ml of 1:6 dilution	5–8	0.21 ml of 1:6 dilution
	7	0.16 ml of 1:6 dilution	9	0.18 ml of 1:6 dilution
	8–9	0.14 ml of 1:6 dilution		
	10	0.16 ml of 1:6 dilution		
	11–16	0.12 ml of 1:6 dilution		

[1] One exact unit.

[2] Five rats were used in each pool.

toneally with 10 ml of inflammation-inciting fluid. This fluid was a mixture of sterile Locke's solution and double strength nutrient broth in the ratio of 85 to 15 on a volume basis.

Twelve hours after the intraperitoneal injections, the rats were anesthetized with ether and bled to death by the cardiac puncture technique. The blood specimens so obtained were allowed to clot and the sera were collected, pooled, and utilized for the determination of complement activity as described by Wertman, Smith and O'Leary ('54). The results of these

determinations are recorded in table 3 as the volume of diluted pooled sera representing one exact unit of complement.

At the same time as the cardiac bleeding, the peritoneal exudates were collected from the anesthetized animals by washing the peritoneal cavity with heparinized Locke's solution (Wertman et al., '54). The exudates and the washings were centrifuged to remove cellular elements which were then resuspended in 2 ml of Locke's solution for total and differential leucocyte counts. Standard techniques were used for the total counts. The differential counts were performed on smear preparations of the cell suspensions which were stained by Wright s method. Two hundred cells were counted on each slide using the methods of Menkin ('40) and Maximow and Bloom ('44) in classification. The results of the total and differential counts of the exudate cells appear in table 4.

Samples of each cell-free exudate supernatant were pooled in groups of 5 and concentrated 10-fold by evaporation of water from dialysis bags as described in a previous paper (Wertman et al., '54). In an effort to determine the presence and activity of "leukotaxine" in the concentrated exudate supernatant, Menkin's ('40) intradermal dye-accumulation technique was employed. Two-tenths milliliter of pooled exudate concentrate was injected intradermally into shaved rabbit abdomens and along with control injections of saline, heparinized Locke's solution and inciting fluid. Ten milliliters of 1% trypan blue were injected into the marginal ear vein of each rabbit immediately after the intradermal injections were completed. The sites of the intradermal injections were observed and measurements of the areas of dye accumulation made 15 and 30 minutes after the injection.

After the cardiac bleedings and removal of peritoneal exudates, bone marrow specimens were taken from each rat by cutting through the proximal end of the tibia and removing a portion of the marrow so exposed. The marrow was mixed with a drop of normal rabbit serum and smeared across a slide (Endicott, '45). The resultant films were stained with Wright's stain and differential counts made. Three hundred

TABLE 4

Total and differential leucocyte count of peritoneal exudates in vitamin B_{12}- and folic acid-deficient and control rats

EXUDATE CELLS		AD LIBITUM CONTROL	INANITION CONTROL	VITAMIN B_{12} DEFICIENT	AD LIBITUM CONTROL	INANITION CONTROL	FOLIC ACID DEFICIENT
Total leucocytes, cells/mm (1×10^3)	Median	30.2	22.4	16.6	17.8	19.8	2.8
	M.D.[1]	1.73	1.87	1.68	3.2	2.7	1.0
	Range	28.7–32.9	20.2–26.9	13.1–19.6	14.2–26.8	0.5–5.3	10.3–30.4
Granulocytes, %	Median	24.9	21.5	13.4	37.5	41.0	11.5
	M.D.	4.8	6.0	4.4	4.0	4.0	2.7
	Range	14–36	8.5–43	4.5–30	28–43	34–50	4–17
Lymphocytes, %	Median	70.2	74.3	82.2	28.5	17.5	25
	M.D.	6.1	4.5	5.1	2.9	2.8	3.2
	Range	54–84	53–89	66–94.5	22–33	13–24	17–32
Monocytes, %	Median	4.8	4.3	4.4	35	40	65
	M.D.	0.7	0.6	1.2	4.4	4.3	5.1
	Range	2–10.5	2–10	0.5–8	26–45	30–48	52–76

[1] Mean deviation.

cells were observed on each slide using a modification of the method of Endicott and Ott ('45) for classification. The results of these counts appear in tables 5 and 6.

RESULTS AND DISCUSSION

The results of this investigation indicated that there was no alteration of the peripheral erythrocyte count due to either inanition or vitamin B_{12} deficiency. However, a vitamin B_{12} deficiency produced a leucopenia in the rat. Inanition likewise resulted in a leucopenia as compared with ad libitum controls, but not as severe as in the deficient rats. The differential counts performed with circulating blood revealed no significant qualitative or quantitative difference between deficient animals and controls.

The results indicated that neither folic acid deficiency nor inanition affected the total number of erythrocytes in the peripheral blood. The deficiency of folic acid resulted in a reduction of the total number of leucocytes in the peripheral blood. Inanition likewise resulted in a decrease but to a lesser degree than the folic acid deficiency. A relative decrease of polymorphonuclear neutrophiles was observed in the blood of the folic acid-deficient animals.

Complement titrations were performed on the pooled sera from 5 rats. Sixteen pools of sera from vitamin B_{12}-deficient animals were titrated and these results compared with results of titrations performed on 8 pools of sera from inanition controls and 8 pools of sera from ad libitum controls. These titrations indicated that vitamin B_{12} deficiency and inanition had no significant effect on the activity of rat complement.

The sera of the folic acid-deficient and inanition rats were found to have a reduction of complement activity. The exact unit of complement of a 1:6 dilution of the ad libitum rat sera was approximately 0.15 ml and that of the inanition and deficient rat sera was 0.21 ml of a 1:6 dilution.

The leucopenia of the circulating blood of vitamin B_{12}-deficient animals was reflected in the total leucocyte counts of the peritoneal exudate. Cellular migration to the inflamed area

was reduced in vitamin B_{12}-deficient animals as compared to ad libitum controls. Inanition produced a slight decrease in the cellular migration but not nearly as great as the decrease in deficient animals. Differential counts of the peri-

TABLE 5

Cellular composition of bone marrow in vitamin B_{12}-deficient and control rats

BONE MARROW CELLS		DIET		
		Ad libitum	Inanition	Vitamin B_{12} deficient
Total granulocytes, %	Median	35.2	37.3	33.5
	Range	22.6–45.5	26.6–49.6	20.6–45.9
Promyelocytes and myelocytes, %	Median	9.6	10.5	9.2
	Range	7–13.6	8–14	6–12.6
Metamyelocytes and segmenters, %	Median	22.3	23	21.4
	Range	14.3–27.6	17–28.3	13.3–27.3
Eosinophiles, %	Median	3.3	3.8	2.9
	Range	1.3–4.3	1.6–7.3	1.3–6
Nucleated red blood cells, %	Median	51.3	48.4	52.8
	Range	45–64	41.3–60.3	45.3–66
Lymphocytes, %	Median	8.9	9.8	8.3
	Range	6.3–12.6	8–12.6	5.3–12.6
Monocytes, %	Median	1.8	2.4	1.6
	Range	1–3.2	1–4	0.6–3
Blast cells, %	Median	0.9	0.5	0.8
	Range	0–7.3	0–2.3	0.7
Mast cells, %	Median	0.5	0.7	0.4
	Range	0–1.9	0–1.6	0–1.9
Plasma cells, %	Median	0.3	0.2	0.4
	Range	0–1.6	0–1.9	0–1.6
Unclassified, %	Median	0.2	0.4	0.2
	Range	0–1.3	0–1.3	0–1.3

toneal exudate revealed a lymphocytosis with a concomitant granulocytopenia. The granulocytes are the most active in phagocytosis (Menkin, '40, '50), so it appears that the resistance of the deficient animals might be reduced in this instance.

The total leucocyte counts of the inflammatory exudates removed from folic acid-deficient groups demonstrated a significant reduction over the ad libitum and inanition control groups. The percentage cellular composition of the folic

TABLE 6

Cellular composition of bone marrow in folic acid ad libitum-control, inanition-control and vitamin-deficient rats

BONE MARROW CELLS		DIET		
		Ad libitum	Inanition	Deficient
Total granulocytes, %	Median	34.3	37.0	21.0
	Range	26.7–38	30.3–47	14.3–26.3
Promyelocytes and myelocytes, %	Median	21.0	21.0	17.5
	Range	12–25	16–25	11–24
Metamyelocytes and segmenters, %	Median	13.0	14.0	1.7
	Range	6–16	11–22	0.7–5.0
Eosinophiles, %	Median	1.0	2.0	1.0
	Range	0.3–2.3	0.7–3.0	0–1.7
Nucleated red cells, %	Median	58.5	57.0	65.6
Lymphocytes, %	Median	9.2	3.5	2.0
	Range	5–15	1–5	0–6
Blast cells, %	Median	1.7	1.0	1.5
	Range	0–3	0—2.7	0–2.7
Mast cells, %	Median	1.0	1.7	1.2
	Range	0–3	0.7–4.7	0.3–3
Plasma cells, %	Median	0.0	0.0	0.0
	Range	0	0–1.0	0–1.0
Monocytes, %	Median	0.3	0.7	0.5
	Range	0–3.3	0.3–2.7	0.3–2.3
Unclassified, %	Median	0.8	1.5	6.5
	Range	0.3–2.3	0.3–3.3	0.7–12

acid-deficient rats demonstrated a reduction of 26% in the relative number of polymorphonuclear leucocytes. The lymphocyte count was comparable in folic acid-deficient and ad libitum-control rats. However, the inanition-control exudate showed a reduction in lymphocytes when compared to

the ad libitum-control rat exudate. The mononuclear leucocyte count of peritoneal exudates in folic acid-deficient rats was 25% greater than in the inanition controls and 30% greater than in the ad libitum-control animals.

Experiments were performed to determine whether or not "leukotaxine" activity was present in vitamin B_{12}-deficient, folic acid-deficient and control animals. No alteration in capillary permeability could be detected by the Menkin dye accumulation technique. In every instance, the cell-free peritoneal exudate from all groups of animals produced the same type and degree of reaction in the skin of the rabbit after injection of dye. These results are in complete agreement with those of the three previous papers in this series (Wertman et al., '53, '54, and '55).

No significant difference in the cellular composition of the bone marrow of the vitamin B_{12}-deficient, inanition-control and ad libitum-control rats was noted. These results correspond with those obtained by Wang, Scheid and Schweigert ('54) in their experiment using varying levels of vitamin B_{12}. No marked changes were found in the bone marrow composition of the vitamin B_{12}-deficient rats as compared to the normal rats (table 5).

The bone marrow preparations of the folic acid-deficient rats exhibited a relative decrease in granulocytes as compared to the ad libitum and inanition controls. This decrease was caused primarily by a severe decrease in the percentage of older forms of the granulocytic elements, such as metamyelocytes and segmenters. There was a significant decrease in the percentage of lymphocytes in the marrow of the deficient and inanition-control animals. Nucleated erythrocytes in the marrow of the deficient rats decreased 7.0% over the ad libitum controls, and 8.5% over the inanition controls. An increase in reticulocytes was also noted in the bone marrow of folic acid-deficient rats. These were placed in the "unclassified" group (table 6).

SUMMARY

Male white rats were maintained on a well-defined diet deficient in vitamin B_{12} and folic acid and various physiological factors of resistance to infection were studied. Adequate inanition- and ad libitum-control animals were included in the investigation. The following physiological factors were studied: (1) cellular composition of the peripheral blood; (2) complement activity; (3) cellular migration in inflammation; (4) cellular composition of the exudate in inflammation; (5) "leukotaxine" activity; and (6) cellular composition of bone marrow.

The following observations were made from these studies:

1. Vitamin B_{12} deficiency had no effect on the number of erythrocytes per cubic millimiter in the peripheral blood. A severe leucocytopenia was observed in deficient animals. Inanition, however, also caused a decrease in leucocyte counts. Inanition counts were intermediate between those of deficient and ad libitum controls. Vitamin B_{12} deficiency produced no significant difference in differential leucocyte counts. No significant difference was noted in the number of erythrocytes per cubic millimeter of blood in the folic acid-deficient, inanition- and ad libitum-control groups. Inanition which accompanied folic acid deficiency resulted in a severe leucopenia. The differential cell counts of the blood film preparations showed a decrease in both percentage and absolute number of neutrophiles in the folic acid-deficient animals.

2. Vitamin B_{12} deficiency and inanition had no apparent effect on the activity of rat complement. However, complement activity was reduced in the sera of the folic acid-deficient rats.

3. Cellular migration to an inflamed area was reduced in the vitamin B_{12}-deficient animals as compared to the ad libitum controls. A slight decrease in the cellular migration was shown in the inanition-control rats, but this was not as great as in deficient rats. A deficiency of folic acid resulted in a severe reduction of the migration of leucocytes to an inflamed area.

4. The vitamin B_{12} deficiency appeared to have a marked effect upon the types of leucocytes migrating to the site of inflammation. There was a marked lymphocytosis and granulocytopenia in the exudates of the vitamin B_{12}-deficient rats. The inflammatory exudates of the folic acid-deficient rats showed a relative decrease in number of polymorphonuclear leucocytes and a relative increase of mononuclear leucocytes.

5. No alteration in capillary permeability, as measured by Menkin dye accumulation technique, was noted in either vitamin B_{12}- or folic acid-deficient rats.

6. No significant change was observed in the cellular composition of the bone marrow of the vitamin B_{12}-deficient or inanition-control animals as compared to the ad libitum-control rats.

7. The bone marrow preparations from the tibias of the inanition and folic acid-deficient rats presented a relative decrease of lymphocytes. A severe decrease in relative numbers of metamyelocytes and segmenters. a slight decrease in promyelocytes and myelocytes, and an increase of reticulocytes were noted in the bone marrow removed from the tibias of folic acid-deficient rats.

LITERATURE CITED

ENDICOTT, K. M. 1945 Plasma or serum as diluting fluid for thin smears of bone marrow. Stain Tech., *20:* 5.

ENDICOTT, K. M., AND M. OTT 1945 The normal myelogram in albino rats. Anat. Rec., *92:* 61.

GRIFFITH, J. O., AND E. FARRIS 1949 The Rat in Laboratory Investigetions. Lippincott, Philadelphia, Pa.

MARTIN, G. J. 1942 Folic acid in nutritional achromotrichia. Proc. Soc. Exp. Biol. Med., *51:* 353.

MAXIMOW, A. A., AND W. A. BLOOM 1944 Textbook of Histology, 4th Ed. W. B. Saunders, Philadelphia, Pa.

MENKIN, V. 1940 Dynamics of Inflammation. Macmillan Company, New York, New York.

———— 1950 Newer Concepts of Inflammation. Charles C Thomas, Springfield, Ill.

WANG, H., H. E. SCHEID AND B. S. SCHWEIGERT 1954 Proc. Soc. Exp. Biol. Med., *85:* 382.

WELCH, A. D. 1942 Succinylsulfathiazole as an inhibitor of bacterial synthesis in nutrition experiments. Fed. Proc., *1:* 171.

WERTMAN, K., W. M. O'LEARY AND L. W. SMITH 1955 The effects of vitamin deficiencies on some physiological factor of importance in resistance to infection. II. Pyridoxine deficiency. J. Nutrition, *57:* 203.

WERTMAN, K., R. ROTUNDO AND R. YEE 1953 Blood and bone marrow study of vitamin deficient rats. Ibid., *50:* 479.

WERTMAN, K., L. W. SMITH AND W. M. O'LEARY 1954 The effects of vitamin deficiencies on some physiological factors of importance in resistance to infection. I. Niacin-tryptophane deficiency. J. Immunology, *73:* 196.

WRIGHT, L. D., AND A. D. WELCH 1944 Folic acid biotin, and pantothenic acid deficiency and the liver storage of various vitamins in rats fed succinylsulfathiazole in highly purified rations. J. Nutrition, *27:* 55.

NUTRITIONAL STUDIES ON RATS ON DIETS CONTAINING HIGH LEVELS OF PARTIAL ESTER EMULSIFIERS [1]

II. REPRODUCTION AND LACTATION

BERNARD L. OSER AND MONA OSER
Food Research Laboratories, Inc., Long Island City, New York

(Received for publication March 9, 1956)

The stresses imposed by reproduction and lactation would be expected to accentuate such pathological effects as might result from the addition to the diet of unphysiologic test substances. Breeding studies were therefore included in this investigation of the effect of chronic feeding of high levels of partial fatty acid ester emulsifiers. In the introductory report of the series (Oser and Oser, '56) are described the general plan of the study, the 6 emulsifiers, the basal and test diets, and the general procedures employed.

The experiments were started with approximately 800 weanling rats, each dosage group consisting initially of 12 male and 20 female rats. The fertility and lactation responses of the parent generation (F_0) as well as those of the descendent generations, each consisting of approximately 500 rats, are presented in this report.

PROCEDURE

The reproduction and lactation program was initiated shortly after the 12th week on the test diets when the rats were approximately 110 days old and, of course, sexually mature. The scheme shown in figure 1 was adopted for breed-

[1] This investigation was supported by a grant from the Atlas Powder Company, Wilmington, Delaware.

ing studies through successive generations. Matings were set up with one male and two female rats per cage. When pregnancy was recognized visually, by palpation, or from weight increments, the females were transferred to individual cages. If pregnancy was not established by the third week, the male was replaced. Following three (or occasionally 4) unproductive trials with females of known fertility, males were considered sterile and retired. Females were continued

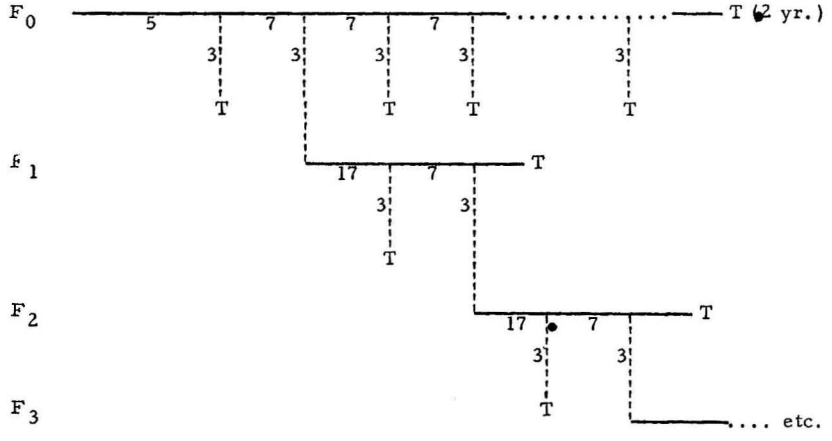

Fig. 1 Chronological scheme of reproduction and lactation. The horizontal lines represent the generations of rats through their successive matings and the dotted vertical lines indicate litters; termination of a litter or a generation is shown by the letter T; the figures indicate the number of weeks elapsed at each stage, beginning with the first mating in Fo.

for a minimum of 6 matings with fertile males, even though some failures may have intervened.

Lactation was permitted for three weeks. Following weaning, death, or destruction of their litters, the females were allowed a one-week rest period before remating. In successive matings, the males were rotated among the females within their respective test groups.

As indicated in figure 1, matings continued in the F_0 generation throughout the entire two-year period. First litters were discarded at weaning. From the second litters of as many different mothers as possible, 10 rats of each sex were

selected whose individual weights approximated the averages for their respective litters. These F_1 generation animals were raised to maturity and mated like the parent generation. The second litters of the F_2 generation were carried through the same breeding program. Similarly, representative rats of the F_3 generation were raised to maturity for growth observations (reported elsewhere) but not mated because the entire study was terminated when the F_0 rats reached two years on test.

During the reproduction phase of the experiment body weights were recorded biweekly; females were also weighed at the time of mating and at appropriate intervals to confirm pregnancy. Pups were weighed at 4, 12 and 21 days of age at which time they were weaned. From the records of performance of the rats, the following indexes of reproductive and lactating efficiency were calculated: fertility index (F.I.), the percentage of matings resulting in pregnancy; gestation index (G.I.), the percentage of pregnancies resulting in the birth of live litters; viability index (V.I.), the percentage of rats born that survived 4 days or longer; and lactation index (L.I.), the percentage of rats alive at 4 days that survived the 21-day lactation period.

RESULTS

Fertility and gestation. The data summarized in table 1 show the responses of the initial generation in terms of the first 6 matings, i.e. through the period of maximum reproductive efficiency. The total number of matings, 2593, resulted in the birth of 1755 litters. Thus the overall F.I. was approximately 68%. A total of 15,789 rats were born as a result of these first 6 matings, an average of 9.0 pups per litter.

The values for F.I. indicate that the emulsifier groups responded quite the same, on the average, as the control and Primex groups, approximately 7 out of 10 matings resulting in pregnancy. With the possible exception of the Myrj 52 group there appeared to be no diminution in fertility with the increase of emulsifier level from 10 to 20%. That practically

TABLE 1

Summary of reproduction and lactation data of F_0 generation rats for six matings

EMULSIFIER OR FAT	NUMBER OF MATINGS	LITTERS BORN ALIVE	PUPS BORN ALIVE	AVERAGE NUMBER PUPS/LITTER		AVERAGE WEIGHT PUPS AT WEANING	FI [1]	GI [2]	VI [3]	LI [4]
				Born	Weaned					
						gm				
None	119	76	654	8.6	6.5	43.3	66	98	83	91
5% level										
Myrj 45	106	71	616	8.7	6.2	39.7	67	99	83	85
Myrj 52	114	89	875	9.8	6.4	39.7	79	99	71	91
Span 60	109	82	714	8.7	6.5	40.5	79	97	81	92
Tween 60	102 [5]	69	616	8.9	5.9	40.2	67	97	75	88
Tween 65	102	70	661	9.4	6.9	38.5	69	100	78	93
Tween 80	106	75	644	8.6	5.9	41.3	73	97	75	92
Mixture	88	71	624	8.8	6.5	41.8	82	99	80	93
Primex	116	83	761	9.2	5.9	44.1	72	99	73	89
10% level										
Myrj 45	112	83	765	9.2	5.7	40.3	75	99	66	94
Myrj 52	106	73	610	8.4	5.8	38.4	71	97	78	89
Span 60	112	84	735	8.8	5.3	37.5	79	94	64	94
Tween 60	111	79	752	9.5	5.6	38.0	79	100	67	88
Tween 65	109	70	684	9.8	5.1	40.0	65	99	60	88
Tween 80	112	68	617	9.1	6.6	41.0	63	97	75	96
Mixture	103	66	663	9.0	7.8	38.7	66	97	82	95
Primex	107	78	725	9.3	6.4	43.2	76	96	75	92
20% level										
Myrj 45	106	73	686	9.4	2.3	26.5	71	97	33	74
Myrj 52	118 [5]	61	534	8.8	4.3	37.0	53	98	55	90
Span 60	109	78	684	8.8	3.7	30.7	73	98	51	83
Tween 60	103	59	464	7.9	2.5	35.9	58	98	35	92
Tween 65	122 [5]	77	590	7.7	1.5	32.2	65	98	26	75
Tween 80	98	57	504	8.9	4.9	36.1	61	95	55	100
Mixture	103	66	590	9.0	3.4	32.9	64	100	45	84

[1] Fertility Index, (Pregnancies/Matings) 100.
[2] Gestation Index, (Litters born/Pregnancies) 100.
[3] Viability Index, (Pups surviving at 4 days/Pups born) 100.
[4] Lactation Index, (Pups weaned/Pups at 4 days) 100.
[5] These groups consisted of 21 females each; all other groups consisted of 20.

all pregnancies, once established, went to term, is indicated by the G.I. values which ranged from 94 to 100%. Hence it can be concluded that insofar as may be indicated by these responses in the first 6 matings of the F_0 generation, no evidence was observed of impairment of the reproductive function.

TABLE 2

Litter production through entire F_0 generation

(20 to 21 females per group)

EMULSIFIER	PER CENT	NUMBER OF LITTERS PER FEMALE (Range)	NUMBER OF FEMALES HAVING 4 OR MORE LITTERS	MEAN NUMBER OF LITTERS (± S.E.)[1]
None		1–8	15	4.15 ± 0.42
Myrj 45	5	0–7	12	3.67 ± 0.49
	10	0–7	16	4.65 ± 0.41
	20	0–7	11	3.85 ± 0.45
Myrj 52	5	1–8	17	4.95 ± 0.42
	10	1–7	13	4.05 ± 0.39
	20	0–6	10	3.29 ± 0.37
Span 60	5	0–7	14	4.60 ± 0.46
	10	1–9	12	4.60 ± 0.45
	20	1–8	12	4.40 ± 0.47
Tween 60	5	0–7	10	3.43 ± 0.33
	10	1–7	12	4.05 ± 0.36
	20	1–5	8	3.11 ± 0.28
Tween 65	5	1–8	12	4.05 ± 0.51
	10	0–6	12	3.75 ± 0.40
	20	1–7	13	4.00 ± 0.41
Tween 80	5	0–7	14	3.85 ± 0.44
	10	0–8	11	3.95 ± 0.48
	20	1–6	6	3.05 ± 0.31
Mixture	5	0–7	12	3.75 ± 0.46
	10	0–9	11	3.65 ± 0.49
	20	0–6	11	3.50 ± 0.55
Primex	5	1–6	15	4.30 ± 0.33
	10	0–6	14	4.00 ± 0.36
	20	1–4[2]	—[2]	—[2]

[1] S. E. $= \sqrt{\frac{d^2}{n\,(n-1)}}$.

[2] Discontinued at about one year.

Additional support for this conclusion may be seen in table 2. Here are shown the ranges in total number of litters cast throughout the two-year period by the females in the F_0 generation, not limited, as in table 1, to the first 6 matings. Some produced as many as 9 litters whereas others, distributed rather uniformly among the test and control groups alike, proved to be sterile or nearly so. It may further be seen in table 2 that at least half of the females in each group (with two exceptions) gave birth to 4 or more litters. However, there was a tendency toward diminished productivity (in terms of numbers of litters) in groups receiving certain of the emulsifiers at the 20% level. The emulsifiers concerned, in increasing order of this effect, were Tween 60, Tween 80, and Myrj 52. It is pertinent to note that in these groups a distinct laxative response was seen. The feces of the affected rats were of varying degrees of softness, sometimes sufficiently fluid to be characterized as frankly diarrheal. Concomitantly, variable degrees of perianal inflammation and irritation developed.

Since the rats in the F_0 generation were permitted to mate as long as they were productive, additional data on reproductive efficiency were obtained, namely the age at which loss of fertility occurred. This was estimated on the basis of two successive mating failures, specifically as of the day when the second mating was definitely established to be non-productive. While the precision of this estimate is far from exact, the data are nevertheless of interest. Table 3 shows that the F_0 females were roughly 450 ± 50 days old when their sterility was established. No significant differences were observed among the control groups and those receiving either 5 or 10% of emulsifier in their diets. While some tendency for a reduction in duration of fertility was noted with an increase in the emulsifier level to 20%, this was not marked, the average duration being in no case less than 376 days.

Viability of the offspring. Returning to table 1, it can be seen that the size of the litters, which ranged from 7.7 to

9.8 pups at birth (average 9.0) was substantially reduced at weaning in all groups. As pointed out by Mirone et al. ('48) it is necessary to distinguish losses in litters due to neglect or to poor viability of the newborn from those due to lactation failure. The former are considered to result from inadequate prenatal nourishment or lack of maternal interest whereas the latter is due to qualitative or quantitative insufficiency of the milk supply. In this report therefore the efficiency of lactation is expressed in terms of the ratio of the number of young weaned to the number surviving 4 days after birth

TABLE 3

Average age in days at loss of fertility in F_0 generation females as estimated from successive mating failures

FAT OR EMULSIFIER	LEVEL, PER CENT			
	0	5	10	20
None	> 402			
Myrj 45		> 419	478	396
Myrj 52		> 450	410	410
Span 60		> 462	> 474	440
Tween 60		414	432	376
Tween 65		469	420	398
Tween 80		454	> 492	397
Mixture		469	> 471	440
Primex		412	421	...

> = more than.

whereas the percentage of the newborn which survive through 4 days (viability index) is regarded as a reflection of either prenatal nutrition, maternal interest, or both.

From the relative magnitude of the V.I. and L.I. values shown in table I, it is apparent that the drop in litter size occurred principally during the few days immediately after birth. A somewhat greater proportion of infant deaths was noted at the 10% level of a few emulsifiers than at 5% (cf. Myrj 45, Span 60, Tween 65), whereas at the 20% level a significant diminution in survival of newborn was observed in all emulsifier groups.

Lactation. At the 5% level of emulsifiers the L.I. values varied from 85 to 93%; at the 10% level the range was 88 to 96%, and at 20% it was 74 to 100%. Thus lactation efficiency, while quite high at all levels of emulsifiers, was moderately reduced in a few of the 20% groups, notably Myrj 45 and Tween 65. An indication of possible impairment of lactation at this feeding level may be seen in the somewhat lower weaning weights of the young. That neglect of the litters was a more significant factor than lactation failure *per se* is evident from the relatively greater drop in the V.I. values than in those for L.I.. This was manifest to some extent even at the 10% emulsifier level. A much larger proportion of deaths among the newborn occurred shortly after birth, i.e. up to 4 days of age, than during the remainder of the 21-day nursing period. It would appear at least likely that the laxative effect with concomitant posterior ventral irritation at the high dosage level of some of the surfactants may also have had an adverse influence on the interest of the dams in caring for their offspring.

Reproduction and lactation in successive generations. The effect of the emulsifier diets on reproduction and lactation were made in rats of three generations descended from the first or parent generation (F_0). As previously described, groups of 10 males and 10 females selected from the second litters of each generation constituted the progenitors. The data for the F_1 and F_2 generations are summarized in tables 5 and 6, respectively. Since the breeding experiments after the F_0 generation were terminated when the second litters were weaned, the figures shown in these tables represent not more than two litters from each female, i.e. the product of 20 matings per group. For comparison, the responses to the first two matings in the F_0 generation are shown in table 4; these observations are quite similar to those shown in table 1 for the first 6 matings in the initial generation, except for fertility which, as might be expected, tended to diminish as the rats grew older. In fact, comparison of tables 1 and 4 demonstrates that much of the relevant information on

TABLE 4

Summary of reproduction and lactation data of F_0 generation rats for two matings

EMULSIFIER OR FAT	NUMBER OF MATINGS	LITTERS BORN ALIVE	PUPS		NUMBER PUPS PER LITTER		AVERAGE WEIGHT PUPS AT WEANING	F.I.[1]	G.I.[2]	V.I.[3]	L.I.[4]
			Born alive	Weaned	Born	Weaned					
							gm				
None	40	31	276	215	8.9	6.9	41.1	83	94	82	95
5% level											
Myrj 45	39	35	332	233	9.5	6.7	37.8	90	100	87	80
Myrj 52	40	37	394	254	10.6	6.9	39.0	93	100	71	91
Span 60	39	36	356	272	9.9	7.6	38.1	95	97	82	93
Tween 60	39	33	318	232	10.0	7.0	38.0	85	97	90	81
Tween 65	36	33	341	248	10.3	7.5	36.7	92	100	80	91
Tween 80	38	28	274	225	9.8	8.0	37.5	76	97	89	92
Mixture	36	34	297	236	8.8	6.9	42.0	97	97	85	94
Primex	40	36	352	240	9.8	6.7	43.9	90	100	75	91
10% level											
Myrj 45	39	33	332	217	10.1	6.6	36.9	85	100	66	99
Myrj 52	37	33	331	220	10.0	6.7	39.1	89	100	67	100
Span 60	40	35	329	216	9.4	6.2	37.5	90	97	67	97
Tween 60	40	33	355	192	10.7	5.8	36.5	83	100	64	84
Tween 65	38	27	279	148	10.3	5.5	38.6	74	97	54	99
Tween 80	39	32	315	242	9.9	7.6	39.0	82	100	79	98
Mixture	38	34	361	275	10.6	8.1	36.7	92	97	82	94
Primex	39	36	356	267	9.9	7.4	42.3	95	97	80	94
20% level											
Myrj 45	39	33	343	110	10.4	3.3	27.4	87	97	41	77
Myrj 52	42	24	220	108	9.2	4.5	34.7	57	100	52	94
Span 60	39	36	349	154	9.7	4.3	26.8	92	100	57	78
Tween 60	38	24	226	59	9.4	2.5	34.3	63	100	29	89
Tween 65	42	33	291	59	8.8	1.8	32.6	71	100	24	83
Tween 80	38	31	308	182	9.9	5.9	37.1	82	100	65	91
Mixture	39	26	251	102	9.7	3.9	30.2	67	100	51	80
Primex	40	33	319	208	9.7	6.3	40.6	83	100	70	93

[1] Fertility Index, (Pregnancies/Matings) 100.
[2] Gestation Index, (Litters born/Pregnancies) 100.
[3] Viability Index, (Pups surviving at 4 days/Pups born) 100.
[4] Lactation Index, (Pups weaned/Pups at 4 days) 100.

TABLE 5

Summary of reproduction and lactation data of F_1 generation rats for two matings

EMULSIFIER OR FAT	NUMBER OF MATINGS	LITTERS BORN ALIVE	PUPS		NUMBER PUPS PER LITTER		AVERAGE WEIGHT PUPS AT WEANING	F.I.[1]	G.I.[1]	V.I.[1]	L.I.[1]
			Born alive	Weaned	Born	Weaned					
							gm				
None	20	18	182	107	10.1	6.0	40.9	90	100	86	69
5% level											
Myrj 45	20	19	196	125	10.3	6.6	38.4	95	100	82	78
Myrj 52	20	18	164	95	9.1	5.3	37.2	90	100	79	73
Span 60	20	19	199	139	10.5	7.3	37.8	95	100	79	89
Tween 60	19	15	154	92	10.3	6.1	36.3	85	92	69	86
Tween 65	20	18	159	113	8.8	6.3	42.7	90	100	84	84
Tween 80	20	15	149	126	10.0	8.4	37.7	75	100	93	91
Mixture	19	18	174	116	9.7	6.5	36.8	95	100	78	86
Primex	20	15	131	92	8.8	6.1	44.8	80	94	70	100
10% level											
Myrj 45	20	20	205	99	10.3	5.0	37.9	100	100	64	76
Myrj 52	20	19	222	100	11.7	5.3	36.4	95	100	73	62
Span 60	20	18	188	127	10.4	7.1	31.8	100	90	82	82
Tween 60	20	18	200	101	11.1	5.6	36.2	90	100	61	83
Tween 65	20	17	185	100	10.8	5.9	37.7	85	100	59	92
Tween 80	18	18	191	144	10.6	8.0	35 ?	90	100	87	87
Mixture	20	14	142	75	10.1	5.4	38.9	70	100	68	78
Primex	20	15	161	116	10.7	7.7	36.7	80	93	80	90
20% level											
Myrj 45	22	13	142	28	10.9	2.2	28.1	59	100	28	70
Myrj 52	20	14	131	83	9.3	5.9	33.5	75	93	68	93
Span 60	20	17	145	75	8.5	4.4	31.5	85	100	60	86
Tween 60	20	12	103	36	8.5	3.0	37.4	60	100	41	86
Tween 65	20	13	147	31	11.3	2.4	33.1	75	87	35	60
Tween 80	20	12	133	87	11.0	7.2	32.3	60	100	71	92
Mixture	20	13	130	22	10.0	1.7	30.5	65	100	28	59

[1] See footnotes, table 4.

TABLE 6

Summary of reproduction and lactation data of F_2 generation rats for two matings

(10 females per group)

EMULSIFIER OR FAT	NUMBER OF MATINGS	LITTERS BORN ALIVE	PUPS		NUMBER PUPS PER LITTER		AVERAGE WEIGHT PUPS AT WEANING	F.I.[1]	G.I.[1]	V.I.[1]	L.I.[1]
			Born alive	Weaned	Born	Weaned					
							gm				
None	19	15	138	101	9.2	6.7	39.1	84	94	84	87
5% level											
Myrj 45	20	10	93	38	9.3	3.8	43.7	50	100	89	46
Myrj 52	20	12	90	48	7.5	4.0	40.1	65	92	66	81
Span 60	20	14	127	69	9.1	4.9	34.4	70	100	68	80
Tween 60	20	19	215	109	11.3	5.7	33.6	95	100	63	81
Tween 65	20	12	112	61	9.3	5.1	38.0	60	100	63	86
Tween 80	20	13	126	72	9.7	5.5	35.8	65	100	70	82
Mixture	20	11	97	25	8.8	2.3	37.0	55	100	44	58
Primex	20	13	105	55	8.1	4.2	46.0	65	100	61	86
10% level											
Myrj 45	19	12	110	53	9.2	4.4	39.2	63	100	51	95
Myrj 52	20	9	84	49	9.3	5.4	36.6	45	100	70	83
Span 60	20	16	150	88	9.4	5.5	36.1	80	100	67	88
Tween 60	20	11	87	25	7.9	2.3	36.4	60	92	33	86
Tween 65	20	13	146	75	11.2	5.8	33.0	65	100	55	94
Tween 80	19	13	101	27	7.8	2.1	38.5	68	100	45	60
Mixture	20	9	92	30	10.2	3.3	37.6	50	90	63	52
Primex	19	11	89	39	8.1	3.5	40.0	58	100	57	76
20% level											
Myrj 45	20	13	106	18	8.2	1.4	29.8	65	100	40	43
Myrj 52	20	9	88	62	9.8	6.9	28.8	45	100	86	82
Span 60	20	6	55	19	9.2	3.2	30.2	30	100	58	59
Tween 60	20	11	63	21	5.7	1.9	36.2	55	100	41	81
Tween 65	20	17	184	22	10.8	1.3	25.9	85	100	20	61
Tween 80	20	7	72	44	10.3	6.3	32.0	40	88	64	96
Mixture	20	15	144	17	9.6	1.1	36.2	75	100	21	57

[1] See footnotes, table 4.

reproduction and lactation can be obtained from only the first two litters in a generation.

Comparison of the reproduction data in tables 4, 5, and 6 shows that the responses in the three successive generations were quite similar. The proportion of matings resulting in pregnancy tended to be lower at the 20% emulsifier level although this effect was less noticeable in the case of Myrj 45 and Span 60 than in the other emulsifier groups. That the third generation was generally less productive than the first two may be seen in the lower F.I. values for the Primex as well as the emulsifier groups.

Nearly, if not exactly, 100% of pregnancies went to term in all generations. The trend toward higher mortality during the 4 days post partum as the level of emulsifier increased, was not as marked in the F_1 and F_2 as in the F_0 generation. The proportion of nurslings surviving the lactation period was reduced in some cases in the F_2 generation at the 20% emulsifier level (e.g. Myrj 45, Span 60, Tween 65).

Compared with the other 20% emulsifier groups, the one receiving Tween 80 evidenced a striking superiority in respect to survival of young from birth to weaning age; whereas the Myrj 45, Tween 65 and mixed emulsifier groups appeared to respond most poorly.

Reproductive performance in general appeared to be inferior in the third generation rats compared to their progenitors, in both the emulsifier and Primex series.

The reproductive performance of the F_3 generation was not investigated because the rats in this generation were sacrificed either at weaning or at the end of the 12-week growth period.

The effect of increasing the fat level of the basal diet on post-partum survival. In order to determine whether the effects observed at the 20% emulsifier levels, especially in relation to fertility and viability, might have been due to the low (4.0%) fat content of the basal, unsupplemented diet, further tests were conducted on the F_2 generation rats after their second litters were weaned. These groups of about 10

TABLE 7

Effect of addition of Primex (9%) to diets containing emulsifiers at 20% levels [1]

EMULSIFIER	PRIMEX	NUMBER OF MATINGS	TOTAL NUMBER PUPS BORN	AVERAGE WEIGHT OF PUPS AT WEANING	FERTILITY INDEX [2]		GESTATION INDEX [2]		VIABILITY INDEX [2]		LACTATION INDEX [2]	
					1–2	3–4	1–2	3–4	1–2	3–4	1–2	3–4
20%	*%*			*gm*								
Myrj 45	0	10	36	30.7	(65)	40	(100)	100	(40)	50	(43)	94
	9	9	25	32.0		44		100		80		90
Myrj 52	0	10	55	36.3	(40)	50	(100)	100	(86)	43	(82)	92
	9	9	43	43.8		67		100		30		54
Span 60	0	11	88	34.3	(30)	91	(100)	100	(58)	33	(59)	83
	9	9	70	30.2		89		100		53		92
Tween 60	0	8	50	34.7	(55)	88	(100)	100	(41)	30	(81)	93
	9	10	52	40.6		90		100		67		97
Tween 65	0	10	76	38.7	(85)	80	(100)	100	(20)	21	(64)	50
	9	9	58	62.0		67		100		10		16
Tween 80	0	10	21	— [3]	(40)	20	(88)	100	(64)	14	(96)	0
	9	10	4	— [3]		10		100		50		0
Mixture	0	10	41	40.0	(75)	40	(100)	100	(21)	39	(57)	75
	9	10	73	40.0		70		100		29		81

[1] Each subgroup represents the results of the third and 4th matings of 5 males and 5 females from F_2 generation. (cf. table 6 for responses to first and second matings of the full groups.)

[2] Shown in parentheses for first two matings (cf. table 6).

[3] No survivors at 21 days.

males and 10 females were divided into two subgroups. One was allowed to continue on the same diet, whereas the other received an addition of 9% of Primex (the level computed as isocaloric with 20% Myrj 45), in replacement of an equal proportion of the wheat and corn component of the basal diet. These subgroups were then mated as before to produce two additional litters from each female.

Table 7 shows the number of matings actually set up in each subgroup and the results of these matings. There was no consistent relation between the responses of the 20% emulsifier groups to the third and 4th matings as compared to the first two (table 6). The F.I. values rose in two cases (Span 60 and Tween 60), fell in three groups (Myrj 45, Tween 80 and the Mixture), and were practically unchanged in the other two groups (Myrj 52 and Tween 65). The V.I. values dropped in three instances (Myrj 52, Span 60 and Tween 80) and showed little or no change in the remaining groups. However improvement in lactation was observed in all groups with the exception of the Tween 65 group which fell off somewhat and the Tween 80 group which, quite surprisingly in view of its previously high L.I. values, showed complete failure.

As regards the influence of the addition of dietary fat on the reproductive responses, it is apparent from the F.I. values in table 7 that little if any increase on the proportion of successful matings was observed. Post partum survival was improved in 4 of the emulsifier groups (Myrj 45, Span 60, Tween 60, and Tween 80) as a result of the addition of fat, while the remainder showed little change. No striking effects on the L.I. values were observed from the addition of fat to the 20% emulsifier diets with the exception of the Myrj 52 and Tween 65 groups where decreases were noted.

In considering these somewhat erratic responses to fat supplementation in the third and 4th matings it is necessary to take into account the fact that the subgroups were of smaller size than the original groups. In any event it can be stated that the most common finding appeared to be improved via-

bility of the newborn wnen the fat level of the 20% emulsifier diets was increased by the addition of 9% of vegetable fat.

DISCUSSION

By comparison of the corresponding values in tables 4, 5 and 6, it can be seen that no adverse or cumulative effect on mating instinct, fertility or gestation occurred in any of the groups of rats receiving the same test or control rations through three successive generations. A reduction in the F.I. occurred in certain groups of the F_2 generation, but since they included the groups receiving 5 and 10% of Primex, and several low as well as high level emulsifier groups, this effect can not be ascribed to the emulsifiers *per se*. As a matter of fact fertility in the 20% Tween 65 and Mixture groups was slightly better in the third than in the preceding generations. Hence, so far as the emulsifiers are concerned, no particular significance is attached to the changes in the F.I. in the F_2 generation.

A frequent observation was a diminution in viability of the newborn at the highest level of emulsifier in the diet. This was noted not only in all three generations on the Myrj 45 and Tween 65 diets and to a less pronounced degree on Tween 60 and Mixture diets, but also in at least one generation on all the diets, including the basal and Primex groups. As mentioned above in relation to the F_0 generation, it is possible that the added stress of perianal irritation in certain of the higher level emulsifier groups contributed to post partum mortality of the young.

The effect of some of the 20% emulsifier diets on fertility (Myrj 52 and Tween 60) and on viability of the young, appeared to be attributable in part at least to the fact that the basal diet was relatively low in fat since these responses were improved by the addition of neutral vegetable fat to the diets.

That the young which survived 4 days or more were adequately nourished is shown by the figures for L.I. which are, in general, higher than those for the V.I. A few exceptions

were noted, however. For example, the relatively low L.I. values for the basal control group in the F_1 generation, for the 10% Primex group in the F_2 generation, and for the 5 and 20% (but not the 10%) Myrj 45 groups in the F_2 generation. These scattered observations were not limited to the emulsifier groups nor were they graded to dosage level. They may therefore be regarded as falling within the range of normal biological variation.

SUMMARY AND CONCLUSIONS

Breeding studies were undertaken in successive generations of rats on diets containing partial ester emulsifiers (Myrj 45 and 52, Span 60, and Tween 60, 65 and 80) to determine whether their chronic ingestion at levels up to 20% might induce cumulative or subtle effects manifested only under the conditions of physiological stress thus imposed. The responses were assessed, *inter alia,* in terms of indexes representing the proportions of matings resulting in pregnancy (fertility), pregnancies resulting in live litters (gestation), young remaining alive at 4 days (viability), nurslings weaned in relation to the number alive at 4 days and their weights at weaning (lactation).

On the average, 7 out of 10 matings were successful in both control and emulsifier groups, regardless of the level of dietary supplementation. Practically all pregnant rats cast live litters. The reproduction and lactation responses in all emulsifier groups at the 5% level were no different from those of the controls. Probably because of maternal neglect, survival of newborn litters was somewhat diminished in several of the emulsifier groups at the 10% level (Myrj 45, Span 60, and Tween 65) and in all of them at 20%. At the highest level some impairment in lactation efficiency was evidenced in most groups by the lower weaning weights; and in the Myrj 45, Tween 65, and mixed emulsifier groups also by greater mortality of the nurslings.

Similar responses with respect to survival and lactation were noted in the two succeeding generations. Despite the

fact that the general level of reproductive performance (in the Primex as well as emulsifier series) was somewhat lower in the third generation, the effects noticeable particularly at the 20% dosage levels of emulsifiers were not markedly more severe in the third than in the initial generation.

Increasing the fat level in the basal diet from its original concentration of 4% by adding 9% of Primex, had little if any effect on the proportion of successful matings in the 20% emulsifier groups. However, better survival of the young was observed in the Myrj 45, Span 60, Tween 60, and Tween 80 groups following the addition of fat. This suggests that the presence of more nearly normal levels of dietary fat would diminish such adverse effects as might be induced by the excessively high concentration of "undiluted" emulsifier in these experimental diets.

LITERATURE CITED

MIRONE, L., F. P. PANZARELLA AND L. R. CERECEDO 1948 A new method for reporting data on reproduction and lactation in the mouse. Science, *108:* 139.

OSER, B. L., AND M. OSER 1956 Nutritional studies on rats on diets containing high levels of partial ester emulsifiers. I. General plan and procedures: growth and food utilization. J. Nutrition, *60:* 367–390.

MEASUREMENT OF THE NET UTILIZATION OF HEAT-PROCESSED PROTEINS BY MEANS OF THE PEPSIN DIGEST-RESIDUE (PDR) AMINO ACID INDEX[1,2]

A. LEONARD SHEFFNER, RICHARD ADACHI AND HARRY SPECTOR
Nutrition Branch, Quartermaster Food and Container Institute for the Armed Forces, Chicago, Illinois

(Received for publication July 9, 1956)

It has long been recognized that the nutritional value of a protein is dependent primarily upon its constituent amino acids. Nonetheless, only since the publication in recent years of reliable and relatively simple methods for amino acid analysis has it been feasible to develop *in vitro* procedures for the measurement of protein quality (Mitchell and Block, '46; Kühnau, '49; Oser, '51; and Mitchell, '54). These procedures, based upon chemical analysis of proteins for their essential amino acids, yielded figures which were well correlated with biological values.[3] However, for certain proteins the calculated values did not agree with the results of animal assay. In addition, an obvious fault with these methods lay in the fact that the biological value of many proteins was considerably changed by heat processing in the absence of dis-

[1] This paper reports research undertaken by the Quartermaster Food and Container Institute for the Armed Forces and has been assigned number 644 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

[2] A preliminary report of this work was presented before the American Society of Biological Chemists at Atlantic City, New Jersey, April 16–20, 1956 (Sheffner, Adachi and Spector, '56).

[3] The term biological value is used in this paper in accordance with the formula introduced by Mitchell ('24) after Thomas.

cernible destruction of amino acids. Consequently, several procedures were developed to take into account the enzymatic availability of component amino acids (Dunn and Rockland, '47; Anderson and Williams, '51; Horn et al., '52; and Halevy and Grossowicz, '53). The results obtained with these methods generally did not correlate well with the biological value of proteins as determined by rat assay. The values reported by Horn et al. ('52) were in good agreement with the relative protein efficiency of heat processed cotton seed meals; however, no evidence was presented concerning the general applicability of the method.

A procedure for the *in vitro* estimation of the net utilization of proteins was reported by Sheffner et al. ('55, '56). This method referred to as the Pepsin Digest-Residue (PDR) index was derived by integration of the pattern of amino acids released by *in vitro* pepsin digestion with the amino acid pattern of the remainder of the protein. The new index gave excellent correlation with the net utilization value of the proteins studied. The present study demonstrates that the PDR index also measures changes in net protein utilization which occur during heat processing and storage.

METHODS AND MATERIALS

Acid and alkaline hydrolysates and enzyme digests were prepared as previously described (Sheffner et al., '56), except that pancreatin (USP) was used where trypsin was formerly indicated; also, alkaline hydrolysis for tryptophan and tyrosine was extended to 8 hours at 120°C. with 5 N NaOH. Nitrogen was measured by a macro-Kjeldahl procedure in which mercuric oxide was used as the digestion catalyst. Individual amino acid analyses were performed by the microbiological procedures of Sheffner et al. ('48) as subsequently modified ('56).

The test protein materials used in this study were: vitamin-free casein,[4] low-temperature solvent-extracted soybean meal

[4] Labco brand, The Borden Company.

and raw soybean meal.[5] The raw soybean meal was finely ground with solid CO_2 in a Wiley mill before use. For heat treatment, the finely divided casein and soybean samples were spread in a Petri dish to a depth of 0.5 inch and heated in a thermostatically controlled electric oven or in an autoclave. Steaming of samples was done in the autoclave at atmospheric pressure. Casein and soy samples which were heat treated in the autoclave were subsequently dried under vacuum for 24 hours at room temperature and then finely ground with a mortar and pestle. The preparations were then thoroughly blended in a kitchen mixer.[6]

The beef used in the study was from boneless cuts from the low end of the loin in U. S. Good beef. Steaks were cut one-half inch in thickness and trimmed of fat to three-eighths inch. Pan-fried beef was obtained by frying the steaks without added fat at 375°F. for a total of two and one-half minutes, the steaks being turned every one-half minute. Before analysis, the meat samples were finely ground in an electric meat grinder and mixed thoroughly. Beef used in the canned ground meat and spaghetti was of utility, cutter, or canner grade, trimmed and boned and carrying not more than 10% of trimmable fat. The spaghetti was a semolina farina-egg albumin blend containing not less than 12.2% ($N \times 5.7$) of protein. The ratio of meat to spaghetti in the product was 5 to one. The meat and spaghetti were pre-cooked, the meat being braised without burning. Heat processing was done at 240°F. for 140 minutes.

RESULTS AND DISCUSSION

The effect of heat treatment upon the PDR index and net utilization of casein is presented in table 1. Heating casein in the electric oven at 350°F. resulted in a progressive decrease in PDR index values from 68, initially, to 23 after 5 hours. The net utilization values of the oven-heated caseins as de-

[5] Nutrisoy 7B and raw soybean meal were obtained from The Archer-Daniels-Midland Company, through the courtesy of Dr. J. W. Hayward.

[6] Hobart Kitchen Aid.

termined by the Mitchell ('24) method of nitrogen balance in rats were similarly lowered. The PDR index of casein was not appreciably changed when the casein was autoclaved at 250°F. for 30 minutes or for 20 hours. Net utilization values were not obtained for these samples; however, Chick et al. ('35) reported little, if any, change in the biological value or digestibility of casein heated at 250°F. for as long as 72 hours.

The effect of processing and storage upon beef and a beef with spaghetti mixture is presented in table 2. For fresh raw beef, a PDR index value of 76 was obtained. This checks closely with the net utilization values obtained by Mitchell

TABLE 1

The effect of heat treatment upon the PDR index and net utilization of casein

TREATMENT	PDR INDEX	NET UTILIZATION
None	68	82
Oven, 350°F., 40 min.	60	
Oven, 350°F., 1 hr.	39	44
Oven, 350°F., 5 hrs.	23	24
Autoclave, 250°F., 30 min.	71	
Autoclave, 250°F., 20 hrs.	66	

and co-workers ('49) and Mayfield and Hedrick ('49). Pan-fried beef was not significantly different from the control. In this respect, both Mitchell et al. and Mayfield and Hedrick have reported that roasting does not reduce the net utilization of beef protein. In the case of the mixed beef and spaghetti, there was a decrease in the PDR index from 72 to 66 following processing and a further decrease to 60 after storage for 6 months at 118°F. These changes in PDR index reflected the drop in net utilization as measured by rat assay.

The PDR index and net utilization value of raw and heated soybean meals are presented in table 3. The PDR index of soybean meal steamed for 30 minutes was the same as the net utilization value. Soybean meal autoclaved for 8 hours showed an equivalent decrease in both the PDR index and the net

utilization value. The PDR index of low-temperature solvent-extracted soybean meal also was very close to the net utilization value. However, contrary to the PDR index results, the rat assays indicated that the raw soybean meal had a net protein utilization value which was significantly below that of the steamed soybean meal. Since, in the calculation of the

TABLE 2

Effect of processing and storage upon the PDR index and net utilization of beef products

PRODUCT	TREATMENT	PDR INDEX	NET UTILIZATION
Beef	None (raw)	76	74,[1] 76 [2]
Beef	Roasted, 5 hrs., 300°F.	..	74 [1]
Beef	Roasted, open pan, 325°F. to internal temp. of 176°F.	..	77 [2]
Beef	Pan fried, 2½ min., 375°F.	77	
Beef with spaghetti	Precooked	72	77
Beef with spaghetti	Precooked, processed, 240°F., 140 min.	66	66
Beef with spaghetti	Processed, 240°F., 140 min., stored 6 mos., 118°F.	60	58

[1] Mitchell, Hamilton and Beadles ('49).
[2] Mayfield and Hedrick ('49).

TABLE 3

The effect of heat treatment upon the PDR index and net protein utilization of soybean meal

TREATMENT	PDR INDEX	NET UTILIZATION
Raw	70	58
Steamed, 212°F., 30 min.	69	69
Autoclaved, 250°F., 8 hours	44	47
Low temp. solvent extracted	65	63
Low temp. solvent extracted, steamed, 212°F., 30 min.	70	..
Low temp. solvent extracted, autoclaved, 250°F., 8 hours	42	..

PDR index, only the pepsin digest and total amino acid results are used, the question arose as to whether a correction for trypsin digestion should be introduced into the PDR index to account for the effects of anti-tryptic factors in raw soybean meal.

In an attempt to answer this question, the soybean samples were treated with pepsin as usual, then adjusted to pH 8.2 and incubated with pancreatin for 24 hours at 37°C. The

TABLE 4

Effect of optimal heating upon the enzymatic release of amino acids from soybean meal

AMINO ACID	COMPLETE HYDROLYSIS		PEPSIN		PEPSIN PLUS PANCREATIN	
	Raw	Steamed [1]	Raw	Steamed	Raw	Steamed
	mg/gm	*mg/gm*	*per cent liberation*		*per cent liberation*	
Cystine	12.8	12.4	2.3	1.6	4.7	21.0
Lysine	59.1	59.5	2.0	1.7	20.6	68.9
Histidine	29.2	30.8	2.4	2.0	17.1	33.8
Valine	57.8	56.5	16.3	15.4	36.7	56.8
Methionine	13.2	13.5	15.9	14.1	36.4	51.1
Isoleucine	54.0	54.5	47.6	47.5	68.2	89.9
Leucine	77.3	78.0	57.6	60.3	77.8	96.4
Tyrosine	31.0	30.9	13.9	13.9	66.8	81.6
Tryptophan	16.8	17.0	22.6	22.4	43.4	51.2
Phenylalanine	57.0	59.7	17.7	16.8	44.9	50.2
Threonine	37.9	39.0	53.8	48.7	74.9	84.1

[1] Steamed at atmospheric pressure (212°F.).

total amino acid composition of the proteins and the percentage liberation of amino acids from the raw and steamed soybean meals by the pepsin and the pepsin plus pancreatin treatments are presented in table 4. Whereas there is no change in the quantity of amino acids in the completely hydrolyzed protein nor in the amount of amino acids released by pepsin, there is a considerable increase in the amount of amino acids released from the steamed soybean meal by the pepsin plus pancreatin treatment. The results also show that this increased liberation following pancreatin treatment varies with the individual amino acids, and in this respect

they are in agreement with the work of Melnick, Oser and Weiss ('46). Thus, while the increase in geometric mean for the 11 amino acids was only 42.3%, the increase for cystine was 347% and for lysine 234%. However, contrary to the results obtained by Melnick et al. with pancreatin alone, the increased liberation of methionine from the steamed soybean meal was found to be no greater than that of the mean increase.

The non-uniform suppression of the pancreatic release of amino acids by anti-tryptic factors has been suggested as a major cause of the lower biological value of raw soybean meal (Melnick et al., '46). If this were true, supplementation of optimally heated soy meal with the amino acids limiting in

TABLE 5

Effect of optimal heating and amino acid supplementation upon the biological value of raw soybean meal

SUPPLEMENTATION	TREATMENT	BIOLOGICAL VALUE
None	None	68
None	Steamed, 30 min.	75
Methionine and lysine	None	79
Methionine and lysine	Steamed, 30 min.	86

the trypsin digest, namely lysine and cystine (or methionine), should not result in a biological value greater than that of the supplemented raw meal. Consequently, in the present study, raw soybean meal was supplemented to correct for the deficiency of these amino acids both at the tryptic stage of digestion and in the total protein. Another sample of the raw soy meal was similarly supplemented after optimal heat treatment. The biological values of these preparations as determined by rat assay are presented in table 5. The results show that an equivalent increase in biological value due to heating occurred whether or not the raw soybean meal was supplemented with lysine and methionine. Since lysine and methionine (or cystine) were the amino acids most affected by the anti-tryptic factors, the data suggest that although the

anti-tryptic factors of raw soybean meal introduce differences in the rate of release of individual amino acids, these differences do not significantly influence the biological value of the raw protein. On the basis of the results reported here, a correction for tryptic digestion would not be expected to improve the accuracy of the PDR index for predicting the net utilization value of raw soybean meal.

Overestimation by the PDR index of the net utilization value of raw soybean meal is probably best explained as being due to the presence in the meal of a toxic factor or factors. Such toxic factors have been experimentally demonstrated and shown to be sensitive to heat. (Liener et al. '49; Liener, '53; Desikachar and De, '47; Klose et al., '48; Borchers et al., '48; Westfall et al., '48). Consequently, the PDR index should also be an accurate indicator of the net protein utilization in soybean preparations in which the toxic factor has been destroyed by heat treatment.

The particular advantage of the nitrogen balance method for measuring biological value (Thomas, '09; Mitchell, '24) over other biological assay methods is that it determines directly the storage of protein in growth rather than assuming that this storage is proportional to body weight gains. The procedure also distinguishes between loss of nitrogen in the digestive process, i.e., undigested plus secretory protein, and losses due to the remaining metabolic processes of the animal body. However, for purposes of appraising the value of a food as a source of dietary protein, a single figure for the net protein utilization has distinct advantages (Mitchell, '44). For most food proteins the distinction between biological value and net utilization is academic since their coefficients of digestibility are very high. However, in the case of heat-processed foods in which protein digestibilities are significantly reduced it is important for practical nutritional considerations to measure the net utilization rather than the biological value. The PDR index which measures the net utilization of proteins directly is a useful procedure for estimation of the nutritional quality of both natural and processed proteins.

SUMMARY

The pepsin digest-residue (PDR) amino acid index was found to reflect the effects of heat processing upon the net protein utilization of proteins and mixed protein foods.

Data are also presented which indicate that the discrepancy between the PDR index and the net utilization value of raw soybean meal is due to the presence in raw soybeans of substances which exert effects apparently unrelated to the enzymatic release of amino acids. The PDR index can be used to predict the net utilization of soybean meals accurately if these "toxic" factors are inactivated.

ACKNOWLEDGMENT

The authors wish to thank Dr. D. H. Calloway for determining the biological value and net utilization of the proteins used in this study. We also express our appreciation to Mr. John McMullen for supplying the proximate analyses and Mr. Lawrence Wills for aiding in the amino acid analyses.

LITERATURE CITED

ANDERSON, M. E., AND H. H. WILLIAMS 1951 Microbiological evaluation of protein quality. 1. A colorimetric method for the determination of the growth of *Tetrahymena geleii W* in protein suspensions. J. Nutrition, *44:* 335.

BORCHERS, R., C. W. ACKERSON, F. E. MUSSEHL AND A. MOEHL 1948 Trypsin inhibitor. VIII. Growth inhibiting properties of a soybean trypsin inhibitor. Arch. Biochem., *19:* 317.

CHICK, H., M. A. BOAS-FIXSEN, J. C. D. HUTCHINSON AND H. M. JACKSON 1935 The biological value of proteins. 7. The influence of variation in the level of protein in the diet and of heating the protein on its biological value. Biochem. J., *29:* 1712.

DESIKACHAR, H. S. R., AND S. S. DE 1947 Role of inhibitors in soybean. Science, *106:* 421.

DUNN, M. S., AND L. B. ROCKLAND 1947 Biological value of proteins determined with *Tetrahymena geleii H.* Proc. Soc. Exp. Biol. Med., *64:* 377.

HALEVY, S., AND N. GROSSOWICZ 1953 A microbiological approach to nutritional evaluation of protein. Ibid., *82:* 567.

HORN, M. J., A. E. BLUM, M. WOMACK AND C. E. F. GERSDORFF 1952 Nutritional evaluation of food proteins by measuring availability of amino acids to microorganisms. I. Cottonseed protein. J. Nutrition, *48:* 231.

KLOSE, A. A., B. HILL AND H. L. FEVOLD 1948 Food value of soybean protein as related to processing. Food Technology, *2:* 201.

KÜHNAU, J. 1949 The biochemistry of natural proteins. Angew. Chemie., *61:* 357.

LIENER, I. E. 1953 Soyin, a toxic protein from the soybean. I. Inhibition of rat growth. J. Nutrition, *49:* 527.

LIENER, I. E., H. SPECTOR, H. L. FEVOLD AND G. H. BERRYMAN 1949 The effect of soybean growth inhibitors on the availability of metheonine for growth and lipotropism. Arch. Biochem., *24:* 299.

MAYFIELD, H. L., AND M. T. HEDRICK 1949 The effect of canning, roasting and corning on the biological value of the proteins of western beef, finished on either grass or grain. Ibid., *37:* 487.

MELNICK, D., B. L. OSER AND S. WEISS 1946 Rate of enzymic digestion of proteins as a factor in nutrition. Science, *103:* 326.

MITCHELL, H. H. 1924 The nutritive value of proteins. Physiol. Rev., *4:* 424.

———— 1944 Determination of the nutritive value of the proteins of food products. Ind. Eng. Chem., Anal., *16:* 696.

———— 1954 Biological value of proteins and amino acid interrelationships. In Symposium on Methods for Evaluation of Nutritional Adequacy and Status. pp. 13–28. Ed. by H. Spector, M. S. Peterson, and T. E. Friedemann, National Research Council, Washington.

MITCHELL, H. H., AND R. J. BLOCK 1946 Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem., *163:* 599.

MITCHELL, H. H., T. S. HAMILTON AND J. R. BEADLES 1949 The nutritional effects of heat on food proteins, with particular reference to commercial processing and home cooking. J. Nutrition, *39:* 413.

OSER, B. L. 1951 Method for integrating essential amino acid content in the nutritional evaluation of protein. J. Am. Dietetic Assn., *27:* 396.

SHEFFNER, A. L., G. A. ECKFELDT AND H. SPECTOR 1955 Release of amino acids during peptic digestion as a determining factor in the biological value of proteins. Fed. Proc., *14:* 279.

———— 1956 The pepsin digest-residue (PDR) amino acid index of net protein utilization. J. Nutrition, *60:* 105.

SHEFFNER, A. L., J. B. KIRSNER AND W. L. PALMER 1948 Studies on amino acid excretion in man. I. Amino acids in urine. J. Biol. Chem., *175:* 107.

THOMAS, K. 1909 Über die biologische Wertigkeit der Stickstoffsubstanzen in verschiedenen Nahrungsmitteln. Arch. Anat. u. Physiol., Physiol. Abth., p. 219.

WESTFALL, R. J., D. K. BOSSHARDT AND R. H. BARNES 1948 Influence of crude trypsin inhibitor on utilization of hydrolyzed protein. Proc. Soc. Exp. Biol. Med., *68:* 498.

SERUM CHOLESTEROL LEVELS OF YOUNG AND OF ELDERLY WOMEN CONSUMING AN INSTITUTION DIET[1]

GEORGIANNA R. WALKER, ELLEN H. MORSE AND MARTHA POTGIETER
School of Home Economics and Storrs Agricultural Experiment Station, University of Connecticut, Storrs[2]

(Received for publication June 27, 1956)

Many studies have been made of the effects of age and of diet on the cholesterol content of the blood, but conflicting results have been reported. Messinger et al. ('50) and Keys et al. ('50) found it necessary to use very high or very low amounts of cholesterol intake to raise or lower the cholesterol level in the blood of human subjects. Mayer et al. ('54) reported that, in young men subjects, a diet low in fat and cholesterol lowered the plasma cholesterol. Addition of cholesterol to the diet caused no change, but an increase in percentage of calories from fat, either animal or vegetable, caused a rise in plasma cholesterol. Hildreth et al. ('51) and Anderson and Keys ('54) also found a rise in serum cholesterol when fat was added to the diet. Gillum et al. ('55), in their study of a large number of elderly men and women, reported a positive correlation between serum cholesterol and fat intake and between serum cholesterol and cholesterol intake, both significant at the 5% level. Wilkinson et al. ('50), however, studying 83 persons of all ages from families with familial hypercholesteremia, found no relationship between

[1] Supported in part by funds from a Regional Project, Relationship of Nutrient Intake to Nutritional Status in Human Subjects; a cooperative study involving Agricultural Experiment Stations in the Northeastern Region.

[2] Credit is due Dr. Geoffrey Beall, Professor of Statistics, and Dr. Mary L. Greenwood, Associate Professor of Foods and Nutrition, University of Connecticut, for verification of the statistical treatment of the data.

fat, carbohydrate, protein or cholesterol intake and the serum cholesterol level.

In the studies relating age and serum cholesterol most workers agree that in women, at least, the serum cholesterol level rises with age. Sperry and Webb ('50), who studied the serum cholesterol levels of 14 men and 8 women in 1934–'36 and again in 1949, reported that in 6 of the men and in 6 of the women increases of 15 to 30% were found in the second survey. In the work of Gram and Leverton ('49) and of Garcia et al. ('55), with women on a self-selected diet, a significant rise in serum cholesterol with age was shown. Hobson et al. ('53) reported that the mean serum cholesterol levels of their subjects (elderly men and women living at home) were significantly higher than those of control groups of younger people.

Work with animals has suggested a relationship between cholesterol and ascorbic acid in the body metabolism. Booker et al. ('51) found an increase in serum cholesterol in both rats and dogs after administration of ascorbic acid, and Consuelo Mendoza ('52) demonstrated that injection of rabbits with ascorbic acid also resulted in a rise in serum cholesterol. Gillum et al. ('55), in their study of older women, reported a slight positive relationship between serum cholesterol and serum ascorbic acid.

In the present study a comparison was made of the serum cholesterol levels of women of two different age groups offered the same institution diet. The effect of supplementation of the diet with various levels of ascorbic acid on the serum cholesterol in the two groups was also studied.

PROCEDURE

The subjects for this study were 29 women in a state training school for the handicapped [3]; all were in good physical health. They were divided into two groups according to age. In the younger group of 15 subjects the average age was 31 years, with a range of 28 to 34. In the older group of 14 subjects the average age was 64 years, with a range of 56 to 77.

During the 4 months of the study the subjects ate together and all were served the usual institution diet. Calculations showed that, with the exception of iron, their diet on the average contained at least 100% of the daily allowances for nutrients recommended by the National Research Council ('53). It contained over 96% of the iron allowance. As these women were also serving as subjects in an ascorbic acid study, the high-vitamin C foods, such as citrus fruits, tomatoes, pineapple, and raw cabbage, were omitted from their diet. When these foods appeared on the institution menu, other fruits and vegetables were always served to the subjects. The ascorbic acid intake was calculated and found to average 32 ± 1 mg per day. After 7 weeks on the restricted diet, ascorbic acid supplements were given to each subject daily starting with 15 mg per day. The supplement was increased to levels of 25, 40, 50, and 75 mg per day, each for a two week period. As these supplements were in addition to the 32 mg received in the food each day, the total intake of ascorbic acid at each level was 47, 57, 72, 82, and 107 mg per day.

The food intake of each subject was recorded on 16 scattered days during the study, as well as during the 24 hour period preceding the collection of blood samples for cholesterol determinations. Food consumption was recorded in terms of the number of servings or fraction of serving of each food. Servings were weighed at intervals to determine size of portions. The fat, protein, carbohydrate, calories, minerals, and vitamins (except for vitamin C) were calcu-

[3] Mansfield State Training School and Hospital, Mansfield Depot, Connecticut. Thanks are extended to the medical and dietary staffs of the school as follows: To Dr. Gail F. Moxon, M.D., and Dr. Harriet Bixby, M.D., resident doctors, for the physical examinations; to Dr. Joseph E. Nowrey, M.D., resident doctor, and his assistants for taking the venous blood samples; to Dr. Luke Grotano, D.D.S., resident dentist, for the dental examinations; to Mrs. Pauline Duckett, chief dietitian and to the dietary staff of the women's dining room, for cooperation in the collection of dietary data and food samples; to the 29 mentally retarded women who served so cheerfully and cooperatively as subjects; and to Dr. Neil A. Dayton, M.D., Superintendent of the Training School, for making the institution available for the study and for his continued interest and encouragement in research work.

lated for the diets, using the U.S.D.A. Agriculture Handbook No. 8 (Watt and Merrill, '50). The cholesterol content of the diets was calculated from the tables of Okey ('45) and from data provided by Gillum ('55).[4] Analysis of food samples collected in the dining room from time to time throughout the experimental period yielded information for calculating the vitamin C content of the diets. The method used in these determinations was the 2,4-dinitrophenylhydrazine method of Roe and Kuether ('43), using norit oxidation.

Venous blood samples for serum cholesterol determinations were taken after 7 weeks on the 32-mg level of ascorbic acid intake, and following the 25-, 50-, and 75-mg supplement levels. The first three samples were taken at intervals of one month. The 4th sample was taken two weeks after the third. The samples were always taken at 10:00 A.M., three hours after breakfast. A modification of the method of Kibrick, Roberts and Skupp ('51)[5] was used to determine the total serum cholesterol in duplicate 150-mm^3 samples of serum. The samples were read in a Coleman Jr. spectrophotometer. Serum ascorbic acid was determined according to the method outlined in the Northeast Regional Publication on Techniques ('51).

Height and skeletal build were recorded during a physical examination of the women made at the beginning of the study. Each subject was weighed every month. The percentage deviation from the desirable weight was calculated for each subject by means of the Metropolitan Life Insurance Tables (Metropolitan Life Insurance Co., '42).

RESULTS

The women in the older group had a higher serum cholesterol level than the younger women throughout the study. The mean serum cholesterol level for the older women was

[4] Cholesterol values for foods provided by Dr. Helen L. Gillum were from data compiled by the California Agricultural Experiment Station for use in the Western Regional Research Project W4.

[5] Dr. Mary M. Clayton of the Maine Agricultural Experiment Station assisted in the modification of this method.

230 ± 9 mg per 100 ml of serum and for the younger women, 172 ± 8 mg. The correlation between age of subjects and serum cholesterol level was positive: $r = 0.625$, which is significant at the 1% level. The regression equation of serum cholesterol (Y) on age (X) was $Y = 1.55\,X + 127.06$. The serum cholesterol levels of the older subjects in the present study were lower than those observed by Gillum et al. ('55) and by Hobson et al. ('53) in older women living at home. However, they were comparable to the average value (237 mg per 100 ml) found by Kountz et al. ('45) in women living in institutions.

Two of the younger women in the present study had consistently higher serum cholesterol levels (over 230 mg per 100 ml) than the others in their group. One of these proved to have a low basal metabolic rate. This condition has been associated with high serum cholesterol levels (Turner and Steiner, '39; Wisniewski, '55). The other woman was mongoloid. High cholesterol levels have been reported in young mongoloids under 25 years of age (Benda and Mann, '55; Simon et al., '54).

During the rise in the serum ascorbic acid level of the younger women with increased intake of vitamin C, no significant change occurred in their serum cholesterol level. In the older group, as the serum ascorbic acid level was rising with increased intake of the vitamin, the serum cholesterol rose from an average of 219 ± 9 mg per 100 ml on the lowest level of vitamin C intake to 238 ± 10 mg per 100 ml on the highest level. A *t* test indicated that this rise was significant (P lies between 0.01 and 0.001). However, the correlation between serum cholesterol and serum ascorbic acid was only 0.212, which is not significant for this number of subjects.

Calculations from the food records for the 16 days showed that the two groups consumed on the average very nearly the same amounts of all nutrients (table 1). Fat and cholesterol intake values were studied particularly because high intake of these two substances has been implicated in high serum cho-

lesterol values. The average fat intake of the older group was 75 ± 3 gm per day and for the younger group was 76 ± 3 gm per day. This consisted of about 55 gm of animal fat from butter, milk, meat, bacon and eggs. The remainder was vegetable fat including salad oils, peanut butter and hydrogenated fats used in frying and in making pies, cakes and biscuits. The average cholesterol intake of the older group was 472 ± 17 mg per day and of the younger group, 447 ± 36 mg per day. Correlations between serum cholesterol and fat intake, and serum cholesterol and cholesterol intake were not significant.

TABLE 1

Average daily nutrient intakes of 15 women with an average age of 31 years and of 14 women with an average age of 64 years, living in an institution

NUTRIENT	YOUNGER GROUP Mean ± S.E.[1]	OLDER GROUP Mean ± S.E.[1]
Calories	2018 ± 78	2003 ± 44
Protein, gm	72 ± 3	72 ± 2
Fat, gm	76 ± 3	75 ± 2
Carbohydrate, gm	253 ± 9	251 ± 4
Calcium, mg	1002 ± 82	997 ± 75
Iron, mg	11.6 ± 0.4	11.7 ± 0.2
Vitamin A, I.U.	7972 ± 728	7054 ± 467
Thiamine, mg	1.22 ± 0.05	1.22 ± 0.02
Riboflavin, mg	1.91 ± 0.13	1.83 ± 0.10
Niacin, mg	13.7 ± 0.5	13.3 ± 0.2
Ascorbic acid, mg	32 ± 1	32 ± 1
Cholesterol, mg	447 ± 36	472 ± 17

[1] Standard error.

The younger women were found to be, on the average, 7% overweight and the older women, 20% overweight. No significant correlation appeared between percentage deviation from desirable weight and serum cholesterol. The younger women lost, on the average, 0.8 lb during the study. The greatest loss was 8 lb and the greatest gain was 6 lb. The older women had an average weight change of zero, with a range from — 7 lb to + 7 lb.

DISCUSSION

Results of this study indicate that the higher serum cholesterol levels found in older women were related to age rather than to diet. Cholesterol is thought to be a precursor of steroid hormones (Long, '47). Gillum et al. ('55) suggest that a decrease in the production of these hormones in old age might account for the higher level of cholesterol found in the blood serum of older women. This difference in use of cholesterol by older and younger women might also explain the difference in rise in serum cholesterol level in the two groups in response to increased intake of ascorbic acid. The younger women may use any extra cholesterol, produced by the body when ascorbic acid intake is increased, in hormone synthesis or in some other way, while the older women do not dispose of it by this method.

SUMMARY

The serum cholesterol and the dietary intakes of two age groups of women living in an institution were studied. The average age of the 15 women in the younger group was 31 years (range 28 to 34) and of the 14 women in the older group, 64 years (range 56 to 77).

Their diet was restricted in ascorbic acid to an average daily intake of 32 mg, but was adequate in all other nutrients. Samples for serum cholesterol analysis were taken at the end of the period of restricted diet and following supplementation of the diet with 25, 50, and 75 mg of ascorbic acid.

The mean serum cholesterol level for the older women was 230 $\pm$ 9 mg per 100 ml of serum and for the younger, 172 $\pm$ 8 mg. The correlation between age of subjects and serum cholesterol level was positive and significant at the 1% level. Calculations from the 16-day food records showed that the two groups consumed very nearly the same amounts of all nutrients. Results of this study indicate that the higher serum cholesterol levels found in older women were related to age rather than to diet.

A slight rise in serum cholesterol with rise in serum ascorbic acid was noticed in the older group, but not in the younger group.

The correlation between serum cholesterol and percentage deviation from desirable weight was not significant.

LITERATURE CITED

ANDERSON, J. T., AND A. KEYS 1954 Food fats and serum cholesterol. Fed. Proc., *13:* 449.

BENDA, C. E., AND G. V. MANN 1955 The serum cholesterol and lipoprotein levels in mongolism. J. Pediat., *46:* 49.

BOOKER, W. M., F. M. DENT AND R. L. HAYES 1951 Hypercholesterolemia as a possible adrenal response to ascorbic acid. Fed. Proc., *10:* 17.

CONSUELO MENDOZA C., A. 1952 Effect of ascorbic acid on blood cholesterol. An. Fac. Farm. Bioquim., Lima, *3:* 232. Nutrition Abstr. Rev., 1954, *24:* 573.

FOOD AND NUTRITION BOARD 1953 Recommended dietary allowances, revised 1953. Natl. Acad. of Sciences — Natl. Research Council Pub. 302.

GARCIA, P., C. RODERUCK AND P. SWANSON 1955 The relation of age to fat absorption in adult women together with observations on concentration of serum cholesterol. J. Nutrition, *55:* 601.

GILLUM, H. L. 1955 Personal communication.

GILLUM, H. L., A. F. MORGAN AND D. W. JEROME 1955 Nutritional status of the aging. IV. Serum cholesterol and diet. J. Nutrition, *55:* 449.

GRAM, M. R., AND R. M. LEVERTON 1949 Interrelation of age, serum cholesterol and basal metabolism of women. Fed. Proc., *8:* 384.

HILDRETH, E. A., S. M. MELLINKOFF, G. W. BLAIR AND D. M. HILDRETH 1951 An experimental study of practical diets to reduce the human serum cholesterol. J. Clin. Invest., *30:* 649.

HOBSON, W., A. JORDAN AND C. ROSEMAN 1953 Serum-cholesterol levels in elderly people living at home. Lancet., *265:* 961.

KEYS, A., O. MICHELSEN, E. O. MILLER AND C. B. CHAPMAN 1950 The relation in man between cholesterol levels in the diet and in the blood. Science, *112:* 79.

KIBRICK, A. C., T. ROBERTS AND S. S. SKUPP 1951 Determination of cholesterol in blood plasma or serum by hydrolysis with benzyltrimethylammonium hydroxide. Arch. Biochem. Biophys., *32:* 9.

KOUNTZ, W. B., A. SONNENBURG, L. HOFSTETTER AND G. WOLFF 1945 Blood cholesterol levels in elderly patients. Biol. Symposia, *11:* 79.

LONG, C. N. H. 1947 The conditions associated with the secretion of the adrenal cortex. Fed. Proc., *6:* 461.

MAYER, G. A., W. F. CONNELL, M. S. DE WOLFE AND J. M. R. BEVERIDGE 1954 Diet and plasma cholesterol levels. Am. J. Clin. Nutrition, *2:* 316.

MESSINGER, W. J., Y. POROSOWSKA AND J. M. STEELE 1950 Effect of feeding egg yolk and cholesterol on serum cholesterol levels. Arch. Int. Med., *86:* 189.

METROPOLITAN LIFE INSURANCE COMPANY 1942 Ideal weights for women. Statistical Bull., *23:* 6.

NORTHEAST REGION 1951 Cooperative nutritional status studies in the Northeast Region. I. Techniques. Northeast Reg. Pub. no. 5. Memoir 307. Cornell Univ. Agr. Exp. Sta.

OKEY, R. 1945 Cholesterol content of foods. J. Am. Dietet. Assn., *21:* 341.

ROE, J. H., AND C. A. KUETHER 1943 The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem., *147:* 399.

SIMON, A., C. LUDWIG, J. W. GOFMAN AND G. H. CROOK 1954 Metabolic studies in mongolism: Serum protein-bound iodine, cholesterol, and lipoprotein. Am. J. Psychiat., *111:* 139.

SPERRY, W. M., AND M. WEBB 1950 Effect of increasing age on serum cholesterol concentration. J. Biol. Chem., *187:* 107.

TURNER, K. B., AND A. STEINER 1939 A long term study of the variation of serum cholesterol in man. J. Clin. Invest., *18:* 45.

WATT, B. K., AND A. L. MERRILL 1950 Composition of Foods — Raw, Processed, Prepared. U. S. D. A. Agriculture Handbook No. 8.

WILKINSON, C. F. JR., E. BLECHA AND A. REIMER 1950 Is there a relation between diet and blood cholesterol? Arch. Int. Med., *85:* 389.

WISNIEWSKI, B. 1955 Cholesterol and its fractions in the plasma and erythrocytes of healthy persons and in hyper- and hypothyroidism. Bulletin de L'Academie Polonaise des Sciences, *3:* 225.

ALTERATIONS IN THE NUTRITIVE VALUE OF CASEIN BY EXPOSURE TO ETHYLENE OXIDE

H. G. WINDMUELLER,[1] C. J. ACKERMAN
AND R. W. ENGEL
Virginia Agricultural Experiment Station, Virginia Polytechnic Institute, Blacksburg

(Received for publication July 11, 1956)

The germicidal and fungicidal properties of ethylene oxide (ETO) have long been recognized (Cotton and Roark, '28), and use of this volatile agent in the fumigation of various food products has found considerable favor (Anon, '54). The rapid diffusion of the gas and the detectability of only minute residues in products after fumigation promote its use. The first literature reference to nutritional damage of foods when fumigated with ETO was a publication by Hawk and Mickelsen ('55) reporting severe growth depression of weanling albino rats when their diet, either stock or purified, had been exposed to the gas. Analysis for thiamine revealed almost complete destruction of this vitamin in the fumigated diets, but when neither thiamine nor complete vitamin supplementation materially improved animal growth, the above workers were led to suspect the destruction of other essential nutrients.

In our laboratory the feeding of stock and purified diets fumigated by a slightly different method yielded results similar to those of the above workers. In subsequent experiments,

[1] This study was part of a dissertation in partial fulfillment of requirements for the M.S degree. A preliminary report of these studies was presented at the 20th Annual Meeting of the American Institute of Nutrition, Fed. Proc., *15:* Part 1, page 386, 1956.

it was found that feeding a purified diet in which only the protein (casein) had been fumigated resulted in a similar severe growth inhibition. The present paper describes some of the preliminary experiments and those which led to the finding that the histidine and methionine of casein are affected by ETO fumigation.

EXPERIMENTAL

The albino rats employed in these studies were 21- to 24-day-old weanlings from our stock colony. The colony originated from the Holtzman strain. The rats were caged individually in wire-bottom cages and were supplied with fresh diet and water daily. The animals were weighed weekly or at the end of the experiment. The young rats were randomized among the experimental groups according to litter, sex and weight.

The stock diet used had the following percentage composition: ground wheat, 56.5; casein,[2] 12.0; meat scrap, 10.0; skim milk powder, 8.0; hydrogenated vegetable oil,[3] 5.0; molasses, 5.0; alfalfa meal, 2.0; vitamin A and D concentrate[4] (5,000 and 625 USP units of vitamins A and D_2 respectively per gram), 1.0; and salt, 0.5. Purified diet 3 contained the following (in per cent): sucrose, 73.0; casein, 18.0; B vitamins in sucrose, 5.0; and minerals (Salmon, '47), 4.0. Purified diet 4 was similar except that the casein was reduced to 9%, the sucrose increased to 72%, and 10% of hydrogenated vegetable oil was included. The B-vitamin supplement was made up to provide the following per kilogram of diet: 2 gm choline-Cl; 200 mg inositol; 50 mg niacin; 20 mg calcium pantothenate; 10 mg riboflavin; 5 mg pyridoxine-HCl; and 5 mg thiamine-HCl. The purified diets were further supplemented per kilogram with 50 mg alpha-tocopherol, 5 mg beta-carotene, and

[2] The casein was Vitamin-Free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Crisco.

[4] Quadrex.

0.125 mg calciferol. Purified diet 2 was the same as diet 3 without thiamine-HCl.

A modification of a published soil fumigation method (Allison, '51) was used to expose diets or dietary constituents to ETO. The material to be fumigated was put into a paper bag and placed into a vacuum desiccator. The desiccator was evacuated to a pressure of about three inches of mercury, and ETO,[5] from a cylinder of the compressed gas, was admitted until the pressure in the desicator returned to ambient. During the course of fumigation, as the pressure fell, as a result of reaction or sorption of ETO by the treated material, additional gas was admitted to maintain nearly atmospheric pressure. The duration of fumigation varied between experiments. Fumigation was terminated by drawing off the gas in the desiccator and flushing several times with air, filtered through cotton. Subsequent heating of the fumigated material in a forced-draft oven for 6 hours at 80°C. was practiced initially but was discontinued when it failed to improve the biological value of the diets so treated. This fumigation procedure probably exposed diets to higher concentrations of ETO than those used commercially. It was felt, however, that the more drastic conditions would speed the identification of the nutritional damage.

RESULTS AND DISCUSSION

Feeding the stock diet fumigated for 4.5 hours, as described, to 7 weanling rats resulted in rapid weight loss averaging 14.3 gm in 18 days with a standard deviation of $\pm$ 3.0 gm. By the 6th day on this diet the rats began to exhibit a stiffness of gait and severe nervous symptoms quite typical of the neuritis associated with a thiamine deficiency. By the 18th day, all 7 rats had been observed in a convulsive state at least once, in some cases followed by a coma lasting 5 to 10 minutes. Subcutaneous injections of 0.3 mg of thiamine-HCl were observed to improve the condition of the rats temporarily. By

[5] The Matheson Co., Inc., East Rutherford, N. J., purity 99.15%.

this time the rats were moribund and were sacrificed for autopsy. Aside from a severe depletion of depot fat, no specific lesions could be detected, even upon microscopic examination of the major tissues. Control animals fed the same diet without prior fumigation performed normally over the experimental period.

When the stock diet, fumigated for 6 hours, was fed to 6 older rats (160 to 240 gm), they lost an average of 10.6 ± 7.3 gm the first week and then gained slowly (av., 21.3 ± 9.3 gm) the three following weeks. No indication of neural damage was observed in these older rats. On autopsy, after 25 days on the fumigated diet, the contents of the large intestine

TABLE 1

Thiamine stimulation of the growth of weanling rats fed a thiamine-deficient basal diet (diet 2) before and after fumigation with ETO

DIET	NO. OF RATS	AV. WT. GAIN OVER DAYS	
		1–7	8–32
		gm	*gm*
Basal + thiamine-HCl	4	15.0 ± 1.4	81.7 ± 19.3
Fumigated basal + thiamine-HCl	4	— 6.5 ± 2.6	17.3 ± 5.6
Basal	3	12.3 ± 1.6	— 27.3 ± 8.8
Fumigated basal [1]	3	— 8.0 ± 1.0	— 26.7 ± 5.0

[1] Fumigated 14 hours.

and cecum was found to be more fluid, richer in mucous, and lighter in color than similar contents from control rats.

The growth inhibition resulting from feeding a fumigated thiamine-deficient diet (diet 2) supplemented with non-treated thiamine is apparent from the data in table 1. Only about one-third of the growth depression resulting from fumigating this diet was reversed by bi-weekly subcutaneous injections of 0.4 mg of thiamine-HCl per 100 gm of food intake. Feeding the thiamine-deficient diet without supplementation resulted in a 59% greater loss in body weight after 32 days when the diet had been previously fumigated with ETO.

Table 2 presents the results of feeding a purified diet in which only the protein (casein) had been fumigated. The

casein sample had been used previously in a gas-sorption study, and therefore had been fumigated longer than samples used in later experiments. When, after nearly 5 weeks on the fumigated casein diet, rats were returned to the untreated diet, they began to gain weight immediately.

When fumigated and non-fumigated casein were subjected to the reduced sulphur test (Koch and Hanke, '48), the markedly greater PbS precipitate observed with the non-fumigated sample suggested the involvement of sulphur amino acids. Consequently, sulphur amino acids were added to diet 3, containing 18% casein fumigated for 24 hours, in an effort to improve its quality. Neither 0.5% of DL-methionine nor

TABLE 2

Growth inhibition in weanling rats resulting from the ETO-fumigation of the protein component (casein) of the purified diet (diet 3)
(4 rats per treatment)

DIETARY	AV. WT. GAIN OVER DAYS	
	1–33	34–54
	gm	*gm*
Non-fumigated casein	109.5 ± 25.6	36.8 ± 12.8
Fumigated casein [1]	— 17.3 ± 1.5	65.3 ± 19.2

[1] Fumigated 168 hours.

0.2% of L-cysteine-HCl improved growth on this diet. Triple extraction of the casein with 95% ethanol after fumigation and before incorporation into the diet also failed to improve the biological value of the protein. None of the 24 rats on the diets containing the fumigated casein were able to grow during the 4-week experiment while 6 control rats consuming the diet containing non-fumigated casein gained an average of 98.0 ± 11.9 gm over this interval.

Exploratory experiments indicated that the biological value of fumigated casein was markedly improved when non-fumigated casein, or a mixture of the 10 essential amino acids, was added to the diet. On the strength of this finding, studies were undertaken to find which of the essential amino acids

could improve growth when used as a supplement in a fumigated casein diet. The results of the first experiment are shown in table 3. Phase 1 (1 to 14 days) indicates the growth obtained when the proportion of fumigated casein to untreated casein in the diet is progressively increased. All diets in this phase contained a total of 9% of casein, but growth was inhibited progressively as the proportion of fumigated casein was increased.

TABLE 3

Stimulatory effect of some amino acids on the growth of weanling rats fed a purified basal diet (diet 4) containing graded levels of casein fumigated for 24 hours. (4 rats per treatment)

GROUP	CASEIN IN DIET		AVERAGE WT. GAIN OVER DAYS			
	Fumigated	Untreated	1–14	15–21 [1]	22–28 [2]	29–34 [3]
	%	%	*gm*	*m*	*gm*	*gm*
1	0.0	9.0	19.5 ± 5.3	10.3 ± 2.6	9.0 ± 2.1	...
2	1.5	7.5	14.2 ± 1.5	18.3 ± 1.3	12.8 ± 3.6	...
3	3.0	6.0	9.0 ± 1.8	20.8 ± 4.1	13.8 ± 2.9	...
4	4.5	4.5	6.2 ± 3.3	14.3 ± 5.3	22.5 ± 4.4	...
5	6.0	3.0	1.8 ± 1.5	1.2 ± 1.0	3.8 ± 1.1	19.8 ± 8.3
6	7.5	1.5	— 2.2 ± 1.5	— 3.8 ± 1.5	1.0 ± 0.0	17.5 ± 5.2
7	9.0	0.0	— 3.8 ± 1.7	— 5.3 ± 0.5	2.3 ± 0.5	14.8 ± 1.7

[1] Diet supplemented with methionine, cystine and threonine.

[2] Diet further supplemented as follows: group 4, histidine and arginine; group 5, isoleucine and lysine; group 6, leucine and tryptophan; and group 7, valine and phenylalanine.

[3] Diet supplemented with methionine, cystine, threonine, histidine and arginine.

During the third week (15 to 21 days) all the diets containing fumigated casein were supplemented with the three indicated amino acids at the level at which these amino acids would be expected (Block and Bolling, '45) in the fumigated casein component of the diet had it not been fumigated. Supplementation in subsequent phases of this experiment and similar experiments was controlled likewise. Thus the diet with 9% of ETO-fumigated casein received three times the supplementation of the 3% treated casein diet.

Methionine, cystine and threonine greatly enhanced the growth of rats on the lowest two levels of ETO-fumigated casein, improved the growth of two of the 4 rats on the 4.5% fumigated casein, but had no stimulatory value for diets in which more than one-half of the casein had been fumigated. Further supplementation with arginine and histidine (days 22 to 28) improved growth while the remaining amino acid pairs failed to improve their respective diets. These diets were improved, however, by substituting the non-effective pairs of amino acids with the arginine-histidine pair (days 29 to 34).

TABLE 4

Stimulatory effect of amino acid supplements on the growth of weanling rats fed a purified basal diet (diet 4) containing 9% ETO-fumigated casein [1]
(3 rats per treatment)

AMINO ACID SUPPLEMENTS IN %					AV. WT. GAIN, 14 DAYS
L-cystine	DL-methionine	DL-threonine	L-arginine	L-histidine	
					gm
0.032	0.315	0.351	0.423	0.279	
+	+	+	+	+	25.3 ± 5.7
—	+	+	+	+	28.0 ± 8.7
—	+	—	+	+	27.0 ± 4.6
+	+	+	+	—	— 8.0 ± 1.0
+	+	+	—	+	24.3 ± 2.5
—	—	+	+	+	— 3.3 ± 7.8

[1] Fumigated 24 hours.

From the data in table 4 it becomes apparent that methionine and histidine were the two amino acids responsible for the previously observed growth stimulation. It was only when histidine or the methionine-cystine pair were omitted from the amino acid supplement that no growth resulted. The omission of cystine alone did not alter growth, however; therefore, methionine and histidine were assumed to be the two stimulatory compounds.

The extent of the growth stimulation from histidine and methionine supplementation of the 9% ETO-fumigated casein diets is seen in table 5. Neither amino acid alone sufficed to support growth. When included together at the indicated

levels, the growth observed was fully comparable with that on an untreated casein diet. It would appear that if any other essential amino acid is affected when casein is fumigated as described it must be one which is not growth limiting under the conditions of this experiment.

TABLE 5

Ability of histidine and methionine to stimulate growth of weanling rats fed a purified diet (diet 4) containing 9% ETO-fumigated casein [1] *(6 rats per treatment)*

PROTEIN SOURCE	SUPPLEMENT TO DIET		AV. WT. GAIN, 21 DAYS
	L-histidine	DL-methionine	
	%	%	*gm*
Unfumigated casein	0.279	0.315	48.8 ± 6.4
Fumigated casein	0.279	0.315	39.8 ± 5.6
Unfumigated casein	....	....	30.5 ± 4.5
Fumigated casein	....	....	2.6 ± 1.5
Fumigated casein	0.279	....	2.2 ± 1.6
Fumigated casein	....	0.315	—1.4 ± 1.7

[1] Fumigated 24 hours.

TABLE 6

Growth inhibition of weanling rats fed a purified basal diet (diet 4) containing 9% casein variably fumigated with ETO (3 rats per treatment)

DURATION OF ETO FUMIGATION OF CASEIN	AV. WT. GAIN, 21 DAYS
	gm
0 min.	27.7 ± 7.3
15 min.	25.0 ± 5.3
30 min.	19.7 ± 7.0
1 hr.	19.7 ± 7.6
4 hr.	6.3 ± 2.2
24 hr.	—8.7 ± 1.6

The effect of the duration of the ETO fumigation of casein upon its biological value is seen in table 6. Growth depression was severe after 4 hours of fumigation, but maximum protein damage was not achieved until fumigation had proceeded for much longer periods, possibly 24 hours, or more.

Microbiological assay for histidine and methionine in 24-hour ETO-fumigated and non-fumigated casein samples indi-

cated 71% destruction of the former amino acid and 56% destruction of the latter; i.e., only 29% and 44% respectively of the amino acids were available to the bacterium, *Lactobacillus mesenteroides*. The possibility exists, of course, that factors other than amino acid availability were operative in these assays, e.g., a toxic factor in the fumigated casein may have been limiting microbial growth. The close correlation of the microbiological assay with the results from the rat experiments makes the hypothesis of unavailability the more likely one, however.

The ETO effect on the histidine and methionine of casein is particularly interesting in that these amino acids in the purified dry powdered form [6] show no tendency to react with the gas. Reactivity has been tested by three methods; namely, their ability to sorb or absorb the fumigant, their mobility on paper chromatograms following fumigation, and their nutritive value following fumigation. Of 16 amino acids, histidine and methionine included, only cysteine-HCl showed any appreciable ETO uptake when exposed to the gas in a Warburg tissue respirometer (Umbreit et al., '49). Cysteine-HCl reacted readily with ETO to yield, in 24 to 36 hours, a brown, viscous, water-soluble liquid which was toxic to weanling rats upon subcutaneous injection ($LD_{50} = 13$ mg fumigated cysteine-HCl per 50 gm body weight). The mobility on paper chromatograms (water-saturated phenol solvent used) of histidine or methionine was not altered after fumigation of the amino acids. After a 24-hour fumigation, a histidine and methionine supplement had not lost its ability to reverse the growth inhibition produced by a 9% ETO-fumigated-casein purified diet.

The apparent chemical changes in casein upon ETO fumigation are accompanied by physical changes, the most conspicuous of which is an increase in mass. This increase, determined from the measured dilution of nitrogen, was progressive during the course of fumigation and totalled 10.5% after 24 hours' exposure to the fumigant.

[6] Amino acids from Nutritional Biochemicals, Inc., Cleveland, Ohio.

The effect of ETO on proteins other than casein remains to be determined. There is no indication to date that the lability of protein histidine and methionine is a general phenomenon. It is interesting to speculate, however, that ETO-protein (enzyme) reactions are involved in the lethal action of the fumigant on microorganisms.

Some reactions of ETO with protein have been described earlier (Fraenkel-Conrat, '44). ETO was found to react with most of the available reactive groups, namely, carboxyl, amino, sulphydryl and phenol groups. The reactions were studied only in aqueous solution, however, and their application to the conditions described in this paper remains to be investigated.

SUMMARY

Weanling rats failed to grow when fed a purified diet containing 9 or 18% of casein as the only protein source when this casein had been previously fumigated with ethylene oxide. A histidine and methionine supplement was active in reversing this inhibition. Only 29% of the histidine and 44% of the methionine of casein appeared to be available to the bacterium, *Lactobacillus mesenteroides* after 24 hours of fumigation of the intact protein with ethylene oxide.

ACKNOWLEDGMENTS

The authors are indebted to Dr. J. R. Rooney, II, Animal Pathology Section, Virginia Agricultural Experiment Station, Blacksburg, Virginia, for performing the gross and histological examinations of the sacrificed animals, to Mr. Howard Bakerman, of the Laboratory of Biochemistry and Nutrition, National Institutes of Health, Bethesda, Maryland, for the microbiological assays, and to Merck and Company, Rahway, New Jersey, for the B vitamins used in these studies.

LITERATURE CITED

ALLISON, L. E. 1951 Vapor phase sterilization of soil with ethylene oxide. Soil Sci., *72:* 341.

ANON 1954 Cool Killer. Chemical Week, Oct. 2, p. 96.

BLOCK, R. J., AND D. BOLLING 1945 The Amino Acid Composition of Proteins and Foods. Charles C Thomas, Springfield, Ill., p. 303.

COTTON, R. T., AND R. C. ROARK 1928 Ethylene oxide as a fumigant. Ind. Eng. Chem., *20:* 805.

FRAENKEL-CONRAT, H. 1944 The action of 1,2-epoxides on proteins. J. Biol. Chem., *154:* 227.

HAWK, E. A., AND O. MICKELSEN 1955 Nutritional changes in diets exposed to ethylene oxide. Science, *121:* 442.

KOCH, F. C., AND M. E. HANKE 1948 Practical Methods in Biochemistry. Williams and Wilkins, Baltimore, p. 54.

SALMON, W. D. 1947 Some physiological relationships of protein, fat, choline, methionine, cystine, nicotinic acid and tryptophane. J. Nutrition, *33:* 155.

UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER 1949 Manometric Techniques and Tissue Metabolism. Burgess Publishing Co., Minneapolis, Minn.

ADDED DIETARY INORGANIC SULFATE AND ITS EFFECT UPON RATS FED MOLYBDENUM [1,2]

RUSSELL F. MILLER, N. O. PRICE AND R. W. ENGEL
Department of Biochemistry and Nutrition, Virginia Agricultural Experiment Station, Virginia Polytechnic Institute, Blacksburg

(Received for publication July 11, 1956)

INTRODUCTION

The toxicity of trace amounts of molybdenum for cattle and sheep has been described by many workers (Ferguson et al., '38, '40, '43; Dick and Bull, '45; Britton and Goss, '46; Cunningham, '46; Comar et al., '48). Marston ('52) has reviewed the literature with particular reference to large animals. The literature on the toxicity of molybdenum for experimental animals has been reviewed by Fairhall et al. ('45). In subsequent studies with laboratory rats the toxic effects of molybdenum have been reported by Neilands et al. ('48), Comar et al. ('49), Gray and Ellis ('50), Gray and Daniel ('54), Jeter and Davis ('54) and Van Reen ('54). In general these workers found that the molybdenum-induced growth inhibition could be alleviated to a considerable extent by the addition of copper salts to the diet. Neilands et al. ('48) reported, however, that whole liver powder had a mitigating effect upon this condition that could not be attributed to its copper content; while Gray and Daniel ('54) found that methionine supplementation to a methionine-adequate ration relieved the condition.

[1] Preliminary results were presented at the 20th annual meeting of the American Institute of Nutrition, Atlantic City, New Jersey. Fed. Proc. Part 1 no. 1 pp. 1564 (1956).

[2] Supported in part by a grant from the Nutrition Foundation Inc., New York, N. Y.

Dick ('52) reported that a factor present in two different types of forage was involved in the copper-molybdenum imbalance in sheep, and in subsequent studies ('53a) identified this factor as inorganic sulfate. Results of the additions of inorganic sulfate to high-molybdenum rations for sheep have been further investigated by Dick ('53b, '54) who reported that the addition of sulfate to the high copper-molybdenum-containing ration increased the storage of copper in the liver. Furthermore, at a constant level of molybdenum supplementation liver copper levels increased as the level of sulfate increased. Similar results were observed when the blood copper concentration was considered. Observations of the fleece character of these sheep by Dick ('54) led him to conclude that these sheep were copper deficient while blood and liver copper levels were elevated.

The present report summarizes studies that illustrate the beneficial effects of dietary sulfate in molybdenum-fed rats. Data are also presented on the effect of varying sulfate and molybdenum levels in the diet on copper and molybdenum concentration in blood and liver.

EXPERIMENTAL

Equal numbers of male and female 21-day-old albino rats (35 to 50 gm) were used in these studies. The rats in experiment I (Holtzman strain) were from the stock colony of this laboratory while those in experiment II were obtained commercially.[3] All rats of the same sex were allotted at random to their respective treatments. The basal ration used in all experiments had the following percentage composition: sucrose 80.25, crude casein 12.0, cottonseed oil 5.0, low-sulfate salts 2.55, L-cystine 0.2.

Vitamins were added as follows (milligrams per kilogram of ration): choline chloride, 1,000; inositol, 100; calcium pantothenate, 20; niacin, 10; menadione, 10; thiamine·HCl, 5; riboflavin, 3; pyroxidine · HCl, 3; folic acid, 0.2; biotin, 0.1

[3] Holtzman Company, Madison, Wisconsin.

and vitamin B_{12}, 0.01. Vitamins A, D and E were supplied by two drops/rat/week of 50% percomorph liver oil [4] in cottonseed oil (containing 0.5 gm of α-tocopherol acetate/10 ml). The composition of the salts used in grams per kilogram of ration follows: $Ca_3(PO_4)_2$, 12.5; NaCl, 6.5; KCl, 5.5; MgO, 0.7; Fe_2O_3, 0.15; MnO_2, 0.10; KI, 0.03; $ZnCO_3$, 0.02; $CuSO_4 \cdot 5 H_2O$, 0.013. All salts were C. P. or A. R. The only sulfate salt used was copper sulfate.

The basal ration averaged 4 p.p.m. in copper and less than 0.2 p.p.m. in molybdenum. The highest level of sulfate added increased the copper concentration of the ration by less than 0.2 p.p.m.

The first experiment was designed to investigate the effect of molybdenum when the sulfate was held constant at 2,200 p.p.m. in the diet. The design of this experiment is summarized in table 2. The second experiment was planned to study the effect of varying the sulfate when the molybdenum was held constant at 100 p.p.m. in the diet. The experimental design is given in table 3.

All rats were sacrificed after 6 weeks. At this time as much blood as possible was withdrawn by heart puncture. Blood hemoglobin concentration in each rat was determined by the acid hematin method. Because of the small volume, the blood from all rats in each lot was pooled. The molybdenum (Evans et al., '50) and copper (A.O.A.C., '50) concentration of the whole blood was determined. Analyses for liver copper and molybdenum were made. The values were expressed as micrograms per gram of dry fat-free liver. Calculations of copper and molybdenum concentration on a liver-nitrogen basis were also made but are not included since they did not vary from the results found on the dry fat-free liver basis.

RESULTS AND DISCUSSION

Five separate trials were conducted to establish the effects of sulfate and molybdenum supplementation. The results are

[4] Abbott Laboratories, North Chicago, Illinois.

TABLE 1

The alleviation of molybdenum-induced rat growth inhibition with inorganic sulfate (22 rats per treatment, 5 replicates)

DIET	AV. 6 WEEK BODY WEIGHT GAIN
	gm
Basal	109 ± 18 [3]
Basal + SO_4 [1]	112 ± 23
Basal + Mo [2]	60 ± 15
Basal + SO_4 + Mo	102 ± 27

[1] 2,000 p.p.m. SO_4 as 1:1 Na_2SO_4 and K_2SO_4.

[2] 100 p.p.m. Mo as Na_2MoO_4.

[3] $\sqrt{\frac{\Sigma(x)^2}{n-1}}$.

TABLE 2

Dietary molybdenum level and the effect of inorganic sulfate upon rat growth and blood and liver levels of molybdenum and copper

(Experiment I, 4 rats/treatment)

LOT AND TREATMENT	AV. 6 WEEK GAIN	WHOLE BLOOD		LIVER (DRY, FAT-FREE)	
		Copper conc.	Molybdenum conc.	Copper conc.	Molybdenum conc.
	gm	*μg/ml*	*μg/ml*	*μg/gm*	*μg/gm*
1. Basal	78 ± 22	0.6	Trace	10.0 ± 1	1.6 ± 0.5
2. Basal + 75 p.p.m. Mo [1]	47 ± 11	4.0	13.4	16.5 ± 4	29.8 ± 1.7
3. Basal + 300 p.p.m. Mo	17 ± 8	6.5	13.4	38.0 ± 15	51.6 ± 12
4. Basal + 2,200 p.p.m. SO_4 [2]	76 ± 17	0.5	Trace	8.0 ± 1.4	1.4 ± 0.2
5. Basal + 75 p.p.m. Mo + 2,200 p.p.m. SO_4	84 ± 22	2.5	5.7	12.3 ± 1	9.2 ± 1.6
6. Basal + 300 p.p.m. Mo + 2,200 p.p.m. SO_4	53 ± 12	3.0	9.3	16.0 ± 1.6	19.0 ± 5.5

[1] As H_2MoO_4.

[2] As equimolar mixture of Na_2SO_4 and K_2SO_4.

summarized in table 1. The data were analyzed statistically using the "t"-test and the analysis of variance (Snedecor, '46). Molybdenum significantly suppressed growth only when the diet was low in sulfate. Sulfate supplement in the absence of molybdenum was without effect.

Experiment I. The alleviating effect of dietary inorganic sulfate upon the molybdenum-induced growth inhibition can be seen in table 2. Molybdenum alone exerted a growth inhibition at both the 75 and 300 p.p.m. level. At the 75 p.p.m. level, 2,200 p.p.m. of sulfate, as the sodium and potassium salts, were able to reverse this growth inhibition. The growth of rats fed 300 p.p.m. of molybdenum plus 2,200 p.p.m. of sulfate was approximately equal to that of rats receiving 75 p.p.m. of molybdenum without added sulfate.

The addition of molybdenum alone to the basal diet increased the blood and liver molybdenum and copper levels (table 2). The addition of 2,200 p.p.m. of sulfate reduced these elevated levels of molybdenum and copper. The addition of sulfate alone did not appear to alter either the growth or the concentration of copper or molybdenum in the liver and blood. In these experiments it appeared that inorganic sulfate functioned only when added molybdenum was fed. Inasmuch as sulfate supplementation increased dietary sodium and potassium it could be argued that the effect could be due to these minerals. Dick ('53a), however, noted that non-sulfate salts of sodium and potassium were without effect in the ruminant. Furthermore, the amount of sodium and potassium supplied by the sulfate supplements used in these studies was small compared with that provided by the salt mixture in the basal diet.

Hemoglobin levels appeared to be depressed in lots 2, 3 and 6. The values in these lots ranged from 8 to 10 gm/100 ml while in all other lots the hemoglobin levels ranged from 10 to 12 gm/100 ml.

An enlargement of the femoro-tibial joint and a thickening of the epiphysis of the femur and tibia were observed at the time of sacrifice in lots 2, 3 and 6. Histological examination

of the femurs of these rats indicated a chondro-dystrophy of the epiphysial cartilages. The femurs of the rats in the other lots were normal when examined grossly and histologically. The percentage of femur ash was determined on the opposite

TABLE 3

The effect of dietary inorganic sulfate level upon the molybdenum-induced rat growth inhibition and upon blood and liver levels of molybdenum and copper

(Experiment II, 4 rats/treatment)

LOT AND TREATMENT	AV. 6 WEEK GAIN	WHOLE BLOOD		LIVER (DRY, FAT-FREE)	
		Copper conc.	Molybdenum conc.	Copper conc.	Molybdenum conc.
	gm	*μg/ml*	*μg/ml*	*μg/gm*	*μg/gm*
Basal	98 ± 10	0.6	Trace	10.0 ± 2	2.6 ± 1.4
Basal + 100 p.p.m. Mo [1]	47 ± 6	5.7	16.6	40.6 ± 15	48.3 ± 12
Basal + 400 p.p.m. SO_4 [2]	102 ± 14	0.45	Trace	10.6 ± 1	2.4 ± 1.1
Basal + 400 p.p.m. SO_4 + 100 p.p.m. Mo	66 ± 18	4.9	10.6	28.6 ± 5	20.4 ± 6
Basal + 800 p.p.m. SO_4	80 ± 11	0.4	Trace	10.8 ± 2	2.3 ± 0.8
Basal + 800 p.p.m. SO_4 + 100 p.p.m. Mo	83 ± 26	3.8	9.9	22.2 ± 4	17.9 ± 4
Basal + 2,200 p.p.m. SO_4	95 ± 25	0.5	Trace	10.6 ± 0.8	1.8 ± 0.6
Basal + 2,200 p.p.m. SO_4 + 100 p.p.m. Mo	100 ± 17	4.7	10.0	27.2 ± 2.5	19.0 ± 3
Basal + 3,300 p.p.m. SO_4	95 ± 7	0.5	Trace	11.6 ± 2	1.6 ± 0.4
Basal + 3,300 p.p.m. SO_4 + 100 p.p.m. Mo	96 ± 28	3.8	7.6	23.8 ± 5	14.9 ± 3

[1] As Na_2MoO_4.

[2] As equimolar mixture of Na_2SO_4 and K_2SO_4.

femur and found to average 61% (dry, fat-free basis) in lots 2, 3 and 6 and over 65% in the femurs of all other lots.

Experiment II. On the basis of the results of experiment I, 100 p.p.m. of molybdenum (as Na_2MoO_4) were used as a supplement to the basal ration. The results obtained in experiment I were confirmed. In addition, it appeared that a sulfate level between 800 and 2,200 p.p.m. was required to alleviate a considerable degree of the molybdenum-induced growth inhibition (table 3). There appeared to be no beneficial effect upon growth by increasing the sulfate content of the molybdenum-containing ration to 3,300 p.p.m. A similar pattern was noted when the analytical data were considered. Four hundred parts per million of added sulfate appeared sufficient to reduce the elevated blood and liver molybdenum and copper levels observed when molybdenum was fed. The absolute values appeared to vary between experiments but the results appeared qualitatively similar.

In experiment II no reduction in hemoglobin was noted nor was any bone abnormality observed. Recent work by the authors indicates that as the copper level in the ration decreased the incidence and severity of anemia and the bone abnormality increased. Small differences in copper levels in the ration and in initial copper stores of the rat could explain the above variations.

SUMMARY

Under the conditions of these experiments dietary inorganic sulfate had an alleviating effect on the molybdenum-induced rat growth inhibition. The inclusion of molybdenum in the diet caused an increase in the liver and blood levels of molybdenum and copper. This increase was reduced when inorganic sulfate was added to the molybdenum-containing ration.

In these experiments the growth inhibition caused by 75 p.p.m. of molybdenum could be overcome by the addition of 2,200 p.p.m. of inorganic sulfate. Sulfate was partially effective in diets containing as much as 300 p.p.m. of molyb-

denum. When 100 p.p.m. of molybdenum was added to the diet it appeared that a level of sulfate between 800 and 2,200 p.p.m. exerted its maximum growth-protective effect.

ACKNOWLEDGMENTS

The authors wish to thank Dr. J. R. Rooney II, Department of Animal Pathology, who conducted the histological examinations; Mrs. Josephine McCown for technical assistance: Abbott Laboratories, North Chicago, Illinois, which supplied the Percomorph Liver Oil and Merck and Company, Rahway, New Jersey for furnishing the other vitamins.

ADDENDUM

Since this manuscript was submitted, R. Van Reen and M. A. Williams have published results indicating that sulfur compounds alleviated the toxicity of molybdenum for the rat. Archives of Biochem. Biophysics, *63:* 1 (1956).

LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1950 *Official Methods of Analysis.* 7th Ed. pp. 100.

BRITTON, J. W., AND H. GOSS 1946 Chronic molybdenum poisoning in cattle. J. Am. Vet. Med. Assn., *108:* 176.

COMAR, C. L., G. K. DAVIS AND L. SINGER 1948 The fate of radioactive copper administered to the bovine. J. Biol. Chem., *174:* 905.

———— 1949 Molybdenum metabolism and interrelationships with copper and phosphorus. Ibid., *180:* 913.

CUNNINGHAM, I. J. 1946 Copper deficiency in cattle and sheep on peat lands. N. Z. J. Sci. Tech., *27* (A): 381.

DICK, A. T. 1952 The effect of diet and of molybdenum on copper metabolism of sheep. The Australian Vet. J., *28:* 30.

———— 1953a The effect of inorganic sulphate on the excretion of molybdenum in the sheep. Ibid., *29:* 18.

———— 1953b The control of copper storage in the liver of sheep by inorganic sulphate and molybdenum. Ibid., *29:* 233.

———— 1954 Preliminary observations on the effect of high intakes of molybdenum and of inorganic sulphate on blood copper and on fleece character in crossbred sheep. Ibid., *30:* 196.

DICK, A. T., AND L. B. BULL 1945 Some preliminary observations on the effect of molybdenum on copper metabolism in herbivorous animals. Ibid., *21:* 70.

EVANS, H. J., E. R. PURVIS AND F. E. BEAR 1950 Colorimetric determination of molybdenum by means of nitric and perchloric acids. Anal. Chem., *22:* 1568.

FAIRHALL, L. T., R. C. DUNN, N. E. SHARPLESS AND E. A. PRITCHARD 1945 Toxicity of molybdenum. U. S. Public Health Bull., *293:* 1–36.

FERGUSON, W. S., A. H. LEWIS AND S. J. WATSON 1938 Action of molybdenum in nutrition of milking cattle. Nature, *141:* 553.

FERGUSON, W. S., A. H. LEWIS AND S. J. WATSON 1940 The teart pastures of somerset, cause of teartness and its prevention. Jealott's Hill Research Station Bull., No. 1.

———— 1943 The teart pastures of somerset. I. The cause and cure of teartness. J. Agr. Sci., *33:* 44.

GRAY, L. F., AND L. J. DANIEL 1954 Some effects of excess molybdenum on the nutrition of the rat. J. Nutrition, *53:* 43.

GRAY, L. F., AND G. H. ELLIS 1950 Some interrelationships of copper, molybdenum, zinc and lead in the nutrition of the rat. Ibid., *40:* 441.

JETER, M. A., AND G. K. DAVIS 1954 The effect of dietary molybdenum upon growth, hemoglobin, reproduction and lactation of rats. Ibid., *54:* 215.

MARSTON, H. R. 1952 Cobalt, copper and molybdenum in the nutrition of animals and plants. Physiol. Rev., *32:* 66.

NEILANDS, J. B., F. M. STRONG, AND C. A. ELVEHJEM 1948 Molybdenum in the nutrition of the rat. J. Biol. Chem., *172:* 431.

SNEDECOR, G. W. 1946 Statistical Methods Applied to Experiments in Agriculture and Biology. Collegiate Press, Inc., Ames, Iowa.

VAN REEN, R. 1954 The influence of excessive dietary molybdenum on rat liver enzymes. Archives of Biochem. and Biophysics, *53:* 77.

NITROGEN BALANCES OF WOMEN MAINTAINED ON VARIOUS LEVELS OF LYSINE [1]

EVELYN M. JONES,[2] C. A. BAUMANN AND MAY S. REYNOLDS
Department of Foods and Nutrition, School of Home Economics, and Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication June 26, 1956)

Since the demonstration of man's need for the 8 essential amino acids (Rose, '47), attention has been focused on the determination of the quantitative requirements for these nutrients. Lysine is of particular interest since it occurs in relatively low concentrations in vegetables and cereals which supply a major portion of the protein in many diets. Rose et al. ('55) have reported that the minimal intakes of L-lysine which permitted nitrogen balance in 6 young adult men on a synthetic ration ranged from 0.4 to 0.8 gm per day. The present paper presents nitrogen balance data of women maintained on a semi-synthetic diet furnishing various levels of lysine.

EXPERIMENTAL

Experimental plan. A diet of natural foods (Jones, '56) was fed for 10 to 16 days, and daily nitrogen balances were determined during two or three periods of 4 days each in

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a U. S. Department of Agriculture contract, sponsored by the Human Nutrition Research Branch, Agricultural Research Service. Presented in part before the 19th Meeting of the American Institute of Nutrition, San Francisco, April, 1955 (Fed. Proc., *14:* 438, 1955). Part of the data in this paper are taken from a thesis submitted by Evelyn M. Jones in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Human Nutrition.

[2] Present address: Department of Foods and Nutrition, College of Home Economics, Michigan State University, East Lansing.

order to demonstrate that all subjects were capable of maintaining nitrogen equilibrium on an adequate diet and to establish a level of nitrogen intake for use throughout the experiment. In a transition period (three to 4 days) the items of food in the normal diet were gradually replaced by those of the semi-synthetic diet which was then fed throughout the remainder of the experiment. For the first 12 to 16 days on the semi-synthetic diet, lysine was supplied at the level found in 20 gm of egg protein by a so-called "complete" amino acid mixture. In series I and II, after it had been established that all subjects were in nitrogen balance on this regimen, the need for lysine was demonstrated by their inability to attain nitrogen equilibrium during 10 to 11 days when all of the lysine in the supplements was replaced by isonitrogenous amounts of glycine. Six different levels of lysine were fed successively in series I, and 4 in series II. The subjects in series II participated in a methionine study for a 30-day interval between the time the complete amino acid supplements were fed and the initial feeding of the lysine-deficient supplements.

In series III, the "complete" amino acid mixture was fed as before, but thereafter lysine and methionine were fed at levels which had appeared to be adequate in series I and II and in certain other studies by Reynolds ('56); the daily amino acid supplements supplied 300 mg of lysine, 250 mg of methionine and 480 mg of cystine for two periods of 4 days each. Other variations in procedure have been recorded elsewhere (Jones, '56).

Subjects. The subjects were women students or staff members 19 to 43 years of age who maintained their usual academic pursuits throughout the experiment (table 1). All were in normal health as determined by physical examinations at the Department of Student Health, University of Wisconsin. In series I and II, the subjects were housed in an apartment under the direction of a graduate dietitian, whereas in series III, they lived in dormitories or private homes, but ate all

of their meals in the metabolism unit of the School of Home Economics.

Diets. A normal diet (Jones, '56) consisting of bread, butter, cheese, meat, milk, fruits and vegetables was fed prior to the start of the experimental period. This diet supplied about 10 gm of nitrogen and 4 gm of lysine per day (analysis)

TABLE 1

Vital statistics of the subjects

SUBJECT	AGE AT START OF STUDY		WEIGHT		HEIGHT
			Initial [1]	Final [2]	
	years	*months*	*kg*	*kg*	*cm*
Series I					
5	43	1	67.4	67.2	166
6	36	5	54.6	55.8	157
7	34	8	54.4	54.7	168
8	31	2	59.5	58.8	168
9	31	6	49.5	49.7	156
Series II					
10 [3]	36	11	58.2	59.7	157
11	31	2	65.9	66.7	164
12	30	10	62.2	61.8	171
Series III					
15	25	11	65.9	65.4	157
16	21	6	60.8	60.2	175
17	18	7	70.2	70.5	172
18	18	7	60.0	60.3	168
19	21	10	67.9	68.0	164
20	21	5	60.0	59.8	164

[1] Mean for the normal diet.

[2] Mean for the final experimental period.

[3] Same as subject 6.

and about 2,000 calories (calculated from data in Agriculture Handbook No. 8 by Watt and Merrill, '50).

The semi-synthetic diet consisted of a few low-protein foods plus butter oil, cornstarch, sucrose and vegetable oil,[3] as well as amino acids, diammonium citrate, purified hemicellulose [4] and mineral and vitamin supplements. The semi-synthetic

[3] Wesson Oil.

[4] Mucilose Flakes, Winthrop-Stearns, Inc.

TABLE 2

Semi-synthetic diet exclusive of the nitrogen supplements

ITEM	WEIGHT	ITEM	WEIGHT
	gm		*gm*
Applesauce, canned, sweetened	200	Sanka [2]	
Butter oil	43	Sucrose	180
Carrots, raw	25	Tomatoes, canned	100
Grape juice, canned	100	Wafers: [3]	1 recipe
Jelly	40	Butter oil	10
Lemon juice, canned	75	Cornstarch	50
Orange juice, frozen, reconstituted	100	Hemicellulose [4] (Mucilose flakes)	3
Peaches, canned, freestone	100	Salt	4
Peach syrup, canned	50	Sucrose	20
Pudding: [1]	1 recipe	Wesson oil	7
Butter oil	13	Water	57
Cornstarch	8	Baking powder — Mineral mixture [5]	9.4
Salt	1		
Sucrose	30		
Water	90		

[1] Basic recipe was obtained from Leverton ('53). Vanilla or peppermint pudding was prepared by adding one or two drops of the extract. Lime or lemon pudding was made by substituting 15 gm of the fresh juice (strained) for 15 gm of the water. Twenty grams of sucrose was replaced by brown sugar in the butterscotch pudding.

[2] Sanka was served at breakfast and dinner. Quantities were based on individual preference, but were constant for any given individual.

[3] Basic recipe was obtained from Leverton ('53).

[4] Mucilose Flakes, Winthrop-Stearns, Inc.

[5] The mixture contained 1.8 gm of mineral supplement and 7.6 gm of the baking powder. The composition of the baking powder and of the mineral supplements were given by Leverton et al. ('56).

diet used in series II and III is presented in table 2. In series I the diet contained an additional 100 gm of grape juice, 25 gm of lettuce and 100 gm of potato, or an additional 50 gm of potato. In series I the basal portion of the semi-synthetic diet supplied from 0.6 to 1.0 gm of nitrogen and from 0.10 to 0.25 gm lysine per day depending upon the foods which were included.[5] In series II and III the basal portion of the diet

[5] The results of the analysis of potatoes were variable; one variety yielded 4.7 mg of nitrogen and 1.41 mg of lysine, whereas a second contained 2.7 mg of nitrogen and 0.86 mg of lysine per gram of potato.

contained about 0.5 gm of nitrogen and 0.1 gm of lysine per day.

The mineral supplement [6] of Leverton et al. ('56) was used. One-half (1.8 gm) was incorporated into the wafers (table 2); the remainder was added to 75 ml of lemon juice and equal portions served at each meal. The daily vitamin supplement [7] was given by capsule at breakfast. It supplied 180 mg choline dihydrogen citrate, 3 mg thiamine hydrochloride, 3 mg riboflavin, 4.5 mg calcium pantothenate, 3 mg pyridoxine hydrochloride, 9 mg niacinamide, 0.6 mg folic acid, 0.15 mg biotin, 0.001 mg vitamin B_{12}, 4,500 U.S.P. Units vitamin A (synthetic) and 4.5 mg *dl*-alpha-tocopherol acetate.

The caloric intakes of the individual subjects were adjusted to meet their particular energy requirements by the addition of appropriate amounts of butter oil, candy (plain fondant), pudding, sucrose or a plain carbonated beverage, or by the omission of part of the pudding or wafers. For one subject in series I, the potato was reduced by 50 gm per day. The subjects maintained their initial weights throughout the entire experiment.

Amino acid supplements. Most of the nitrogen of the semisynthetic diet was supplied as mixtures of amino acids and diammonium citrate (table 3). The 8 essential amino acids plus arginine, cystine, histidine and tyrosine were fed at the levels at which they occur in 20 gm of egg protein. The nitrogen content of these supplements was raised to 10 gm per day by the addition of glycine and diammonium citrate in isonitrogenous amounts. With the exception of DL-isoleucine the natural isomers of all amino acids were used; in addition DL-valine was used as a source of L-valine in the final periods of series III, in order to determine the effect of this substitution on nitrogen balance. Sufficient racemic mixture was used to supply an equivalent amount of L-valine and

[6] Nutritional Biochemicals, Inc.

[7] Corresponded to Litrison of Hoffmann-LaRoche, Inc., except that DL-methionine was omitted.

the total nitrogen content was maintained constant by a suitable reduction of the glycine.

A mixture of all the amino acids except cystine and tyrosine was ball-milled over night and sieved. Any material which did not readily pass through the sieve was ground in a mortar

TABLE 3

Individual daily allotments of the amino acids and diammonium citrate

AMINO ACID	AMOUNT	NITROGEN
	gm	*gm*
Solution supplement:		
L-Arginine hydrochloride	1.549	0.412
L-Histidine hydrochloride	0.519	0.114
DL-Isoleucine	3.200	0.342
L-Leucine	1.840	0.197
L-Lysine hydrochloride [1]	1.800	0.276
L-Methionine	0.820	0.077
L-Phenylalanine	1.260	0.107
L-Threonine	0.930	0.115
L-Tryptophan	0.300	0.041
L-Valine	1.460	0.175
Glycine	21.473	4.009
SUBTOTAL	35.201	5.865
Diammonium citrate	32.357	4.009
Dry powder supplement:		
L-Cystine	0.480	0.056
L-Tyrosine	0.900	0.070
SUBTOTAL	1.380	0.126
TOTAL		10.000

[1] Since this compound was only 95% pure, 1.895 gm was used.

and the entire batch was returned to the ball mill for additional mixing. One-fourth of the day's quota of the mixture was fed at breakfast, and three-eighths each at luncheon and dinner. The precise amounts were weighed for each subject for each meal. Sixty grams of sugar were added to each portion and sufficient hot distilled water was used to put all of the amino acids in solution. A diammonium citrate solution

of appropriate concentration was pipetted into each amino acid serving so that each meal supplied one-third of the diammonium citrate nitrogen. The solutions were chilled before serving.

Because of the low solubilities of cystine and tyrosine, the daily allotment of these amino acids (ball-milled and sieved) was weighed for each subject, thoroughly blended with 100 gm of applesauce, and approximately one-third was consumed at each meal.

When a specific amino acid was under study, it was omitted from the amino acid mixture and the desired level added as an adjustment solution so that one-third of the day's total was given at each meal. In all cases the nitrogen intake was kept constant by appropriate changes in glycine. Supplementary glycine was added as an adjustment solution with one-third of the day's total at each meal.

Samples and analytical determination. The individual foods were sampled daily and composites were prepared for each period with the foods in the proportions fed to the subjects. These aliquots were kept frozen until analyzed. Nitrogen and lysine contents were determined in the homogenized composites. Beef from the normal diet and butter oil, candy, carbonated beverage, coffee, lemon juice, pudding, Sanka, sucrose, tea and wafers from the semi-synthetic diet were not included in the composites, but representative samples were analyzed for nitrogen with the exception of the butter oil, candy and sucrose.

Daily urine samples were collected under toluene and were kept refrigerated. The pH of the 24 hour urine sample was brought to between 5 and 6 (hydrion paper) with hydrochloric acid. The acidified urine was diluted to a convenient volume, usually 2 l, and samples were preserved with toluene and held under refrigeration until analyzed.

Fecal samples were collected in waxed cartons and immediately frozen. The feces from an entire balance period, as established by carmine markers, were placed in jars and hydrochloric acid (one volume diluted to 5 volumes) was

added to cover. After the gases had been exhausted from the samples by holding the jars in a hot-water bath for about 12 hours, they were autoclaved for three hours at 15 pounds of pressure, cooled, weighed, and sampled for analysis.

The nitrogen contents of the amino acid mixtures and adjustment solutions and of the food, urine and fecal samples were determined by a boric acid modification of the Kjeldahl method (Scales and Harrison, '20). The lysine contents of the foods and amino acid supplements were determined by microbiological assay (Jones, '56). Creatinine determinations were made on the daily urine samples with either the Peters ('42) or the Klett-Summerson adaptations of the Folin ('14) method. The constancy of the creatinine concentration was considered an indication of the completeness of collection of the daily urines.

RESULTS AND DISCUSSION

In agreement with the observations of others (Rose, Coon and Lambert, '54; Pratt et al., '55), a larger caloric intake was required to maintain the weights of the subjects on the semi-synthetic regimen, wherein most of the nitrogen was supplied by amino acids and diammonium citrate, than on the normal diet of natural foods. On the normal diet the mean daily caloric intakes of the individual subjects ranged from 1540 to 2115 with a mean of 1974; whereas, on the semi-synthetic regimen, they varied from 1761 to 2585 with a mean of 2286. The low values were for a relatively small subject. Expressed as calories per kilogram of body weight, the mean values for the subjects on the normal diet ranged from 28.3 to 36.0 with a mean of 32.8. The comparable figures for the semi-synthetic diet were 32.7 to 43.0 with a mean of 37.6 Cal. per kilogram.

Detailed metabolism data for a representative subject are summarized in table 4 which demonstrates the experimental plan and shows the fluctuations associated with this type of study. For this subject the mean daily nitrogen balances of the periods which supplied 0.10, 0.18, 0.22, 0.25 and 0.64 mg

of lysine per day were — 0.81, — 0.42, — 0.21, — 0.28, and + 0.25 gm, respectively. The mean daily nitrogen balance during the final period on the complete amino acid supplements (1.60 gm lysine per day) was + 1.07. The nitrogen balances of another representative subject (no. 8) at different levels of lysine intake in the sequence fed, are shown in figure 1.

The mean daily nitrogen balances during the final 4 days on a given lysine intake for all subjects are presented in table

TABLE 4

Metabolism data for subject 6

PERIOD [1]	DIET	DAILY L-LYSINE INTAKE	CALORIES PER KG	MEAN DAILY NITROGEN INTAKE	MEAN DAILY NITROGEN ELIMINATION		MEAN DAILY NITROGEN BALANCE
					Urinary	Fecal	
days		*gm*		*gm*	*gm*	*gm*	*gm*
1 (4)	Normal		34.4	10.15	7.79	1.26	+ 1.10
2 (4)	Normal		35.6	9.96	7.76	1.26	+ 0.94
3 (4)	Normal		36.8	9.76	8.02	1.26	+ 0.48
4 (4)	Transition		...	...	...	...	...
5 (4)	Semi-synthetic	1.60	41.6	10.82	9.46	0.76	+ 0.60
6 (4)	Semi-synthetic	1.60	41.8	10.97	9.66	0.76	+ 0.55
7 (4)	Semi-synthetic	1.60	42.4	10.92	9.20	0.76	+ 0.97
8 (4)	Semi-synthetic	1.60	43.5	10.98	9.16	0.76	+ 1.07
9 (5)	Semi-synthetic	0.25	43.6	10.98	10.54	0.72	— 0.28
10 (6)	Semi-synthetic	0.18	43.6	11.00	10.64	0.77	— 0.42
11 (6)	Semi-synthetic	0.64	43.4	10.96	9.93	0.77	+ 0.25
12 (7)	Semi-synthetic	0.22	43.1	10.90	10.33	0.77	— 0.21
13 (5)	Semi-synthetic	0.10	43.2	10.95	10.99	0.77	— 0.81

[1] Samples from periods 1 to 3, 5 to 8, and 10 to 13 were run as totals for these respective groups of periods.

5. The lysine intake varied slightly from period to period and from subject to subject. These variations have been recorded elsewhere (Jones, '56). The averages of the mean daily nitrogen balances for all of the subjects were — 0.62, — 0.84, — 0.36, — 0.46 gm for lysine intakes of 0.10, 0.18, 0.22 and 0.25 gm per day. When 9 of the subjects were studied on a daily lysine intake of 0.40 gm, the average of the mean daily nitrogen balances was 0.00. On this level of lysine intake, subject 15 exhibited a strongly negative nitrogen balance

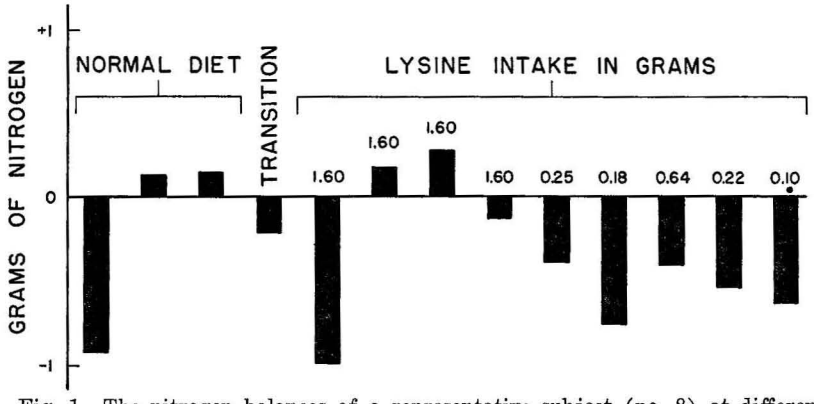

Fig. 1 The nitrogen balances of a representative subject (no 8) at different levels of lysine intake, in sequence used.

TABLE 5

Summary of nitrogen balances at different levels of lysine intake

SUBJECT	MEAN DAILY NITROGEN BALANCE ON INDICATED DAILY INTAKE OF LYSINE, GM							
	0.10	0.18	0.22	0.25	0.40	0.50	0.64	1.60
	gm N	*gm N*	*gm N*	*gm N*	*gm N*	*gm N*	*gm N*	*gm N*
Series I								
5	...	— 1.42	— 0.35	— 1.16	...	...	+ 0.08	+ 0.49
6	— 0.54	— 0.41	— 0.08	— 0.40	...	...	+ 0.29	+ 1.07
7	...	— 0.78	— 0.36	— 0.11	...	...	0.00	+ 0.09
8	— 0.62	— 0.75	— 0.53	— 0.38	...	...	— 0.40	— 0.12
9	— 0.75	— 0.83	— 0.46	...	...	...	+ 0.05 [1]	+ 0.45
Series II								
10 [2]	— 0.23	...	...	— 0.04	+ 0.03	...	...	+ 0.56
11	— 0.71	...	...	— 0.24	— 0.04	...	...	+ 0.65
12	— 0.88	...	...	— 0.87	— 0.05	...	...	+ 0.01
Series III								
15	...	...	...	...	— 0.79 [3]	+ 0.07 [3]	...	+ 0.34
16	...	...	...	...	— 0.26 [3]	0.00 [3]	...	+ 0.43
17	...	...	...	...	+ 0.54 [3]	...	...	+ 1.27
18	...	...	...	...	+ 0.59 [3]	...	...	+ 0.71
19	...	...	...	...	— 0.19 [3]	...	...	+ 0.14
20	...	...	...	...	+ 0.16 [3]	...	...	+ 0.27
Mean	— 0.62	— 0.84	— 0.36	— 0.46	0.00	+ 0.04	0.00	+ 0.45

[1] Studied at a lysine level 0.56 gm per day.

[2] Same as subject 6.

[3] The daily methionine intake was 0.29 gm.

(— 0.79 gm) and subject 16 had a negative nitrogen balance of — 0.26 gm per day. When the lysine allotment of these two subjects was increased to 0.50 gm daily, both of them attained nitrogen equilibrium. The averages of the mean daily nitrogen balances of the 5 subjects on lysine intakes of 0.64 (one subject was studied at 0.56 gm intake) and 1.60 gm were 0.00 and + 0.45 gm of nitrogen respectively.

Although there was considerable individual variation, it appears that a daily lysine intake of 0.40 to 0.50 gm would be adequate for most women under circumstances similar to those described in the present experiment. These values are of the same magnitude as the minimum daily lysine requirements of young men (namely 0.4, 0.4, 0.6, 0.7, 0.7 and 0.8 gm) as reported by Rose et al. ('55). This suggests that there is little or no sex difference in the lysine requirement of adult humans. No correlation could be established between the lysine requirement of an individual and the height, weight, body surface area, age or creatinine excretion. Moreover, since these values obtained on a semi-synthetic diet are comparable to those established by Rose et al. ('55) on a highly purified regimen, it is likely that the results of such reference experiments are applicable to the usual normal diet.

The exact lysine requirement of these subjects is dependent in part upon the concept of nitrogen balance which is applied to these data. Rose has repeatedly stated that the nitrogen balance must be slightly positive. Leverton et al. ('56) have defined nitrogen equilibrium as that state in which the nitrogen output (urinary plus fecal) is within 95 to 105% of the total nitrogen intake. According to the Rose criterion our subject 8 was not in balance even on the highest level of lysine intake, 1.60 gm per day. However, the nitrogen loss of this subject was not very great, and she had been in balance on the same regimen for the two previous periods of 4 days each.

That the average American diet probably contains adequate lysine is evident from available data. The normal diet in the present study supplied about 4 gm of lysine per day. Self-

selected diets of women furnished 1.7 to 8.0 gm of lysine daily (Futrell et al., '52; Reynolds, Futrell and Baumann, '53). Moreover Block and Bolling ('51) have calculated that the "average" American diet supplies 5.2 gm of lysine per day, and that even the diet of the lowest income urban group yields 4.0 gm of lysine.

DL-*Valine.* With all other factors constant for a given subject the use of the racemic mixture as the source of L-valine in series III (table 6) had little effect on the nitrogen balances of these subjects. The averages of the mean daily

TABLE 6

Mean daily nitrogen balances on 1.46 gm of L-*valine and on 2.92 gm of* DL-*valine in the amino acid supplements*

SUBJECT	MEAN DAILY NITROGEN BALANCE [1]	
	1.46 gm L-Valine	2.92 gm DL-Valine
	gm	*gm*
15	+ 0.07	+ 0.16
17	+ 0.54	— 0.29
18	+ 0.59	+ 0.06
19	— 0.19	— 0.26
20	+ 0.16	+ 0.31
Mean	+ 0.23	0.00

[1] All subjects received 0.29 gm of methionine and about 0.5 gm of cystine per day. Subject 15 received 0.50 gm of lysine per day, while the remaining subjects received 0.40 gm per day.

nitrogen balances for all subjects were + 0.23 gm (— 0.19 to + 0.54) on the L-valine and 0.00 gm (+ 0.29 to + 0.31) on the DL-valine.

SUMMARY

The effects of various levels of lysine intake on nitrogen balance were studied in 14 women maintained on a semi-synthetic diet in which about 95% of the total nitrogen was furnished by pure amino acids and diammonium citrate. From data obtained in this experiment, it appears that 0.40 to 0.50 gm lysine per day is adequate for the establishment of nitrogen balance in women under these conditions.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the students and staff for serving as subjects and for aiding in the execution of this study, and in particular to thank Eleanor Halter, Miriam McTeer, Saranya Reddy and Gertrude Skerski for their technical assistance. Grateful acknowledgment is made to Dr. Ruth Leverton for many helpful suggestions in planning the project, to Hoffmann-LaRoche, Inc., for supplying the vitamins, and to E. I. duPont deNemours and Company for contributing lysine hydrochloride.

LITERATURE CITED

BLOCK, R. J., AND D. BOLLING 1951 The Amino Acid Composition of Proteins and Foods. Charles C Thomas Publishing Co., Springfield.

FOLIN, O. 1914 On the determination of creatinine and creatine in urine. J. Biol. Chem., *17:* 469–473.

EUTRELL, M. F., R. N. LUTZ, M. S. REYNOLDS AND C. A. BAUMANN 1952 Studies on amino acids in self-selected diets. J. Nutrition, *46:* 299–311.

JONES, E. M. 1956 Studies on the microbiological determination of amino acids and on methionine and lysine requirements of women. Thesis for Ph.D. degree. University of Wisconsin, Madison.

LEVERTON, R. M. 1953 Personal communication.

LEVERTON, R. M., M. R. GRAM, M. CHALOUPKA, E. BRODOVSKY AND A. MITCHELL 1956 The quantitative amino acid requirements of young women. I. Threonine. J. Nutrition, *58:* 59–81.

PETERS, J. H. 1942 The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter. J. Biol. Chem., *146*. 179–186.

PRATT, E. L., S. E. SNYDERMAN, M. W. CHEUNG, P. NORTON, L. E. HOLT, JR., A. E. HANSEN AND T. C. PANOS 1955 The threonine requirement of the normal infant. J. Nutrition, *56:* 231–251.

REYNOLDS, M. S. 1956 Unpublished data.

REYNOLDS, M. S., M. F. FUTRELL AND C. A. BAUMANN 1953 Nitrogen balances, and amino acid content of self-selected diets of women. J. Am. Diet. Assn., *29:* 359–364.

ROSE, W. C. 1947 The role of the amino acids in human nutrition. Proc. Am. Phil. Soc., *91:* 112–116.

ROSE, W. C., A. BORMAN, M. J. COON AND G. F. LAMBERT 1955 The amino acid requirements of man. X. The lysine requirement. J. Biol. Chem., *214:* 579–587.

ROSE, W. C., M. J. COON AND G. F. LAMBERT 1954 The amino acid requirements of man. VI. The role of the caloric intake. J. Biol. Chem., *210:* 331–342.

SCALES, F. M., AND A. P. HARRISON 1920 Boric acid modification of the Kjeldahl method for crop and soil analysis. J. Ind. Eng. Chem., *12:* 350–352.

WATT, B. K., AND A. L. MERRILL 1950 Agriculture Handbook no. 8: Composition of Foods — Raw, Processed, Prepared. U. S. Department of Agriculture, Washington, D. C.

EFFECTS OF RUBIDIUM IN PURIFIED DIETS FED RATS [1,2,3]

B. L. GLENDENING,[4] W. G. SCHRENK AND D. B. PARRISH
Department of Chemistry, Kansas Agricultural Experiment Station, Kansas State College, Manhattan

(Received for publication June 14, 1956)

Only some 14 mineral elements are recognized as dietary essentials for animals, but nutritionists are interested in investigations of the possible nutritional and physiological effects of other mineral elements. One element which has received only limited attention is rubidium.

Mitchell, Wilson and Stanton ('21) substituted rubidium for potassium in the diets of white rats and found that they died in 10 to 17 days. Heppel and Schmidt ('38) studied the potassium metabolism of rats during pregancy, lactation and growth. They reported that the addition of 0.28% of rubidium to a ration containing 0.58% of potassium had no influence on growth and that the animals were still in good condition after receiving the diet for 72 to 86 days. When the ration contained 0.01% of potassium and 0.28% of rubidium, growth was 50 to 60% of normal until a few days before death; all but two animals died within 27 days.

Follis ('43) found that rats fed a potassium-deficient diet grew better when rubidium chloride was added, but that they survived only a short time. Rats receiving both potassium

[1] This study was supported in part by funds contributed by The Nutrition Foundation.

[2] Contribution no. *543* of the Department of Chemistry.

[3] Portion of a dissertation presented by the senior author in partial fulfillment of the requirements for the degree Doctor of Philosophy in Biochemistry at Kansas State College.

[4] Present address, Laboratory Division, Kansas State Board of Health, Topeka, Kansas.

and rubidium did not gain so well as those receiving rubidium alone, indicating that when potassium was present rubidium acted as a poison. He observed nervous conditions in rubidium-fed rats. Certain histological changes were found in rats receiving potassium-deficient diets and also in those receiving diets containing both rubidium and potassium, but not in those receiving rubidium alone.

The biological effects of rubidium assume an added interest since radioactive rubidium now is used as a tracer for potassium (Love, Romney and Burch, '54; Threefoot, Ray and Burch, '55; Burch, Threefoot and Ray, '55). Love and Burch ('53), who used Rb^{86} as a tracer in an *in vitro* study of erythrocyte electrolyte metabolism, pointed out that absolute reliance on the metabolic similarity of rubidium and potassium was not justified.

In view of the limited information on the effects of ingested rubidium and of the somewhat conflicting findings on rubidium when used with or in place of potassium, this study of feeding different levels of rubidium in various combinations with potassium and sodium in a purified diet was undertaken.

EXPERIMENTAL

Experiment 1. The first experiment was on the effects of feeding different levels of rubidium in the purified diet with and without sodium. The 13 experimental groups used in this study consisted of random selections of two male and two female weanling rats, 21 to 27 days of age, weighing between 33 and 63 gm. The rats were individually housed in cages constructed with open-mesh floors which allowed droppings to pass through. The animal room was kept at a temperature of 75 to 80°C.

The synthetic basal diet used was patterned after those of Sporn et al. ('47) and Meyer et al. ('50). Alterations were made in the proportions of the alkali metals as shown in table 1.[5] This basal diet proved to be reasonably adequate

[5] Analysis of the basal diet revealed it was not so free of sodium as expected. It contained 0.006% of sodium, mostly from the casein used.

for rats when 0.20% sodium was added: good growth and reproduction were obtained; more than half the young from the first mating survived and were weaned.

Experimental diets with 6 different levels of rubidium were prepared by adding calculated amounts of rubidium chloride to the basal diet, as shown in table 2. For each of the 6 food mixtures another was made identical in composition, except that it contained 0.20% of sodium as the chloride. A control

TABLE 1

Composition of basal ration used in experiment I

BASAL DIET	PARTS BY WT.	MINERAL MIXTURE	%	VITAMINS	MG/KG BASAL
Sucrose	73.0	$K_2HPO_4 \cdot 3H_2O$	21.45	Thiamine	3.0
Casein, alcohol		$CaHPO_4$	34.45	Riboflavin	3.0
extracted	18.0	$CaCO_3$	22.55	Niacin	20.0
Corn oil (Mazola)	5.0	$MgSO_4 \cdot 7H_2O$	17.90	Pyridoxine	2.0
Mineral mixture	4.0	Fe Citrate	3.17	Ca pantothenate	20.0
Vitamins		$MnSO_4 \cdot H_2O$	0.27	Folic acid	0.25
		$ZnSO_4$	0.05	Biotin	0.1
		$CuSO_4 \cdot 5H_2O$	0.05	Inositol	100.0
		KI	0.11	Choline Cl	1000.0
			100.00	*p*-Amino benzoic acid	250.0
				A.P.F. conc.[1]	1000.0
				2-methyl naphthoquinone	0.3

[1] Animal protein factor concentrate.

group of rats was fed a complete laboratory chow.[6] One of the 13 experimental groups of rats was assigned at random to each of the 13 diets. Both food and distilled water were given ad libitum. Weekly doses of oil-soluble vitamins, as fish oil, and synthetic α-tocopherol, were administered to all rats.

Analyses of diets for sodium and potassium were made using the Beckman D.U. spectrograph with flame, and for rubidium using the large Littrow quartz spectrograph (Glendening, Parrish and Schrenk, '55).

[6] Purina Laboratory Chow, Ralston Purina Co., St. Louis, Missouri.

TABLE 2

Sodium, potassium and rubidium contents of diets; average weights, and survival times of rats

Experiment 1

DIET [1]	CONTENTS IN DIETS			NO. RATS	AVERAGE WEIGHT						SURVIVAL TIME		
	Na	K	Rb		0 days	10 days	20 days	40 days	80 days	120 days	Min.	Max.	Mean
	%	%	%		*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*days*	*days*	*days*
A	0.39 (0.36)[2]	0.60 (0.76)	... (0.004)	4	55	102	138	200	268	288	180	300+	
B	0.00 (0.006)	0.29 (0.28)	0.00 (0.000)	4	47	63	71	92	139	202	262	300+	
C	0.20 (0.20)	0.29 (0.32)	0.00 (0.000)	4	46	81	139	177	242	273	180	300+	
D	0.00 (0.009)	0.29 (0.27)	0.01 (0.009)	4	48	65	76	106	152	196	141	300+	
E	0.20 (0.19)	0.29 (0.28)	0.01 (0.011)	4	45	84	120	188	259	300	300+	300+	
F	0.00 (0.008)	0.29 (0.29)	0.10 (0.14)	4	47	64	71	92	134	185	200	300+	
G	0.20 (0.19)	0.29 (0.28)	0.10 (0.14)	4	50	79	118	175	218	239	176	300+	
H	0.00 (0.010)	0.29 (0.29)	0.20 (0.21)	4	44	60	70	90	111[3]	127[4]	64	140	101
I	0.20 (0.19)	0.29 (0.28)	0.20 (0.21)	4	53	85	116	141[3]	171[4]	...	24	102	74
J	0.00 (0.008)	0.29 (0.30)	0.30 (0.30)	4	51	63	70	74[5]	...	...	29	54	37
K	0.20 (0.18)	0.29 (0.32)	0.30 (0.30)	4	49	76	106[4]	...	...	...	15	28	17
L	0.00 (0.007)	0.29 (0.30)	0.40 (0.37)	4	47	61	62	...	...	...	21	38	27
M	0.20 (0.15)	0.29 (0.34)	0.40 (0.40)	4	52	76	...	...	...	...	12	14	12

[1] Diet A was Purina Laboratory Chow. Diets B through M were composed of purified ingredients.

[2] Number outside parentheses is calculated quantity added. Number within parentheses is content by analysis.

[3] Only three surviving rats at this stage.

[4] Only one surviving rat at this stage.

[5] Only two surviving rats at this stage.

Average weights of rats fed the various diets and survival times are shown in table 2. Group A (controls), fed laboratory chow, gained faster than any group fed purified diets. However, at 80 days on the experiment, the rats of group E (0.20% of sodium and 0.01% of rubidium) had attained over 96% of the weight of those of group A (control). The addition of rubidium in amounts of 0.2%, 0.3%, and 0.4% had a deleterious effect on both growth and survival time, as shown in table 2. This was true whether or not sodium was added to the diet. When receiving the foregoing levels of rubidium in the diet, groups receiving 0.2% of sodium grew better than, but did not survive so long as, those not receiving sodium. At rubidium levels of 0.3 and 0.4% the mean survival times were more than doubled when sodium was not added to the diet. All 4 rats receiving 0.4% of rubidium and 0.2% of sodium died in 12 to 14 days.

Rubidium toxicity was indicated first by failure to gain weight normally. Then as feeding the diets containing toxic amounts of rubidium continued, the skin, especially around the neck and ankles, sometimes changed to a blue or purple tinge. The nose and mouth became sore. Whiskers became matted with food or body secretions and the hair was rough and stood out. The rats assumed a humped-up appearance. Sores developed on the tails. The animals were sensitive to noise and touch; they often bit and squealed when handled. The excitment of handling caused many animals to have convulsions, often leading to death. In general these observations are similar to those reported by Follis ('43), Mitchell, Wilson and Stanton ('21) and Heppel and Schmidt ('38).

Rats in experiment 1 that survived were allowed to mate and reproduce. The two females given a diet containing 0.1% of rubidium gave birth to young but the young failed to survive. Generally with 0.1% of rubidium in the diet of parents, the progeny did not survive to weaning age.

A reproduction study was made on another group of rats receiving the basal diet with 0.02% each of sodium and rubidium added. Although growth was subnormal, three of 5 males

and all 6 females lived more than 300 days All mothers killed their first litters but 11 of 30 young of the second litters survived. Five second generation females had young resulting from the first mating, but only one litter was raised. In a second mating, three of 4 females gave birth to young, but only one litter was raised. Two females had a third litter, but no young were raised. One 4th generation litter was born to a female in this dietary group.

Experiment 2. A second experiment was conducted using a basal diet lower in content of alkali metals. In this study, diets contained different levels of sodium, potassium and rubidium, both alone and in combination. In experiment 1, no variation was made in potassium content.

The basal diet was the same as shown in table 1, with the following modifications. Vitamin-free casein was substituted for alcohol-extracted casein, since the former was found to contain less sodium and potassium. Potassium salts were not used in the mineral mix. NH_4I was substituted for KI. Calcium and phosphorus were supplied by 48.90% of $Ca(H_2PO_4)_2 \cdot H_2O$, and 31.76% of $CaCO_3$ in the mineral mixture. Diets and tissues were analyzed for sodium, potassium and rubidium as in experiment 1.

In experiment 2 the groups of rats are designated by symbols indicating the kinds and amounts of alkali metals added to the basal diet (table 3). A single symbol indicates a content of 0.25% of that element and a double symbol twice that amount (e.g. Rb, 0.25% rubidium; NaK, 0.25% sodium and 0.25% potassium; KK, 0.50% potassium). B.O. designates basal diet only and L.C., laboratory chow (control group). Equimolar quantities of the alkali metals were not used since an amount of rubidium equimolar to desirable experimental levels of potassium and sodium could be tolerated but a few days.

In experiment 2 weanling rats from the stock colony, 24 to 28 days of age, were randomized into 12 groups, each consisting of 4 males and 4 females. The rats were placed in individual cages, kept in air conditioned space, and given

TABLE 3

Sodium, potassium and rubidium contents of diets; average weights and survival times of rats

Experiment 2

DIET [1]	CONTENTS IN DIETS			NO. RATS	AVERAGE WEIGHT						SURVIVAL TIME		
	Na	K	Rb		0 days	8 days	16 days	32 days	64 days	100 days	Min.	Max.	Mean
	%	%	%		*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*days*	*days*	*days*
B.O.	0.00 (0.0005)[2]	0.00 (0.0032)	0.00 (0.000)	8	44	46	46	45	37 [3]	...	41	71	53
Na	0.25 (0.20)	0.00 (0.0050)	0.00 (0.000)	8	46	48	52	73 [4]	...	...	28	56	37
K	0.00 (0.0007)	0.25 (0.22)	0.00 (0.000)	8	43	55	61	69	61 [5]	57 [6]	58	100+	99
Rb	0.00 (0.0005)	0.00 (0.0096)	0.25 (0.24)	8	45	54	59	...	...	...	16	31	23
NaNa	0.50 (0.48)	0.00 (0.0064)	0.00 (0.000)	8	43	48	48	48 [4]	...	...	18	45	33
NaK	0.25 (0.22)	0.25 (0.24)	0.00 (0.000)	8	44	74	113	185	250	291	100+	100+	100+
NaRb	0.25 (0.23)	0.00 (0.13)	0.25 (0.25)	8	40	56	67	...	...	...	16	28	21
KK	0.00 (0.0013)	0.50 (0.45)	0.00 (0.000)	8	44	58	65	72	71	71 [7]	65	100+	100+
KRb	0.00 (0.0011)	0.25 (0.24)	0.25 (0.26)	8	44	57	64	71 [7]	64 [8]	67 [3]	29	131	55
RbRb	0.00 (0.0006)	0.00 (0.0176)	0.50 (0.51)	8	44	57	68 [3]	...	...	...	10	17	13
L.C.[9]	0.38 (0.36)	0.60 (0.66)	... (0.004)	8	45	92	138	204	273	301	100+	100+	100+

[1] See text, page 568, for ration designations.

[2] Number outside parentheses is calculated quantity added. Number within parentheses is content by analysis.

[3] Only 2 surviving rats at this stage.

[4] Only 5 surviving rats at this stage.

[5] Only 6 surviving rats at this stage.

[6] Only 4 surviving rats at this stage.

[7] Only 7 surviving rats at this stage.

[8] Only 3 surviving rats at this stage.

[9] Numbers outside parentheses are manufacturer's data.

daily care. They were fed ad libitum, as in experiment 1, and a record of food consumption was kept. Live rats were weighed every 4 days, and those that died were weighed soon after death.

Average weights of each group of rats at certain periods and survival times are in table 3. Rats receiving basal diet only (B.O.) failed to grow, those receiving sodium (Na and NaNa) grew little; those receiving rubidium (Rb and RbRb) grew at early stages comparable to those receiving potassium (K and KK). The sodium-rubidium (NaRb) and potassium-rubidium (KRb) diets produced comparable early growth. Rats receiving the sodium-potassium combination (NaK) grew better than those of any other group, except for the controls (L.C.), which averaged a little heavier.

Rats eating a diet practically devoid of sodium, potassium and rubidium (B.O.) survived a mean of 53 days. Adding rubidium (Rb and RbRb) decreased mean survival time to 23 days and 13 days, respectively. Adding sodium (Na and NaNa) alone, decreased survival time to 37 and 33 days, respectively. Adding potassium (K and KK), however, increased survival time to 99 days or more. Survival time on the sodium-rubidium (NaRb) diet and the rubidium (Rb) diet was approximately the same. Rats receiving the potassium-rubidium combination (KRb) survived longer than those receiving rubidium (Rb) alone but died earlier than those receiving potassium (K) alone.

When an animal died, it was dissected and gross examination made of internal organs. Symptoms of rubidium toxicity observed before death of the rats were the same as described in experiment 1. Post mortem findings were not conclusive as to the cause of death. Animals receiving no alkali metal except sodium were bloated before death, and post mortem examination revealed abnormal quantities of a free, watery fluid in the abdominal and thoracic cavities.

After death of each animal the following organs were removed, weighed and preserved for analysis: lungs, heart, liver, kidney, brain. In addition, samples of bone and muscle

were taken. Rats that did not succumb during the experiment were sacrificed at 300 days and the aforementioned tissues removed for analyses.

Analyses were made on either the whole organ or a representative sample which, of course, included residual blood. However, hearts were split and blood clots rinsed out. Sodium, potassium and rubidium contents of the composite samples from the 8 animals in each group are shown in table 4. It should be remembered that early deaths and longevity probably affected depletion or retention of certain elements.

As would be expected, an increase of an element in the diet generally resulted in an increase of that element in the tissues. Traces of rubidium were found in organs of rats fed the control diet (LC). The presence of potassium in the diet markedly reduced the sodium content of tissues. In general, rubidium had a similar but less marked effect on sodium content. Rubidium in the diet greatly increased rubidium content of the tissues and reduced potassium content somewhat, except in the brain. Rubidium did not appear to accumulate to a marked degree in any particular organ or tissue studied, but seemed to be disseminated throughout the body, as were sodium and potassium. On a dry matter basis, however, bone contained a smaller concentration of rubidium than did the soft tissues examined.

Rubidium, sodium and potassium contents of tissues (table 4) were calculated on a molar basis. When rubidium and sodium were fed simultaneously in the diet at equal concentrations (0.25%) the molar concentrations of rubidium in the tissues were higher than those of sodium. The same thing occurred when rubidium and potassium were fed. When the diet contained rubidium it was found that there often was a small reduction in the total molar concentration of sodium plus potassium in the tissues of the rats, but total molar concentration of the alkali metals was increased markedly, most of the increase being due to rubidium. Sodium in the diet increased molar concentration of alkali metals somewhat in most tissues.

TABLE 4

Average sodium, potassium and rubidium contents of various tissues and organs of rats fed diets containing different amounts of alkali metal cations in experiment 2

DIETARY GROUP [1]	APPROXIMATE DIETARY CONTENT			CONTENT, DRY BASIS						
	Na	K	Rb	Lungs	Heart	Liver	Kidneys	Muscle	Brain	Bone
	%	%	%	%	%	%	%	%	%	%
							Sodium			
B.O.	0.0	0.0	0.0	0.19	0.32	0.15	0.26	0.24	0.37	0.35
Na	0.25	0.0	0.0	0.73	0.63	0.55	0.77	0.91	0.57	0.57
K	0.0	0.25	0.0	0.25	0.21	0.14	0.21	0.15	0.33	0.26
Rb	0.0	0.0	0.25	0.38	0.43	0.27	0.44	0.24	0.50	0.46
NaNa	0.50	0.0	0.0	0.79	0.70	0.64	0.76	0.66	0.51	0.53
NaK	0.25	0.25	0.0	0.39	0.26	0.19	0.42	0.12	0.37	0.43
NaRb	0.25	0.0	0.25	0.84	0.59	0.43	0.57	0.38	0.56	0.49
KK	0.0	0.50	0.0	0.13	0.09	0.06	0.16	0.04	0.13	0.24
KRb	0.0	0.25	0.25	0.24	0.27	0.16	0.28	0.23	0.40	0.34
RbRb	0.0	0.0	0.50	0.41	0.63	0.24	0.33	0.17	0.40	0.32
L.C.	0.38	0.60	0.0	0.41	0.25	0.30	0.42	0.40	0.38	0.48
							Potassium			
B.O.	0.0	0.0	0.0	0.75	0.82	0.77	0.85	0.80	1.02	0.28
Na	0.25	0.0	0.0	0.89	0.93	0.82	0.97	0.88	0.97	0.37
K	0.0	0.25	0.0	0.86	0.85	0.54	0.91	1.03	1.06	0.30
Rb	0.0	0.0	0.25	0.43	0.43	0.48	0.50	0.63	0.87	0.19
NaNa	0.50	0.0	0.0	0.72	0.70	0.78	0.84	0.81	1.05	0.39
NaK	0.25	0.25	0.0	0.91	0.97	0.86	0.83	1.20	1.43	0.38
NaRb	0.25	0.0	0.25	0.73	0.58	0.64	0.69	0.57	1.34	0.23
KK	0.0	0.50	0.0	0.93	1.03	0.97	0.90	1.06	1.17	0.29
KRb	0.0	0.25	0.25	0.85	0.89	0.90	0.91	0.88	1.05	0.55
RbRb	0.0	0.0	0.50	0.57	0.55	0.59	0.56	0.58	1.05	0.29
L.C.	0.38	0.60	0.0	1.03	1.06	0.97	0.85	1.26	1.43	0.43
							Rubidium			
B.O.	0.0	0.0	0.0	0.03	0.03	0.04	0.03	0.00	0.03	0.00
Na	0.25	0.0	0.0	0.01	0.00	0.02	0.02	0.00	0.02	0.00
K	0.0	0.25	0.0	0.02	0.00	0.01	0.02	0.02	0.01	0.00
Rb	0.0	0.0	0.25	0.89	0.82	1.18	1.19	.52	1.04	0.42
NaNa	0.50	0.0	0.0	0.03	0.03	0.06	0.05	0.04	0.05	0.00
NaK	0.25	0.25	0.0	0.01	0.00	... [2]	..	...	0.00	0.00
NaRb	0.25	0.00	0.25	1.54	1.15	1.50	1.71	1.36	1.62	0.42
KK	0.0	0.50	0.0	0.04	0.00	0.03	0.03	0.04	0.02	0.00
KRb	0.0	0.25	0.25	1.08	1.24	1.32	1.34	1.29	1.23	0.74
RbRb	0.0	0.0	0.50	1.55	1.66	1.56	1.51	1.70	1.85	0.77
L.C.	0.38	0.60	0.00	0.02	0.02	0.02	0.01	0.02	0.01	0.00

[1] See text, page 568, for ration designations.
[2] Measurable but less than 0.01%.

The concentration of rubidium was greater in the blood cells than in the plasma. Average rubidium content of the cell fraction of 1 ml of heart blood from two rats fed 0.1% of rubidium for 300 days in experiment 1 was 0.54 mg, while that of the plasma fraction was 0.028 mg. The blood cells of two rats under the same conditions except that the diet contained 0.01% of rubidium, were found to contain 0.043 mg rubidium and rubidium was not detected in the plasma fraction.

Balance study. An investigation of rubidium metabolism was made through a balance study. Six experimental groups, each composed of two normal male albino rats 25 to 35 days old, were used. The purified diet was similar to that of experiment 2, and alkali metals were added to various diets as indicated in table 5. Diets are designated as in experiment 2 These diets were fed ad libitum. Distilled water was supplied and the consumption measured. Animals were caged individually. Rats and food were weighed daily, and urine and feces were collected separately for analysis during the experimental period of 14 days. Table 5 shows weights gained, feed eaten, water consumed, waste products collected, and balance data on sodium, potassium, and rubidium during the 14-day trial.

The addition of one of the three alkali elements to the basal diet resulted in accumulation of the that element in the tissues. Sites of retention of sodium, potassium and rubidium in the body and paths of elimination were similar.

DISCUSSION

The effects of dietary rubidium and the interrelationships with sodium and potassium have been studied more completely than heretofore: more than 170 rats were observed, some of them for more than 300 days.

Soybeans contain the highest concentrations of rubidium (0.02%) of any feeds or foods studied (Glendening, Parrish and Schrenk, '55). Data of the present studies indicate that

TABLE 5

Weight gains and balance data on rats during a 14-day trial [1]

DIET [2]	CONTENTS IN DIET [3]			WT. GAIN 14 DAYS	FEED EATEN	WATER INTAKE	FECES (Dry wt.)	URINE (Est.) [4]
	Na	K	Rb					
	%	%	%	*gm*	*gm*	*ml*	*gm*	*ml*
LC	.36	.76	.004	88	195	331	65.2	68
BO	.001	.003	.000	1	81	321	4.07	130
Rb	.001	.001	.24	13	97	232	4.81	64
NaRb	.23	.013	.25	33	119	145	5.16	18
KRb	.001	.24	.26	21	121	271	5.82	42
NaKRb	.25	.25	.27	44	127	197	5.43	35

DIET	QUANTITY MINERAL INGESTED	EXCRETED IN URINE	EXCRETED IN FECES	TOTAL EXCRETED	TOTAL RETAINED
	mg	*mg*	*mg*	*mg*	*mg*
			Sodium		
LC	700	370	70	440	260
BO	0.5	* [5]	*	0.00	0.5
Rb	0.5	*	*	0.00	0.5
NaRb	273	90	5	95	178
KRb	1.3	*	*	0.00	1.3
NaKRb	318	115	4	119	198
			Potassium		
LC	1479	117	186	303	1176
BO	2.6	0	2	2	.6
Rb	9.3	15	2	17	(—7.7)
NaRb	15.4	14	4	18	(—2.6)
KRb	290	37	8	45	245
NaKRb	313	39	9	48	265
			Rubidium		
LC	7.8	*	*	0.00	7.8
BO	0.0	*	*	0.00	0.0
Rb	228	13	3	16	212
NaRb	296	14	5	19	277
KRb	314	68	8	76	238
NaKRb	343	63	5	68	275

[1] All data are averages from 2 rats.

[2] Diets designated in same manner as in experiment 2.

[3] Contents by analysis of diets.

[4] Some volume lost by evaporation.

[5] Not detected in analysis.

0.02% of rubidium in presence of other nutrients in feeds is not toxic to rats.

No reports have been found in the literature on whether small amounts of rubidium are a dietary essential or a stimulatory agent for animals. Although in one experiment of the present study the best growth was obtained when the purified diet contained 0.01% rubidium the increase was without significance. As judged by data of this study, rubidium does not appear to have any dietary value for rats under normal conditions. If, however, trace quantities should be found advantageous, it poses no practical problem as many foods contain traces of rubidium (Glendening, Parrish and Schrenk, '55).

These studies supply information on the metabolic similarity of rubidium and potassium, and the propriety of using Rb^{86} as a tracer for potassium. Previous work on this problem (Love and Burch, '53; Love, Rommey and Burch, '54; Threefoot, Ray and Burch, '55) was on short-time studies of uptake of injected rubidium, whereas the present report is on the feeding of rubidium for various lengths of time. Ingested rubidium was distributed rather generally throughout the animal body, as was potassium. However, on a dry matter basis, bone contained less rubidium than the soft tissues examined. Under conditions different from these studies (short time uptake of injected Rb^{86}) Freedberg, Pinto and Zipser ('52) reported that the tissues differed in rubidium content. In the present study, rubidium, like potassium, was found in a higher concentration in blood cells of rats than in serum. This is consistent with findings of Bertrand and Bertrand ('51) and Freedberg, Pinto and Zipser ('52) on human blood.

When added alone to purified diets, rubidium or potassium produced better early growth of rats than did sodium. Rats receiving a rubidium-sodium combination grew better than those receiving sodium alone; however, they did not grow nearly so well as those receiving a potassium-sodium combination. This indicates that rubidium possibly substitutes for potassium to some extent, but has effects on growth and

longevity that precludes full metabolic interchangeability. Heppel and Schmidt ('38) expressed the same idea.

When a total of less than 400 mg of alkali metals was fed to rats during a 14-day balance study, rubidium resembled potassium more than sodium in that the major part was retained in the body. Ingested rubidium was similar to sodium and potassium in that the main path of elimination was by the kidneys rather than by the digestive tract. This is in accord with findings of Mendel and Closson ('06) and Freedberg, Pinto and Zipser ('52).

Purified diets that contained as little as 0.1% of rubidium in presence of 0.25% of potassium were toxic, as judged by growth and reproductive performance. Toxicity increased as the rubidium content of the diets was increased, and 0.20% of rubidium markedly decreased survival time. Toxicity also was increased by the addition of sodium to the diets, although growth up to a short time before death was improved by sodium. Diets containing 0.25% of rubidium were more toxic in the absence of potassium than those that also contained 0.25% of potassium.

The more toxic effects of dietary rubidium in the absence of potassium, or presence of only small amounts, is in agreement with the findings of Mitchell et al. ('21) and Heppel and Schmidt ('38). The present observations that rats lived longer on a diet containing both potassium and rubidium than on one containing rubidium alone is not in agreement with the report by Follis ('43). However, it is difficult to compare present results with those of other workers cited because of the differences in experimental conditions.

Heppel and Schmidt ('38) analyzed carcasses of rats fed two levels of rubidium and potassium and reported that in both groups there were differences in molar concentrations of rubidium and of potassium but that the sum of these two elements was about the same in both groups. One might expect a similar finding in this study if the sums of molar contents of rubidium plus potassium in the tissues were calculated. All tissues from rats eating diets containing rubidium

had increased total molar contents of alkali metals. Rubidium did not replace more than a small part of the sodium and potassium, and apparently was retained in addition to these two normal tissue constituents. When sodium and potassium or sodium and rubidium were fed, each at 0.25% of the diet, the intake of rubidium on a molar basis was only one-fourth that of sodium and one-half that of potassium, but molar concentrations of rubidium in the tissues were higher than those of either of the other two elements. This indicates that rubidium is preferentially taken up and retained by the tissues.

SUMMARY

Using more than 170 rats, a study was made of the physiological effects of different dietary levels of rubidium alone and in combination with various amounts of sodium and potassium.

Under conditions of this study trace quantities of rubidium did not appear to be a dietary essential or a stimulatory agent for rats.

Purified diets containing 0.02% of rubidium or less were not toxic to rats, but diets containing 0.1% of rubidium or more were toxic. Toxicity, as measured by decreased growth, general condition, reproductive performance and survival time, increased with increasing concentrations of rubidium in the diet. No rats receiving 0.2% of rubidium or more in the diet reproduced; some of those receiving 0.1% of rubidium gave birth to young, but the young did not survive when fed the same diet.

Other evidences of toxicity when diets contained 0.1% or more of rubidium were poor hair coat, sore noses, sometimes red deposits on whiskers, sensitivity, extreme nervousness leading to convulsions in advanced stages, and finally death.

Small concentrations of rubidium were found in tissues of rats fed a commercial chow. When diets contained added rubidium, the concentration of this element increased in the tissues and potassium generally was reduced, except in the brain. Rubidium was not concentrated in any particular tissue or organ studied, except that bone contained less than the soft

tissues. There were small reductions of the molar concentrations of the sum of sodium plus potassium in the tissues of rats eating diets containing rubidium, but the sum of molar concentrations of alkali ions was increased markedly. Rubidium replaced only a small amount of the sodium and potassium of tissues and apparently was retained in addition to them.

The inclusion of sodium in diets containing rubidium increased early growth of rats but decreased survival time. The presence of potassium in diets containing rubidium caused better growth of rats and longer survival than rubidium alone. Rubidium appeared to substitute only partially for potassium.

In blood, rubidium was found in much higher concentrations in the cells than in the serum fraction. The kidney was the main path for elimination of rubidium.

LITERATURE CITED

BERTRAND, G., AND D. BERTRAND 1951 Sur la repartition du rubidium du sang entre le plasma et les globules. Comp. rend., *232:* 131.

BURCH, G. E., S. A. THREEFOOT AND C. T. RAY 1955 The rate of disappearance of Rb^{86} from the plasma, the biological decay rates of Rb^{86}, and the applicability of Rb^{86} as a tracer of potassium in man with and without chronic congestive heart failure. J. Lab. Clin. Med., *45:* 371.

FOLLIS, R. H. 1943 Histological effects in rats resulting from adding rubidium or cesium to a diet deficient in potassium. Am. J. Physiol., *138:* 246.

FREEDBERG, A. S., H. B. PINTO AND ALBERT ZIPSER 1952 Distribution of administered rubidium^{86} carbonate in mouse, guinea pig, dog and man. Fed. Proc., *11 (1):* 49.

GLENDENING, B. L., D. B. PARRISH AND W. G. SCHRENK 1955 Spectrographic determination of rubidium in plant and animal tissues. Anal. Chem., *27:* 1554.

HEPPEL, L. A., AND C. L. A. SCHMIDT 1938 Studies on potassium metabolism of the rat during pregancy, lactation and growth. Univ. Calif. Pub. Physiol., *8:* 189.

LOVE, W. D., AND G. E. BURCH 1953 A comparison of potassium^{42}, rubidium^{86} and cesium^{134} as tracers of potassium in the study of cation metabolism of human erythrocytes in vitro. J. Lab. Clin. Med., *41:* 351.

LOVE, W. D., R. B. ROMNEY AND G. E. BURCH 1954 A comparison of the distribution of potassium and exchangeable rubidium in the organs of the dog, using Rb^{86}. Circulation Res., *2:* 112.

MENDEL, L. B., AND O. E. CLOSSON 1906 The paths of excretion for inorganic compounds. III. The excretion of rubidium. Am. J. Physiol., *16:* 152.

MEYER, J. H., R. H. GRUMMER, P. H. PHILLIPS AND G. BOHSTEDT 1950 Sodium, chlorine, and potassium requirements of growing pigs. J. Animal Sci., *9:* 300.

MITCHELL, P. H., J. W. WILSON AND R. E. STANTON 1921 The selective absorption of potassium by animal cells. II. The cause of potassium selection as indicated by absorption of rubidium and cesium. J. Gen. Physiol., *4:* 141.

SPORN, E. M., W. R. RUEGAMER AND C. A. ELVEHJEM 1947 Growth and reproduction in rats on synthetic rations. Proc. Soc. Exp. Biol. Med., *65:* 5.

THREEFOOT, S. A., C. T. RAY AND G. E. BURCH 1955 Study of the use of Rb^{86} as a tracer for the measurement of Rb^{86} and K^{39} space and mass in intact man with and without congestive heart failure. J. Lab. Clin. Med., *45:* 395.

VARIATION IN RIBOFLAVIN EXCRETION[1]

D. M. HEGSTED, S. N. GERSHOFF, M. F. TRULSON AND D. H. JOLLY

Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts, and the Massachusetts Department of Mental Health, Wrentham State School, Wrentham, Massachusetts

(Received for publication June 29, 1956)

The urinary excretion of various nutrients or their metabolic products is related to the quantities consumed, and urinary excretion measurements may be the methods of choice in attempting to evaluate nutritional status. However, the difficulties of obtaining accurately timed samples or achieving other standard conditions in the field are well known. The advantages of using randomly collected samples, particularly small samples, are obvious provided methods are available for their evaluation. Since creatinine excretion is known to be relatively constant and more or less proportional to muscle mass, this appears to be the most suitable parameter for evaluating such samples (Adamson et al., '45; Lowry, '52). Urine volume appears to be a less suitable baseline, but it has been reported that riboflavin excretion was more closely related to urine volume than to the time during which collections were made (Feder, Lewis and Alden, '44).

Clearly, the value of urine analysis in the assessment of the nutritional status of an individual or a population depends upon how accurately the sample obtained estimates the true level of excretion. As far as we are aware, there has been

[1] Supported in part by grants-in-aid from the National Institute of Mental Health (No. M-611C), Public Health Service, Bethesda, Maryland; Merck and Company, Rahway, New Jersey; National Biscuit Company, New York, N. Y., and General Mills, Inc., Minneapolis, Minnesota.

no attempt to determine the accuracy of single urine samples as estimates of the true excretion rate. The advantages or disadvantages of the three parameters — time, creatinine, and urine volume — to which the excretion may be compared are not known in quantitative terms. We have attempted to make such estimates in the present paper with regard to riboflavin excretion. Urine was collected every two or 4 hours from a series of subjects and the variation in riboflavin excretion per hour, per milligram of creatinine, and per milliliter of urine has been compared.

Regardless of the method of expressing the results, it may be assumed that the longer the collection period, the more accurately the sample will estimate the true average rate of excretion. From the data obtained, it was also possible to estimate the improvement in accuracy obtainable by increasing the time of urine collection.

EXPERIMENTAL

Data were obtained in two separate experiments. In the first, the subjects were 4 mentally-subnormal men between the ages of 20 and 43 years. Two had mongolism and the other two were mentally subnormal following brain damage. We have been unable to show a significant difference in the excretion of riboflavin in mongoloids as compared to other types of mentally deficient patients. The subjects in this study consumed their usual diets in the same institutional dining room and records were kept of the kind and weight of food consumed. Estimates of the riboflavin intake were made using standard food tables. The diets contained considerable amounts of milk and were accordingly high in riboflavin. The usual intakes were from 2.5 to 3.5 mg of riboflavin per day and on the two days during which urine collections were made the mean intakes were between 3.2 and 3.4 mg per day. Meal hours were uniform with breakfast at 7 A.M., lunch at 12:30, and supper at 5 P.M. The three meals provided approximately 25, 45, and 30% of the total riboflavin

intake, respectively Urine samples were collected every two hours, day and night, for two days.

Since the subjects in the first experiment were consuming a high riboflavin diet under rather unvarying conditions, it seemed possible that their excretion might be more variable from sample to sample than when a low riboflavin diet was eaten. Accordingly, data were obtained on three presumably normal adults, two men and a woman, who during the course of the experiment continued their regular activities but eliminated nearly all high riboflavin foods from their diets. No milk, cheese, eggs or green vegetables and only small amounts of meat were eaten during a 7-day period. Twenty-four-hour collections were made during the first three days. During the last 4 days the urine was collected every 4 hours day and night. The total riboflavin intake calculated from the dietary records varied from 0.3 to 0.8 mg per day and the average intake was 0.5 mg per person per day.

The urine volume of each collection was measured in the smallest conveniently graduated cylinder. Riboflavin analyses were done microbiologically using *L. casei* and the media of Snell and Strong ('39). Creatinine determinations were made using a slight modification of the method of Clark and Thompson ('49).

The correlation and partial correlation coefficients relating riboflavin excretion, creatinine excretion and urine volume were calculated for each subject. Significant correlations exist between riboflavin and creatinine excretion as well as between riboflavin excretion and urine volume, but in most instances the association was not close and these calculations are not presented. The standard deviation of the riboflavin excretion for two-hour samples regardless of how expressed was seen to be large, such that two standard deviations were greater than the mean excretion. This would imply that excretions of zero were not uncommon whereas these were never found. Hence, in comparing the relative constancy of riboflavin excretion per hour, per unit creatinine and per unit volume, logarithms were used to calculate the standard

deviations. Other reasons for the use of logarithms were found in the observation that the distribution of the data tended to be skewed toward high values, and that the coefficient of variation for different subjects was of similar magnitude although the mean excretion rate varied. The values for the points, two standard deviations of either side of the geometric mean, were obtained and the antilogarithms of these values expressed as percentage of the mean value. Approximately 95% of the values are expected to fall within $\pm$ 2 standard deviations of the mean value.

Logarithms were not used for calculation of the standard deviations of the creatinine output.

We also desired to compare the relative variation in two-hour collections as compared to 4-hour collections and longer periods. For this purpose the riboflavin and creatinine excretions and urine volumes for each pair of two-hour periods were added together to give 4-hour periods, etc., and the excretion per milligram of creatinine and per milliliter of urine recalculated. Logarithms of the values were used for the statistical calculations as described above, and the results expressed in the same way. It is realized that these are not independent estimates but the findings are of practical interest.

Finally, in the subjects who consumed the low-riboflavin diet, the urinary excretion of riboflavin gradually declined during the collection period. In order to obtain a better estimate of the variation due to unknown causes, the deviations from the mean which could be accounted for by linear regression (due to the gradual decline in excretion) were subtracted. It is realized that the regression may not be linear but more extensive manipulation of the limited data available is probably not justified. Also, the expression of the variation about the line of regression as percentage of the mean excretion may be criticized. These factors may explain the somewhat greater variation seen in the data from the subjects consuming low riboflavin diets than in the subjects in the first experiment.

RESULTS AND DISCUSSION

Creatinine excretion. Since creatinine is one of the baselines used for estimating the rate of riboflavin excretion, the constancy of creatinine excretion itself is of interest. The standard deviations of the two-hour collection periods were large. In the 4 subjects in experiment I, the coefficient of variation (standard deviation expressed as percentage of the mean value) was 22.8% in the most constant subject and

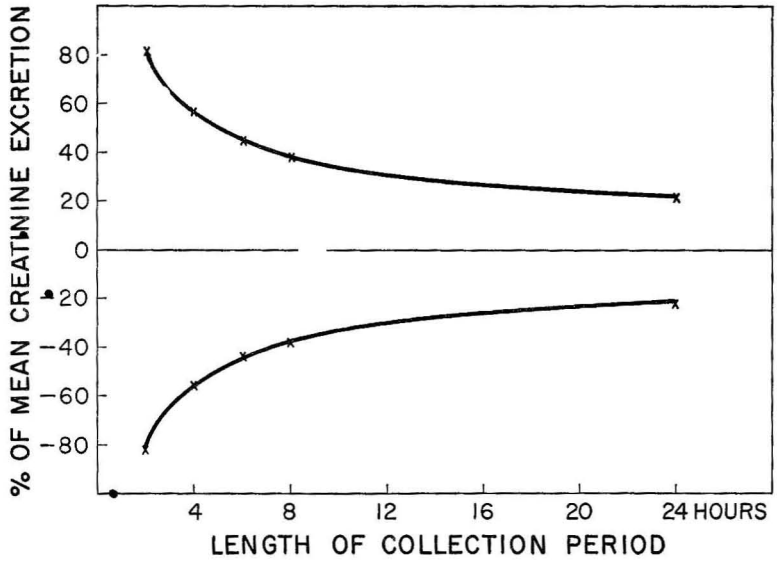

Fig. 1 The effect of the length of the collection period on constancy of creatinine excretion.

52% in the most variable. The average value was 40.3%. Thus, it may be expected that single two-hour collections will fall within $\pm$ 80% of the average two-hour excretion.

As the sample size increases, the accuracy of the estimate also increases. Whereas data were available on only 4 subjects for two-hour periods, data from 7 subjects were used to calculate the 4-, 8-, and 24-hour excretion. As seen in figure 1, great improvement is achieved by increasing the collection period over two hours, but the improvement ob-

tained by lengthening the collection above 12 hours is relatively small. Since the standard error of a mean is $\frac{\text{st. dev.}}{\sqrt{N}}$, where N is the number of samples, the number of samples of any particular size required to give the degree of accuracy desired may be estimated from figure 1.[2]

Textbooks widely quote the original observations of Shaffer ('08–'09) to the effect that the excretion of creatinine from hour to hour is as constant as it is from day to day. A calculation using the values obtained by Shaffer upon subject M.S. where the urines were collected in periods of two to two and one-half hours show a coefficient of variation of only 5.6%. The data from subject P.A.S. are similar with a coefficient of variation of about 6%. The original data of Folin ('05) taken from table XI, p. 116 of Hunter ('28) show a coefficient of variation on the 24-hour samples of about 4%. Thus, Shaffer's conclusion appears entirely justified. On the other hand, various authors have failed to find such constant rates of excretion (Albanese and Wangerin, '44; Clark et al., '51; Addis et al., '51). The data from table 214, p. 708 and table 327, p. 1034 of Macy's publication ('46) obtained with the children Jimmy and Frank give coefficients of variation of the 24-hour samples of 8.2 and 17.9% respectively of the mean daily excretions. A part of the increased variability observed by us, by Macy and others might be partially explained by meat consumption which may influence creatinine excretion somewhat (Karambelkar et al., '52) or by less than complete urine collections. The latter presumably show up when rather low values are followed by high values or the reverse. As Shaffer ('08–'09) says "It is by no means an easy matter without some practice to empty the bladder

[2] It would be expected that the standard deviation of the 4-, 8- and 24-hour samples could be obtained by dividing the standard deviation of the two-hour samples by $\sqrt{2}$, $\sqrt{4}$, and $\sqrt{12}$, respectively, since 2, 4, and 12 two-hour collections are combined to obtain these samples. Such calculations essentially duplicate the values of figure 1. Since the number of subjects was only 4 for the two-hour samples and 7 for the 4-hour samples, and the total number of observations at each period was not large, the experimentally determined values were used to construct the figure.

completely, especially at frequent intervals . . ." It appears that our estimate of a coefficient of variation of about 10% in 24-hour collections (fig. 1) is in line with recent experience. Approximately 65% of the individual 24-hour collections

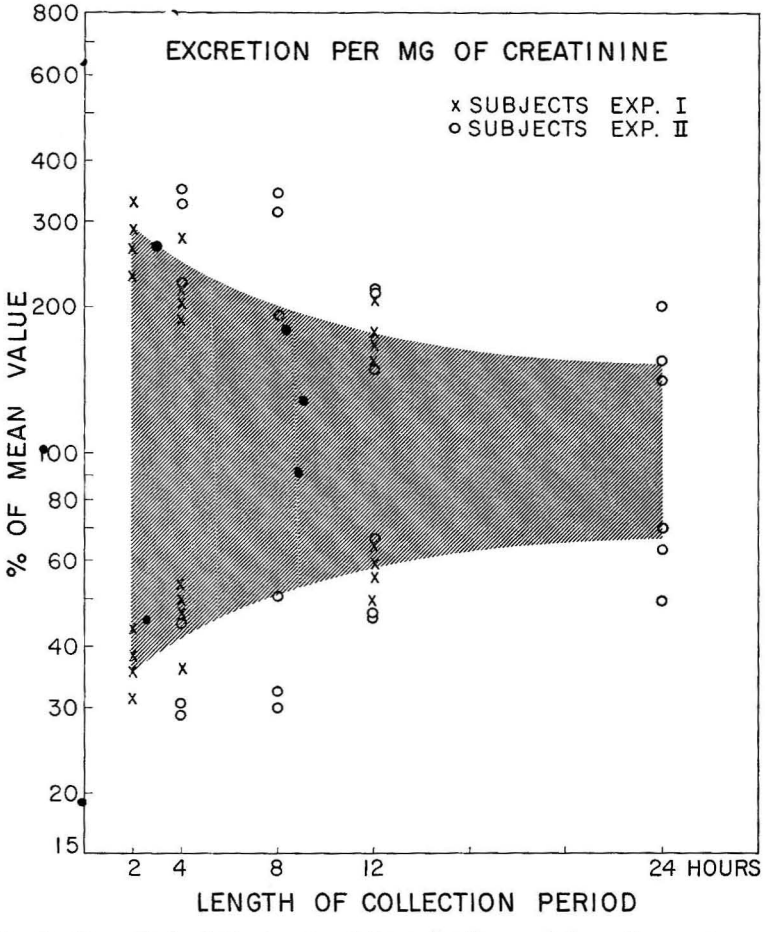

Fig. 2 The effect of the length of the collection period on the constancy of riboflavin excretion per milligram of creatinine.

should fall within this range and 95% will be expected to fall within $\pm$ 20% of the mean daily excretion.

Shorter collections are subject to greater error as indicated in figure 1. Whether this is due to incomplete collections

or not is immaterial for the present purposes since the samples were obtained from unpracticed persons and greater accuracy would not be expected in the field. Thus, a measure of the creatinine excretion does not appear to provide a

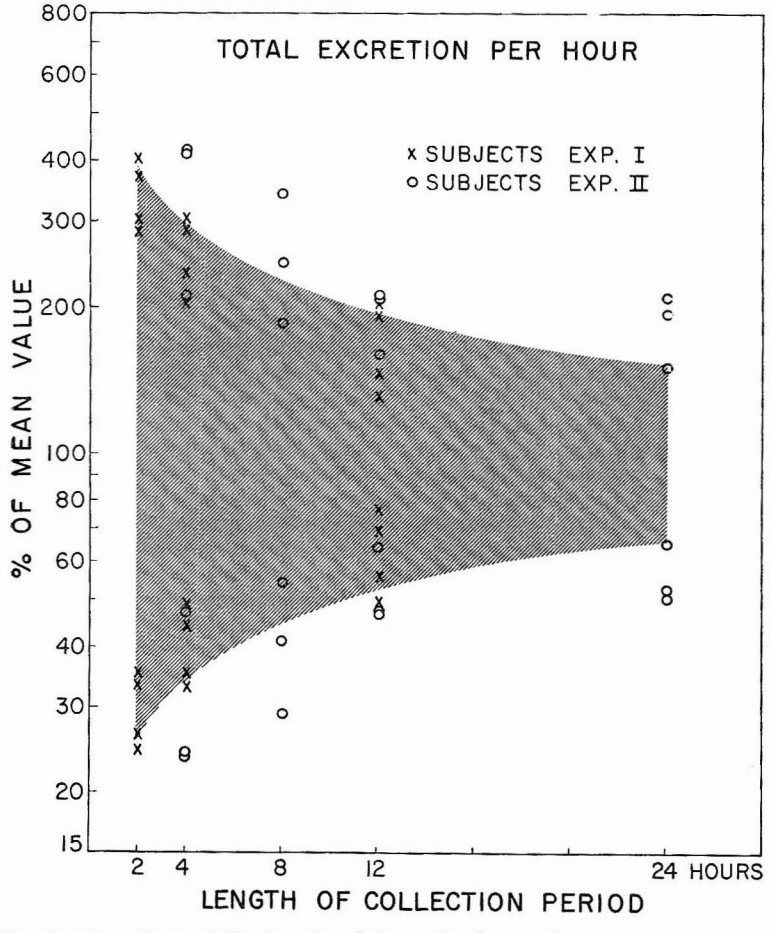

Fig. 3 The effect of the length of the collection period on the constancy of riboflavin excretion expressed on an hourly basis.

particularly accurate estimate of the time during which the urine was collected. Whether it is a suitable parameter for estimating the nutritional status, however, depends upon whether or not other baselines provide more accurate esti-

mates of the daily excretion. If the variation is due largely to incomplete collections, this should not be particularly important since the excretion of the nutrient in question would probably be similarly affected.

Riboflavin excretion. In figures 2, 3, and 4, two standard deviations on either side of the geometric mean are indicated for each subject at two-hour, 4-hour and longer collection periods. The points represented by $\pm$ 2 standard deviations are expressed as a percentage of the mean value. The shaded area may represent the range within which samples of different size may be expected to fall. It is apparent that this is an under-estimate of the variation in the data actually presented. However, it is likely that the consumption of monotonous diets may lower the variation in riboflavin excretion and, since individuals or populations consuming such diets are of primary interest in dietary surveys, we do not wish to prejudice the value of excretion data unduly. In any event, it should be clear that the shaded area has been drawn free hand and represents something approaching an average of the values plotted on either side of the mean. We would not expect that average random samples would be more constant than indicated by this area. Thus, from figure 2 we assume that 95% of single two-hour samples will be expected to fall within 35 and 380% of the true two-hour value of the subject. Similarly, a single 12-hour sample may be expected to fall within 67 and 175% of the mean value for 12-hour samples from a subject.[3]

[3] It will be observed that the standard deviation of the different collection periods estimated from the data in figures 1, 2 and 3 closely approximate those expected from the calculation of $\frac{s}{\sqrt{N}}$, where s is the standard deviation of the two-hour samples and N is the number of such samples combined in the different collection periods. We have preferred, however, in view of the limited data and great variation between subjects, to present the crude data obtained. The fact that we have used the estimate indicated by the limits of the shaded area in figure 2 for the calculations in table 2 also explains why the standard deviation of one 12-hour sample is not the same as the standard error of 6 two-hour samples (see table 2) and so on, when theoretically these should agree.

It may be seen from these figures that single samples provide a very poor estimate of the actual rate of excretion regardless of whether the results are expressed on a time, creatinine, or urine volume basis. When the riboflavin ex-

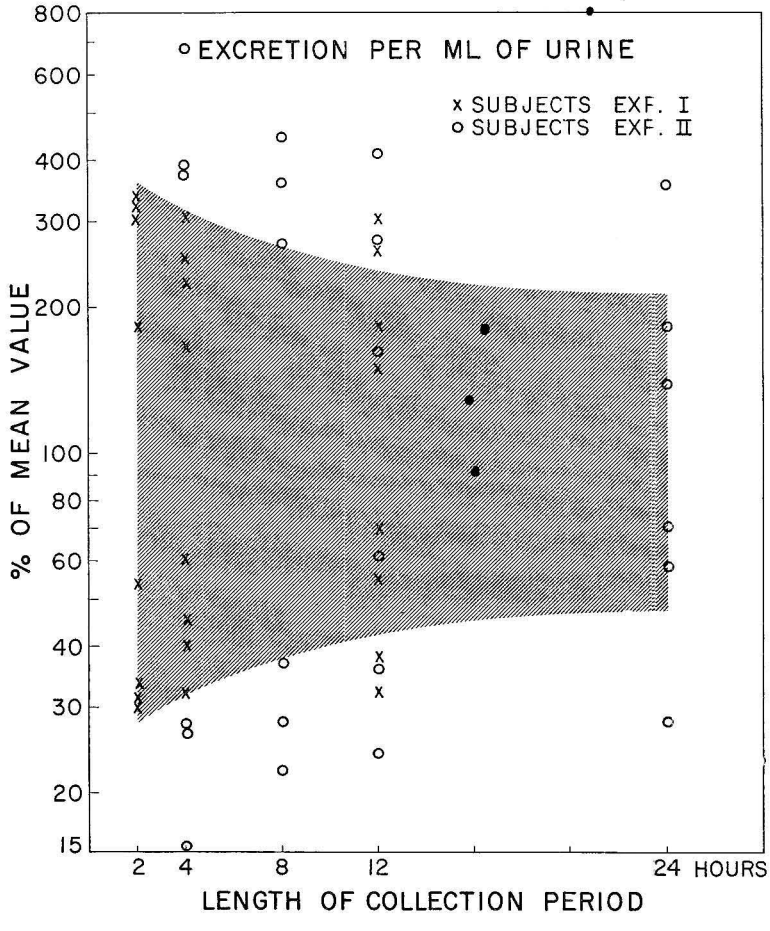

Fig. 4 The effect of the length of the collection period on the constancy of riboflavin excretion per milliliter of urine.

cretion is expressed per hour or per milligram of creatinine, the variation appears to be of the same order of magnitude. The difference between individuals appears to be much larger when the riboflavin is related to urine volume. Whereas some

subjects show a reasonably constant excretion per milliliter of urine, others do not.

The variation in riboflavin excretion is undoubtedly due to a multitude of causes, some of which may be the time and amounts of riboflavin consumed during the day and the rates of absorption. If this is true, samples collected during the night when foods are not eaten should be somewhat less variable than those collected during the day. In table 1 are

TABLE 1

A comparison of the variation in samples collected during the entire 24 hours with those collected from 7 P.M. to 7 A.M.

MEASUREMENT	SUBJECTS				MEAN
	La	Lu	LaF	Ri	
All samples					
Mean excretion, μg/2 hrs.	50.2	85.8	46.4	44.2	56.7
Mean + 2 σ, % of mean	400	284	300	374	340
Mean — 2 σ, % of mean	25	36	34	27	31
Mean excretion, μg/mg creatinine	0.432	0.619	0.458	0.587	0.524
Mean + 2 σ, % of mean	322	260	284	231	274
Mean — 2 σ, % of mean	31	38	37	43	37
Evening samples					
Mean excretion, μg/2 hrs.	38.5	74.7	36.9	41.5	47.9
Mean + 2 σ, % of mean	394	180	272	361	302
Mean — 2 σ, % of mean	25	55	37	28	36
Mean excretion, μg/mg creatinine	0.337	0.521	0.341	0.440	0.410
Mean + 2 σ, % of mean	254	164	226	155	200
Mean — 2 σ, % of mean	39	61	44	64	52

shown the results from the 4 subjects in experiment I in which the variation in two-hour samples collected during the entire day are compared to those collected only from 7 P.M. to 7 A.M. It will be seen that the range between two standard deviations is somewhat less for the evening samples. This is particularly true when the results are related to creatinine excretion. The estimated range for all samples was from 36 to 302% of the mean, whereas in the evening samples the spread was from 52 to 200% of the mean value.

DISCUSSION

In view of the great advantages of using randomly collected urine samples in field surveys, the results we have obtained are discouraging, but also thought provoking. From these data it appears that single urine samples provide a very poor estimate of the actual riboflavin excretion regardless of whether the excretion is related to time of collection, creatinine excretion or urine volume. Some satisfaction may be taken from the observation that riboflavin excretion per unit of creatinine is as constant, or perhaps more constant, than riboflavin excretion per hour. Thus, it will ordinarily be more satisfactory to measure creatinine than attempt to determine the time during which the urine is collected.

Since the accuracy of single samples improves as the collection periods become longer, emphasis should be placed upon getting a sample accumulated over the longest period possible. Also, it would appear that the influence of meals and variations in riboflavin intake can be at least partially eliminated by collecting evening samples. The urine sample upon rising in the morning should be the most satisfactory since it will ordinarily represent a fairly long collection and be less variable than those collected during the daytime. Johnson et al. ('45) have previously concluded that the values from a urine sample before breakfast were more reliable than those taken during the day.

From the data presented by Brewer et al. ('46) and Horwitt et al. ('50) it would appear that the urinary excretion of riboflavin changes rather markedly when the intake rises above about 1.1 mg per day in the adult subject. Below this level of intake the excretion is approximately 9% of the intake and rises to about 30% of the intake at higher levels of intake. Thus, in a dietary survey one is apparently interested in attempting to determine the number of individuals whose excretion falls below 100 μg per day. The creatinine excretion of the average adult (male and female) is approximately 1.2 gm per day. However, for practical purposes we may assume that the critical level is about 100 μg per gram

of creatinine. This level of excretion apparently estimates the degree of "saturation" since clinical symptoms probably do not result until the excretion is considerably lower (Horwitt, '54). Lowry ('52) has suggested that riboflavin excretion above 150 μg/gm of creatinine be considered indicative of an adequate intake.

Since the average adult excretes about 50 mg of creatinine per hour, it may be of more interest to relate the variation in figure 2 to creatinine rather than time. Thus, two-hour collections should represent samples containing about 100 mg of creatinine and 4-hour samples, 200 mg, and so on.

It is of some interest to determine the effect of the number of samples on the accuracy of the estimated excretion.[4] As shown in table 2, if the range between two standard deviations on either side of the mean of two-hour samples is from 36 to 280% of the mean value, the collection of 10 samples should supply a mean value that falls within 72 and 138% of the true mean. The improvement in accuracy as the number of samples is increased is evident, as well as the relative worth of larger samples.

From our own results it is apparent that the variation within a population consuming similar diets will be somewhat more variable, but to an unknown degree, than samples taken from a single individual. Table 2 probably underestimates the number of samples required to define the mean excretion of a population within specified limits. Nevertheless, it is apparent that the mean excretion of a population can be reasonably well defined with small random samples provided the number is sufficiently large. The interpretation, however, as to the number of individuals which may be classified as in the "deficiency" range is difficult. In the Newfoundland Survey (Adamson et al., '45) 39% of the subjects from outposts were found to be excreting less than 200 μg/gm of creatinine and the average excretion was 380 μg/gm of creatinine. On the basis of the variation we have encountered

[4] Standard error of a mean $= \frac{s}{\sqrt{N}}$ (see footnote 3, page 589.

in our two-hour samples we would expect at least 11% of the values from a single individual with a mean excretion of 380 μg/gm to be less than 200 μg/gm. Since presumably the data from Newfoundland were skewed similarly to our data,

TABLE 2

The effect of the number of samples upon the accuracy of the estimated riboflavin excretion

NO. OF SAMPLES	LENGTH OF COLLECTION PERIOD					
	2 hrs.		4 hrs.		12 hrs.	
	-2σ	$+2\sigma$	-2σ	$+2\sigma$	-2σ	$+2\sigma$
	% of mean		*% of mean*		*% of mean*	
1	36	280	41	242	57	175
2	48	208	54	187	67	148
3	55	181	59	169	72	138
4	60	167	65	155	76	132
5	63	158	67	148	78	128
6	66	152	70	142	80	126
10	72 [1]	138	76	132	84	119
15	77	130	80	126	87	116
20	79	126	82	122	88	113
100	90	111	92	109	95	106

[1] Sample calculation:

Mean excretion, 100 μg	log 2.0000
Mean + 2 st. dev.= 280 μg	log 2.4472
diff., 2 st. dev.	0.4472
1 st. dev.	0.2236
Standard error for mean of 10 samples,	$\frac{0.2236}{\sqrt{10}} = 0.0706$

2.0000 + 2 (0.0706) = 2.1412; antilog = 138%
2.0000 — 2 (0.0706) = 1.8588; antilog = 72%

the value of 39% is probably greater than would have been found had logarithms of the data been used. For comparative purposes we have estimated (table 3) the percentage of the samples collected which should be expected to fall below 100 or 200 μg/gm of creatinine at various mean riboflavin outputs when different size samples are used. It should be noted

at the outset that the values presented probably underestimate the variation which would be encountered in the field. The variation indicated in table 3 is presumably due principally to variation in excretion of riboflavin. In a survey sample the mean excretion obtained will be due not only to differences in excretion but also to actual differences in riboflavin intakes. The distribution of samples from two individuals, one with an excretion rate of 300 μg/gm of creatinine, will, of course, be much different than if both individuals were excreting 200 μg/gm although the average output would

TABLE 3

Percentage of samples estimated to fall below 100 and 200 μg of riboflavin per gram of creatinine at different mean excretion rates and with different size samples

MEAN EXCRETION	2-HOUR SAMPLES % LESS THAN		4-HOUR SAMPLES % LESS THAN		12-HOUR SAMPLES % LESS THAN	
	100 μg	200 μg	100 μg	200 μg	100 μg	200 μg
creatinine μg/gm						
100	50	91	50	94	50	99
150	22	81	18	74	7.3	85
200	8.9	50	5.8	50	.6	50
250	3.7	33	1.9	31	...	21
300	1.7	22	.7	19	...	8.7
400	.4	8.9	...	5.8	...	.6
500	...	3.7	...	2.0	...	...

be the same. The values in table 3 allow a somewhat better evaluation of survey data than has been possible in the past. Clearly, the evaluation of survey data must ultimately be made by comparison of the number of individuals in the deficiency zone compared to the number which may be expected to fall in this zone as the result of variation in samples alone.

The data presented indicate the severe limitation of urinary excretion rates of riboflavin in the diagnosis of riboflavin deficiency either in the individual or in a population. More thorough statistical examination of the excretion data obtained in field surveys than has yet appeared are obviously

needed, since average values and the percentage below a given excretion rate do not adequately describe the situation. In future field studies it would be of great advantage to obtain duplicate random samples from each individual. The proportions of the total variance due to differences in individuals (presumably dietary differences or differences in need) and to variation in samples from the same individual could then be determined.

SUMMARY

The variation in the excretion of riboflavin per hour, per gram of urinary creatinine, and per milliliter of urine has been studied in several subjects in which urine samples were collected every two or 4 hours.

Regardless of the method of expressing the riboflavin excretion, single urine samples provide a poor estimate of the average excretion rate.

The variation in excretion per hour and per gram of creatinine is of the same order of magnitude while the excretion per milliliter of urine is more variable in most subjects.

The improvement in the estimates of the average excretion obtained by increasing the collection period or by multiple sampling has been calculated. Since the accuracy of the estimate improves as the length of the collection period is increased, field studies should attempt to collect urine over the longest convenient period. The variation from sample to sample is somewhat less in samples collected during the night than during the daytime. Thus, the most valuable sample should be that obtained upon rising in the morning.

The limitations of urinary excretion data in the assessment of the nutritional status with regard to riboflavin for individuals or population groups have been discussed.

ACKNOWLEDGMENTS

We wish to acknowledge the cooperation of the staff of the Wrentham State School which made the collections of urine

possible and the technical assistance of Mrs. Janet Owens and Miss Doris Hedrick.

LITERATURE CITED

ADAMSON, J. D., N. JOLLIFFE, H. D. KRUSE, O. H. LOWRY, P. E. MOORE, B. S. PLATT, W. H. SEBRELL, J. W. TICE, F. F. TISDALL, R. M. WILDER AND P. C. ZAMECNIK 1945 Medical survey in nutrition in Newfoundland. Can. Med. Assn. J., *52:* 227.

ADDIS, T., E. BARRETT, L. J. POO, H. J. UREEN AND R. W. LIPPMAN 1951 The relation between protein consumption and diurnal variations of the endogenous creatinine clearance in normal individuals. J. Clin. Invest., *30:* 206.

ALBANESE, A. A., AND D. M. WANGERIN 1944 The creatine and creatinine excretion of normal adult males. Science, *100:* 58.

BREWER, W., T. PORTER, R. INGALLS AND M. A. OHLSON 1946 The urinary excretion of riboflavin by college women. J. Nutrition, *32:* 583.

CLARK, L. C., AND H. L. THOMPSON 1949 Determination of creatine and creatinine in urine. Anal. Chem., *21:* 1218.

CLARK, L. C., H. L. THOMPSON, E. I. BECK AND W. JACOBSON 1951 Excretion of creatine and creatinine by children. Am. J. Dis. Child., *81:* 774.

FEDER V. H., G. T. LEWIS AND H. S. ALDEN 1944 Studies on the urinary excretion of riboflavin. J. Nutrition, *27:* 347.

FOLIN, O. 1905 Laws governing the chemical composition of urine. Am. J. Physiol., *13:* 66.

HORWITT, M. K. 1954 Page 398 in "The Vitamins," edited by W. H. Sebrell and R. S. Harris. Academic Press, Inc., New York.

HORWITT, M. K., C. C. HARVEY, O. W. HILLS AND E. LIEBERT 1950 Correlation of urinary excretion of riboflavin with dietary intake and symptoms of ariboflavinosis. J. Nutrition, *41:* 247.

HUNTER, A. 1928 "Creatine and Creatinine." Longsman, Green and Co., London.

JOHNSON, R. E., C. HENDERSON, P. F. ROBINSON AND F. C. CONSOLAZIO 1945 Comparative merits of fasting specimens, random specimens and oral loading tests in field nutritional surveys. J. Nutrition, *30:* 89.

KARAMBELKAR, P. V., V. N. PATWARDHAN AND A. SREENIVASAN 1952 Studies in protein metabolism: Creatinine excretion in urine in relation to sources of dietary proteins. Ind. J. Med. Res., *40:* 89.

LOWRY, O. H. 1952 Biochemical evidence of nutritional status. Physiol. Rev., *32:* 431.

MACY, I. G. 1946 "Nutrition and Chemical Growth in Childhood. Vol. II." Charles C Thomas, Springfield, Ill.

SHAEFER, P. A. 1908–1909 The excretion of kreatinin and kreatin in health and disease. Am. J. Physiol., *23:* 1.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed., *11:* 346.

THE INFLUENCE OF METHIONINE SUPPLEMENTATION OF 12, 14 AND 16 PERCENT PROTEIN CORN-SOYBEAN OIL MEAL DIETS UPON NITROGEN BALANCE OF GROWING SWINE [1]

R. J. MEADE

Department of Animal Husbandry, Nebraska Agricultural Experiment Station, Lincoln

(Received for publication October 28, 1955)

Ferrin ('46) and Robison ('51) reported that methionine supplementation of all-plant type of diets containing 17.5 and 15.0% of crude protein, respectively, did not improve the performance of growing pigs. Almquist et al. ('42) had reported earlier that high soybean oil meal diets were deficient in methionine for the chick. Dyer et al. ('49) used corn-expeller soybean oil meal diets and reported a significant improvement in the rate of gain of growing pigs due to methionine supplementation. Bell et al. ('50) fed growing pigs a 10% crude protein semi-purified type of diet in which soybean oil meal was the source of protein and reported that methionine supplementation improved the diet. They suggested that the methionine requirement of the pig was between 0.07 and 0.27% of the diet.

Shelton et al. ('51a) reported that growing pigs required 0.3% of methionine in the diet when 0.3% of cystine was also present. These workers had no test levels of methionine between 0.1 and 0.3% of the diet. Curtin et al. ('52) fed grow-

[1] Published with the approval of the Director as Paper 715, Journal Series, Nebraska Agricultural Experiment Station. A part of the thesis submitted by the author to the graduate college, University of Illinois, Urbana, in partial fulfillment of the requirements for the Doctor of Philosophy Degree.

ing pigs a 22% corn-solvent soybean oil meal diet containing a 0.31% of methionine and reported that supplemental methionine did not improve the diet. They suggested that the method of processing soybean oil meal might have contributed to apparent discrepancies in reported responses of pigs to methionine supplementation of corn-soybean oil meal diets.

The calculated amino acid composition of 12 to 14% crude protein corn-soybean oil meal diets, based on published values of Baumgarten et al. ('46) and Williams ('55) indicates that such diets may be deficient in tryptophan, methionine and lysine if the presently reported requirements of the pig are accepted. However, Becker et al. ('54) recently reported that diets containing as low as 0.13, 0.23 and 0.63%, respectively, of tryptophan, methionine and lysine were adequate to support satisfactory rate of gain of 40- to 100-pound pigs.

This investigation was conducted to determine the influence upon nitrogen metabolism of growing pigs of supplementing typical corn-soybean oil meal diets containing approximately 12, 14, and 16% of crude protein, and adequate in non-protein dietary factors, with DL-methionine. The nitrogen balance method was employed as it was considered to provide an excellent method by which to measure utilization of dietary protein as affected by methionine supplementation and protein level.

EXPERIMENTAL PROCEDURES

Growing barrow pigs of Hampshire or Hampshire × Yorkshire breeding and which had been weaned at approximately 8 weeks of age were used in this investigation. The live weight range of the pigs for the duration of the experiment was from 23.4 to 66.5 kg, weights which corresponded to metabolic size values of 10.11 to 21.78. Prior to each preliminary feeding period pigs were randomly assigned to each of the 24 diets used. The test diet was then fed throughout the entire preliminary period and the collection period. All pig weights were reduced to a standard basis of $W_{kg}^{0.734}$ in an attempt to eliminate insofar as possible the influence of difference in

body weight, W, between different pigs and different periods (Brody and Procter, '32).

All animals were fed twice daily and the feed intake was maintained at a constant level closely approximating 4% of body weight throughout the final 4 days of the preliminary period and the entire collection period. Nitrogen balance trials were conducted using 8– to 10-day preliminary feeding periods followed by 6-day collection periods. Total urine and fecal collections were made for the 6-day collection periods using cylindrical swine metabolism cages patterned after those designed by Bell ('48).

Typical corn-soybean oil meal diets, the composition of which is shown in table 1, were used as the basal diets in this study. Sufficient L-lysine was added to provide a final level of lysine equivalent to 5% of the crude protein in those diets containing 11.8% and 13.8% of protein. The 15.8% protein diet contained at least the desired level of lysine as a result of the contribution of lysine from the added soybean oil meal. Brinegar et al. ('50a) indicated that the lysine requirement of the pig was approximately 5.5% of the dietary protein. The final lysine levels are all well below the reported requirement of Shelton et al. ('51b) and the suggested requirement of Williams et al. ('54). Each level of methionine feeding was carried out in the presence and absence of 0.04% of additional DL-tryptophan as the tryptophan content of the 11.8 and 13.8% protein diets was somewhat below a level of 0.132% of tryptophan which appeared to be adequate for growing pigs in other investigations conducted at this station (Meade, '55).

Data from 4 nitrogen balance trials for each of the 24 experimental diets have been used in summarizing this study. When responses to protein level were considered, the data for all pigs on a given protein level were used, ignoring amino acid supplementation. Results are presented showing the average daily nitrogen metabolism of the pigs used on the various diets and the daily nitrogen metabolism per unit of metabolic size ($W_{kg}^{0.734}$). All conclusions are based on daily

TABLE 1

Composition of basal diets

INGREDIENTS	AMOUNTS		
	%	%	%
Ground yellow corn	86.25	81.00	75.65
Solvent soybean oil meal	10.70	16.00	21.35
Dicalcium phosphate	1.50	1.50	1.50
Ground limestone	1.00	1.00	1.00
Iodized salt	0.50	0.50	0.50
Vitamin-antibiotic-trace element mixture [1]	+	+	+
Percentage of crude protein	11.8	13.8	15.8
Percentages of amino acids [2]			
Tryptophan	0.10	0.126	0.153
Methionine	0.22	0.25	0.27
Cystine [3]	0.17	0.22	0.26
Lysine [4]	0.58	0.69	0.80
Isoleucine	0.53	0.64	0.74
Histidine	0.30	0.35	0.40
Leucine	1.18	1.32	1.46
Phenylalanine	0.58	0.68	0.79
Threonine	0.45	0.54	0.63
Valine	0.60	0.70	0.81

[1] Provided 2.2 mg of riboflavin, 8.8 mg of calcium pantothenate, 22 mg of niacin, 220 mg of choline chloride, 11 μg of vitamin B_{12}, 4400 I.U. of vitamin A, 220 I.U. of vitamin D_2, 22 mg of procaine penicillin, 33 mg of iron, 17.6 mg of manganese, 3.3 mg of zinc, 3.3 mg of copper and 1.1 mg of cobalt per kilogram of diet.

[2] Amino acid assays of basic feed ingredients generously supplied by Dr. Ruth M. Leverton and associates, Human Nutrition Laboratory, University of Nebraska.

[3] Calculated cystine content. (National Research Council, '53.)

[4] The calculated total lysine, including added L-lysine.

nitrogen retention per unit of metabolic size. Data were treated statistically using covariance analysis as set forth by Snedecor ('46).

RESULTS AND DISCUSSION

Average nitrogen metabolism data for the 11.8, 13.8 and 15.8% crude protein diets are shown in table 2. As is evident from these data, it was only with the addition of 0.1% of DL-methionine to the 11.8% protein diet in the presence of 0.04% of supplemental DL-tryptophan that there was a marked

TABLE 2

Average nitrogen metabolism data of pigs fed 11.8, 13.8 and 15.8% crude protein diets supplemented with DL-*methionine* [1]

PER CENT OF TRYPTOPHAN IN DIET	FINAL PER CENT METHIONINE IN DIET	MEAN BODY WEIGHT	AVERAGE DAILY INCREASE	AIR DRY FEED CONSUMED DAILY	NITROGEN METABOLISM PER DAY				NITROGEN METABOLISM PER DAY PER W $^{0.734}$ kg		
					Intake	Feces	Urine	Balance	Absorbed	Urine	Balance
		kg	*kg*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm* [2]	*gm*	*gm* [3]
11.83% protein diet											
0.10	0.22	44.04	0.49	1849	35.26	8.13	13.75	13.38	1.69	0.85	0.84
	0.245	46.20	0.38	1771	33.85	7.64	12.82	13.39	1.58	0.76	0.84
	0.27	40.94	0.57	1705	32.65	8.29	11.87	12.49	1.60	0.77	0.82
	0.32	39.78	0.49	1666	32.07	7.91	10.88	13.28	1.62	0.72	0.89
0.12	0.22	39.41	0.54	1666	31.87	7.73	10.96	13.18	1.63	0.73	0.88
	0.245	42.45	0.44	1788	34.27	8.62	12.26	13.39	1.64	0.76	0.88
	0.27	41.97	0.58	1777	34.14	9.11	12.26	12.77	1.62	0.78	0.83
	0.32	43.78	0.54	1862	35.95	10.17	16.39	9.39	1.60	1.00	0.61
13.81% protein diet											
0.126	0.25	41.71	0.63	1720	38.11	8.34	16.59	13.18	1.89	1.01	0.88
	0.275	45.72	0.50	1903	42.25	9.55	17.45	15.25	1.96	1.01	0.98
	0.30	35.70	0.44	1524	33.89	7.82	13.67	12.40	1.88	0.95	0.89
	0.35	36.40	0.43	1496	33.40	7.34	13.48	12.58	1.86	0.95	0.88
0.146	0.25	41.37	0.45	1625	36.10	9.67	13.56	12.87	1.71	0.86	0.85
	0.275	49.18	0.44	1982	44.11	9.49	19.58	15.04	1.98	1.10	0.93
	0.30	42.93	0.52	1784	39.79	9.75	15.35	14.69	1.90	0.94	0.97
	0.35	38.14	0.32	1563	34.99	8.17	13.97	12.85	1.84	0.94	0.88
15.84% protein diet											
0.153	0.27	44.58	0.46	1804	45.73	9.33	20.70	15.70	2.23	1.25	0.98
	0.295	43.08	0.60	1732	43.95	9.40	16.58	17.97	2.18	1.04	1.14
	0.32	45.77	0.56	1892	48.11	10.07	22.73	15.31	2.28	1.34	0.94
	0.37	42.50	0.54	1701	43.38	8.84	16.53	18.01	2.20	1.05	1.15
0.173	0.27	47.38	0.66	1933	49.10	9.99	24.94	14.17	2.29	1.43	0.86
	0.295	47.13	0.73	1930	49.11	10.15	24.05	14.91	2.28	1.38	0.90
	0.32	40.94	0.48	1670	42.56	8.96	17.65	15.95	2.20	1.16	1.04
	0.37	42.97	0.65	1750	44.72	8.90	19.01	16.81	2.25	1.18	1.07

[1] Mean values for 4 pigs per treatment.
[2] Apparently absorbed nitrogen.
[3] Adjusted mean values obtained by application of within protein level error regression coefficient.

influence upon nitrogen retention. Covariance analysis of the data within protein level indicated that there was a highly significant ($P < 0.01$) depression in nitrogen retention of pigs on this particular treatment.

Almquist ('52) reported that the relative proportion of amino acids was a more important attribute than the level of protein in the diet of chicks. If this hypothesis is applicable to swine diets, an imbalance of amino acids may have been created when the 11.8% protein diet was supplemented to provide final levels of at least 0.12 and 0.32% of tryptophan and methionine, respectively. Lysine may have become limiting in this instance as it was present at only 0.58% of the diet. Isoleucine, present at 0.53% of the diet, may have been limiting if the 0.70% of the diet requirement reported by Brinegar et al. ('50b) is accepted as the absolute requirement. However, this explanation is not tenable if the isoleucine requirement is considered to be a function of dietary protein at levels below the optimum. Isoleucine was present at 4.5% of the dietary protein as contrasted to the suggested requirement of 3.2% of the dietary protein by the above workers.

There was considerable variability in the grams of nitrogen retained per unit of metabolic size by the pigs fed the 15.8% protein diet with the various amino acid supplementations, and less variation with the pigs fed the 13.8% protein diet. The differences in grams of nitrogen retained by the pigs on either level of protein and because of amino acid supplementation were not statistically significant. As might have been expected, highly significant ($P < 0.01$) differences resulted in grams of nitrogen retained due to diet, 24 experimental diets being considered. Likewise, highly significant differences in nitrogen retention resulted from the level of dietary protein. The mean nitrogen balance values obtained for pigs fed the 11.8 and 13.8% protein diets were significantly improved through application of within protein level error regression coefficients. The adjusted mean nitrogen balance values are shown in table 2.

Inspection of the mean nitrogen metabolism data shown in table 2, and of the nitrogen metabolism data for the individual pigs, indicated that pigs fed the diets containing the two higher levels of protein apparently absorbed more nitrogen, but they also excreted more nitrogen in the urine. This was particularly true of the pigs fed the 15.8% protein diet which tends to indicate that this diet contained excess nitrogen which was not efficiently utilized. The 13.8% protein diet apparently contained enough dietary nitrogen for the lighter weight pigs and a surplus of dietary nitrogen for the heavier pigs.

Because of the overlapping of nitrogen retention values obtained for pigs fed the 13.8 and 15.8% protein diets the 13.8% protein diet fed unsupplemented, or supplemented with 0.025% of DL-methionine, was assumed to have provided satisfactory amino acid nutrition of growing swine. Pigs fed the 15.8% protein diet would have been expected to retain more total nitrogen because of their greater protein intake. DL-Methionine supplementation of the diets at either of these levels of protein intake, and in the presence or absence of supplemental DL-tryptophan, did not significantly influence the nitrogen balance of the pigs. The amino acid content of the 13.8% protein diet would have been 0.126, 0.25 to 0.275, and 0.69% of tryptophan, methionine and lysine, respectively. When the reported amino acid requirements of swine are expressed as a percentage of diet, these amino acid values are in good agreement with those suggested to be adequate as a result of a previous study by the author ('55) and by other investigators.

SUMMARY AND CONCLUSIONS

The nitrogen balances of growing pigs fed an 11.8% crude protein corn-soybean oil meal diet containing 0.10 to 0.12 and 0.58% of tryptophan and lysine, respectively, were not improved by supplementation of the diet with 0, 0.025, 0.05 and 0.10% of DL-methionine to provide final methionine levels of 0.22, 0.245, 0.27 and 0.32% of the diet. It was suggested that lysine may have been inadequate to the extent that it limited

the response of growing pigs to methionine supplementation in the presence of supplemental tryptophan. This lower protein diet also contained less than 0.7% of isoleucine which could have limited nitrogen retention if the requirement for isoleucine is not a function of protein content of the diet.

Although nitrogen retention values for pigs fed a 15.8% crude protein diet supplemented with several levels of DL-methionine and DL-tryptophan were greater, on the average, than those values obtained for pigs fed a 13.8% crude protein diet supplemented in an identical manner, there was some overlapping of mean nitrogen retention values for the various treatments on the two levels of dietary protein. The addition of 0, 0.025, 0.05 or 0.1% of supplemental DL-methionine to a 13.8% protein diet with or without 0.04% of supplemental DL-tryptophan did not result in significant differences in nitrogen balances of growing swine.

The levels of 0.126, 0.25 to 0.27 and 0.69% tryptophan, methionine and lysine, contained in the 13.8% protein diet, when 0 or 0.025% of supplemental DL-methionine was added, appeared to be adequate to promote satisfactory nitrogen retention by growing pigs fed the diet at approximately 4.00% of their body weights.

The 15.8% protein diet used in these investigations appeared to supply dietary nitrogen in excess of the needs of growing pigs, particularly heavier weight pigs, as evidenced by a marked tendency toward increased loss of nitrogen by urinary excretion.

ACKNOWLEDGMENTS

This study was supported in part by grants-in-aid, and supplies of experimental materials, from Dow Chemical Company, Midland, Michigan; E. I. duPont de Nemours and Company, Wilmington, Delaware; Merck and Company, Inc., Rahway, New Jersey; and Swift and Company, Chicago, Illinois. Mr. Arthur Speece, Department of Biochemistry and Nutrition, assisted in making nitrogen determinations. Messrs. W. S. Teter, D. M. Nelson, W. Lingo and Fred Krieger assisted in

caring for experimental animals and preparing excreta for chemical analysis.

LITERATURE CITED

ALMQUIST, H. J., E. MECCHI, F. H. KRATZER AND C. R. GRAU 1942 Soybean oil meal as a source of amino acids for the chick. J. Nutrition, *24:* 385.

ALMQUIST, H. J. 1952 Amino acid requirements of chickens and turkeys — a review. Poultry Sci., *31:* 966.

BAUMGARTEN, W., A. N. MATHER AND L. STONE 1946 Essential amino acid composition of feed materials. Cereal Chem., *23:* 135.

BECKER, D. E., J. W. LASSITER, S. W. TERRILL AND H. W. NORTON 1954 Levels of protein in practical rations for the pig. J. Animal Sci., *13:* 611.

BELL, J. M. 1948 An adjustable cylindrical cage for use in metabolism studies with young swine. J. Nutrition, *35:* 365.

BELL, J. M., H. H. WILLIAMS, J. K. LOOSLI AND L. A. MAYNARD 1950 The effect of methionine supplementation of a soybean oil meal purified ration for young pigs. Ibid., *40:* 551.

BRINEGAR, M. J., H. H. WILLIAMS, F. H. FERRIS, J. K. LOOSLI AND L. A. MAYNARD 1950a The lysine requirement for the growth of swine. Ibid., *42:* 129.

BRINEGAR, M. J., J. K. LOOSLI, L. A. MAYNARD AND H. H. WILLIAMS 1950b The isoleucine requirement for the growth of swine. Ibid., *42:* 619.

BRODY, S., AND R. C. PROCTER 1932 Growth and development with special reference to domestic animals. XXIII. Relation between basal metabolism and mature body weight in different species of mammals and birds. Missouri Agr. Exp. Sta. Res. Bull. 166.

CURTIN, L. V., J. K. LOOSLI, J. P. WILLMAN AND H. H. WILLIAMS 1952 Methionine as a supplement to soybean oil meal for weanling pigs. J. Animal Sci., *11:* 459.

DYER, I. A., J. L. KRIDER AND W. E. CARROLL 1949 Known and unidentified factors supplement a corn-soybean meal ration for weanling pigs. Ibid., *8:* 541.

FERRIN, E. F. 1946 Addition of synthetic nutrients to protein supplemental feeds in swine rations. Ibid., *5:* 42.

MEADE, R. J. 1955 The influence of amino acids and other dietary factors upon nitrogen utilization by growing swine. Ph.D. thesis. University of Illinois.

NATIONAL RESEARCH COUNCIL 1953 Nutrient requirements for domestic animals. II. Nutrient requirements for swine. Publication 295.

ROBISON, W. L. 1951 Soybean oil meal for pigs. Ohio Agr. Exp. Sta. Res. Bull. 190.

SHELTON, D. C., W. M. BEESON AND E. T. MERTZ 1951a The effect of methionine and cystine on the growth of weanling pigs. J. Animal Sci., *10:* 57.

———— 1951b Quantitative L-lysine requirement of the weanling pig. Arch. Biochem., *30:* 1.

SNEDECOR, G. W. 1946 Statistical methods applied to experiments in agriculture and biology, 4th edition. Iowa State College Press. Ames, Iowa.

WILLIAMS, H. H. 1955 "Essential" amino acid content of animal feeds. Cornell University Memoir 337.

WILLIAMS, H. H., L. V. CURTIN, J. ABRAHAM, J. K. LOOSLI AND L. A. MAYNARD 1954 Estimation of growth requirements for amino acids by assay of the carcass. J. Biol. Chem., *208:* 277.

THE INFLUENCE OF L-LYSINE SUPPLEMENTATION OF 12, 14 AND 16 PERCENT PROTEIN CORN-SOYBEAN OIL MEAL DIETS UPON NITROGEN BALANCE OF GROWING SWINE [1]

R. J. MEADE AND W. S. TETER

Department of Animal Husbandry, Nebraska Agricultural Experiment Station, Lincoln

(Received for publication October 28, 1955)

The lysine requirement of growing swine has been reported to be 1.0% of the diet by Shelton et al. ('51), 1.2% of the diet or 5.5% of the dietary protein by Brinegar et al. ('50a) and 1.1% of the diet based on carcass assays by Williams et al. ('54). It is well recognized that many of the 14 to 16% crude protein diets used in routine swine production contain considerably less lysine than these reported requirements.

Becker et al. ('54a) reported acceptable body weight gain of 5- to 9-week-old pigs fed a 12% protein diet containing 0.72% of lysine. In a later report, Becker et al. ('54b) reported that diets containing only 0.63% of lysine were adequate to produce acceptable weight gain of 40- to 100-pound pigs. Meade ('56) has demonstrated satisfactory nitrogen retention by growing swine fed 15.9% crude protein diets containing 0.69% of lysine, and also when a 13.8% crude protein diet containing 0.69% of lysine was employed. Catron et al. ('53) reported that corn-soybean oil meal rations con-

[1] Published with the approval of the Director as Paper 732, Journal Series, Nebraska Agricultural Experiment Station. A part of the thesis submitted by the senior author to the graduate college, University of Illinois, Urbana, in partial fulfillment of the requirements for the Doctor of Philosophy degree.

taining 14 or 16% of crude protein were not improved for pigs by lysine supplementation. If the values for the lysine content of corn and soybean oil meal reported by Williams ('55) and the National Research Council ('53) are used for purposes of calculation, the diets used by Catron and co-workers would have contained an estimated 0.66 and 0.81% of lysine. Pfander and Tribble ('55) were unable to demonstrate a significant increase in growth rate of growing swine fed 14, 16 and 18% protein corn-soybean oil meal diets due to the addition of 0.1% of L-lysine. They reported that lysine equivalent to 5% of the dietary protein appeared to be adequate for growing swine.

Miner et al. ('55) reported a depression in rate of gain of growing pigs fed a corn-cottonseed meal diet when amounts of DL-lysine in excess of 0.1% of the diet were added. Similar results were observed at this station (Meade, '54) when growing pigs were fed a 14% protein corn-soybean oil meal diet supplemented with 0.15% of L-lysine. The lack of consistency in suggested requirements may indicate that the initially reported requirements were too high. The reported depression in performance due to higher levels of lysine may indicate that balance of amino acids is important. Almquist ('52) has reported that the relative proportion of amino acids is a more important attribute than level of protein in the diet of chicks.

These studies were conducted to determine the influence of lysine supplementation of typical swine diets fed at three levels of protein upon nitrogen utilization by growing swine. The nitrogen balance method has not been widely used in studying amino acid nutrition of growing swine, and it was felt that differences in nitrogen retention of growing swine which might result from lysine supplementation of the diets would help to clarify the lysine requirement. If the addition of an excess of lysine interfered with nitrogen metabolism by disrupting the relative proportion of amino acids in the various diets it should have been reflected in the nitrogen retention values.

EXPERIMENTAL PROCEDURES

Typical corn-soybean oil meal diets containing approximately 12, 14 and 16% of protein were employed in this investigation. The composition of the diets is shown in table 1. The corn used in this experiment contained 8.75% of crude

TABLE 1

Composition of basal diets

INGREDIENT	AMOUNTS		
	%	%	%
Ground yellow corn	87.75	82.35	77.75
Solvent soybean oil meal	9.10	14.50	19.10
Dicalcium phosphate	1.50	1.50	1.50
Ground limestone	1.00	1.00	1.00
Iodized salt	0.50	0.50	0.50
Vitamin-antibiotic-trace element mixture [1]	0.15	0.15	0.15
Percentage of crude protein	12.1	14.2	16.0
Percentages of amino acids: [2]			
Tryptophan	0.11	0.137	0.159
Methionine [3]	0.42	0.48	0.56
Lysine	0.48	0.62	0.74
Histidine	0.29	0.34	0.38
Isoleucine	0.52	0.63	0.72
Leucine	1.20	1.33	1.44
Phenylalanine	0.55	0.66	0.75
Threonine	0.44	0.52	0.60
Valine	0.62	0.72	0.81

[1] Supplied 2.2 mg of riboflavin, 8.8 mg of calcium pantothenate, 22 mg of niacin, 11 μg of vitamin B_{12}, 220 mg of choline chloride, 4400 I.U. of vitamin A, 220 I.U. of vitamin D_2, 22 mg of terramycin, 33 mg of iron, 17.6 mg of manganese, 3.3 mg of zinc, 3.3 mg of copper and 1.1 mg of cobalt per kilogram of diet.

[2] Amino acid values for soybean oil meal furnished by Dr. Ruth M. Leverton and associates, Human Nutrition Laboratory, University of Nebraska.

[3] The calculated total methionine, including added methionine.

protein and the soybean oil meal contained 45.7% of protein. The amino acid content of the various diets is calculated on the basis of the determined amino acid content of the soybean oil meal used and amino acid values for the corn reported by Block and Mitchell ('46).

L-Lysine monohydrochloride was added to the diets to provide final lysine levels equivalent to approximately 5 and 6% of the dietary protein. Levels of lysine equivalent to exactly 4% of the diet could not be achieved in the cases of the 14.2 and 16.0% crude protein diets as lysine in excess of 4% of the protein was contributed by the natural feed ingredients. The lower levels of lysine represent 3.96, 4.37 and 4.64% of the protein in the 12.1, 14.2 and 16.0% protein diets, respectively. Each level of lysine feeding was carried out with diets containing zero and 0.04% of additional DL-tryptophan because the 0.11% tryptophan content of the 12.1% crude protein diet was considered to be slightly inadequate, at least for light-weight pigs. DL-Methionine was added to all diets to supply final levels of methionine equivalent to 3.5% of the dietary protein.

The experimental animals used in this investigation were Hampshire $\times$ Duroc and purebred Duroc barrows which weighed approximately 20 kg at the start of the experiment. Some of the heavier pigs attained weights as great as 58 kg prior to the termination of the experiment. Barrows were randomly assigned to the experimental diets from which the collections were to be made prior to the start of the 8 to 10 day preliminary feeding period and no consideration was given to the pig's previous treatment

All animals were fed twice daily at a constant level of feed intake closely approximating 4% of body weight throughout the final 4 days of the preliminary feeding period and the entire collection period. Nitrogen balance trials were conducted using 6-day collection periods. Three separate total fecal and urine samples were collected for pigs fed each experimental diet. Cylindrical metabolism cages patterned after those designed by Bell ('48) were used for making collections of excreta.

All data have been summarized to present animal weights as mean weight, W, expressed in kilograms for the time that the individual pigs were on urine and fecal collections. The mean weights of the animals have been raised to the power

of 0.734 to eliminate insofar as possible the influence of differences in body weight, W, between different pigs and different periods (Brody and Procter, '32). The average daily nitrogen metabolism data per unit of metabolic size ($W_{kg}^{0.734}$) for the pigs used on the various diets are presented in table 2. Covariance analysis as set forth by Snedecor ('46) has been used in the statistical analysis of the data, and conclusions are based on daily nitrogen retention per unit of metabolic size

RESULTS AND DISCUSSION

It is apparent from the average nitrogen metabolism data shown in table 2 that the 12.1% crude protein diet failed to yield satisfactory nitrogen retention values of growing pigs even when the diet contained lysine equivalent to 5.94% of the crude protein, and contained 0.13 and 0.42% of tryptophan and methionine, respectively. The differences in nitrogen retention of pigs due to amino acid supplementation of the diets containing 12.1% of crude protein approach significance ($P = 0.05$). The adjusted mean nitrogen retention values, shown in table 2, were obtained by application of the within protein level regression coefficient (0.014), and these values indicate a definite trend toward decreased nitrogen retention values of pigs when the diets contained 0.72% of lysine (5.94% of the dietary protein).

Disrupting the relative proportion of amino acids by the addition of the highest level of lysine and supplemental DL-tryptophan, caused an increased excretion of urinary nitrogen which resulted in the lower nitrogen retention values. Sheffner and Bergeim ('53) have shown that the rate of L-amino acid oxidation in rat kidney increases with an excess of dietary amino acids. It is recognized that the 12.1% protein diet contained only 0.52% of isoleucine which was below the 0.70% of diet requirement of growing pigs reported by Brinegar et al. ('50b), but which represented 4.3% of the dietary protein and was in excess of their suggested requirement of 3.2% of dietary protein. The lower nitrogen retention

TABLE 2

Average nitrogen metabolism data of pigs fed a 12.1, 14.2, and 16.0% crude protein diet supplemented with L-*lysine*

CALCULATED PER CENT OF AVAILABLE TRYPTOPHAN IN DIET	FINAL LYSINE CONTENT AS PER CENT OF PROTEIN	MEAN BODY WT.	AV. DAILY INCREASE	AIR DRY FEED CONSUMED DAILY	NITROGEN METABOLISM PER DAY [1]				NITROGEN METABOLISM PER DAY PER $W^{0.734}_{kg}$		
					Intake	Fæces	Urine	Balance	Absorbed	Urine	Balance
		kg	*kg*	*kg*	*gm*	*gm*	*gm*	*gm*	*gm* [2]	*gm*	*gm*
12.1% crude protein											
0.11	3.96	43.77	0.47	1724	33.42	8.14	11.16	14.12	1.57	0.69	0.85 [3]
	4.95	33.75	0.38	1370	26.78	8.06	7.65	11.07	1.40	0.57	0.85 [3]
	5.94	34.16	0.52	1383	27.25	7.14	9.94	10.17	1.50	0.74	0.77 [3]
0.13	3.96	37.23	0.50	1530	29.74	7.72	10.98	11.04	1.55	0.78	0.77 [3]
	4.95	29.25	0.44	1185	23.24	6.20	7.23	9.81	1.43	0.61	0.85 [3]
	5.94	39.65	0.48	1585	31.34	6.97	12.64	11.73	1.58	0.85	0.71 [3]
14.2% crude protein											
0.137	4.37	40.56	0.53	1611	36.56	8.66	13.63	14.27	1.84	0.88	0.96
	4.94	36.58	0.57	1419	32.33	8.01	11.92	12.40	1.73	0.82	0.91
	5.92	40.59	0.59	1616	37.42	8.28	10.58	18.56	1.94	0.71	1.23
0.157	4.37	38.48	0.49	1537	34.94	8.03	12.21	14.70	1.86	0.85	1.01
	4.94	35.94	0.45	1432	32.70	6.90	11.77	14.03	1.86	0.85	1.01
	5.92	36.32	0.47	1444	33.57	7.45	10.07	16.05	1.87	0.72	1.15
16.0% crude protein											
0.159	4.64	33.14	0.50	1328	33.92	7.52	12.07	14.33	2.01	0.92	1.09
	5.01	32.98	0.53	1321	33.84	8.04	10.48	15.32	1.99	0.80	1.19
	6.02	43.92	0.64	1785	46.09	10.25	18.10	17.74	2.22	1.08	1.14
0.179	4.64	40.14	0.67	1641	42.01	10.38	14.54	17.09	2.09	0.95	1.14
	5.01	44.87	0.69	1737	44.58	11.98	14.78	17.82	2.00	0.90	1.10
	6.02	43.92	0.44	1713	44.32	10.62	15.00	18.70	2.10	0.94	1.16

[1] Mean values for three pigs per treatment.

[2] Apparently absorbed nitrogen.

[3] Adjusted mean values obtained by application of within protein level error regression coefficient.

values obtained when pigs were fed the 12.1% protein diet would have been due in part to the lower intake of total nitrogen.

Covariance analysis of the data indicated a highly significant difference in grams of nitrogen retained per metabolic unit by pigs when all diets were considered. A highly significant difference in nitrogen retention values also resulted from the level of protein in the diets. Such a difference would have been expected when the wide difference between mean nitrogen retention values of pigs fed the 16.0 and 12.1% crude protein diets is considered. However, it appears doubtful that a significant difference existed between nitrogen retention values of pigs fed the two higher protein diets as there was some overlapping of mean nitrogen retention values of pigs fed the different experimental diets within each of these two levels of protein.

A significant difference in nitrogen retention values resulted from amino acid supplementation of the 14.2% protein diet. This difference cannot be explained on the basis of a consistent improvement, or increase, in nitrogen retention due to increased lysine, or supplemental DL-tryptophan. It appears that it may be the more direct result of using small numbers of animals on the several treatments. All pigs fed the 14.2% protein diet containing 0.137 and 0.84% of tryptophan and lysine, respectively, showed consistent and high nitrogen retention values, while the usual greater variation was encountered with pigs fed the other diets. Thus, little attention has been given this significant effect when evaluating the response to amino acid supplementation of all pigs fed the 14.2% protein diets.

Many of the nitrogen retention values obtained with this medium protein diet equalled or exceeded values obtained when the 16.0% protein diet was supplemented in a similar manner. It is evident from the data shown in table 2 that satisfactory nitrogen retention values were realized when growing pigs were fed the 14.2% protein diet containing as low

as 0.137, 0.62 and 0.63% of tryptophan, lysine and isoleucine, respectively. The 0.48% of methionine contained in the 14.2% protein diet is considered to be in excess of the pigs' needs since a calculated excess of methionine was intentionally added to the diet.

Nitrogen retention values of pigs obtained when the two higher protein diets were fed do not indicate that increasing levels of lysine had a depressing effect upon nitrogen retention. The relative proportion of lysine to other essential amino acids did not remain constant at the two higher levels of protein. These results indicate that amino acid requirements may be a function of protein content of the diet and that relative proportion of amino acids may be important until an adequate amount of dietary protein is included in the diet.

SUMMARY AND CONCLUSIONS

A 12.1% crude protein diet was inadequate to support satisfactory nitrogen retention by growing pigs, due in part to inadequate total nitrogen intake. This diet should have been adequate in tryptophan, methionine and lysine, after supplementation. This diet contained less isoleucine than has been reported to be required by growing pigs.

Nitrogen retention values ranging from 0.96 to 1.23 gm of nitrogen retained per unit of metabolic size resulted when a 14.2% protein corn-soybean oil meal diet containing 0.137 to 0.157, 0.62 to 0.84 and 0.63% of tryptophan, lysine and isoleucine, respectively, was fed to growing pigs.

The use of higher levels of lysine in conjunction with a 12.1% crude protein diet appeared to contribute toward a decrease in nitrogen retention of growing pigs, perhaps because lysine was not in the correct proportion to other essential amino acids in the diet. Supplementation of higher protein diets with additional lysine when these diets apparently contained adequate amounts of all other essential amino acids did not significantly influence nitrogen balance of growing pigs.

ACKNOWLEDGMENTS

These studies were supported in part by grants-in-aid or supplies of experimental materials from Dow Chemical Company, Midland, Michigan; E. I. duPont de Nemours and Company, Wilmington, Delaware; Merck and Company, Inc., Rahway, New Jersey; and Chas. Pfizer and Company, Inc., Brooklyn, New York. The authors are grateful to Mr. Arthur Speece, Department of Biochemistry and Nutrition, for assistance in carrying out chemical analyses. Messrs. L. D. VanVleck, Fred Krieger and P. F. Cunningham assisted in caring for experimental animals and preparation of excreta for chemical analysis.

LITERATURE CITED

ALMQUIST, H. J. 1952 Amino acid requirements of chickens and turkeys — a review. Poultry Sci., *31:* 966.

BECKER, D. E., D. E. ULLREY AND S. W. TERRILL 1954a Protein and amino acid intakes for optimum growth rate in the young pig. J. Animal Sci., *13:* 346.

BECKER, D. E., J. W. LASSITER, S. W. TERRILL AND H. W. NORTON 1954b Levels of protein in practical rations for the pig. Ibid., *13:* 611 .

BELL, J. M. 1948 An adjustable cylindrical cage for use in metabolism studies with young swine. J. Nutrition, *35:* 365.

BLOCK, R. J., AND H. H. MITCHELL 1946 The correlation of the amino acid composition of proteins with their nutritive value. Nutrition Abstr. and Rev., *16:* 249.

BRINEGAR, M. J., H. H. WILLIAMS, F. H. FERRIS, J. K. LOOSLI AND L. A. MAYNARD 1950a The lysine requirement for the growth of swine. J. Nutrition, *42:* 129.

BRINEGAR, M. J., J. K. LOOSLI, L. A. MAYNARD AND H. H. WILLIAMS 1950b The isoleucine requirement for the growth of swine. Ibid., *42:* 619.

BRODY, S., AND R. C. PROCTER 1932 Growth and development with special reference to domestic animals. XXIIII. Relation between basal metabolism and mature body weight in different species of mammals and birds. Missouri Agr. Exp. Sta. Res. Bull. 166.

CATRON, D. V., D. C. ACKER, R. C. ASHTON, H. M. MADDOCK AND V. C. SPEER 1953 Lysine and methionine supplementation of corn-soybean oil meal rations for pigs in drylot. J. Animal Sci., *12:* 910.

MEADE, R. J. 1954 Uupublished data.

MEADE, R. J. 1956 The influence of tryptophan, methionine and lysine supplementation of a corn-soybean oil meal diet on nitrogen balance of growing swine. J. Animal Sci., *15:* 288.

MINER, J. J., W. B. CLOWER, P. R. NOLAND AND E. L. STEPHENSON 1955 Amino acid supplementation of a corn-cottonseed meal diet for growing-fattening swine. Ibid., *14:* 24.

NATIONAL RESEARCH COUNCIL 1953 Nutrient requirements for domestic animals. II. Nutrient requirements for swine. Publication 295.

PFANDER, W. F., AND L. F. TRIBBLE 1955 Some effects of adding supplements of lysine, methionine and tryptophan to practical swine rations. J. Animal Sci., *14:* 545.

SHEFFNER, A. L., AND O. BERGEIM 1953 The effect of amino acid levels upon oxidation of L and D amino acids by kidney tissues. J. Nutrition, *50:* 141.

SHELTON, D. C., W. M. BEESON AND E. T. MERTZ 1951 Quantitative L-lysine requirement of the weanling pig. Arch. Biochem., *30:* 1.

SNEDECOR, G. W. 1946 Statistical methods applied to experiments in agriculture and biology, 4th edition. Iowa State College Press, Ames, Iowa.

WILLIAMS, H. H. 1955 "Essential" amino acid content of animal feeds. Cornell University Memoir 337.

WILLIAMS, H. H., L. V. CURTIN, J. ABRAHAM, J. K. LOOSLI AND L. A. MAYNARD 1954 Estimation of growth requirements for amino acids by assay of the carcass. J. Biol. Chem., *208:* 277.

HISTIDINE REQUIREMENT OF BABY PIGS

M. RECHCIGL, JR., J. K. LOOSLI, D. J. HORVATH AND H. H. WILLIAMS
Department of Animal Husbandry, Cornell University, Ithaca, N. Y.

(Received for publication June 21, 1956)

Considerable progress has been made in the past 6 years in establishing the amino acid requirements of growing pigs (Brinegar et al., '50a, b; Shelton et al., '51a, b, c; Curtin et al., '52; Sewell et al., '53; Beeson et al., '53; Jackson et al., '53; Eggert et al., '54; Mertz et al., '54; Becker et al., '55a, b; Firth, '56). Most of these studies have been done with weanling pigs, only limited data being available for younger pigs, which have a higher protein requirement.

The qualitative dietary requirement for histidine for the growth of young pigs was clearly demonstrated by Eggert et al. ('55). Loosli ('50) suggested that the growing pig needed histidine at a level not exceeding 1.9% of the protein, on the basis of a preliminary trial by Brinegar ('51). By carcass analysis, Williams et al. ('54) estimated that the histidine requirement of the growing pig would be 1.7% of the dietary protein or approximately 0.34% of a diet containing 20% protein. Becker et al. ('54) calculated a value of 0.58% of the diet for the histidine requirements of the suckling pig on the basis of the minimum need for protein (22%) of high biological value, namely dried skim milk. More recently Mertz et al. ('55) reported that weanling pigs require a total of 0.2% of L-histidine in a diet containing 13% crude protein. The work reported in this paper was undertaken to determine the quantitative histidine requirement of the baby pig.

EXPERIMENTAL PROCEDURE

Three experiments were conducted in this series.

Experiment 1. This experiment was designed to see if a

basal "milk-type" diet similar to that used in the studies of the leucine requirement of the suckling pig (Eggert et al., '54), which is capable of supporting normal growth of young pigs, would be low enough in histidine content to be useful for a quantitative study of the histidine requirement. Supplementary amounts of the 10 amino acids known to be required for the growth of the weanling rat were added to one diet, while the same amino acids minus histidine were supplied to the other diet. One per cent of monosodium glutamate was supplied in each diet and sufficient diammonium citrate was added to equalize the nitrogen content of each diet at a level equivalent to 20% of the air-dry diet as crude protein (N $\times$ 6.25) instead of the 25% level used in the leucine experiments.

Eight litter-mate Yorkshire pigs were removed from the sow at two days of age. They were fed a stock diet containing 25% of protein (casein) for 5 days, at which time they were divided into two groups on the basis of weight and sex, and shifted gradually over the course of 4 feedings to the two experimental diets. All pigs were individually fed essentially ad libitum, and weight gains measured at weekly intervals for a 21-day period were used as the criteria of judging the adequacy of the experimental diet.

Experiment 2. In order to reduce the attending difficulties encountered with a liquid diet, dry diets were used in experiments 2 and 3.

Eight Berkshire $\times$ Yorkshire pigs were removed from the sow at 10 days of age and placed in a heated battery unit. For the initial 7-day period they were fed a low-fat, dry feed mixture described by Crampton and Ness ('54) with some modifications. Then they were divided on the basis of weight into two replicates of 4 each, and randomized to treatments and pens. The treatments were 4 levels of histidine; 0.1, 0.2, 0.3, and 0.4% of the total diet. The basal experimental ration was compounded to provide 20% protein equivalent, 10% fat and 2% fiber. It consisted of 33.33% dried whey, 4.45% amino acid mixture, 12.32% diammonium citrate, 13.00% glucose, 25.23% dextrinized starch, 9.67% corn oil and 2.00% fiber source.

Vitamins and minerals were included in amounts recommended by the National Research Council ('53) for 25 lb. pigs. The experimental rations were fed 18 days and then lots 1, 2 and 3 were discontinued and lot 4 changed to 0.3% histidine for an additional week.

Experiment 3. Sixteen Yorkshire pigs were removed from the sow at 10 to 12 days of age and placed in a heated battery unit similarly to the previous experiment. The pigs were fed a dry diet (Crampton and Ness, '54). Following the 14-day transitional period on this diet the pigs were divided at ran-

TABLE 1

Composition of basal diet for experiment 3

INGREDIENTS	AMOUNT
	%
Dried whey [1]	20
Amino acid mixture [2]	4.76
Diammonium citrate	12.35
Glucose (Cerelose)	20
Dextrinized starch [3]	24.69
Lard	5
Corn oil [4]	5
Fiber [5]	2
Inverted molasses	2
Minerals [6]	4.20
Vitamins [7]	+

[1] Supplied by Midwest Dried Milk Co., Dundee, Illinois, through the courtesy of R. F. Van Poucke.

[2] See table 2.

[3] Prepared by cooking raw starch in a steam-jacketed kettle for 10 minutes followed by drying in an oven and grinding.

[4] Mazola.

[5] Solka-floc.

[6] The following ingredients were included in the mineral mixture: KCl, 248 gm; $MgCO_3$, 180 gm; $FeSO_4 \cdot 7H_2O$, 7.47 gm; $CuSO_4 \cdot 5H_2O$, 786 mg; $CoCl_2 \cdot 6H_2O$, 356 mg; $MnSO_4 \cdot H_2O$, 5.544 gm; $ZnSO_4 \cdot H_2O$, 12.375 gm; iodinated salt, 228 gm; dicalcium phosphate, 1226 gm.

[7] The following vitamins were added to each 100 lb. of basal ration: thiamine-HCl, 50 mg; riboflavin, 120 mg; niacin, 800 mg; Ca-pantothenate, 250 mg; pyridoxine-HCl, 60 mg; folic acid, 30 mg; 0.1% vitamin B_{12}, 700 mg; vitamin A, 125,000 I.U.; vitamin D, 9,000 I.U., supplied as viosterol; alpha tocopherol acetate, 300 mg; menadione, 100 mg; choline chloride, 30 gm.

dom into 4 groups of 4, in such a way that each group contained one pig from each litter, and placed on the experimental diets.

TABLE 2

Amino acid composition and nitrogen content of dried whey and amino acid mixture, used in experiment 3

	AMOUNT SUPPLIED BY				TOTAL AVAILABLE AMINO ACID OF BASAL DIET	
	Whey [1]	Amino acid mixture				
			Form	N content		
	%	%		%	% of diet	% of crude protein [5]
Arginine	0.06	0.22	L·HCl	0.06	0.24	1.5
Histidine [2]	0.03		L·HCl		0.03	0.2
Isoleucine	0.16	0.80	DL	0.09	0.56	3.5
Leucine	0.26	0.54	L	0.06	0.80	5.0
Lysine	0.22	0.73	L·HCl	0.11	0.80	5.0
Methionine [3]	0.03	0.39	DL	0.04	0.42	2.6
Phenylalanine [3]	0.07	0.43	DL	0.04	0.50	3.1
Threonine	0.14	0.84	DL	0.10	0.56	3.5
Tryptophan [3]	0.03	0.13	DL	0.02	0.16	1.0
Valine	0.13	0.70	DL	0.08	0.48	3.0
Cystine [4]	0.06				0.06	0.4
Tyrosine [4]	0.06				0.06	0.4
Total	1.25	4.78		0.60	4.81	29.2

[1] Per cent of amino acid × % digestibility of whey protein (90%). Figures for the amino acid composition of dried whey were obtained from Williams ('55) and figure for the digestibility of whey protein was obtained from Morrison ('51).

[2] The histidine content of dried whey was determined by column chromatography (Moore and Stein, '51).

[3] Methionine, phenylalanine and tryptophan are utilized in both forms.

[4] Cystine has a sparing action on methionine and tyrosine on phenylalanine.

[5] Crude protein = 16.0% of diet.

The basal diet, shown in table 1, contained 16.0% total crude protein, 2.77% being supplied by dried whey (N × 6.44),[1] 3.75% by amino acids (N × 6.25) and 9.48% by diammonium citrate (N × 6.25). The nitrogen and amino acid contents of the amino acid mixture are summarized in table 2. The dietary contents of the individual amino acids were based on values which had been determined for baby pigs, where available.

[1] The N factor of 6.44 was suggested by Perlmann and Longsworth ('48) and is based on β-lactoglobulin, the main constituent of whey protein.

The remaining values were based on the requirements of weanling pigs. These calculations were made on the assumption that the requirements of growing swine for all essential amino acids are directly proportional to the level of protein in the diet. L-Histidine was added to the different diets at various levels so as to give 4 experimental diets of different histidine content. The added histidine replaced an equivalent amount of diammonium citrate.

Growth response and efficiency of feed utilization were used as measures for comparing the various levels of histidine over a 21-day period.

TABLE 3

Average data for pigs receiving two levels of histidine[1]

Experiment 1

	SUPPLEMENTAL L-HISTIDINE (%)	
	None	0.45%
Total L-histidine, % of diet[2]	0.30	0.75
Total L-histidine, % of protein	1.50	3.75
Number of pigs	4	4
Days on trial	21	21
Initial weight, kg	2.2	2.2
Final weight, kg	6.5	6.8
Daily gain, gm	205 ± 5[3]	216 ± 20
Daily gain, % of initial weight	9.23	9.69
Air-dry feed/kg of gain, kg	1.27	1.21

[1] Data in this table are cited from Eggert ('54).

[2] The histidine content was determined by microbiological analysis of an acid hydrolysate of the protein, using *Leuconostoc mesenteroides*. (Williams, '55.)

[3] Standard deviation.

RESULTS

The results of the first experiment are shown in table 3. The daily gains and efficiency of feed utilization were good for both diets. The average daily gain was slightly better for the pigs receiving the supplemental histidine, but the difference was only slight. These data indicated that the basal diet was not low enough in histidine content for an extensive study of the histidine requirement of the young pig and that the requirement of the suckling pig for histidine is probably

not much, if any, higher than 0.3% of such a diet. This would be equivalent to a requirement of approximately 1.5% of the dietary protein.

The results of the second experiment are summarized in table 4. The 0.3% level of total L-histidine appeared to be optimum and 0.2% produced gains of essentially the same magnitude. The 0.1% level was associated with a less uniform response of a lesser magnitude. In terms of both gains and appetite of the pigs the 0.4% level was poorest. When the

TABLE 4

Average data for pigs receiving various levels of histidine

Experiment 2

	SUPPLEMENTAL LEVEL OF DL-HISTIDINE (%)			
	0.06	0.26	0.46	0.66
Total L-histidine, % of diet [1]	0.10	0.20	0.30	0.40
Total L-histidine, % of protein	0.50	1.0	1.50	2.00
Number of pigs	2	2	2	2
Days on trial	18	18	18	18
Initial weight, kg	3.8	3.6	3.5	3.6
Final weight, kg	4.5	4.4	4.4	3.8
Daily gain, gm	35 ± 7 [2]	46 ± 2	51 ± 7	11 ± 24
Daily gain, % of initial weight	0.91	1.28	1.48	0.32

[1] See footnote 2, table 3.

[2] Standard deviation.

pigs in lot 4 were transferred to the 0.3% level for an additional 5 days, they gained rapidly, the average daily gain being 115 gm. The abrupt gain in one pig from lot 4 in the three days prior to changing the ration cannot be explained.

A summary of the data obtained in the third experiment is presented in table 5. That the basal diet is deficient in histidine (0.03% of the diet) is indicated by the slow growth, poor appetite and low feed utilization. Increasing the level of histidine in the diet improved the pigs' appetites and produced significantly higher daily gains. In terms of growth, appetite and efficiency of feed utilization the 0.19% level of total L-histidine appears to be approximately optimum. However, the

differences in daily gains between the pigs receiving the higher levels of histidine (0.11, 0.19 and 0.27) in their diets did not prove to be statistically significant, as measured by the Duncan multiple range test ('53).

TABLE 5

Average data for pigs receiving various levels of histidine

Experiment 3

	SUPPLEMENTAL LEVEL OF L-HISTIDINE (%)			
	None	0.08	0.16	0.24
Total L-histidine, % of diet [1]	0.03	0.11	0.19	0.27
Total L-histidine, % of protein	0.19	0.69	1.19	1.69
Number of pigs	4	4	4	4
Days on trial	21	21	21	21
Initial weight, kg	4.1	4.1	4.4	4.6
Final weight, kg	4.4	5.4	6.2	6.1
Daily gain, gm	16 ± 8	63 ± 23 [2]	86 ± 23 [2]	72 ± 16 [2]
Daily gain, % of initial weight	0.39	1.54 [2]	1.95 [2]	1.56 [2]
Daily feed intake, kg	0.18	0.22	0.26	0.24
Daily feed intake, % of body weight	4.39	5.37	5.91	5.22
Feed intake/kg of gain, kg	13.66	3.51	3.07	3.53

[1] See footnote 2, table 2.

[2] Statistically significant over the basal lot at the 1% level.

DISCUSSION

The data show that the 0.03% level of L-histidine is inadequate to support satisfactory growth of baby pigs. The optimum level is not less than approximately 0.2% of L-histidine in a ration containing 16% of crude protein. This is equivalent to 1.2% of the protein. If one expresses the data as the percentages of the dietary protein, as was done in figure 1, there is a close agreement between the last two experiments. There is also moderately good agreement between this value and the value of 0.2% of L-histidine for the weanling pig (Mertz et al., '55) as well as the value of 0.34% obtained by the indirect method of carcass analysis as reported by Williams et al. ('54). The requirement of baby pigs also approaches the chick requirement of 0.15% (Almquist, '52) and to a lesser degree the young rat requirement of 0.4% (Rose et al., '49).

A comparison of the last two experiments suggests that the D form of histidine is poorly utilized, if at all, when it is fed in a racemic mixture, since in experiment 2 the DL form was fed but the response was proportional to the level of the L form as judged by the results of experiment 3. Similar findings were reported in studies with dogs (Abderhalden and Buadze, '31), guinea pigs (Edlbacher et al., '41), rabbits (Abderhalden, '22), man (Albanese et al., '45) and the mouse

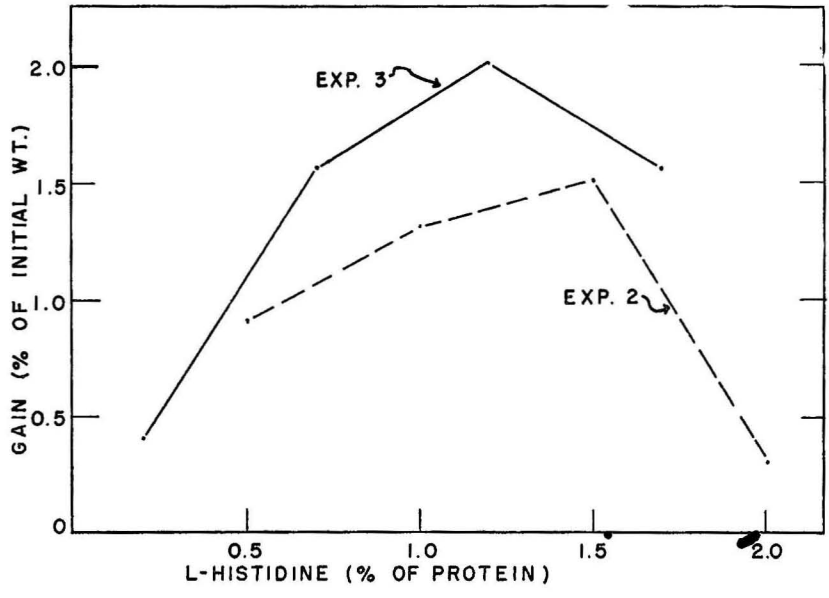

Fig. 1 Relative growth response of baby pigs fed different levels of L-histidine.

(Celander and Berg, '53). However, the rat seems to utilize the unnatural isomer both for growth (Cox and Berg, '34) and for the maintenance of nitrogen equilibrium (Nasset and Gatewood, '54).

It is seen that the daily gains in the first experiment were much higher than those in the latter two experiments. It seems probable that the liquid nature of the first diet was not entirely responsible for the greater gains, the dietary components of that diet could have been a factor. It contained

three times as much fat, had a higher ratio of essential amino acids to diammonium citrate and contained chlortetracycline. The question arises then as to the optimum ratio between essential and non-essential amino acids as well as to the utilization of diammonium citrate. We have observed that rats on essentially the same diet as was used in the third experiment grew better if the diammonium citrate was decreased while the amounts of essential amino acids were kept constant. There is a need for controlled experiments investigating the utilization of diammonium citrate as well as other simple non-protein nitrogen substances by the young pig and other monogastric animals.

It has been customary to express the amino acid requirement in terms of percentage of the diet or of the crude protein. Since the quality of protein depends upon its content of essential amino acids and their proper balance, it would be more precise to express amino acid requirements as percentage of the crude protein or nitrogen supplied by the essential amino acids. By this procedure inter- and intra-species requirements for amino acids could be put on an equivalent basis regardless of the nitrogen (or crude protein) content of the diet or the ratio of essential and non-essential amino acids.

SUMMARY

Three experiments involving a total of 32 pigs were conducted to study the histidine requirement of baby pigs.

The first experiment showed that a simulated "milk-diet" used in earlier studies of the leucine requirement of suckling pigs was not low enough in histidine to be useful in studying the histidine requirement of the young pig.

In two experiments baby pigs were fed a semi-purified, dry diet compounded to provide 10% fat, 2% fiber and two different levels of protein, namely 16% and 20%. The dietary nitrogen was supplied by dried whey, amino acids and diammonium citrate. The data indicate that the optimum level is approximately 0.2% of L-histidine in a 16% protein ration. This requirement is equivalent to 1.2% of the dietary protein.

ACKNOWLEDGMENT

These studies were supported largely through a grant from the Herman Frasch Foundation.

LITERATURE CITED

ABDERHALDEN, E. 1922 Physiologisches Praktikum. Berlin, 3rd ed., p. 134; quoted by Albanese et al. ('45).

ABDERHALDEN, E., AND S. BUADZE 1931 Weitere Studien über das Schicksal des Histidins im tierischen Organismus. Z. Physiol. Chem., *200:* 87.

ALBANESE, A. A., J. E. FRANKSTON AND V. IRBY 1945 The utilization of D-amino acids by man. V. Histidine. J. Biol. Chem., *160:* 441.

ALMQUIST, H. J. 1952 Amino acid requirements of chickens and turkeys — A review. Poultry Sci., *31:* 966.

BECKER, D. E., A. H. JENSEN, S. W. TERRILL AND H. W. NORTON 1955a The methionine-cystine need of the young pig. J. Animal Sci., *14:* 1086.

BECKER, D. E., R. A. NOTZOLD, A. H. JENSEN, S. W. TERRILL AND H. W. NORTON 1955b The tryptophan requirement of the young pig. Ibid., *14:* 664.

BECKER, D. E., D. E. ULLREY AND S. W. TERRILL 1954 Protein and amino acid intakes for optimum growth rate in the young pig. Ibid., *13:* 346.

BEESON, W. M., H. D. JACKSON AND E. T. MERTZ 1953 Quantitative threonine requirement of the weanling pig. Ibid., *12:* 870.

BRINEGAR, M. J. 1951 The lysine, tryptophan, histidine and isoleucine requirements of swine with observations on the biological utilization of several proteins. Ph.D. thesis, Cornell University, Ithaca, N. Y.

BRINEGAR, M. J., J. K. LOOSLI, L. A. MAYNARD AND H. H. WILLIAMS 1950a The isoleucine requirement for the growth of swine. J. Nutrition, *42:* 619.

BRINEGAR, M. J., H. H. WILLIAMS, F. H. FERRIS, J. K. LOOSLI AND L. A. MAYNARD 1950b The lysine requirement for the growth of swine. Ibid., *42:* 129.

CELANDER, D. R., AND C. P. BERG 1953 The availability of D-histidine, related imidazoles, and D-tryptophan in the mouse. J. Biol. Chem., *202:* 339.

COX, G. J., AND C. P. BERG 1934 The comparative availability of D- and L-histidine for growth. Ibid., *107:* 497.

CRAMPTON, E. W., AND O. M. NESS 1954 A meal mixture suitable as the entire ration to be self-fed dry to pigs weaned at ten days of age. J. Animal Sci., *13:* 357.

CURTIN, L. V., J. K. LOOSLI, J. ABRAHAM, H. H. WILLIAMS AND L. A. MAYNARD 1952 The methionine requirement for the growth of swine. J. Nutrition *48:* 499.

DUNCAN, D. B. 1953 Multiple Range and Multiple F Tests. Virginia Agr. Exp. Sta. Tech. Report no. 6.

EDLBACHER, S., H. BAUER AND H. R. STAEHELIN 1941 Über das Verhalten von L- und D-histidin im Tierkörper. Z. Physiol. Chem., *270:* 165.

EGGERT, R. G. 1954 Studies of the leucine and histidine requirements for growth of suckling pigs. Ph.D. thesis, Cornell University, Ithaca, N. Y.

EGGERT, R. G., L. A. MAYNARD, B. E. SHEFFY AND H. H. WILLIAMS 1955 Histidine — an essential nutrient for growth of pigs. J. Animal Sci., *14:* 556.

EGGERT, R. G., H. H. WILLIAMS, B. E. SHEFFY, E. G. SPRAGUE, J. K. LOOSLI AND L. A. MAYNARD 1954 The quantitative leucine requirement of the suckling pig. J. Nutrition, *53:* 177.

FIRTH, J., AND B. C. JOHNSON 1956 Quantitative relationships of tryptophan and nicotinic acid in the baby pig. Ibid., *59:* 223.

JACKSON, H. D., E. T. MERTZ AND W. M. BEESON 1953 Quantitative valine requirement of the weanling pig. J. Nutirtion, *51:* 109.

LOOSLI, J. K. 1950 The amino acid requirements of swine. Proc. Cornell Nutr. Conf. for Feed Mfg., p. 30.

MERTZ, E. T., J. N. HENSON AND W. M. BEESON 1954 Quantitative phenylalanine requirement of the weanling pig. J. Animal Sci., *13:* 927.

MERTZ, E. T., O. C. DELONG, D. M. THRASHER AND W. M. BEESON 1955 Histidine and leucine requirements of the weanling pig. (Abs.). Ibid., *14:* 1217.

MOORE, S., AND W. H. STEIN 1951 Chromatography of amino acids on sulfonated polystyrene resins. J. Biol. Chem., *192:* 663.

MORRISON, F. B. 1951 Feeds and Feeding. Twenty-first edition. Morrison Publishing Co., Ithaca, New York.

NASSET, E. S., AND V. H. GATEWOOD 1954 Nitrogen balance and hemoglobin of adult rats fed amino acid diets low in L- and D-histidine. J. Nutrition, *53:* 163.

NATIONAL RESEARCH COUNCIL 1953 Nutrient requirements for domestic animals. No. 2 Nutrient requirements for swine. Washington, D. C.

PERLMANN, G. E., AND L. G. LONGSWORTH 1948 The specific refractive increment of some purified proteins. J. Am. Chem. Soc., *70:* 2719.

ROSE, W. C., L. C. SMITH, M. WOMACK AND M. SHANE 1949 The utilization of the nitrogen of ammonium salts, urea, and certain other compounds in the synthesis of non-essential amino acids in vivo. J. Biol. Chem., *181:* 307.

SEWELL, R. F., J. K. LOOSLI, L. A. MAYNARD, H. H. WILLIAMS AND B. E. SHEFFY 1953 The quantitative threonine requirement of the suckling pig. J. Nutrition, *49:* 435.

SHELTON, D. C., W. M. BEESON AND E. T. MERTZ 1951a Quantitative DL-tryptophan requirement of the weanling pig. J. Animal Sci., *10:* 73.

———— 1951b Quantitative L-lysine requirement of the weanling pig. Arch. Biochem. Biophys., *30:* 1.

———— 1951c The effect of methionine and cystine on the growth of weanling pigs. J. Animal Sci., *10:* 57.

WILLIAMS, H. H. 1955 "Essential" amino acid content of animal feeds. Cornell University Memoir 337.

WILLIAMS, H. H., L. V. CURTIN, J. ABRAHAM, J. K. LOOSLI AND L. A. MAYNARD 1954 Estimation of growth requirements for amino acids by assay of the carcass. J. Biol. Chem., *208:* 277.

BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1957 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. The award is usually given to one person, but if in their judgment circumstances and justice so dictate, the Jury of Award may recommend that it be divided between two or more collaborators in a given research. The Jury may also recommend that the award be omitted in any given year if in its opinion the work submitted does not warrant the award. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award. Employees of the Borden Company are not eligible for this honor.

Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award

Chairman, Nominating Committee:
C. G. MACKENZIE
Department of Biochemistry
University of Colorado School of Medicine
4200 E. 9th Avenue, Denver 7, Colorado

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award.

Chairman, Nominating Committee:
R. V. BOUCHER
Agricultural and Biological Chemistry
Pennsylvania State University
University Park, Pennsylvania