Cardiovascular Lesions, Blood Lipids, Coagulation and Fibrinolysis in Butter-induced Obesity in the Rat [1]

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ABSTRACT A study was made of obesity in the rat, induced by feeding a high butter fat diet over a long period and possible effects of obesity on the development of cardiovascular lesions. A group of 17 male Wistar albino rats was fed a diet that contained 40% butter by weight (providing about 65% of the calories). To evaluate the independent contribution of obesity to the development of cardiovascular disease, repeated measurements were made of other parameters that might be affected by fats, namely, blood lipids, coagulation and fibrinolysis. The animals became grossly obese but did not develop any significant changes in blood lipids, coagulation and fibrinolysis throughout the period of the experiment (average of 428 days). Nor were there any significant cardiovascular lesions at the end of this period, beyond those normally observed in the aging rat. It was concluded that under the conditions of this experiment where no significant changes occurred in blood lipids, coagulation and fibrinolysis, butter-induced obesity in the rat does not materially predispose to the development of cardiovascular lesions.

Obesity has been implicated frequently in the development of cardiovascular disease (1, 2), and yet the clinical evidence for this is by no means incontrovertible (3–6). Moreover, in view of the emphasis placed on this aspect of obesity in man, long-term studies of the atherogenic effect of experimental obesity have not received the attention they might. It was therefore considered worthwhile to study experimental obesity in the rat as induced by a high fat diet; and in addition, to study the independent effect of obesity on the development of atherosclerosis by the measurement of some other parameters that might be affected by fats, such as blood lipids, coagulation and fibrinolysis. Also, a high butter fat diet was selected for the study because of the presumed atherogenic effect of saturated fats.

MATERIALS AND METHODS

Two groups of rats were used. Each group consisted of 17 male Wistar albinos. The experimental group received a diet the composition of which appears in table 1. The butter content of this diet was 40% by weight which provided about 65% of the calories. Since the studies involved the measurement of blood lipids, coagulation and fibrinolysis over a period of several months, it was necessary to compare the measurements in the experimental group with that of a normal group to safeguard against any variation in the activity of reagents used at different periods of the experiment. This was particularly important for coagulation studies where different batches of substrates and reagents may show considerable variation in activity. The normal group selected for this purpose was a chow-fed group, since in our previous studies (7), we have had extensive experience with the level of blood lipids, coagulation, fibrinolysis and the normal variation thereof in this group, and since this is the accepted normal diet of laboratory rats about which extensive data have accumulated over the years.

The statistical study both in the initial and the terminal phases of the experiment was carried out between the experimental group and the normal group. However, this was used only as an indirect way to detect the possible changes in the level of blood lipids, coagulation and fibrinolysis in

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TABLE 1

Composition of the diet of the experimental group

	wt %
Powdered cellulose [1]	6.0
Casein [2]	20.0
Choline chloride	0.2
Salt mixture [3]	4.0
Sucrose	27.8
Vitamin mixture [4]	2.0
Butter	40.0

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[2] Casein (purified), Nutritional Biochemicals Corporation.

[3] The salt mixture (Nutritional Biochemicals Corporation) was the Wesson modification of the Osborne and Mendel salt mixture. The composition in per cent by weight is as follows: calcium carbonate, 21.000; copper sulfate ($5H_2O$), 0.039; ferric phosphate, 1.470; manganous sulfate (anhyd.), 0.020; magnesium sulfate (anhyd.), 9.000; potassium aluminum sulfate, 0.009; potassium chloride, 12.000; potassium dihydrogen phosphate, 31.000; potassium iodide, 0.005; sodium chloride, 10.500; sodium fluoride, 0.057; tricalcium phosphate, 14.900.

[4] Each kilogram of the vitamin mixture contained the following triturated in dextrose: vitamin A concentrate (200,000 U/g), 4.5 g; vitamin D concentrate (400,000 U/g), 0.25 g; α-tocopherol, 5.0 g; ascorbic acid, 40.5 g; inositol, 5.0 g; niacin, 4.5 g; riboflavin, 1.0 g; pyridoxine·HCl, 1.0 g; thiamine·HCl, 1.0 g; Ca pantothenate, 3.0 g; biotin, 20 mg; folic acid, 90 mg; and vitamin B_{12}, 1.35 mg.

the experimental group over the many months of the experiment. As such the normal group should not be considered as a control group. The studies on the experimental group in the initial phase of the experiment provide the true control for the studies in this same group toward the end of the experiment.

The average starting weight of the animals in the normal group was 410 g and in the experimental group 390 g (the small differences were not statistically significant). All animals were housed in individual cages and were fed ad libitum.

Pathological studies and methods. Six rats in the experimental group were killed for pathological studies after they had received the diet for 6 months. The rest of the animals in both groups were maintained in the experiment for an average of 428 days (range 200 to 580 days). Complete autopsies were done on all animals, but only heart, aorta, liver and kidney were submitted to histological preparations. These organs were removed and were fixed in 10% calcium formalin after death or killing of the animals. Hearts and aortas were then embedded in gelatin for the preparation of frozen sections. Each heart was cut into at least 5 slices, each of which was used for 2 frozen sections. Thus, a minimum of 10 frozen sections, not more than 5 μ thick, was prepared from each rat heart so that as many cardiac lesions as possible would be detected. The aortas were first stained in the gross and examined for lesions with a magnifying lens. They were then cut longitudinally on the freezing microtome, mounted, and searched for lesions under the microscope. The frozen sections were stained with oil red O in triethylphosphate. Livers and kidneys were embedded in paraffin and stained routinely with hematoxylin and eosin.

Blood coagulation and lipid studies and methods. The studies of blood lipids, coagulation, and fibrinolysis were carried out in both groups simultaneously at monthly intervals, in both the early phase of the experiment, that is, the first 4 months, and the late phase, that is, the last 4 months of the experiment. Rats were fasted for 16 hours before blood was collected from the femoral vein. Since a number of blood coagulation and lipid determinations require relatively substantial volumes of blood, it was not feasible to obtain enough blood from a single animal (unless killed) for all the studies. However, to minimize the possible errors from this source, the same number and type of blood coagulation and fibrinolysis tests were made in both groups at each venipuncture. Each rat was anesthetized with ether, and blood was drawn with a 21-gauge siliconized needle and syringe by a 2-syringe technique. The volume of blood drawn at each venipuncture varied from 3 to 6 ml. Three per cent sodium citrate was used as the anticoagulant throughout, and 9 volumes of blood were added to 1 volume of citrate. Blood was centrifuged at 2500 rev/min for 10 minutes. Blood and plasma were kept in melting ice throughout. Observations were made in duplicate. Since the trends were similar both in the early and the late phase of the experiment, in each phase of the study results were pooled for statistical purposes.

Coagulation methods

Prothrombin time. The one-stage method of Quick (8) was used except that a saline extract of acetone-dried human brain was used as a source of thromboplastin.

Stypven time. Russell viper venom [2] was diluted 1:10,000 with distilled water, then diluted 1:100,000 with 0.9% saline. Platelet-poor plasma, 0.1 ml, was incubated with 0.1 ml of freshly prepared venom for 30 seconds at 37°; then 0.1 ml of 0.02 M calcium chloride was added and the clotting time determined.

Prothrombin levels. Plasma prothrombin was quantitatively determined by a modification of the Owren technique as described by Alexander (9).

Factor V (labile factor). Activity was measured by determining the corrective effect on the prothrombin time of a substrate devoid of factor V but containing the other factors (prothrombin, factors VII and X, and fibrinogen) that affect the prothrombin time (10).

Factors VII and X activity. The procedure used was a modification of the Owren technique for determining proconvertin activity (11). This technique is now known to measure the combined activity of factors VII and X.

Modified thromboplastin generation. The test, as modified by Hicks and Pitney (12) for determining the thromboplastin generation from whole plasma was used. Soybean phospholipid [3] was added as a platelet substitute. This procedure measures the development of plasma or blood thromboplastin.

Fibrinolysis. The method described by Loomis (13) was used as modified by Scott and Thomas (14). Streptokinase-streptodornase [4] was used to activate fibrinolysis. Rat plasma, 0.025 ml, was added to 0.05 ml of a 1:5 dilution in saline of citrated human platelet-poor plasma. The mixture was incubated at 37° for 30 seconds and then 0.1 ml of streptokinase-streptodornase containing 2000 U of streptokinase/ml was added to the mixture. Thirty seconds after the reagents had been thoroughly mixed, 0.1 ml of bovine thrombin [5] containing 50 U/ml was added to the mixture and the stop watch was started. Thirty seconds later, at which time a clot had already formed, the open end of a capillary tube was inserted perpendicularly into the bottom of the clot. The tube was left undisturbed in a glass water bath at 37°. Once fibrinolysis had begun, the liquefied plasma ascended into the capillary tube. The end point was taken at the instant that the liquid level in the capillary tube reached the upper surface of the clot.

Platelet count. The method of Brecher and Cronkite (15) was used.

Plasma fibrinogen. The method of Ratnoff and Menzie as modified by Holburn (16) was used.

Statistical studies. Comparison of the means was carried out by the *t* test method (17). The differences were taken to be statistically significant when *P* was less than 0.05.

Chemical methods

The serum cholesterol and cholesterol esters were determined by the method of Schoenheimer and Sperry (18). The lipid phosphorus was obtained by extracting the serum with the Bloor reagent and petroleum ether. The lipid phosphorus was converted to inorganic phosphorus by digestion and the inorganic phosphorus so obtained was measured by the procedure of Fiske and Subbarow (19). A factor of 25 was used to convert the lipid phosphorus to phospholipids. The total serum fatty acids were determined by the method of Stoddard and Drury (20). For the determinations of ethanolamine phosphatide and serine phosphatide, the method of Axelrod et al. (21) was used.

RESULTS

Course of the experiment. Throughout the experiment the animals of the experimental group gained more weight than those of the normal group. The caloric intake of the experimental group was estimated to be about one-and-a-half times that of the normal group (26 kcal/100 g rat/day in the experimental group compared with 16 in the normal group). The average weight of the rats in the experimental group that were maintained with the diet for the full course of the experiment was 1072 g (range 915 to 1570), and that of the normal group after a

[2] Stypven, Burroughs Wellcome and Company, Tuckahoe, New York; prepared at Wellcome Research Laboratories, Beckenham, England.

[3] Inosithin, Associated Concentrates, Inc., Woodside, New York.

[4] Varidase, Lederle Laboratories, American Cyanamid Company, Pearl River, New York.

[5] Obtained from the Upjohn Company, Kalamazoo, Michigan.

comparable period of time was 553 g (range 410 to 664).

Results of blood lipid studies. The mean results of the blood lipid determinations in the early phase of the experiment appear on table 2. Each result is the average of all the determinations in a particular group. The animals of the experimental group tended to have a somewhat higher level of blood lipids as indicated by total cholesterol, total fatty acids, and total phospholipids including ethanolamine and serine phosphatides. In a number of determinations the differences are statistically significant, yet considered in the context of other experiments in diet-induced lipemia in the rat (7), the increase of blood lipids in the experimental group compared with the normal group is minor in absolute terms.

The mean results of the blood lipid determinations in the late phase of the experiment appear in table 3. Total cholesterol and fatty acids were somewhat lower in the experimental group in this phase of the experiment, but total phospholipids including ethanolamine and serine phosphatides were higher. The differences, although statistically significant in respect to some determinations, are again noted to be small.

Comparing the late phase of the study to the early phase, there was some decrease in certain lipid parameters, namely, total fatty acids, cholesterol, and not quite to the same extent as for total phospholipids, in the experimental group. This has resulted in a decrease in total lipids-to-phospholipid ratio which may be significant. However, a similar decrease occurred in the total lipids-to-phospholipid ratio of the chow-fed group. Conversely there was an increase in the phosphatides in the experimental group during the period of study. These various changes are pointed out since many investigators might consider them significant. However, the observed changes were small in absolute terms compared with the changes we have observed in previous studies of experimental atherosclerosis in the rat (7). For this reason and because some of the changes in the lipids in the chow-fed group showed a similar trend, we did

TABLE 2

Mean of blood lipid determinations in the normal and experimental groups in the early phase of the experiment

	Normal group	Experimental group	SED [1]
Total cholesterol, mg/100 ml	67.4(19) [2]	74.7(19)	5.6
Cholesterol esters, %	79.4(19)	70.6(19)	3.2 *
Total fatty acids, mg/100 ml	205.0(18)	301.0(18)	40.3 *
Total phospholipids, mg/100 ml	133.0(15)	226.0(15)	44.3 *
Ethanolamine phosphatide, mg/100 ml	3.7(15)	5.0(15)	0.6 *
Serine phosphatide, mg/100 ml	4.9(15)	5.8(15)	0.5

[1] SED indicates standard error of the difference.
[2] The number of the determinations on which each mean is based appears in parentheses.
* Statistically significant.

TABLE 3

Mean of blood lipid determinations in the normal and experimental groups in the late phase of the experiment

	Normal group	Experimental group	SED [1]
Total cholesterol, mg/100 ml	86.3(11) [2]	65.3(11)	8.5 *
Cholesterol esters, %	86.0(2)	78.5(4)	4.9
Total fatty acids, mg/100 ml	154.0(8)	122.0(8)	27.9
Total phospholipids, mg/100 ml	179.2(8)	202.3(8)	34.8
Ethanolamine phosphatide, mg/100 ml	3.5(8)	6.8(8)	1.8
Serine phosphatide, mg/100 ml	4.7(8)	8.4(8)	1.7 *

[1] SED indicates standard error of the difference.
[2] The number of the determinations on which each mean is based appears in parentheses.
* Statistically significant.

not consider these changes to be biologically significant and therefore withhold further comment, particularly since different interpretations would be placed on various changes in lipid parameters and ratios thereof by different investigators.

Results of blood coagulation and fibrinolysis studies. The average results of blood coagulation and fibrinolysis studies in the early phase of the experiment appear on table 4. There are no significant differences between the 2 groups as indicated by the Quick prothrombin time, Owren determinations of prothrombin, factor V and factors VII and X, platelet count, hematocrit, and streptokinase-induced fibrinolysis. Russell viper venom clotting time was shorter in the experimental group, the difference being statistically significant. The biological significance of this test, however, is not entirely clear (22). The average results of thromboplastin generation test in the early phase of the experiment appear in table 5 and show no statistically significant differences between the 2 groups.

The results of the studies of blood coagulation and fibrinolysis in the late phase of the experiment appear in table 6. The difference between the 2 groups generally appears to be slight in most of the determinations. If anything, there may be a shift in the balance of blood coagulation and fibrinolysis away from clot formation

TABLE 4

Mean of blood coagulation and fibrinolysis studies in the normal and experimental group in the early phase of the experiment

	Normal group	Experimental group	SED [1]
Quick (8) prothrombin time, sec	18.4(30) [2]	18.1(30)	0.8
Russell viper venom time,[3] sec	23.1(29)	20.1(29)	1.4 *
Owren determinations (9)			
Prothrombin, %	101.8(29)	106.9(29)	4.6
Factor V, %	100.2(29)	93.3(29)	5.3
Factors VII and X, %	96.1(30)	104.1(30)	4.8
Platelet count, in 1000/cm^3	1089.5(26)	1003.0(26)	82.7
Hematocrit, %	39.7(31)	39.5(31)	0.7
Streptokinase [4] fibrinolysis, sec	158.2(28)	166.6(28)	9.2

[1] SED indicates standard error of the difference.
[2] The number of the determinations on which each mean is based appears in parentheses.
[3] Stypven, Burroughs Wellcome and Company, Tuckahoe, New York.
[4] Varidase, Lederle Laboratories, American Cyanamid Company, Pearl River, New York.
* Statistically significant.

TABLE 5

Mean of the modified thromboplastin generation test in the normal and experimental groups in the early phase of the experiment

Incubation time	Clotting time		
	Normal group	Experimental group	SED [1]
min	*sec*	*sec*	*sec*
2	91.0(25) [2]	92.1(25)	10.1
3	63.9(21)	57.3(21)	7.5
4	54.2(24)	50.6(24)	8.1
5	44.3(23)	37.0(23)	6.9
6	38.1(25)	30.9(25)	5.2
7	31.8(24)	27.5(24)	3.0
8	27.8(24)	26.0(24)	3.7
9	26.2(24)	23.7(24)	2.8
10	26.2(21)	24.3(21)	2.7

[1] SED indicates standard error of the difference.
[2] The number of the determinations on which each mean is based appears in parentheses.

in the experimental group. The differences between the 2 groups, however, are not great in absolute terms and are not thought to be significant. The results of average thromboplastin generation in the late phase of the study appear in table 7 and similarly indicate no significant differences between the 2 groups.

To summarize: The comparison between the blood coagulation and fibrinolysis studies of the experimental group in the early phase of the experiment and those in the late phase indicate that there was no significant change in the period of the experiment. (This comparison has to be made indirectly, that is, in each phase of the experiment the simultaneous studies on the normal group should be taken into consideration to make allowance for the differences in measurements due to variation in the activity of different batches of substrates and reagents.)

Results of pathological studies. Pathological studies in 6 rats in the experimental group that were maintained with the diet for 6 months showed them to be free of cardiovascular lesions. Apart from gross obesity and a fatty liver, there were no remarkable findings at the autopsy of these rats.

TABLE 6

Mean of blood coagulation and fibrinolysis studies in the normal and experimental groups in the late phase of the experiment

	Normal group	Experimental group	SED [1]
Quick (8) prothrombin time, sec	17.4(11) [2]	18.1(11)	1.1
Russell viper venom time,[3] sec	32.5(11)	32.7(11)	5.8
Owren determinations (9)			
Prothrombin, %	109.2(11)	122.1(11)	9.5
Factor V, %	110.9(11)	85.8(11)	9.0 *
Factors VII and X, %	128.0(11)	125.0(11)	9.0
Platelet count, in 1000/cm^3	1049.7(10)	881.3(10)	141.0
Hematocrit, %	37.3(11)	36.0(11)	2.0
Streptokinase [4] fibrinolysis, sec	157.2(11)	128.6(11)	24.9
Fibrinogen, mg/100 ml	399.0(5)	382.8(5)	35.8

[1] SED indicates standard error of the difference.
[2] The number of the determinations on which each mean is based appears in parentheses.
[3] Stypven.
[4] Varidase.
* Statistically significant.

TABLE 7

Mean of the modified thromboplastin generation test in the normal and experimental groups in the late phase of the experiment

Incubation time	Clotting time		
	Normal group	Experimental group	SED [1]
min	*sec*	*sec*	*sec*
2	144.7(10) [2]	134.8(10)	26.5
4	97.6(10)	77.1(10)	22.4
6	48.8(10)	47.2(10)	9.5
8	35.3(10)	31.5(10)	6.7
9	33.3(10)	27.8(10)	6.1
10	29.9(10)	24.2(10)	4.6
11	28.9(10)	24.0(10)	3.8
12	27.4(10)	23.3(10)	2.7
13	25.9(10)	23.8(10)	1.9
14	25.7(10)	24.2(10)	1.5

[1] SED indicates standard error of the difference.
[2] The number of the determinations on which each mean is based appears in parentheses.

Pathological studies on 11 animals in each group that continued in the experiment for a longer period of time, that is, for an average of 428 days, showed occasional vascular lesions in both groups, identical in appearance and severity to those observed in aging rats. There were essentially 2 types of lesions. One type of lesion was in 2 animals in each group. It consisted of lipid infiltration of the wall of the coronary artery. The other type of lesion was noted in one rat in the experimental group and 2 rats in the normal group. The most striking feature of this type of lesion was the formation of a plaque containing subintimal fibrin-like material and lipids. These lesions may have been inflammatory in nature as indicated by some round cell infiltration in the adventitia. Occasionally foci of round cell infiltration were observed in the myocardium of both groups representing interstitial myocarditis. This type of lesion has been described previously in aging rats (23).

Histological examination of the liver in the experimental group gave a picture identical to that commonly observed in obese rats (predominantly a periportal type of lipid accumulation).

DISCUSSION

A diet containing a very large amount of animal fat did not, under the circumstances of this experiment, produce in the rat changes in blood lipids or vascular lesions that might have been anticipated. The absence of such adverse changes, despite the development of gross obesity in these animals, may be significant, since both obesity and animal fats have been considered to be associated with lipemia and vascular lesions. It may be suggested that other dietary factors might have protected the experimental group against such changes. Yet, even if this happens to be the case, it should not detract from the significance of the fact that large amounts of saturated fat and obesity are not necessarily associated with lipemia and vascular lesions.

With respect to the possible clinical significance of these results, it must be emphasized that the pathogenesis of atherosclerosis in various species may well be influenced by different factors and hence caution must be used in the extrapolation of the experimental data obtained in the rat to the occurrence of obesity in man. Yet, it is perhaps worth noting that in the clinical field, too, in recent years, the association between obesity per se and increased levels of blood lipids has been questioned. Thus, Ross and Proger (24) reported no significant increase in the incidence of hypercholesterolemia (cholesterol over 250 mg/100 ml) in obese subjects compared with matched controls. More recently Spain and associates (6) have reported similar results in a study of 5000 male subjects in New York. These latter authors conclude that it is the overweight due to increased muscle mass rather than that due to excess fat that is related to both serum cholesterol level and the development of coronary heart disease; as such they make a clear distinction between obesity and overweight.

With respect to the relationship between obesity and cardiovascular disease, our results support the hypothesis that obesity per se is not responsible for the development of cardiovascular disease (5), and that the increased risk of coronary heart disease in obesity may well be accounted for by the common association of hypertension and decreased glucose tolerance with this disorder (24). This is also in accord with the more recent observations of the Framingham Study. This group originally (25) attributed to obesity an increased risk in the development of coronary heart disease. More recently, however, they have noted (26) that once the effect is taken into consideration of other factors that may accompany obesity such as hypercholesterolemia and hypertension, the independent contribution of obesity to the risk of the development of coronary heart disease becomes relatively small.

As to the relationship between lipemia and changes in blood coagulation and fibrinolysis, and the possible relevance of these to the development of cardiovascular lesions, the current studies appear to supplement some of our previous results (7). Previously in the rat fed a diet high in cholesterol, sodium cholate, thiouracil and butter, we observed a concurrence among gross increase of blood lipids, marked

changes in the balance of blood coagulation and fibrinolysis in the direction of clot formation, and the development of severe cardiovascular lesions. In the present investigations, however, we find that a diet that does not lead to lipemia and changes in blood coagulation and fibrinolysis does not result in the development of cardiovascular lesions, despite the development of gross obesity. Thus, in the context of previous experiments the present observations may further support the concept that in the pathogenesis of cardiovascular disease the role of obesity is of little importance beyond that of certain variables that may be associated with it.

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Amino Acid Requirements of Children:
QUANTITATIVE AMINO ACID REQUIREMENTS OF GIRLS BASED ON NITROGEN BALANCE METHOD

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ABSTRACT The problem of whether the minimal requirements of essential amino acids and of total nitrogen established previously for boys were also adequate for girls was examined. For a period of 12 to 19 days, 6 healthy 8- to 13-year-old girls were given minimal quantities of the 8 essential amino acids studied previously with boys of the same age group, but with total nitrogen varied. The youngest of the group maintained positive balance with an intake of 8 g nitrogen. Of the three 12-year-old girls, two remained in positive balance with 8 g, and the other with 12 g. Two 13-year-old girls also maintained positive balance with a nitrogen intake of 12 g. The large variation in total nitrogen requirement is attributed to individual differences in the time of onset and degree of the pubertal growth spurt. Body weight and excretion of creatinine and 17-ketosteroids remained almost constant throughout the experimental period.

In the previous papers of this series (1–6), the requirements for each of the 8 essential amino acids and for total nitrogen for 10- to 12-year-old boys were reported. The present paper extends this work to the amino acid requirements for girls of the same age group.

EXPERIMENTAL PROCEDURE AND RESULTS

Six healthy girls 8 to 13 years of age served as experimental subjects (table 1). The general procedures of the experiment have been described previously (1–4). For the first 3 days, the subjects consumed a normal diet having about the same nitrogen and caloric levels as the experimental basal diet. This period served to accustom the subjects to living in a laboratory and to minimize prior dietary effects. The basal diet contained cornstarch, corn oil, butter fat, mineral and vitamin mixtures, and the amino acid mixture. The same minimal quantity of the 8 essential amino acids which was established for boys, was given to the girls, but the total nitrogen was varied as shown in table 2. The nitrogen balance was measured throughout the experimental period of 19 days. The amount of nitrogen in the diet, urine and feces was analyzed by the semimicro-Kjeldahl method. The average daily excretion of fecal nitrogen was obtained by dividing the total output by the number of days in the period. In addition, urinary creatine and creatinine were determined by the methods reported previously (1, 2), and 17-ketosteroids by our modification of the Zimmermann reaction method (7).

The results obtained are shown in table 3. All children remained healthy and maintained constant body weight throughout the experimental period. Subject Y.S., the youngest girl, maintained positive balance with a daily intake of 8 g of total nitrogen (mixture 1) for 16 days. Two other subjects (K.T. and Y.N.) remained in positive balance not only with the intake of 10 g nitrogen (mixture 2), but also with 8 g. The study on subject K.T. could not be continued because of the occurrence of her menstrual period. Subject T.K., although of the same age as K.T. and Y.N., failed to maintain positive balance with the intake of 10 g, but remained in positive balance with 12 g of nitrogen (mixture 3). Two middle-school girls (K.Y. and S.K.) maintained positive nitrogen balance with 12 g, but not with 10 g. When they were fed 12 g, the balance became positive

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TABLE 1

Age, height, weight, basal metabolism and energy intake of subjects

Subject	Age	Body height	Body wt	Basal metabolism	Avg daily energy intake [1]
	years, months	*cm*	*kg*	*kcal/kg/day*	*kcal/kg*
Y.S.	8 6	128.6	21.1	41	60
K.T.	11 9	149.8	38.9	30	39
Y.N.	11 10	150.7	36.0	31	42
T.K.	11 11	138.7	36.4	30	49
K.Y.	13 3	159.3	44.5	25	34
S.K.	13 3	155.9	36.6	29	37

[1] Calories derived from the amino acid mixture are not included.

TABLE 2

Composition and daily intake of amino acid mixtures

Component	Mixture 1		Mixture 2		Mixture 3	
	Daily intake	Nitrogen content	Daily intake	Nitrogen content	Daily intake	Nitrogen content
	g	*g*	*g*	*g*	*g*	*g*
Essential amino acids						
L-Isoleucine			1.00	0.107		
L-Leucine			1.50	0.160		
L-Lysine			1.60	0.307		
L-Methionine			0.80	0.075		
L-Phenylalanine			0.80	0.068		
L-Threonine			1.00	0.118		
L-Tryptophan			0.12	0.017		
L-Valine			0.90	0.108		
Nonessential amino acids						
L-Alanine	5.66	0.890	7.34	1.154	9.01	1.416
L-Arginine	6.64	2.135	8.60	2.766	10.56	3.396
L-Aspartic acid	6.61	0.695	8.56	0.901	10.51	1.106
L-Glutamic acid	6.61	0.629	8.56	0.815	10.51	1.001
L-Sodium glutamate	7.67	0.635	9.94	0.823	12.21	1.011
Glycine	4.71	0.874	6.08	1.129	7.50	1.393
L-Histidine	1.00	0.271	1.00	0.271	1.00	0.271
L-Proline	2.83	0.344	3.67	0.447	4.50	0.548
L-Serine	4.25	0.567	5.50	0.733	6.76	0.898
Total (essential + nonessential)		8.000		10.000		12.000

again. The excretion of creatinine and 17-ketosteroids remained almost constant throughout the experiment.

DISCUSSION

Amino acid requirements of female adults have been reported by Leverton (8–12), Jones (13), Clark (14) and Swendseid (15, 16), but the requirements of growing girls had not been studied previously. Therefore the problem of whether the mixture of minimal requirements of 8 essential amino acids established previously for boys is also adequate for girls was examined in the present study. The results showed that the minimal amounts of essential amino acids established for boys were also adeqate for girls, although the minimal requirement of individual essential amino acids for girls has not been investigated. It is difficult to define the zone for allowance of retention that is necessary not only for maintenance of positive nitrogen balance, but also for growth and development. We estimate that 0.38 g of nitrogen is a safe daily intake for retention consistent with the demands for growth in 10- to 12-year-old boys.

TABLE 3

Nitrogen balance and urinary excretion of creatinine, creatine and 17-ketosteroids

Subject	Period	Body wt	Daily N intake	Avg daily N output		Avg daily N balance	Avg daily urinary excretion		
				Urine	Feces		Creatinine	Creatine	17-Ketosteroids
	days	*kg*	*g*	*g*	*g*	*g*	*mg*	*mg*	*mg*
Y.S.		21.1 [1]					463 [2]	260 [2]	
	4	21.4	8.16	7.50	0.19	+0.47	474	245	
	4	21.2	8.16	6.43		+1.54	469	220	
	4	21.1	8.15	6.59	0.25	+1.31	550	272	
	4	20.5	8.15	7.15		+0.75	421	333	
K.T.		38.9 [1]					737 [2]	99 [2]	
	4	38.4	10.18	7.61	0.14	+2.43	798	151	1.09
	4	38.8	10.18	8.47		+1.57	747	113	0.88
	4	38.8	8.18	6.54	0.14	+1.50	752	60	0.95
Y.N.		36.0 [1]					563 [2]	136 [2]	
	4	36.3	10.17	6.67	0.15	+3.35	576	227	0.72
	4	37.0	10.17	6.83		+3.19	443	141	0.55
	4	36.2	8.17	5.19	0.22	+2.76	544	188	0.70
	4	35.9	8.17	5.41		+2.54	459	200	0.63
T.K.		36.4 [1]					771 [2]	218 [2]	
	4	36.0	10.18	10.81	0.29	−0.92	917	356	1.16
	4	36.5	10.18	10.12		−0.23	811	290	1.12
	4	37.3	12.18	11.28	0.53	+0.37	783	405	1.05
	4	36.1	12.18	11.36		+0.29	775	472	1.27
K.Y.		44.5 [1]					1074 [2]	31 [2]	
	4	43.8	12.18	10.26	0.29	+1.63	1040	67	1.64
	4	44.2	12.18	11.40		+0.49	1100	166	1.28
	4	44.2	10.16	10.21	0.26	−0.31	1130	256	1.13
	3	44.6	10.16	9.82		+0.08	1088	285	1.14
	4	44.6	12.16	10.98	0.24	+0.94	1000	196	1.10
S.K.		36.6 [1]					810 [2]	13 [2]	
	4	36.5	12.16	8.05	0.26	+3.85	732	11	1.01
	4	37.3	12.16	9.76		+2.20	820	35	1.14
	4	36.9	10.16	9.83	0.44	−0.11	935	128	1.17
	3	36.5	10.16	9.61		+0.11	868	67	1.23
	4	36.4	12.16	11.52	0.51	+0.13	897	47	1.06

[1] Initial body weight.
[2] Average of urinary excretion for 3 days, when consuming a normal diet.

In a previous experiment, boys maintained positive nitrogen balance at levels of 11 and 12 g of nitrogen. Girls of the same age group showed greater individual variation with respect to nitrogen in maintaining positive nitrogen balance. Possibly this is because the growth spurt in girls occurs earlier than in boys of this age. Moreover, individual variation in the onset and degree of the pubertal growth spurt may be related to the difference in requirements among the girls of the same age. Further studies are required to clarify this point. Yoshimura (17) reported that after feeding a low protein diet the urinary excretion of 17-ketosteroids decreased, although the nitrogen equilibrium was maintained. In the present study, however, the excretion of 17-ketosteroids remained almost constant throughout the experiment.

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Effect of Age and Dietary Fat on Serum Protein Components of the Rat

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ABSTRACT The effects of age and dietary fat on the concentration of the protein components in the sera of rats were investigated. Two groups of diets were studied: 1) a semipurified diet with the level and kind of fat varied; and 2) the same semipurified diet but with 25% replaced by dried, cooked egg and the kind but not level of fat varied. Of the serum protein components — pre-albumin, albumin + α_1-globulin, α_2-, β- and γ-globulins — pre-albumin concentration was most dependent on diet. Amount and incidence of pre-albumin varied with age and with diet, particularly with the kind and level of fat. Differences observed with the kind of fat did not appear to relate to any specific characteristic of the fat. The age at which high levels of pre-albumin occurred varied with dietary fat. The albumin and α_1-globulin, the β- and the γ-globulin all tended to be influenced by diet with the differences generally related to level of fat. The α_2-globulin was least influenced by diet. At high levels of pre-albumin, concentration of all except α_2-globulin decreased. With age, albumin + α_1-globulin decreased and the other globulins increased.

Several factors — heredity, environment, age, sex, water intake, nutritional history — may affect the electrophoretic distribution of the major, well-recognized serum protein components (1–3). Proteins migrating more rapidly than albumin (pre-albumin) have also been observed in serum (4). Halliday and Kekwick (5) reported that the concentration of serum pre-albumin was higher in young rats than in older animals. In the sera of normal individuals in a state of alimentary hyperlipemia, Herbst and Hurley (6) have observed significant amounts of this component following intravenous injection of heparin. Investigations in this laboratory have shown the presence of high levels of this component in the sera of rats fed a diet containing 25% whole egg (7). This report deals with further studies to determine the combined effect of diet and age and the extent to which dietary fat may influence the presence or absence of pre-albumin as well as the proportion of the other serum proteins.

EXPERIMENTAL

The diets fed were as follows:

Diet 1: SP 8 HVO, a semipurified diet containing 16 g casein, 8 g lactalbumin, 8 g hydrogenated vegetable oil (HVO),[1] 10 g brewer's yeast, 4 g modified Osborne and Mendel salt mixture (8), 2 g cellulose flour and 52 g sucrose/100 g of diet.

Diet 2: SP 8 HVO containing 0.6 g crystalline cholesterol/100 g of diet.

Diet 3: SP 16 HVO, as no. 1 except 16 g HVO and 44 g sucrose/100 g of diet.

Diet 4: SP 16 Blend, as diet 3 except HVO was replaced by a blend of fats similar in fatty acid composition to diets in the United States based on the Household Food Consumption Survey of 1955 (9). The approximate composition of the blend was 39% saturated fatty acids, 46% oleic acid and 9% linoleic acid.

Diet 5: SPE-HVO, 75 g SP 8 HVO + 25 g dried cooked egg.

Diets 6 to 10: The 6 g HVO in diet 5 was replaced by 6 g corn oil, safflower oil, lard, butter or the blend of fats. Each rat received weekly percomorph oil supplying approximately 3000 units of vitamin A and 400 units of vitamin D and in addition 36 mg of α-tocopheryl acetate in 0.01 ml cottonseed oil; 10 g fresh kale were fed twice a week. The fat content of diets 1 and 2 was 9%, of diets 3 and 4, 17%, and of diets 5 to 10, 19%. The cholesterol content of the egg-containing diets was 0.5%, approximately that of diet 2.

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[1] Crisco, Procter and Gamble, Cincinnati; containing approximately 10% linoleic acid.

Male BHE (10) rats were weaned at 21 days of age and caged individually in a room in which the temperature and humidity were maintained at approximately 26.7° and 40%, respectively. From weaning, each animal was fed ad libitum one of the 10 experimental diets, with water available at all times. At scheduled intervals rats were killed following an overnight fast. At the time of killing, the animal was anesthetized with sodium amytal (10 mg/100 g of body weight), the blood removed from the left ventricle of the heart and tissues excised for histological examination.

Prior to electrophoretic analysis, the serum was diluted 1:5 with veronal buffer (pH 8.6, 0.1 μ) and dialyzed against the buffer overnight at 10°. The protein components of the serum were analyzed by moving boundary electrophoresis (Aminco portable apparatus) and the percentages and mobilities of the individual components were calculated from the ascending Schlieren patterns. The total protein and nonprotein nitrogen content of some of the sera were determined by a micro-Kjeldahl method using a 2-step mixed indicator (11). Significant differences within a group were determined by means of the *t* test and comparisons were made at the 5 and 1% levels of probability.

RESULTS AND DISCUSSION

Total serum protein concentrations (5.7 to 6.9) fell within the range generally reported for the rat (12). Although some differences related to age and diet were significant, they were not sufficiently large to influence interpretation of the results included in this report.

The mobilities of the major protein components — albumin, α_1-, α_2-, β- and γ-globulin — were also comparable with values reported in the literature for the rat (13). Neither diet nor age influenced the mobilities of these components. Although proteins migrating more rapidly than albumin were not always present, they were observed in the sera of some of the rats with each of the experimental diets. Pre-albumin mobility was not influenced by age and was consistently lower when rats were fed SP 8 HVO (6.7 $cm^2\ v^{-1}\ sec^{-1}$) than when fed any of the other diets (7.2 to 7.6 $cm^2\ v^{-1}\ sec^{-1}$).

The effect of diet and age on the occurrence and concentration of serum pre-albumin is shown in table 1. In this table, as well as in succeeding tables, data for the serum components are expressed as percentage of total protein. Data are not included for animals that were losing weight consistently or that were moribund when killed. The results have been combined when there were no consistent differences between age groups.

Pre-albumin was observed in sera from some of the rats, regardless of the diet fed. The percentage of sera containing pre-albumin, as well as the relative concentration of this component varied with diet and with age. The addition of cholesterol to the semipurified diet (8 HVO) caused no significant change in level of pre-albumin nor incidence of pre-albumin in sera of these animals. With these two low fat-containing diets the percentage of pre-albumin was significantly higher ($P < 0.01$) in the sera of 550-day-old rats than in the sera of younger rats.

With the 2 diets containing the higher level of fat without egg, the percentage of pre-albumin tended to be high at all ages and was significantly higher in sera of rats 350 and 450 days old than in rats of comparable age fed at the lower level of fat without egg.

Although the diets containing egg were identical in all respects except for the kind of fat accompanying the egg fat, some significant differences were noted. When the fat was HVO, the results for rats 250 days or younger were similar to those obtained when the diet contained the low level of HVO without egg. By 350 days, the percentage of rats with pre-albumin in their sera [2] as well as the concentration was significantly higher ($P < 0.01$) than was observed in the younger rats and was similar to that observed when the diets contained a high level of fat without egg. With the blend of fats or with safflower oil the results were similar in general to those observed with HVO. When the SPE diet contained butter, lard or corn oil, levels of pre-albumin were generally low, with this component absent in 250-day-old rats fed lard or corn oil. In contrast, pre-albumin

[2] When dealing with percentage of rats with pre-albumin in their sera, significance was based on Chi-square analysis.

TABLE 1

Effect of diet and age on the occurrence and concentration of pre-albumin (PA) in rat serum

Diet and age (days)	Total no. of rats	Rats with pre-albumin	PA, % of protein avg [1]
		%	
SP Diet without egg (SP)			
8 HVO			
150	5	20	3.3
250	6	33	4.9 ± 1.6 [2]
350, 450	10	40	3.0 ± 0.3
550	6	50	10.5 ± 3.6
8 HVO + cholesterol			
250	4	50	2.8 ± 0.2
350, 450	10	40	4.2 ± 0.4
550	4	75	9.3 ± 2.6
16 HVO			
350	9	67	9.3 ± 2.0
450	9	56	8.2 ± 2.2
550	5	40	10.1 ± 2.6
16 Blend			
350	7	71	13.8 ± 2.3
450	11	45	7.4 ± 1.6
SP Diet with egg (SPE)			
HVO			
150	5	0	
250	18	39	5.0 ± 1.0
350	13	100	10.8 ± 1.6
450, 550	10	40	11.6 ± 6.6
Blend			
250	8	75	8.0 ± 2.2
350, 450	12	83	12.8 ± 2.7
Safflower oil			
250	8	75	9.8 ± 4.1
350, 450	10	80	11.3 ± 3.3
Butter			
250	5	100	5.7 ± 1.1
350, 450, 550	11	82	6.1 ± 0.8
Lard			
250	5	0	
350, 450, 550	12	58	5.9 ± 1.2
Corn oil			
250	4	0	
350, 450, 550	11	73	5.0 ± 0.9

[1] Average based on values for rats with PA in their sera.
[2] Mean ± SE.

was present in 100% of the sera from 250-day-old rats fed the butter-containing diet. The level of pre-albumin in the sera of the older rats fed butter or corn oil was significantly lower ($P < 0.05$) than that in the sera of rats of comparable age fed SPE-HVO. Although the results reported indicate that the presence and concentration of a rapidly moving component(s) is influenced by the kind and level of dietary fat, the differences observed do not appear to relate to any specific characteristic of the fat ingested. Lard with a high concentration of saturated fatty acids yielded results similar to those with corn oil, which has a high concentration of polyunsaturated fatty acids. The level of pre-albumin in the sera of rats fed the diets containing corn or safflower oil differed significantly, although both of these oils contained high concentrations of polyunsaturated fatty acids.

Preliminary investigations in this laboratory on the chemical nature of pre-albumin have shown this fraction to contain a lipid moiety.[3] By the use of isotopes and thin-layer chromatography Glover and Joo [4] have determined the kinds of lipid material in a pre-albumin fraction obtained on paper electropherograms. They have concluded that this fraction may play a significant part in the transport and uptake of lipids by tissues since it is a molecular aggregate smaller in size than serum lipoproteins and the lipid components are more labile than those in the lipoproteins. In contrast, Losticky (14) has reported that the lipid pre-albumin of immunoelectropherograms is formed by the binding of unesterified fatty acids to α_1-lipoprotein after the unesterified fatty acids are released by lipase from triglycerides of low-density lipoproteins. More information is needed to establish the chemical nature of these rapidly moving components and to determine their role in the transport of fat.

Nephrosis has been found to be the major cause of death of BHE rats and to be accelerated by certain dietary combinations (15). This condition occurred even in animals that were maintaining weight at the time of killing. Summarized in table 2 are data relating pre-albumin concentration of serum to extent of kidney damage based on microscopic examination. The procedure used for rating damage in the kidney is described in detail elsewhere (15). Although small amounts of pre-albumin were present in the sera of 36% of the animals with kidneys showing no or little evidence of structural change (rating of 1.0 or less), both the incidence and concentration of pre-albumin showed a consistent increase with increasing kidney damage. Significant amounts of a rapidly moving component in sera of some nephrotic patients have also been reported (16, 17).

Data to show the influence of diet and age on the five major protein components are presented in table 3. A combined value for albumin and α_1-globulin is given because of the poor separation of these components in some sera. The results are reported for 2 levels of pre-albumin, zero to 6%, and 6% and over, because the presence of an additional component in appreciable amounts necessarily influences the proportions of the other proteins. The influence of the presence of pre-albumin on the relative concentration of the other serum proteins was too small to be significant when the level of pre-albumin was below 6%. Data for rats more than 450 days old were limited due to poor survival with some of the diets, particularly those containing egg.

The influence of diet on the relative percentage of the various serum proteins was apparent in the results for 250- to 450-day-old rats having less than 6% pre-albumin in their sera. The concentrations of the proteins in the sera of rats fed the 3 diets containing HVO as the chief source of fat were similar except for γ-globulin concen-

[3] Lakshmanan, F. L. 1963 Factors influencing the presence of rapidly migrating serum protein component(s), PA. Federation Proc., 22: 608 (abstract).
[4] Glover, J., and C. N. Joo 1963 Nature and origin of the lipids in the pre-albumin fraction of electropherograms of blood serum. Biochem. J., 88: 65P (abstract).

TABLE 2

Relation of the degree of nephrosis to the occurrence and concentration of pre-albumin (PA) in rat serum

Kidney histology [1]			Total no. of rats	Rats with pre-albumin	PA, % of protein avg [2]
Hyalin	Cystic	Glomerular			
				%	
0.0	0.0	0.0	50	20	3.9 ± 0.4 [3]
1.0	0.0	0.0	79	47	5.5 ± 0.5
2.0	0.0	0.0	14	71	6.3 ± 1.1
1.0	1.2	0.3	11	82	7.7 ± 1.3
2.0	1.0	0.0	25	84	10.3 ± 1.6
2.0	2.4	1.3	22	91	12.1 ± 1.5
3.0	2.7	1.5	20	100	12.3 ± 1.9

[1] Rating based on a maximal rating of 4.0 for each type of damage.
[2] Average based on values for rats with PA in their sera.
[3] Mean ± SE.

TABLE 3

Effect of diet and age on the protein components of sera classified according to level of pre-albumin

Diet	Total no. of rats	Concentration of protein components, % of protein			
		Albumin and α_1-globulin	α_2-Globulin	β-Globulin	γ-Globulin
Zero to 6% pre-albumin, 250 to 450 days of age					
SP 8 HVO	10	65.2 ± 1.6 [1]	8.3 ± 0.4	16.7 ± 0.7	8.6 ± 1.1
SP 8 HVO + cholesterol	10	68.0 ± 1.1	7.9 ± 0.5	16.1 ± 0.5	6.3 ± 0.8
SP 16 HVO	12	69.6 ± 1.8	8.2 ± 0.4	15.6 ± 1.0	4.8 ± 0.7
SP 16 Blend	9	73.0 ± 1.3	7.9 ± 0.7	14.5 ± 0.9	4.2 ± 0.6
SPE-HVO	8	72.9 ± 2.1	6.9 ± 0.5	14.2 ± 1.3	4.4 ± 0.9
SPE-Blend	4	73.3 ± 2.7	7.1 ± 0.6	12.8 ± 3.3	5.2 ± 1.4
SPE-Safflower oil	5	72.3 ± 1.2	7.9 ± 0.9	13.8 ± 0.9	3.5 ± 0.7
SPE-Butter	7	73.4 ± 1.7	8.4 ± 0.7	11.6 ± 1.0	3.3 ± 0.5
SPE-Lard	6	71.4 ± 1.8	8.4 ± 0.6	14.4 ± 1.2	5.2 ± 0.9
SPE-Corn oil	8	65.8 ± 1.0	9.1 ± 0.8	15.6 ± 0.9	6.8 ± 0.3
More than 6% pre-albumin, 250 to 450 days of age					
SP 16 HVO	6	62.2 ± 0.8	9.0 ± 1.3	12.6 ± 1.5	3.9 ± 0.7
SP 16 Blend	9	64.3 ± 1.4	8.7 ± 0.6	12.1 ± 1.0	3.5 ± 1.0
SPE-HVO	13	66.9 ± 1.6	7.8 ± 0.5	9.4 ± 0.7	2.6 ± 0.6
SPE-Blend	8	67.5 ± 2.5	6.8 ± 0.5	8.7 ± 0.6	1.9 ± 0.6
SPE-Safflower oil	5	69.9 ± 2.3	7.1 ± 1.3	6.5 ± 1.2	0.9 ± 0.5
SPE-Lard	2	73.5 ± 2.2	7.0 ± 2.3	8.2 ± 2.4	2.0 ± 0.7
Zero to 6% pre-albumin, over 450 days of age					
SP 8 HVO	3	54.4 ± 2.0	11.2 ± 1.5	21.6 ± 1.3	12.7 ± 1.8
SP 8 HVO + cholesterol	2	56.3 ± 1.5	11.1 ± 1.6	19.6 ± 0.4	10.8 ± 0.4
SP 16 HVO	3	62.8 ± 1.9	9.0 ± 1.4	21.3 ± 1.9	7.0 ± 1.6
SPE-HVO	2	64.5 ± 0.6	10.3 ± 0.8	18.2 ± 0.4	6.9 ± 0.3
SPE-Lard	2	46.0 ± 6.0	12.0 ± 2.2	25.6 ± 0.7	14.4 ± 2.2
SPE-Corn oil	2	59.1 ± 0.3	12.6 ± 0.2	15.9 ± 0.8	9.0 ± 1.4

[1] Mean ± SE.

tration which was significantly higher ($P < 0.01$) in sera of rats fed SP 8 HVO than in sera of rats fed SP 16 HVO. In general, the distribution of proteins in the sera of rats fed SP 16 Blend and the egg-containing diets was similar except for SPE-corn oil. When compared with the results for SP 8 HVO, the major differences were in the higher levels of albumin and α_1-globulin and in the lower levels of γ-globulin, which were significant for all except γ-globulin when the diet was SPE-Blend. In contrast, the results with corn oil resembled those obtained with SP 8 HVO rather than those obtained with the other egg-containing diets. The concentration of β-globulin was significantly lower ($P < 0.01$) in the sera of rats fed SPE-butter than in the sera of rats fed SP 8 HVO with or without cholesterol. The serum protein component least affected by diet was α_2-globulin.

When the level of pre-albumin exceeded 6%, diet comparisons were limited because of the lack of high levels of this component with some diets. With this group of sera having a high concentration of pre-albumin, β-globulin was the component most influenced by diet and tended to be low when the diet contained egg. The concentration of γ-globulin was extremely low with the diet containing safflower oil.

When the concentration of pre-albumin exceeded 6% there was a consistent tendency for the levels of albumin and α_1-globulin, β-globulin and γ-globulin to decrease when compared with the levels in sera of rats fed the same diet but with a concentration of pre-albumin of less than 6%. The decrease in albumin and α_1-globulin concentration was significant ($P < 0.01$) when SP 16 HVO or SP 16 Blend was fed; the decrease in β-globulin concentration was significant ($P < 0.01$) when SPE-safflower oil was fed. The sera of rats fed SPE-HVO had significantly reduced amounts of albumin and α_1-globulin ($P < 0.05$) as well as β-globulin ($P < 0.01$). γ-Globulin concentrations tended to be lower when pre-albumin levels were high.

These differences in concentration may actually represent decreases in the α- or β-lipoproteins instead of the globulins, since each boundary of the electrophoretic pattern is the total amount of protein, lipoprotein, and glycoprotein present. Herbst and Hurley (6) have demonstrated that sera of normal hyperlipemic (alimentary-induced) individuals injected with heparin had decreased amounts of β-globulin in their moving boundary electrophoretic patterns as well as the presence of a component migrating ahead of albumin. In their study simultaneous examination of the lipoprotein and protein patterns obtained by paper electrophoresis revealed that the lipid band, normally present in the β-globulin area, was now in the area of α_2- or α_1-globulin. Instead of the lipid band, normally present in the α_1-globulin area, lipid material migrated ahead of albumin.

Despite the limited numbers in the older age group in this present study, it was obvious that the relative concentration of the various serum proteins was influenced by age. Albumin and α_1-globulin decreased with age; α_2-, β- and γ-globulin increased with age. These differences are similar to those reported in the literature for humans and animals (18–20).

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Effect of High Intakes of Thiamine, Riboflavin and Pyridoxine on Reproduction in Rats and Vitamin Requirements of the Offspring [1]

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ABSTRACT Female rats were fed during pregnancy and lactation a control diet containing 0.25 mg pyridoxine, 0.4 mg thiamine, and 0.4 mg riboflavin/100 g, or a high pyridoxine, high thiamine, or high riboflavin diet containing the specified vitamin at 25 times the level of the control diet. The levels of these vitamins in the carcasses of the young at birth and in the liver at weaning were determined. The effect of maternal vitamin intake on the vitamin requirements of the young was tested in 2 ways: 1) by comparing the rate of depletion of the young of females fed the high vitamin diets with the rate of depletion of the control group, and 2) by the growth response of the depleted young to graded levels of the vitamin fed in excess in the maternal diet. It was concluded that high intakes of thiamine, riboflavin, or pyridoxine during the reproductive period had no effect on the young, as shown by litter size at birth, growth until weaning, or their vitamin requirements after weaning.

Relatively little attention has been given to the effects of high intakes of B vitamins on reproduction. Massive doses of thiamine have been reported to interfere with lactation, produce cannibalism, and decrease fertility in rats (1, 2). Richards (3) also reported that excess thiamine fed to female rats increased mortality and decreased the weights of the young at weaning. However, Morrison and Sarett (4) reported normal reproduction in rats fed high levels of thiamine. An abnormally high need for pyridoxine was reported in a newborn human infant whose mother had received large doses of pyridoxine intramuscularly during the first trimester of pregnancy (5). It was suggested that excess pyridoxine intake during gestation may have increased the pyridoxine requirement of the infant. Later, Hunt (6) reported that the pyridoxine intake of the dam during gestation did not affect the rate of vitamin B_6 depletion or increase the occurrence of convulsions in young rats after birth. Morrison and Sarett (4) also reported that a high intake of pyridoxine during pregnancy did not affect reproduction or increase the rate of vitamin B_6 depletion in young rats in the period from 3 to 5 weeks of age.

The preceding studies have shown that high levels of pyridoxine in the maternal diet did not appear to increase the vitamin B_6 requirement of young rats as indicated by rate of vitamin B_6 depletion. If this had been the case, then vitamin B_6 deficiency should have developed more rapidly in the young from the dams fed the high pyridoxine diets. No test was made, however, of the pyridoxine requirement of the young rats after they had been depleted of pyridoxine. This point is important, since an increased need for pyridoxine in the young during the depletion period may have been masked by the higher liver stores of the young from the dams fed the high pyridoxine diets. Greater liver stores were reported by Morrison and Sarett (4).

The following experiment tested the effect of maternal intake of pyridoxine, thiamine, or riboflavin on the rate at which the young rats were depleted of each vitamin, as well as on the subsequent growth response of the depleted young to graded levels of the vitamins.

METHODS

Animals. Thirty female rats (about 300 g) of the Long-Evans strain, were used for each of 3 experiments. In each experi-

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ment, 15 females were fed the basal diet.[2] The other rats were divided into 3 groups of 5 rats each. One group was fed the high pyridoxine diet, the second group the high thiamine diet, and the third group the high riboflavin diet. The high vitamin diets contained 25 times the level of pyridoxine, thiamine, or riboflavin used in the basal diet. The basal diet contained: (in mg/100 g) pyridoxine, 0.25; thiamine, 0.4; and riboflavin, 0.4 (7). The high vitamin diets contained: (in mg/100 g) pyridoxine, 6.25; thiamine, 10; and riboflavin, 10. Food and water were provided ad libitum. Weight gain and food intake were recorded 3 times weekly.

The females were mated after they had been fed the experimental diets for 2 weeks, and these diets were continued during gestation and lactation. The number and average weight of the newborn were recorded as soon as possible after the birth of each litter. Some of the newborn from each diet group were decapitated, the stomachs and intestinal tracts removed, and the carcasses weighed and frozen. The remaining young were nursed for 21 days, when they were weaned. The young had access to the maternal diet during the nursing period. At weaning, representative young from each group were decapitated, the livers removed, weighed and frozen. A number of the male weanlings, weighing from 40 to 50 g, were saved for the growth study.

The growth study was divided into a depletion period and a supplementation period. The rats were housed individually in galvanized, screen-bottom cages. Food and tap water were given ad libitum. Weight gains and food intakes were recorded 3 times weekly. Four to five animals from the high vitamin maternal groups were fed the basal control diet, with the omission of the vitamin which had been fed in excess in the maternal diet. Weanlings from the dams fed the basal diet were also fed the vitamin-deficient diets. The remaining rats from each maternal group served as controls and were fed the complete basal ration. All of the diets were fed until the deficient groups ceased to gain (weight plateau) or until their rate of gain was significantly less than that of the appropriate control group.

Once depletion was established, the animals were fed the appropriate vitamin at a below-minimal or minimal level. The minimal level for pyridoxine was 15 μg/rat/day (4);[3] for thiamine, 10 μg/rat/day (8); for riboflavin, 24 μg/rat/day (7). The below-minimal levels were one-half of the minimal level. Negative controls (without pyridoxine, thiamine, or riboflavin) also continued to be fed the deficient diets.

Each level of the vitamin was made up in a 20% ethyl alcohol solution. Two milliliters of this solution were fed 3 times a week. The total supplement over a period of one week was equivalent to 7 times the daily amount indicated.

Vitamin analyses. The carcasses of the newborn and the livers of the weanlings were homogenized and analyzed for pyridoxine, thiamine, and riboflavin. Pyridoxine was determined microbiologically with *Saccharomyces carlsbergenesis* in a modification of the method of Rabinowitz and Snell (9). Thiamine was determined by the thiochrome method and riboflavin was determined fluorometrically (10).

RESULTS

Reproduction. Reproduction data are shown in table 1. The reproduction of the control group and the high thiamine group was considerably better (68% for both groups) than that of the high riboflavin or high pyridoxine groups (38 and 47%, respectively). The value of 68% was slightly less than that usually found for the stock colony females of this laboratory (70 to 75%).

The average birth weight, number of young per litter, and average weight of the young at weaning were not signifi-

[2] Composition of basal diet: (g/100 g) vitamin-free casein, 18; sucrose, 61.8; USP Salts 14, 4; Cellu Flour (Chicago Dietetic Supply House, Chicago), 5; cottonseed oil, 9; vitamin-fortified cottonseed oil, 1; vitamin mix in sucrose, 1; choline chloride, 0.15. The fortified oil provided per 100 g diet: vitamin A, 1700 IU; vitamin D_2, 100 IU; α-tocopheryl acetate, 6.7 mg. The vitamin mixture in sucrose provided: (mg/100 g diet) thiamine, 0.4; riboflavin, 0.4; pyridoxine, 0.25; Ca pantothenate, 2.0; inositol, 10.0; biotin, 0.01; folic acid, 0.1; niacinamide, 1.0; vitamin B_{12} (0.1% B_{12} triturate in mannitol), 0.02; menadione, 0.5.

[3] The minimal requirement for pyridoxine for maximal growth in the rat beyond 6 weeks of age has recently been shown to be greater than 30 μg/day (Beaton, G. H., and M. C. Cheney, Federation Proc., 24: 624, 1965; Williams, M. A., Federation Proc., 24: 624, 1965). However 15 μg/day will produce nearly maximal growth in the rat for the first 2 weeks after weaning or the first 2 weeks of repletion of previously depleted rats (Williams, M. A., unpublished results).

TABLE 1

Reproduction data on animals receiving a diet containing levels of thiamine, riboflavin or pyridoxine 25 times that of the control level [1]

Maternal diet	No. of mothers	No. of litters	No. of young	Average birth wt	Survival	Average wt at 21 days
				g	%	*g*
Control [1]	40	27 [2] (68%)	295	6.2	78.6 (214) [3]	45.2
+ thiamine (10 mg/100 g diet)	19	13 (68%)	105	6.6	89.4 (96)	45.6
+ riboflavin (10 mg/100 g diet)	13	5 (38%)	51	6.2	60.0 [4] (40)	47.9
+ pyridoxine 6.25 mg/100 g diet)	15	7 (47%)	64	6.1	83.0 (53)	45.8

[1] Control diet contained: (mg/100 g diet) thiamine, 0.4; riboflavin, 0.4; pyridoxine, 0.25.
[2] Cumulative figure; each experiment included a control group of approximately 10 animals.
[3] Numbers in parentheses refer to number of observations on which percentage is based.
[4] Ten offspring that did not survive in this group were all of one litter; therefore the apparent difference loses significance.

TABLE 2

Effect of the level of thiamine, riboflavin or pyridoxine in the maternal diet on the storage of the vitamin in the fetal carcass and in liver of offspring at weaning

Maternal diet	Fetal carcass storage			Liver storage		
	Thiamine	Riboflavin	Pyridoxine	Thiamine	Riboflavin	Pyridoxine
	μg/g	*μg/g*	*μg/g*	*μg/g*	*μg/g*	*μg/g*
Control [1,2]	0.93 ± 0.27 [3] (5) [4]	3.43 ± 0.28 (8)	0.79 ± 0.15 (7)	5.81 ± 0.81 (8)	21.48 ± 2.53 (5)	7.43 ± 1.84 (10)
+ thiamine (10 mg/100 g diet)	0.95 ± 0.11 (5)	—	—	10.13 ± 0.75 [5] (8)	—	—
+ riboflavin (10 mg/100 g diet)	—	3.26 ± 0.19 (10)	—	—	22.41 ± 3.64 (5)	—
+ pyridoxine (6.25 mg/100 g diet)	—	—	1.23 ± 0.11 [5] (6)	—	—	7.12 ± 0.52 (10)

[1] Control diet contained: (mg/100 g diet) thiamine, 0.4; riboflavin, 0.4; pyridoxine, 0.25.
[2] Each vitamin analysis had its own control group.
[3] Mean ± SE.
[4] Numbers in parentheses indicate number of samples analyzed.
[5] Difference is statistically significant ($P < 0.01$).

cantly influenced by increasing the levels of pyridoxine, thiamine, or riboflavin in the maternal diet. The number of offspring per litter from the dams fed the high thiamine ration was somewhat smaller than that from the other maternal groups. The average birth weight, however, was somewhat greater in the high thiamine group. The higher mortality (birth to weaning) in the high riboflavin group reflected the complete loss of one litter (10 young). Two control litters were also lost because the mothers failed to nurse. More data are needed to determine whether the high riboflavin diet increased mortality.

Tissue vitamin storage. High levels of thiamine or riboflavin in the maternal diet did not increase the levels of vitamins in the newborn (table 2). The level of pyridoxine in the newborn from the dams fed the high pyridoxine diet was significantly higher than the level for the control group ($P < 0.01$). At weaning, the offspring of the females fed the high thiamine diet had nearly twice as high a level of liver thiamine as did the controls. In contrast with the effect of thiamine, high levels of riboflavin or pyridoxine in the maternal diet did not influence the storage of these 2 vitamins in the livers of the weanlings. The difference caused by maternal diet in the tissue concentration of pyridoxine at birth did not appear at weaning (21 days after birth).

Growth studies — depletion and supplementation. Figure 1 shows the growth curves of the different groups when fed diets deficient in pyridoxine, thiamine, or riboflavin, as well as the control groups. The only difference observed during depletion was that the onset of thiamine deficiency was delayed slightly in the rats from the dams fed the high thiamine diet. Maternal diet did not affect the development of riboflavin deficiency, in which both groups reached a plateau in body weight by day 21. The depletion period of vitamin B_6 was arbitrarily ended on day 28 when the weight gains of the deficient groups were significantly less than the gains of the groups fed the control diet. Maternal pyridoxine intake did not affect pyridoxine depletion in the young (fig. 1). Neither did the maternal diet affect the growth response of any of the groups to the control diet.

Figure 2 shows the growth response of the vitamin-depleted rats to graded levels of the corresponding vitamin. All groups received the supplements for 14 days. Growth reflected the level of supplementa-

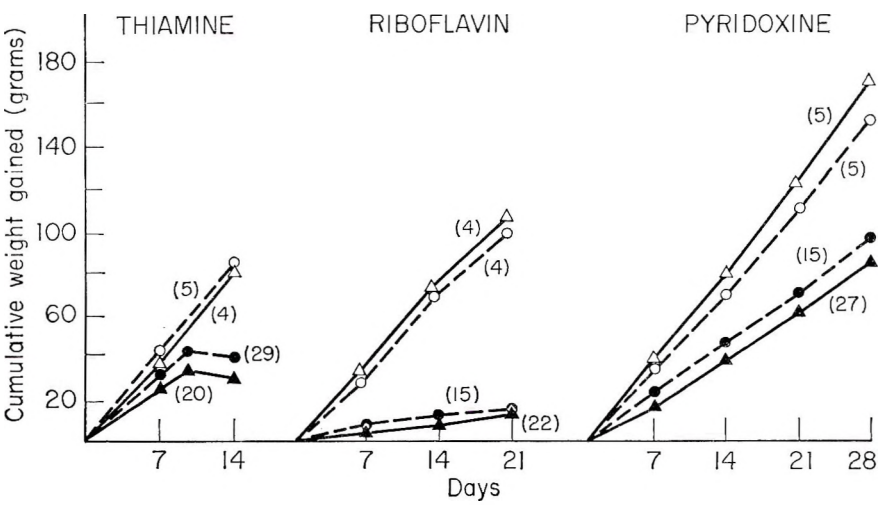

Fig. 1 Influence of excess thiamine, riboflavin, or pyridoxine in maternal diet on weight gain of male weanling rats fed a diet deficient in the respective vitamin. △———△, control young fed adequate diet; ○– –○, experimental young fed adequate diet; ▲———▲, control young fed depletion diet; ●– – –●, experimental young fed depletion diet. Numbers in parentheses indicate number of rats in the group.

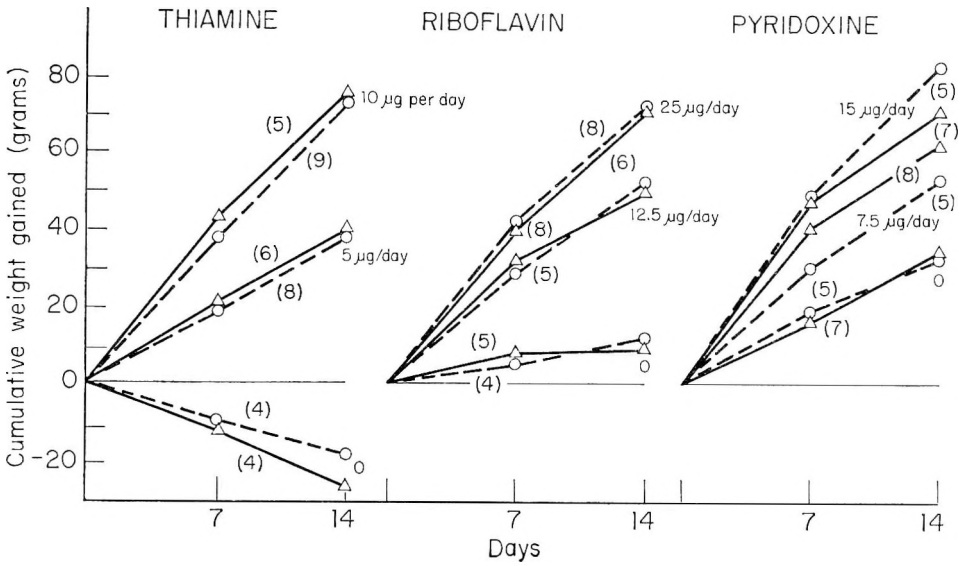

Fig. 2 Influence of excess thiamine, riboflavin, or pyridoxine in maternal diet on weight gain of previously depleted male rats fed graded levels of that vitamin. △——△, control young; ○- - -○, experimental young. Numbers in parentheses indicate number of rats in the group.

tion for each vitamin, and maternal diet had no significant effect. The greater variability in the growth of both pyridoxine-depleted groups to supplements of 7.5 or 15 µg pyridoxine is typical of the response of pyridoxine-depleted rats fed at these levels of the vitamin.[4] Nevertheless, the rats from the dams fed the high pyridoxine diet made weight gains similar to those from the control dams. If the need for pyridoxine had been greater in the young from the high pyridoxine dams, then their weight gains at both levels of pyridoxine would have been less than those of the control young. This lack of a difference in pyridoxine need between the young from the high pyridoxine dams and the control group is supported by the similar weight changes in the pyridoxine-depleted groups that continued to be fed the pyridoxine-deficient diet.

DISCUSSION

The reduced number of litters for the high riboflavin and high pyridoxine groups may be related to the high maternal intake of these vitamins. More data are needed to establish this point. With respect to the number of young per litter and the average birth and weaning weights, all of the high vitamin groups and the control group compared favorably in performance with the laboratory breeding colony. The results from the high thiamine diet confirm the conclusions of Morrison and Sarett (4) that high intakes of thiamine did not affect the overall reproductive performance, in contrast with the report of Richards (3).

The level of thiamine (0.93 µg/g) in the tissues of the newborn from either the control or the high thiamine group was less than that reported by Barrett and Everson (11) and by Brown and Snodgrass (12). Barrett and Everson reported a value of 3.0 µg/g with a maternal diet containing 1.32 mg/100 g. Brown and Snodgrass reported a level of approximately 2 µg/g with a maternal diet containing 0.25 or 0.50 mg/100 g. There is no explanation for these differences except that the analyses were not made on comparable tissues. In the present study, the vitamin analyses were made on the exsanguinated carcass, with the head, stomach, and intestinal tract removed. The other reports imply that the analyses were made on the entire animal. Fetal storage of riboflavin (3.34 µg/g) was similar to

[4] Williams, M. A., unpublished results.

the values of Barrett and Everson (2.5 μg/g). No values for fetal storage of pyridoxine were found in the literature.

At weaning, liver storage of thiamine reflected the thiamine level of the maternal diet although the maternal diet did not affect the carcass thiamine of the newborn. The higher level at weaning may reflect the amount of maternal diet eaten by the young before weaning. The level in the control weanlings (5.8 μg/g) is higher than the value (1.9 μ/g) observed by Morrison and Sarett whose control diet contained only 0.15 μg thiamine/100 g, in contrast with 0.4 mg/100 g in the present study. Ochoa and Peters (13) and Byerrum and Flokstra (14) had reported previously that increasing the dietary thiamine above that needed for maximal growth increased the tissue concentrations of thiamine.

High levels of riboflavin or pyridoxine in the maternal diet did not influence the liver storage of these 2 vitamins at weaning. The value for riboflavin (21.5 μg/g) was similar to the value observed by Decker and Byerrum (15) in rats 30 days after birth (24.5 μg/g). The similarity of values for liver pyridoxine in both the high pyridoxine and the control groups differs from the results of Morrison and Sarett, who observed increased levels of pyridoxine in the livers of weanlings from dams fed high pyridoxine diets (7.5 mg/100 g) in contrast with a control diet containing 0.15 mg/100 g. Their values were 8.8 μg/g for the high pyridoxine group and 4.8 μg/g for the control group. In the present paper the values were 7.1 μg/g for the high pyridoxine group and 7.4 μg/g for the control. Perhaps the level of pyridoxine in the diet of Morrison and Sarett (4) was too low for maximal tissue storage, although this is above the stated requirement (16).

The growth studies gave no evidence for any beneficial effect in the young resulting from high maternal intakes of these vitamins during pregnancy and lactation. In addition, there was no evidence that the vitamin requirements of the offspring were increased, that is, the rats were made more vitamin-dependent, as a result of high maternal intakes. The slight delay in the development of thiamine deficiency in the rats from the high thiamine maternal diet probably reflects the increased tissue thiamine storage at weaning.

In conclusion, high levels of thiamine, riboflavin, or pyridoxine ingested during the reproductive period had no effect on the young, as shown by litter size at birth, growth until weaning, or vitamin requirements after weaning.

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Determination of First Limiting Nitrogenous Factor in Corn Protein for Nitrogen Retention in Human Adults [1]

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ABSTRACT Nitrogen retention of adult men fed isocaloric diets was significantly greater when white corn meal provided 8.0 g nitrogen/subject/day than when either 4.0 or 6.0 g nitrogen were supplied from corn protein. When a suboptimal intake of corn meal was fed (6.0 g nitrogen/subject/day), the optimal nitrogen retention level was re-established by the addition of 2.0 g nitrogen from any of several purified essential amino acids or other purified sources of nitrogen, or from both. Therefore, "non-specific" nitrogen, that is, nitrogen from any metabolically usable, non-toxic source, is the first limiting nitrogenous factor in corn protein for nitrogen retention in adult men.

Cereal grains are the primary source of dietary protein for many peoples throughout the world (1). Most of the knowledge dealing with human utilization and metabolism of cereal proteins has been derived from laboratory studies based on the assumption that the essential amino acid pattern of cereal proteins is the factor that limits their nutritional value. Accordingly, these studies (2–8) have dealt with attempts to achieve optimal nitrogen retention by supplementing cereal proteins with purified essential amino acids or intact proteins to provide a theoretically balanced pattern of amino acids.

The purposes of the present project were to establish the minimal corn protein requirement for adult men to attain positive nitrogen balance and to determine the first limiting nitrogenous factor in corn protein for the maintenance of this degree of nitrogen retention.

PROCEDURE

Pertinent data regarding the 10 men, inmates of the Nebraska Penal and Correctional Complex,[2] who were subjects for the project are shown in table 1. Institution medical records, verified by physical examinations conducted by a physician, certified that all were in good health at the beginning and throughout the project. Subjects were housed and received meals as a group in an isolated section of the institution; however, all maintained their usual work assignments.

The 100-day project was divided into a 3-day introductory period and 3 studies of 31, 50, and 16 days, respectively, conducted consecutively with no lapse in time between studies.

Previous studies (9–10) have shown that subjects attain nitrogen equilibrium more rapidly in response to controlled diets when they are fed a low nitrogen diet for a few days preceding the experimental period. Hence, the purposes of the introductory period were to hasten the attainment of nitrogen equilibrium by the subjects eating experimental diets more readily through an initial use of a very low nitrogen diet, to introduce the subjects to their duties and responsibilities, and to determine the caloric requirements of the individual subjects for weight maintenance. During this time, nitrogen intake was 2.00 g/subject/day, 1.30 from white, degerminated corn meal [3] and the remainder from the basal diet composed of a few low-protein fruits and vegetables. Cornstarch wafers in varying amounts were used to meet individual caloric requirements for maintenance of weight.

Study 1 was divided into 3 experimental periods of 10 days each arranged at ran-

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[2] The feeding phase of this project was pursued at the Reformatory Unit of the Nebraska Penal and Correctional Complex.

[3] White, degerminated corn meal used in this project was a gift of the Gooch Milling and Elevator Company, Lincoln, Nebraska.

TABLE 1

Age, height, weight, and caloric intakes of the subjects

Subject no. [1]	Age	Height	Weight [2]	Daily caloric intake		Calories furnished by fat	
				Studies 1, 2	Study 3	Studies 1, 2	Study 3
	years	*cm*	*kg*	*kcal/kg body wt*	*kcal/kg body wt*	*%*	*%*
50	28	185	90.7	41.0	51.0	25	40
51	29	180	73.9	44.5	54.5	15	31
52	34	171	61.2	50.0	60.0	15	29
53	37	175	72.6	41.5	51.5	7	25
54	32	185	81.6	40.2	50.2	15	—
55	26	190.5	88.4	34.1	44.1	7	28
56	31	185	65.8	50.0	60.0	15	29
57	22	183	84.4	38.9	48.9	15	32
58	35	175	65.8	49.9	59.9	15	29
59	30	179	65.9	40.0	50.0	15	—

[1] Subjects for studies 1, 2, and 3. Subjects 54 and 59 did not participate in study 3. Subject 59 did not participate in periods 7, 8, 9 and 10 of study 2.

[2] Weight at the beginning of the project. All subjects maintained their initial weight or gained weight slightly during studies 1 and 2. During study 3 all subjects showed some gain in weight due to the increased caloric intake.

TABLE 2

Comparison of diets used at three levels of intact corn protein intake

	Intake/subject/day for diet		
	1	2	3
	g	*g*	*g*
Corn meal (white, degerminated) [1]	354	531	708
Cornstarch	345	175	0
Corn oil (as required by each subject for weight maintenance) [2]	4	2	0
Tomato paste	100	100	100
Peaches	100	100	100
Applesauce	100	100	100
Coffee (dry, instant)	11	11	11
Jelly	30	30	30
Vitamin supplement, daily [3]			
Mineral supplement, daily [4]			
Low calorie soft drink, ad libitum			
N/subject/day provided by corn meal	4.0	6.0	8.0
N/subject/day provided by other dietary items	0.7	0.7	0.7

[1] Amino acid composition as follows: (mg amino acid/g of corn nitrogen) tryptophan, 38.5; threonine, 216; isoleucine, 286; leucine, 958; lysine, 135; methionine + cystine, 237; phenylalanine + tyrosine, 637; valine, 307.

[2] The corn meal contained 0.01 g fat/g of meal. Additional corn oil was added for weight maintenance of individual subjects as shown in table 1.

[3] Composed of the following: (mg/subject/day) thiamine·HCl, 1.2; ascorbic acid, 50; riboflavin, 1.5; pyridoxine·HCl, 0.3; niacin, 6.0; vitamin B_{12}, 12.0; and folic acid, 50.0 mEq; vitamin A, 5000 IU; and vitamin D, 400 IU.

[4] Composed of the following: (g/subject/day) Ca, 1.00; P, 1.001; Mg, 0.199; Fe, 0.015; Cu, 0.002; I, 0.00015; Mn, 0.002; Zn, 0.0009.

dom. White, degerminated corn meal was fed in amounts to provide 4.00, 6.00, or 8.00 g of nitrogen/subject/day as shown in table 2. As the amount of corn meal in the diet was increased or decreased, the amount of cornstarch [5] was adjusted so as to maintain a constant caloric intake for each individual. Individual caloric requirements were met by varying the corn oil [6] intake, constant for each individual but varying among the subjects as shown in table 1. Other items in the diet provided 0.70 g of nitrogen/subject/day. Vitamin and mineral supplements and other dietary components were as shown in table 2.

Individual allotments of corn meal cornstarch, corn oil, mineral supplements

[4] See footnote 3.

[5] Cornstarch and corn oil used in this project was a gift of the Corn Products Company, New York, New York.

[6] See footnote 5.

and baking powder for each day were mixed together and then divided into 3 equal portions to be combined with water, cooked and consumed at each of the 3 daily meals.

Study 2 included a preliminary adjustment day and 10 experimental periods of 5 days each arranged in a Latin square design. During this study, nitrogen intake from corn was kept constant at 6.00 g/subject/day, an amount established as suboptimal for nitrogen retention for all subjects in study 1. Various individual amino acids in purified form, thought to be possible first limiting nitrogenous factors, were added in amounts found in 2 g corn protein nitrogen during the 10 experimental periods. These were selected on the basis of results of several other investigators (2, 3, 6, 7) and by comparison of the essential amino acid pattern of corn with the FAO pattern (11), with the minimal requirements of the essential amino acids as reported by Rose (12), and with the essential amino acid pattern of egg protein (11). A mixture composed of 4 parts nitrogen each from glycine and diammonium citrate and 2 parts glutamic acid was used to maintain diets isonitrogenous at 8.70 g/subject/day; that is, 6.00 g nitrogen from corn protein, 2.0 g nitrogen from various purified sources, and 0.70 g nitrogen from the ordinary foods in the diet. The purified nitrogen components of the diets were presented to the subjects in water solutions or suspensions. Other details regarding caloric intake, vitamin and mineral supplementation, and diet preparation were essentially as described in study 1.

Study 3 was divided into 4 experimental periods of 4 days each arranged in a double Latin square design (only 8 of the original 10 subjects participated in this part of the project). Appropriate additions of corn oil to the diet increased caloric intake by 10 kcal/kg of body weight/day over that established during studies 1 and 2 as adequate for weight maintenance for each subject. Total nitrogen intake was maintained at 8.70 g/subject/day; 0.70 from ordinary foods in the diet plus 8.00 g nitrogen from white corn meal or 6.00 g nitrogen from white corn meal and 2.00 g nitrogen from one of 3 purified nitrogen mixtures. The purified nitrogen mixtures were identical to 3 of those used in study 2 and included non-specific nitrogen (as represented by a mixture of glycine, diammonium citrate, and glutamic acid), the essential amino acids and non-essential amino acids as found in 2.0 g corn protein nitrogen, and tryptophan as found in 2.0 g corn protein nitrogen. In all cases, the mixture of glycine, diammonium citrate, and glutamic acid was used to maintain diets isonitrogenous. Other details regarding composition and administration of diets were as previously described.

The amino acid content of the corn was determined microbiologically by the method of Steele et al. (14). Nitrogen in corn, low protein foods, purified nitrogen mixtures, and excreta was determined according to the boric acid modification of the Kjeldahl method (15). Urine samples were preserved under toluene and were analyzed daily. Daily fecal nitrogen values were based on 5-day fecal composites during studies 1 and 2 and on 4-day composites during study 3. Creatinine determinations were made on the 24-hour urine collections by the method of Folin (16).

RESULTS

Study 1. Individual mean daily nitrogen balances of the 10 subjects for the last 5 days of each 10-day experimental period as well as the collective means of all subjects are shown in figure 1. As the amount of nitrogen provided by corn protein was increased from 4.00 to 6.00 to 8.00 g/subject/day, nitrogen retention of each individual increased steadily.

Analysis of variance gave an F value which was significant at the 0.5% level of probability.

No subject was in positive nitrogen balance while receiving 4.00 g, two were in positive balance while receiving 6.00 g, and 8 were in positive nitrogen balance while receiving 8.00 g nitrogen from corn protein. All subjects showed an improvement in nitrogen retention between the 6.00- and 8.00-g corn nitrogen levels, individual increments being 0.51, 0.37, 1.77, 0.60, 1.18, 1.46, 0.33, 0.15, 1.77, and 1.66 g nitrogen. Similarly, increments in nitrogen retention between the 4.00 and 6.00 g corn nitrogen levels were 0.22, 0.33, 0.34,

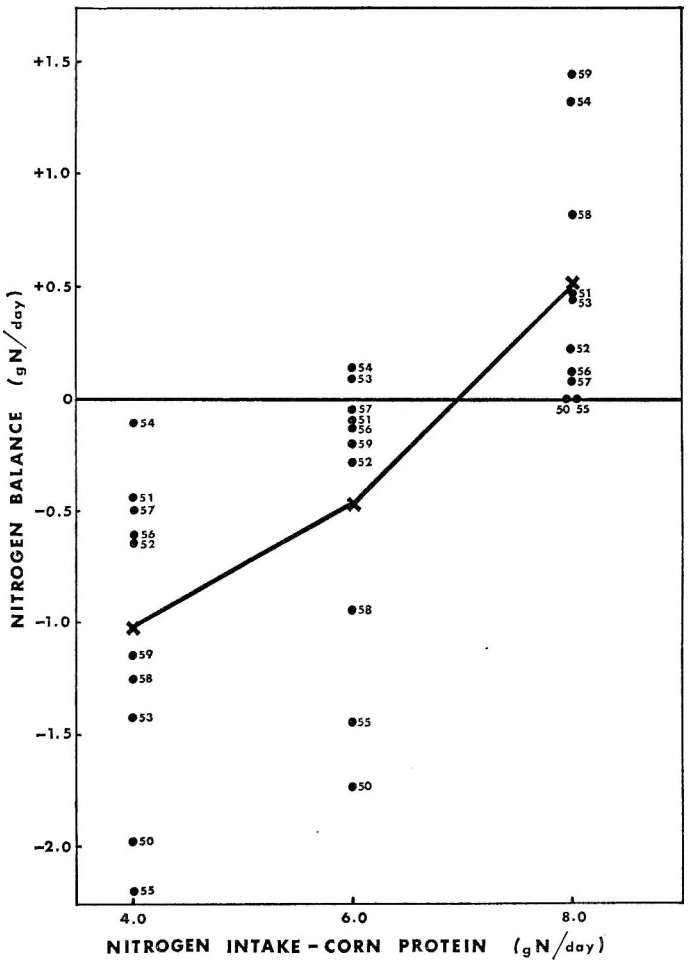

Fig. 1 Average nitrogen balances of subjects fed at 3 levels of nitrogen from white, degerminated corn meal. Points represent the average nitrogen balances of individual subjects for each 5-day period. The line indicates the average nitrogen balances for all subjects at each intake level.

1.51, 0.24, 0.68, 0.49, 0.44, 0.30, and 0.92 g nitrogen.

Fecal nitrogen excretion tended to parallel the level of corn nitrogen in the diet. Average daily fecal nitrogen values for all subjects while receiving 4.00, 6.00, and 8.00 g nitrogen from corn protein were 1.73, 1.99, and 2.28 g nitrogen, respectively. Statistical analysis indicated that these values did not differ significantly.

Study 2. Mean daily nitrogen balances for each 5-day experimental period are shown in table 3. The results indicated no significant differences among the various purified nitrogen mixtures in the promotion of nitrogen retention. A comparison of the results of studies 1 and 2 revealed no differences between nitrogen balances achieved by subjects receiving 8.00 g nitrogen from corn protein and those shown by the subjects while consuming 6.00 g nitrogen from corn protein plus 2.00 g nitrogen from any of the 10 purified nitrogen mixtures. However, nitrogen retention was significantly greater when diets provided 6 g nitrogen from corn protein and 2 g nitrogen from any of several supplementary sources than when 6 g nitrogen from corn alone were provided. Analysis of variance of these differences all gave *F*

TABLE 3

Determination of first limiting nitrogenous factor in corn for nitrogen retention in adult men

Possible first limiting factor [1]	Avg N balance [2]
Essential + non-essential amino acids	+0.65
Essential amino acids	+0.72
Tryptophan	+0.57
Lysine	+0.52
Sulfur-containing amino acids	+0.51
Isoleucine	+0.58
Tryptophan + sulfur-containing amino acids	+0.68
Tryptophan + lysine	+0.74
Isoleucine + threonine	+0.52
"Non-essential" nitrogen (glycine, diammonium citrate, glutamic acid)	+0.69

[1] Limiting factor in purified form fed in amounts found in 2.0 g corn protein nitrogen. Intact corn provided 6.0 g N/day. Diets maintained isonitrogenous at 8.0 g N/day by additions of glycine, diammonium citrate and glutamic acid.

[2] Average nitrogen balance of 10 subjects for 5 days with each diet.

values which were significant at greater than the 0.5% level of probability.

Study 3. Mean daily nitrogen balances for each 4-day experimental period are shown in table 4. All subjects retained slightly more nitrogen with the high caloric diets than with similar diets containing sufficient calories for weight maintenance. Comparison of the nitrogen balances of subjects receiving the 4 dietary variations at the increased caloric intake were similar to results obtained at caloric intakes sufficient to maintain body weight.

DISCUSSION

The concept that the nutritional quality of a protein is dependent upon its amino acid composition has been prevalent for the last half century. However, several papers as reviewed by Synderman (17) have demonstrated an apparent discrepancy between calculated minimal requirements of specific natural proteins based on their amino acid composition and experimentally determined minimal requirements of the same proteins for maintenance of nitrogen equilibrium in humans.

Evidence has been obtained indicating that "unessential" nitrogen (nitrogen from sources other than the essential amino acids) is the first limiting nitrogenous factor in proteins of high biological value such as those contained in milk and eggs by Synderman et al. (17), Clark et al. (18) and Kies.[7]

All of the purified nitrogen mixtures used in the present project were equally effective in the re-establishment of positive nitrogen balance when added to a diet providing insufficient corn protein for supporting nitrogen retention. Apparently "non-specific" nitrogen, that is, nitrogen from any metabolically usable, non-toxic source, is the first limiting nitrogenous factor in corn protein, a protein of low biological value.

At the 6.00-g corn nitrogen level all essential amino acids, except tryptophan, were provided in amounts which met or exceeded the requirements as established by Rose (12). Although this amount provided only 91% of the tryptophan requirement, the addition of purified nitrogen mixtures containing tryptophan were no more effective in the re-establishment of positive nitrogen balance than were the purified mixtures not containing tryptophan. All diets were supplemented with niacin along with other vitamins and minerals (table

[7] Kies, C. V. 1963 Effect of essential to non-essential amino acid relationships in man and in the rat. Ph.D. thesis, University of Wisconsin.

TABLE 4

Effect of caloric intake on determination of first limiting nitrogenous factor in corn

Possible first limiting factor	Avg N balance	
	High caloric diet	Moderate caloric diet
	g N/day	*g N/day*
Essential + non-essential amino acids	+0.88	+0.65
Tryptophan	+0.96	+0.57
Non-essential nitrogen (glycine, diammonium citrate, glutamic acid)	+0.92	+0.69
8 g nitrogen from intact corn diet	+0.66	+0.49

2). Thus, it may be that the tryptophan requirement was spared by the elimination of tryptophan conversion to niacin. Furthermore, in the establishment of the minimal requirements of the essential amino acids, Rose (12) defined adequacy as the amount of each amino acid required by the subject with the greatest apparent need for maintenance of positive nitrogen balance. Individual variation in requirement being great, it is possible that the actual tryptophan requirements for the subjects in the present study were less than the requirement of the one individual whose need, in essence, determined the tryptophan requirement as expressed by Rose.

Supplementation studies in which possible "non-specific" nitrogen requirements of the subjects were not considered cannot be compared directly with the present study. Results reported by Truswell and Brock (7) on the effect of supplementing corn protein with purified amino acids indicated that lysine is the first limiting amino acid in corn. Either arbitrary amounts or the Rose "safe" requirement pattern were used in determining levels of the essential amino acid supplementation, thus markedly altering the corn amino acid pattern. In addition, no attempt was made to keep total nitrogen intake at an optimal level or to determine the possible effect of non-essential nitrogen intake on the results.

The results of a series of experiments carried out by researchers connected with the Institute of Central America and Panama (2–6) and designed to determine the effectiveness of amino acid supplementation of cereal proteins for young children indicate that total protein intake influence the effectiveness of the various supplements and that the effect of amino acid supplementation of particular cereals cannot always be predicted from comparison with a reference amino acid pattern or from the results of animal studies. Using weight gain and nitrogen retention of young children as the criteria of evaluation, tryptophan and lysine were implicated as being the first limiting amino acids in corn-masa. However, these studies also assumed that the corn protein pattern is so imbalanced as to require supplementation to some theoretically balanced pattern in order for nitrogen retention to be achieved.

Since cereal proteins contain a considerably lower percentage of essential amino acids as compared with non-essential amino acids, Jansen and Howe (1) conclude that it is unlikely that total nitrogen can be the limiting factor with a cereal diet, thus suggesting that supplementation of protein-deficient cereal diets with non-essential nitrogen is definitely contra-indicated. The results of the present project offer experimental evidence indicating that, in fact, non-essential or non-specific nitrogen is the first limiting factor in corn protein. Therefore, additional research on the possible beneficial effects of non-essential nitrogen supplementation of cereal proteins is suggested.

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Effect of "Non-specific" Nitrogen Intake on Adequacy of Cereal Proteins for Nitrogen Retention in Human Adults [1]

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ABSTRACT The optimal intake of "non-specific" nitrogen for apparent nitrogen retention of human adults when the essential amino acids are provided by intact corn protein was studied. During 5 periods of 10 days each arranged in a double Latin square design, nitrogen balances of 10 adult men fed 6.0 g nitrogen/day from white degerminated corn meal plus 0.0, 2.0, 4.0, 6.0 or 8.0 g nitrogen/day from "non-specific" nitrogen (an isonitrogenous mixture of glycine and diammonium citrate) were determined. Other foods provided 0.68 g nitrogen/day. Average nitrogen balances for all subjects during the last 5 days of each period when total nitrogen intake was 6.68, 8.68, 10.68, 12.68 or 14.68 g day, were −0.33, +0.44, +0.85, +1.32, and +1.22 g nitrogen/day, respectively. The step-by-step increases in nitrogen retention among the first 4 values were statistically significant at the 0.5% level of probability.

It has been generally accepted that purified diets must provide sufficient nitrogen for synthesis of the non-essential amino acids as well as adequate amounts of the essential amino acids in order to meet human protein requirements (1, 2). Data reported by Snyderman et al. (3) indicate that "unessential" nitrogen, i.e., nitrogen from sources other than the essential amino acids, may be the first limiting nitrogenous factor in milk for growth and nitrogen retention of human infants. Recent evidence indicates that a broader term, "non-specific" nitrogen, i.e., nitrogen from a metabolically useable, non-toxic source including excess essential amino acid nitrogen not utilized in meeting essential amino acid needs, might be more meaningful in defining this suggested requirement (4). Apparent nitrogen retention of adult men fed 6.0 g nitrogen/day from intact corn protein was improved equally by the addition of 2.0 g nitrogen/day from any of several purified essential amino acids or "unessential" nitrogen sources, or both, or from intact corn protein. Thus, "non-specific" nitrogen was implicated as the first factor in corn protein limiting nitrogen retention in human adults.

The possible extent of quantitative improvement of nitrogen retention in human adults with graded increases in "non-specific" nitrogen added to diets providing constant amounts of the essential amino acids as contained in intact corn protein is reported in the present paper.

PROCEDURE

The study was composed of a 3-day preliminary period and 5 experimental periods of 10 days each, arranged in a double Latin square design.

During the preliminary period nitrogen intake per subject per day totaled 1.68 g; that is, 1.00 g nitrogen from white, degerminated corn meal [2] and 0.68 g from the basal diet composed of cornstarch, fat, instant coffee, and a few low protein fruits and vegetables. Purposes of this period included the introduction of subjects to their duties and responsibilities, determination of individual caloric requirements for weight maintenance, and utilization of a very low nitrogen diet to speed the adjustment of subjects to the later experimental diets.

During all experimental periods, intake of the essential amino acids was maintained constant in the amounts shown in table 1 as provided by 6.00 g corn protein nitrogen (472 g white, degerminated corn

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[2] The white, degerminated, unenriched corn meal used in this study was furnished gratis by the Gooch Milling and Elevator Company, Lincoln, Nebraska.

TABLE 1

Composition of diet

	Amount/ subject/ day
	g
Corn meal (white, degerminated, unenriched)	472 [1,2]
Cornstarch, varied [2,3]	
Corn oil or butter oil, varied [2,3,4]	
Jelly	30
Applesauce	100
Tomato paste	100
Instant coffee (powder)	10
Low calorie, carbonated beverages (protein and fat-free), varied	
Vitamin supplement, daily [5]	
Mineral supplement, daily [2,6]	
Glycine and diammonium citrate, varied [7]	

[1] The amino acid analysis of the corn meal gave the following values: (in mg of amino acid/g of corn nitrogen) tryptophan, 38.5; threonine, 216; isoleucine, 286; leucine, 958; lysine, 135; methionine + cystine, 237; phenylalanine + tyrosine, 637; and valine, 307. The corn meal contained 0.0127 g nitrogen/g of corn.

[2] Corn meal, cornstarch, fat source, mineral supplement, and baking powder for each subject for each day were combined, divided by weight into 3 equal portions, mixed with appropriate amounts of water, cooked in several standard forms (mush, muffins, pizza), and served.

[3] Cornstarch and fat were added in amounts needed to meet individual caloric requirements for maintenance of body weight.

[4] Subjects 60–64 received butter oil as the dietary fat source; subjects 65–69 received corn oil. Fat was fed in amounts to provide 20% of the total caloric intake for each individual.

[5] The vitamin supplement in pill form provided the following/subject/day: (in mg) thiamine·HCl, 1.2; riboflavin, 1.5; pyridoxine·HCl, 0.3; ascorbic acid, 50.0; niacin, 6.0; vitamin B_{12}, 12.0; and folic acid, 50.0 mEq. Also, vitamin A, 4000 IU; and vitamin D, 400 IU.

[6] The mineral supplement provided the following/subject/day: (in grams) $CaCO_3$, 1.36; KH_3PO_4, 1.31; $MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$, 0.80; $FeC_6H_5O_7 \cdot 6H_2O$, 0.095; $CuSO_4 \cdot 5H_2O$, 0.008; KI, 0.0002; $MnCl_2 \cdot 4H_2O$, 0.007; $ZnCl_2$, 0.002. In addition, baking powder provided approximately $NaHCO_3$, 2.0 g and $Ca(H_2PO_4)_2 \cdot H_2O$, 2.9 g.

[7] An isonitrogenous mixture of glycine and diammonium citrate was given in solution to provide 0.0, 2.0, 4.0, 6.0, or 8.0 g of nitrogen/subject/day depending upon the experimental period. Each daily allotment was divided into 3 portions of equal size for consumption at each of the 3 daily meals.

meal [3]) per subject per day. A mixture of glycine and diammonium citrate in isonitrogenous amounts provided 0.00, 2.00, 4.00, 6.00, or 8.00 g nitrogen/subject/day during the 5 experimental periods. Thus, with the inclusion of nitrogen provided by the basal diet (0.68 g, daily, as shown in table 1) total nitrogen intake per subject per day was systematically varied according to the Latin square design from 6.67 to 14.67 g. Caloric intake for each individual was kept constant at the level required for weight maintenance (table 2) by varying the intake of cornstarch [4] and fat among the subjects; however, in all

TABLE 2

Vital statistics and the caloric intakes of subjects

Subject	Age	Weight	Height	Caloric intake
	years	*kg*	*cm*	*kcal/day*
60	25	81.8	180	3780
61	30	67.3	173	3119
62	26	67.3	173	3119
63	28	91.4	185	3815
64	25	74.5	178	3447
65	36	77.3	180	3580
66	28	72.3	183	3354
67	33	70.4	180	3264
68	28	71.4	179	3313
69	38	90.4	180	3877

cases fat provided 20% of the calories. As shown in table 1, one-half the subjects received butter oil and one-half received corn oil as the source of dietary fat. Vitamin and mineral supplements were also included (table 1).

Methods used in the preparation and administration of the purified nitrogen mixture, corn meal, cornstarch, and other food items were basically the same as outlined in an earlier paper (4).

Details regarding the 10 men, inmates of the Nebraska Penal and Correctional Complex, who were subjects for the study are shown in table 2. Institutional health records indicated that all were in good health; medical consultation of a physician was available to the subjects throughout the study. Subjects were housed and ate meals together in a separate part of the institution; however, usual work assignments were maintained.

Nitrogen content of corn meal, other foods, glycine and diammonium citrate mixtures, and excreta was determined according to the boric acid modification of the Kjeldahl method (5). Creatinine in urine was analyzed by the method of Folin (6) in order to check the accuracy of collections. Urinary nitrogen and creatinine excretions were determined daily on each 24-hour collection and fecal nitrogen data were obtained from 5-day composite collections for each individual. Amino acid content of the corn meal was determined microbiologically by the method of Steele et al. (7).

[3] See footnote 2.

[4] The cornstarch and corn oil used in this study were supplied gratis by Corn Products Company, Argo, Illinois.

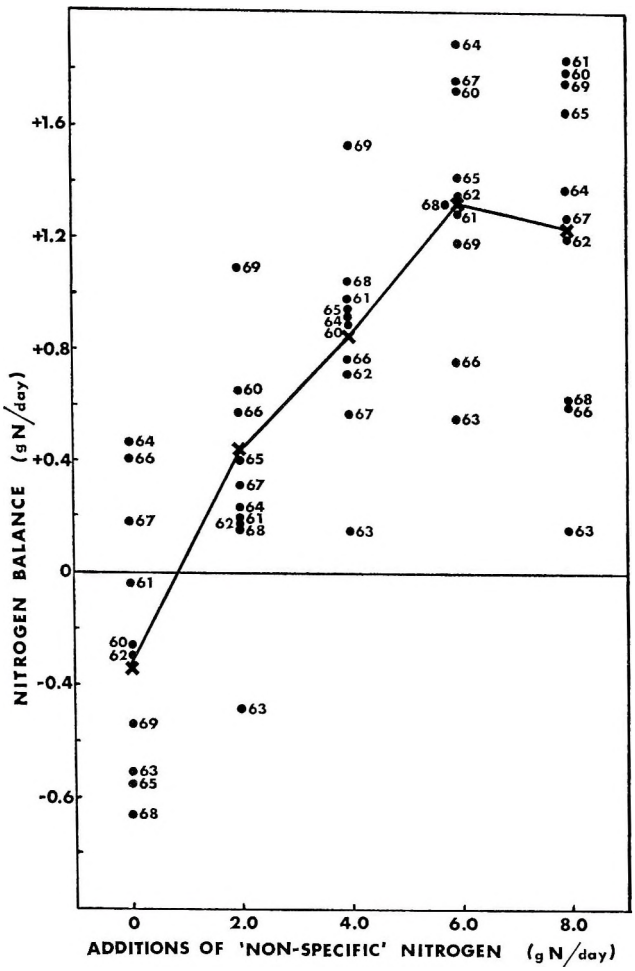

Fig. 1 Effect of several levels of dietary "non-specific" nitrogen on nitrogen balances of adult men fed a constant amount of corn protein (to provide 6.0 g nitrogen/subject/day). Dots represent average nitrogen balances of each individual for the last 5 days of each 10-day experimental period. Crosses represent mean balances of all subjects at each level of "non-specific" nitrogen intake.

RESULTS

Mean nitrogen balances of each individual subject for the last 5 days of each experimental period as well as collective means for all subjects are shown in figure 1.

All subjects showed increases in apparent nitrogen retention when the total nitrogen intake was increased from 6.67 to 8.67 g nitrogen/day, the average increment being 0.72 g nitrogen. Analysis of variance gave an F value significant at greater than the 0.5% level of probability. Similarly, average nitrogen retention was increased when the total nitrogen intake was increased from 8.67 to 10.67 g and from 10.67 to 12.67 g/day, average increments being 0.46 and 0.41 g nitrogen, respectively. Analysis of variance of these data also gave *F* values significant at greater than the 0.5% level of probability. Nitrogen retention was not significantly greater when total nitrogen intake was 14.67 g/day as compared with values obtained on the 12.67 g total nitrogen intake level.

Fecal nitrogen excretion remained relatively constant for all subjects regardless

of total nitrogen intake although differences existed among subjects. Average fecal nitrogen excretion values per day for subjects 60 through 69 were 1.23, 1.42, 1.01, 2.25, 1.73, 2.00, 2.16, 1.56, 1.59, and 1.45 g, respectively.

DISCUSSION

Well-known discrepancies exist among the apparent minimal requirements of the various essential amino acids for human adults reported by several investigators (9). Although some variations existed in experimental procedure and criterion of evaluation, the lack of consistency in level of nitrogen intake from sources other than the essential amino acids may have had a greater effect on the results than formerly supposed. As discussed in an earlier paper (4), several authors have also noticed a discrepancy between calculated minimal requirements of certain intact proteins based on their essential amino acid composition and actual minimal requirements based on the results of nitrogen balance studies. This also may be the result of failure to take the "non-essential" or "non-specific" nitrogen requirements into consideration.

The 472 g of white corn meal fed daily to each subject provided sufficient amounts of all the essential amino acids except tryptophan as judged by the Rose (1) criteria of the minimal requirements of the essential amino acids. Experimental data from the previous study (4) indicated that sufficient amounts of tryptophan (91% of the Rose minimum requirement) were provided at this level of corn protein intake and that higher intakes of tryptophan would result in no improvement in nitrogen retention.

Several authors have reported that adults fed constant amounts of the essential amino acids retain greater amounts of nitrogen with diets containing medium or high levels of "non-specific" nitrogen as compared with those containing relatively low amounts (8–11). In these studies the essential amino acids were provided in accordance with the egg protein proportionality pattern in the form of whole egg, purified amino acids, or cereals plus purified amino acids. In a recent paper, Jansen and Howe (12) stated that the lower level of total essential amino acids and the greatly unbalanced proportions of several amino acids make it extremely unlikely that total nitrogen is the limiting factor in a cereal diet. The results of the present study indicate that nitrogen retention of adults eating a corn diet can be increased greatly by additions of "non-specific" nitrogen, thus by increasing the total nitrogen intake.

Although the results of this study offer interesting possibilities for supplementation of cereal diets on a practical basis, much additional information is needed on the effects of "non-specific" nitrogen supplementation of cereal diets fed children, long-term effects of "non-essential" nitrogen intake, and whether a high degree of apparent nitrogen retention always represents the optimal in protein nutrition.

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B-Complex Vitamin Content of Cheddar Cheese [1,2,3,4]

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ABSTRACT The retention of B-vitamins of milk in Cheddar cheese curd, loss of the vitamins in whey, and the effect of temperature and length of ripening upon the vitamin content of cheese were investigated. The vitamin content of the milk used varied widely. The vitamin content of the cheese curd varied with that of the milk. The significant loss of vitamins in the whey produced during the cheese-making process is indicated by the fact that from 10 to 40% of the vitamin content of the milk was retained in the cheese curd. The temperature and length of ripening markedly affected the vitamin content of cheese. The changes in the vitamin content proceeded most rapidly in the cheese ripened at 15.6°, more slowly at 10°, and slowest at 4.4°. The content of four of the six vitamins increased during the initial period of ripening. The pantothenic acid and vitamin B_{12} content, however, decreased during the initial period and then increased. The increase in at least the niacin and vitamin B_6 content during the early stages of ripening may be related to the lactose metabolism in Cheddar cheese. The data revealed that mild and sharp Cheddar cheese and a mixture of sharp cheese with fresh Cheddar cheese curd as used in the manufacture of process cheese are richer in B-vitamin content than medium Cheddar cheese by itself.

The content of B-vitamins varies widely not only with varieties of cheese, but also with different samples of the same variety. Shahani et al. (1) observed that the niacin content in 278 samples of 23 varieties of cheese varied between 21 and 3,416, with an average of 691 μg/100 g. Similar wide variations were observed in the vitamin B_6, pantothenic acid, biotin, and folic acid content. In 12 varieties of cheese, Sullivan et al. (2) also reported that the niacin, pantothenic acid, and biotin ranged from 30 to 1,600, 130 to 960, and 1.1 to 7.6 μg/100 g, respectively. Karlin (3, 4) observed that the vitamin B_6 content in 19 varieties of cheese ranged between 40 to 198, and the vitamin B_{12} content of 4 varieties ranged between 1.2 and 2.55 μg/100 g of cheese.

Variations in the vitamin content of cheese have been attributed to the differences in the milk and in the cheese-making and curing processes used. The vitamin content of milk, which varies with season, stage of lactation, and feed (5), may affect markedly the resultant vitamin content of fresh cheese curd. Collins and Yaguchi (6) reported that a considerably higher concentration of vitamin B_{12} was lost in rennet whey than in an acid whey. Karlin (3, 4) also observed that 54 to 70% of the vitamin B_6 content and 43 to 60% of the vitamin B_{12} content of milk were lost in the whey produced during the manufacture of cheese.

In general, the proteolytic and mold-ripened cheese varieties such as Limburger, Camembert, and Roquefort contain much higher concentrations of vitamins than the hard and semi-hard varieties, such as Cheddar, Swiss, Mozzarella, and others. The vitamin content of cheese is greatly affected by cultures used in its manufacture and the subsequent ripening conditions (1, 2, 7). Emanuilov and Nachev [5] observed that in 3 types of Kachkaval Bulgarian cheese the vitamin B_{12} content increased during ripening. However, in Camembert, Bleu, Port-Salut, and Gruyere the vitamin B_{12} content decreased during the initial few days of ripening and then increased to beyond the original level (4).

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[3] Part of this paper was presented at the 58th Annual Meeting of American Dairy Science Association, 1963.

[4] Paper no. 3 in a series: B-Complex Vitamin Content of Cheese.

[5] Emanuilov, L, and L. Nachev 1959 Sudurjhanie na vitamin B_{12} v tvurdite sireneta. Dairy Sci. Abstr., 21: 470 (abstract).

Fagen et al. (8) and Sjostrom (9) reported that the small quantity of lactose present in fresh cheese curd is readily metabolized within the initial 3 to 14 days of ripening. Since the nature of fermentation in cheese is greatly influenced by its lactose content (10), the vitamin content of the cheese, particularly during the early stages of ripening, may vary with the amount of lactose present.

The paucity of information concerning the factors affecting the B-vitamin content of Cheddar cheese and the need for standardizing methods to produce cheese rich in vitamins prompted this investigation.

METHODS

The work was directed toward the determination of niacin, vitamin B_6, pantothenic acid, biotin, folic acid, and vitamin B_{12} content of the milk, the Cheddar cheese curd, and the whey produced. The effect of ripening conditions upon the vitamin content of Cheddar cheese was also determined. Also, studies were made to determine the effect of lactose metabolism upon the vitamin content of the cheese during early stages of ripening.

Manufacture of cheese and handling of samples. The milk used in this study was grade A milk produced by the University dairy herd and was pasteurized at the University dairy plant. Using five different lots of milk five separate batches of Cheddar cheese were manufactured by the commercial method. The starter consisted of a commercial Cheddar cheese starter. The cheese curd was hooped in 10-kg Wilson hoops and pressed overnight, after which the cheese was removed from the hoops, cut into approximately 1-kg loaves, wrapped in "Parakote" and pressed for an additional 24 hours. Vitamin assays of fresh samples of milk, whey, and the Cheddar cheese curd were run immediately. Also, each lot of cheese was divided into 3 sublots, ripened at 4.4, 10, and 15.6° and assayed for vitamins at the end of 1 week, 2 weeks, and 1, 2, 4, 6, 9, and 12 months of ripening. Simultaneously with the vitamin assays the cheese samples were examined organoleptically for flavor, and for body and texture.

Vitamin assay methods. The microbiological methods of Gregory (11) and those of Shahani et al. (1) were used for the determination of niacin, vitamin B_6, folic acid, biotin, and pantothenic acid in milk, whey, and cheese. The method reported by Lichtenstein et al. (12) was used for vitamin B_{12}. Vitamin extracts from milk, whey, or cheese were prepared separately for each vitamin and assayed, using *Lactobacillus plantarum* 8014 as the assay organisms for niacin and biotin; *Saccharomyces carlsbergenesis* 9080 for vitamin B_6; *Streptococcus faecalis* 8043 for folic acid; *Lactobacillus casei* 7469 for pantothenic acid; and *Lactobacillus leichmannii* 7830 for vitamin B_{12}.

Effect of lactose metabolism upon the biosynthesis of niacin and vitamin B_6. Since during ripening of cheese most of the lactose is metabolized in the first 2 weeks (8, 9) and since in the preceding phase of work the content of four of the vitamins in cheese increased rapidly during the first few weeks of ripening, it was thought that the increase in vitamin levels during the initial stages of ripening might be associated with the metabolism of lactose in the cheese. Studies were therefore made to investigate the relationship between the lactose metabolism and the vitamin synthesis. In these trials a lactose "feed back" technique was used.

Five additional batches of cheese were manufactured as described above and allowed to ripen at 10°. Samples were taken at 2-day intervals for 14 days and assayed for the lactose, niacin, and vitamin B_6 content. The lactose content was determined by the method of Folin-Wu, as described by Hawk et al. (13). It was found that most of the lactose present in cheese had disappeared at the end of about 14 days of ripening. The cheese samples were then divided into 2 lots. To one lot 2% lactose was added aseptically and the cheese was macerated, put into sterile containers and covered with paraffin. The other lot, serving as the control, was treated the same way except that no lactose was added. The samples were ripened at 10° and assayed for lactose, niacin, and vitamin B_6 every two or three days for the next 6 weeks.

RESULTS

B-vitamin content of milk, whey, and Cheddar cheese curd. In table 1 is presented a summary of the data recorded on the B-vitamin content of milk, whey, and fresh Cheddar cheese curd and the percentage vitamin content of milk retained in the cheese curd.

The 5 lots of milk used for the manufacture of different batches of cheese varied widely in vitamin content. The average content of niacin, vitamin B_6, pantothenic acid, biotin, folic acid, and vitamin B_{12} of the milk were 76, 17, 340, 29, 3.8, and 0.48 μg/100 g, respectively. The average values in whey were 89, 14, 387, 26, 4.3, and 0.41 μg/100 g, respectively, indicating that considerable quantities of the vitamins of milk are lost through the whey produced in the process.

In general, for each vitamin the higher the content in the milk the higher the vitamin content of the cheese curd obtained from it. Average values for the 5 lots showed that 100 g of the cheese curd contained 169 μg of niacin, 33 μg of vitamin B_6, 912 μg of pantothenic acid, 29 μg of biotin, 15.2 μg of folic acid and 0.52 μg of vitamin B_{12}. The percentage vitamin retention in the cheese curd amounted to 22% for niacin, 19% for vitamin B_6, 28% for pantothenic acid, 10% for biotin, 40% for folic acid, and 11% for vitamin B_{12}, based on averages for the 5 lots.

Effect of temperature and ripening time upon the vitamin content of Cheddar cheese. To determine the effect of temperature and ripening time upon the vitamin content of the cheese, each lot of the cheese was divided into 3 sublots and ripened at 4.4, 10, and 15.6°. At predetermined intervals representative samples were withdrawn and assayed for the various vitamins. Simultaneously with the vitamin assays, the cheese samples were judged organoleptically for flavor and for body and texture.

All 5 lots of cheese were of good quality. As the ripening time progressed, the cheese developed typical ripened cheese flavor and texture. The rate of ripening was slightly faster at 15.6° than at 10 or 4.4°. At the end of 2 to 3 months of ripening all the cheese samples possessed a typical ripened cheese flavor.

The data showing the effects of temperature and length of ripening time upon the niacin, vitamin B_6, folic acid, and biotin content of cheese are presented in figure 1. The temperature and time of ripening affected markedly the vitamin content of the cheese. In general, the changes (increase or decrease) in the vitamin content proceeded most rapidly in the cheese samples ripened at 15.6°, more slowly at 10°, and slowest at 4.4°.

Niacin. The changes in the niacin content during ripening are shown in figure 1, block a. In all the 5 trials, there was a progressive increase in the niacin content during ripening. It increased rapidly during the first 3 to 4 weeks at all 3 temperatures; thereafter, it leveled off at the 4.4° temperature, but continued to increase at 10 and 15.6°. There was a marked difference between the maximal level in vitamin content of the cheese ripened at the lowest temperature (210 μg/100 g) compared with that in cheese ripened at the higher temperature (318 μg/100 g).

TABLE 1

B-Vitamin content of milk, whey and Cheddar cheese curd percentage and retention of vitamins of milk in cheese curd

Vitamin	Milk		Whey		Cheese curd		Retention
	Range	Avg [1]	Range	Avg [1]	Range	Avg [1]	
	μg/100 g	*μg/100 g*	*μg/100 g*	*μg/100 g*	*μg/100 g*	*μg/100 g*	*%*
Niacin	61–100	76	42–138	89	140–295	169	22
Vitamin B_6	4–27	17	8–26	14	12–57	33	19
Pantothenic acid	300–380	340	360–412	387	864–960	912	28
Biotin	11–48	29	6–38	26	11–67	29	10
Folic acid	1.0–6.0	3.8	0.3–13.0	4.3	1.0–35.0	15.2	40
Vitamin B_{12}	0.39–0.56	0.48	0.38–0.42	0.41	0.50–0.56	0.52	11

[1] Average of 5 trials.

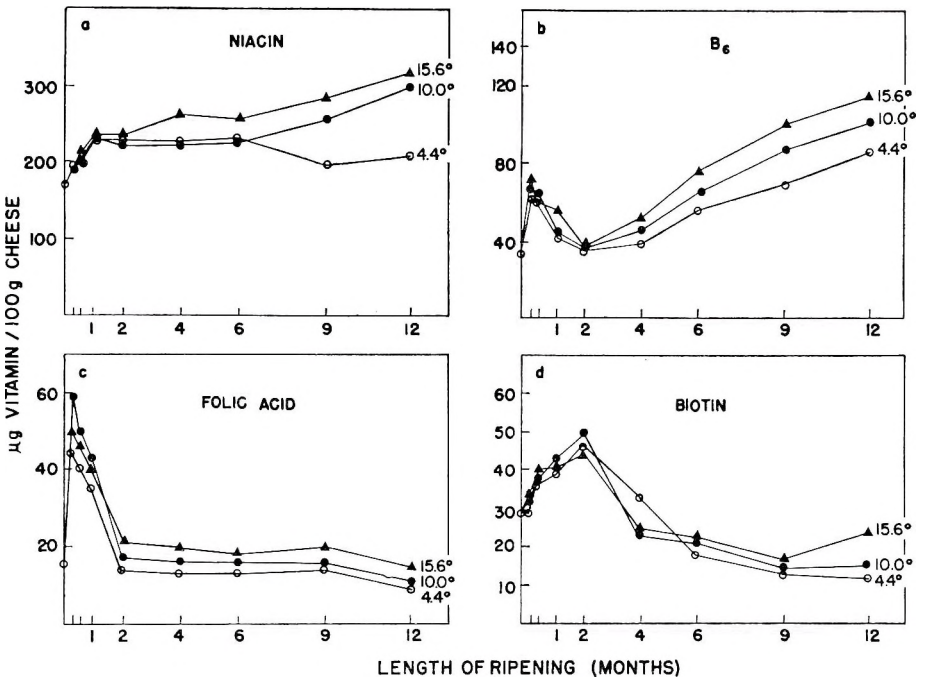

Fig. 1 Effect of temperature and length of ripening upon the niacin, vitamin B_6, folic acid and biotin content of Cheddar cheese (average of 5 lots).

Vitamin B_6. As shown in block b of figure 1, the vitamin B_6 content increased rapidly during the first 1 to 2 weeks from 33 to about 70 μg/100 g and then decreased to a level of 35 to 38 μg/100 g at the end of 2 months' ripening. Thereafter, there was a gradual increase to a range of 87 to 116 μg/100 g at the end of 12 months. The greatest increase was noted at 15.6°, next at 10°, and the least at 4.4°.

Folic acid. Like the vitamin B_6 content, the folic acid content also increased very rapidly at all the three ripening temperatures during the first week of ripening, increasing from 15 to 44 to 60 μg/100 g (fig. 1, block c). Thereafter, it decreased rapidly for 6 weeks and at a slower rate for the remainder of the ripening period.

Biotin. The biotin content (fig. 1, block d), increased for the first 2 months, then decreased sharply to below the original level at the end of the next 2 months, with little change thereafter.

Pantothenic acid and vitamin B_{12} content. The data on the pantothenic acid and vitamin B_{12} content as affected by temperature and length of ripening are presented in figure 2. The changes during ripening were different from those of the 4 vitamins discussed above in that irrespective of the temperature of ripening, a decline in their content was observed during the initial ripening period. The pantothenic acid content decreased sharply during the first 4 to 8 weeks, followed by a slow increase during the remaining period of ripening. The vitamin B_{12} content decreased sharply during the first week (from 0.52 to 0.27 μg/100 g) and thereafter increased slowly up to the end of the 9-month ripening period and then very rapidly during the last 3 months of ripening.

Effect of lactose metabolism upon the niacin and vitamin B_6 synthesis. The data showing the effect of lactose metabolism on vitamin content of the cheese are presented in figure 3. As observed by Fagen et al. (8) and Sjostrom (9), the initial lactose present in cheese was utilized almost completely during the first 14 days (block a). The niacin content (block b) increased during the same period; the

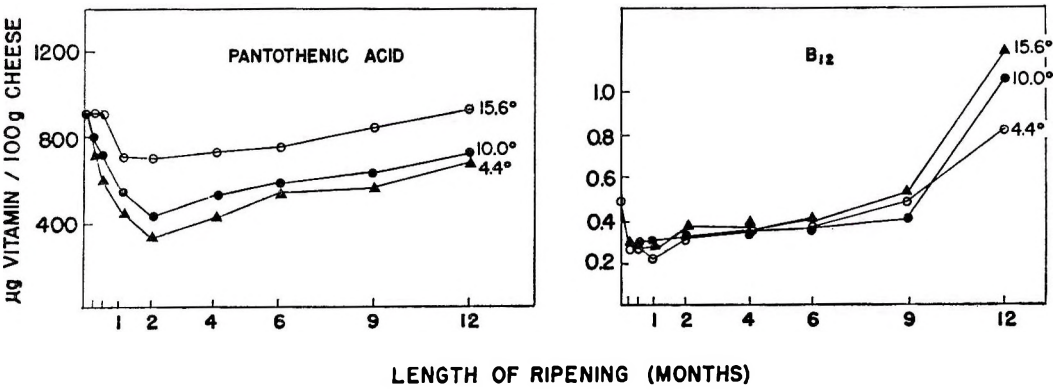

Fig. 2 Effect of temperature and length of ripening upon the pantothenic acid and vitamin B_{12} content of Cheddar cheese (average of 5 lots).

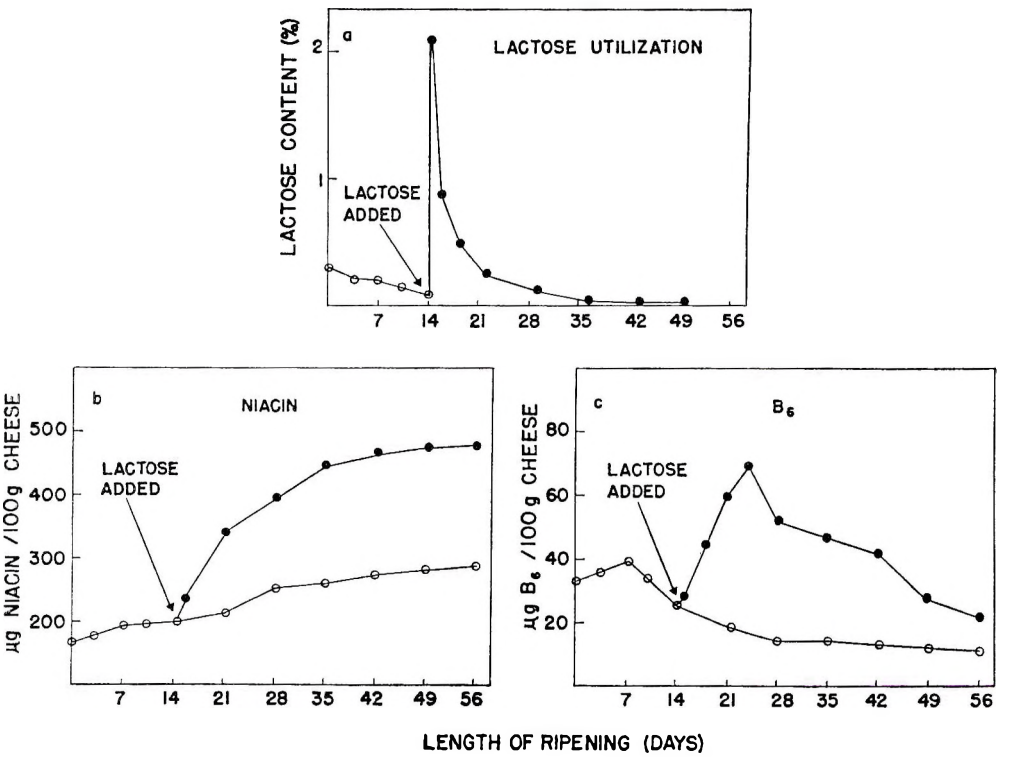

Fig. 3 Relationship between lactose metabolism and the biosynthesis of niacin and vitamin B_6 in Cheddar cheese (average of 5 lots).

vitamin B_6 (block c) also increased but only for 7 days and then decreased. The extra 2% lactose that was "fed back" was also utilized very rapidly. Simultaneously, the niacin content of the lactose-added cheese increased much more rapidly than in the control cheese. At the end of 56 days of ripening (42 days after the addition of extra lactose) the niacin content in the lactose-added cheese had increased from 170 to 472 µg/100 g of cheese as compared with 170 to 282 µg in the con-

trol cheese. Similarly, the vitamin B_6 content of the cheese increased rapidly for 10 days after the addition of lactose, reaching 70 μg/100 g and then decreasing. The vitamin B_6 content of the control cheese continued to decrease, as was observed in the previous trials (fig. 1, block b).

The increase in niacin and vitamin B_6 levels after the addition of lactose to the 14-day-old Cheddar cheese may be attributed to the added lactose. As the lactose was utilized the vitamin content increased, but after the lactose was completely utilized the niacin content tended to level off, and the vitamin B_6 content decreased, as in the case of the regular cheese samples (fig. 1). The data presented herein, therefore, suggest that the increase in the vitamin content of cheese during the initial period of ripening may be related directly to the lactose content and lactose utilization in the cheese. These results are in harmony with the observations of Teply et al. (14) that the addition of lactose to the ration enhanced the synthesis of niacin and folic acid by intestinal microorganisms of rats.

DISCUSSION

The niacin, vitamin B_6, pantothenic acid, biotin, folic acid, and vitamin B_{12} content of milk used in the manufacture of cheese varied widely among different lots of milk. On an average, the milk contained 76 μg of niacin, 17 μg of vitamin B_6, 340 μg of pantothenic acid, 29 μg of biotin, 3.8 μg of folic acid, and 0.48 μg of vitamin B_{12}/100 g of milk. These values are fairly typical of the values reported in the literature (2, 11, 15, 16). Considerable portions of the vitamin content of milk are lost through the whey produced during the manufacture of cheese. The vitamin content of the milk retained in the cheese curd ranged between 10 and 40% for the various vitamins.

The vitamin content of fresh cheese curd varied with the vitamin content of the original milk, in that the higher the vitamin content of the milk, the higher the resultant vitamin content in cheese curd. In addition, as suggested by Burkholder et al. (7) and Sullivan et al. (2), the temperature and length of ripening had a very pronounced effect upon the vitamin content of the cheese. The changes in the vitamin content were greatest at 15.6°, next at 10°, and least at 4.4°. The content of four out of the six vitamins, niacin, vitamin B_6, biotin, and folic acid, increased during the initial period of ripening, and the increase at least in the case of niacin and vitamin B_6 content appears to be related to the available lactose in the cheese.

Cheddar cheese constitutes by far the most widely consumed cheese in the American diet, and is used in considerably higher quantities than any other variety of cheese. It is a good source of the B-vitamins which play an important role in human nutrition. The B-vitamin content of Cheddar cheese, as available to the consumer, will vary depending upon the initial vitamin content of the milk from which it was made and upon the time and temperature of ripening.

No definitive information is available with respect to the amount of Cheddar cheese produced currently in this country that is marketed or consumed as mild, medium, or sharp Cheddar; however, it is estimated that about 31 to 50% is consumed as mild [6] and 33% as sharp (17). The data obtained in this study revealed that, by and large, mild cheese ripened for only 2 months or so and sharp Cheddar ripened for 9 to 12 months contain higher levels of vitamins than medium cheese ripened for 4 to 6 months.

Also, considerable quantities of fresh Cheddar cheese curd, and mild and sharp Cheddar cheese go into the manufacture of "pasteurized process" cheese. Since most of these vitamins in this are heat stable, no great loss of the vitamins would occur during processing. Consequently, the vitamin content of "pasteurized process" cheese would be comparable to that of mild or sharp Cheddar which are richer in B-vitamins than medium Cheddar.

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Effects of Exercise and Diet on Nitrogenous Constituents in Several Tissues of Adult Rats [1,2]

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ABSTRACT The effects of exercise and dietary protein level on amounts of DNA, RNA and total nitrogen in livers, gastrocnemius muscles and skin samples, and on serum albumin-to-globulin ratios were examined. The purpose was to test the stability of DNA and hence the validity of nitrogen-to-DNA and RNA-to-DNA ratios where exercise of various durations was imposed; also to evaluate the hypothesis that exercise might reduce protein reserves by converting labile to stable nitrogen compounds, and that protein in excess of the maintenance requirements might compensate for this conversion. One group of rats was preconditioned by intermittent periods of exercise prior to this test while two other groups underwent no previous exercise. The one-month period of exercise imposed during this test led to reduced serum albumin-to-globulin ratios. Feeding a sub-maintenance protein diet had the same effect, but in combination these treatments did not result in a greater decrease. Preconditioning brought about a marked elevation of plasma globulin level. Preconditioned rats when exercised during this trial had greatly increased amounts of DNA, RNA, and total nitrogen in their gastrocnemius muscles. This increase did not occur in rats exercised daily for 28 days. Liver DNA content was increased in the preconditioned group. Neither exercise nor diet brought about statistically significant changes in skin composition. It was concluded that albumin-to-globulin ratios and DNA ratios could give misleading results in comparisons of exercised and idle rats.

During the past century numerous experiments have been conducted to determine the effect of exercise on dietary protein requirement. The earlier evidence that exercise increased urinary nitrogen output (1, 2) must be discounted because of the observation that this increase is not proportional to severity or duration of exercise, but is a consequence of tissue catabolism resulting from insufficient caloric intake in relation to the increased energy expenditure (3). In fact, where energy intake is adequate, reduced urinary nitrogen loss during exercise has been reported (4). Although there is sufficient evidence to conclude that exercise does not increase protein requirement through increased urinary nitrogen loss, various claims have been made for beneficial effects of supplementary nitrogen during exercise. Kraut and Lehmann (5) reported that additional protein was necessary to maintain efficiency of work and prevent psychic depression. More recently Watkin [4] concluded that increased protein was required to maintain performance of strenuous work. Watkin also observed that exercise led to an increased loss of nitrogen in the sweat and concluded that this contributed to increased nitrogen requirement. This claim undoubtedly has merit; however, the amount of dermal nitrogen loss depends to a large extent upon environment rather than amount of work done (6, 7). Even if this sweat loss is considered as a separate requirement, and it is assumed that exercise does not increase protein requirement by reducing nitrogen retention, it does not necessarily follow that exercise does not increase protein requirement. In fact, Crampton (8) has concluded that most nutrient requirements, including that of protein, are related to energy intake. The experiment reported here was carried out to test our

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[4] Watkin, D. M., J. B. Das and M. C. McCarthy 1964 Protein for strenuous physical work. Federation Proc., 23: 399 (abstract).

hypothesis that exercise might reduce the body's reserve of labile nitrogen compounds through their conversion to more stable compounds, possibly involving translocation to another tissue. If such a reduction in reserve protein occurred it might lead to reduced resistance to intoxication and infection (9, 10). We further hypothesized that an increased protein level in the diet of working individuals might be effective in producing an increase in labile protein which would tend to offset any reduction in reserves induced by work. One of the problems in testing this hypothesis is selection of a sensitive means of expressing the relative amounts of the various nitrogenous components. Davidson (11) observed that under various regimens DNA is the least variable tissue component and concluded that the ratio of tissue constituent to DNA is the most satisfactory means of expression. Mendes and Waterlow (12) arrived at the same conclusion after observing that neither starvation nor protein-deficient diets reduced total liver DNA or DNA per nucleus. Allison (13) has established that the amount of serum albumin is a sensitive index of protein reserves, and because changes in globulin level tend to correct changes in blood volume, albumin-to-globulin ratios are the most sensitive index of the amount of albumin. These criteria have been established for measuring protein deficiency under varied dietary regimens and although effect of exercise on serum protein level has been reported (14) the effects of exercise on DNA and RNA have not been determined in animals fed graded protein levels. The experiment reported here was carried out to measure the effect of exercise on the total amount of these compounds in relation to changes in total nitrogen in representative tissue of adult rats to determine the reliability of DNA as a base for comparing tissue composition, and to reach a preliminary evaluation of our hypothesized effect of exercise on reserve nitrogen.

EXPERIMENTAL

Three groups of 12 female rats were selected. Six rats in each group were exercised as described below, and six were kept as controls. The groups differed in age and preconditioning. The rats in group 1 were 12.5 months old at the end of this 6-week experiment and had been preconditioned by exercising daily as described below during their fifth, seventh and ninth months. Groups 2 and 3 were not preconditioned and were 10.5 and 11.5 months old, respectively, at the end of the experiment. They received a 22% mixed protein diet prior to this experiment.

In each group 3 subgroups comprised of two exercised and two control rats received a diet containing 3.75, 7.5 or 15.0% of casein, respectively. This diet was made up of the following: (in per cent) partially hydrogenated vegetable oil, 14; cellulose,[5] 5; salt mix (USP no. 2), 4; vitamin mix,[6] 1; and starch plus casein, 76. The 7.5% casein diet was designed to meet the essential and non-essential amino acid requirements of adult rats, assuming a gross energy requirement of 121 kcal $\times$ $W^{0.75}$ kg (15). The amino acid composition of the casein was estimated (16).

The exercise equipment consisted of 2 aluminum drums, each 90 cm in diameter and 80 cm long, mounted by means of a single axle supported by bearings on a metal frame. The drums were rotated at 5 rev/min by an electric motor acting through a fixed speed transmission. Twelve individual acrylic plastic (Lucite) cages, fitted with individual watering devices were supported by the frame so that the concave lower edges of the sides were just above the upper surfaces of the drums, the arrangement being such that the drums formed the floor of the cages. Three brass electrodes were mounted in one end of each cage just above the surface of the drums. As the drum rotated, these electrodes were charged intermittently as an aid in training the rats to walk.

The control rats, and, during rest periods, the exercised rats, were housed in cages 20 $\times$ 15 $\times$ 15 cm in length, width and height, respectively.

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[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[6] The vitamin mix had the following composition: (in per cent) vitamin A acetate (500,000 IU/g), 0.488; vitamin D_3 (1654 IU/g), 17.15; *dl*,α-tocopheryl acetate, 2.12; thiamine·HCl, 0.285; riboflavin, 0.285; niacin, 1.27; Ca pantothenate, 0.855; pyridoxine·HCl, 0.285; choline chloride, 21.27; inositol, 1.42; folic acid, 0.258; biotin, 0.006; menadione, 0.634; vitamin B_{12} (0.1%), 0.48; and cellulose (Alphacel, Nutritional Biochemicals Corp., Cleveland), 53.194.

The duration of the experiment was 42 days. During the first 14 days the rats were fed their respective diets ad libitum but were not exercised. Training of the rats in the exercised groups was begun on day 15, and the duration of exercise was gradually increased until day 25. The rate of walking was constant at 14.3 m/minute throughout the experiment. The daily exercise consisted of cycles of 25 minutes of exercise followed by 5 minutes rest. The daily number of these cycles was gradually increased so that on the twenty-fifth day, and thereafter, the rats walked for 180 minutes.

On day 42 of the experiment blood samples were collected following decapitation while the rats were under light ether anesthesia. Exercised rats were decapitated 2 to 3 hours after exercise to avoid short-term effects on blood volume and composition (17, 18).

Just before decapitation the lumbar region of each rat was shaved; immediately following blood collection a uniform area of skin was removed, the liver and the gastrocnemius muscles of the hind legs were quantitatively removed and weighed. These tissues were analyzed for DNA and RNA according to the methods described by Ceriotti (19, 20) and for nitrogen by the micro-Kjeldahl method (21).

The blood plasma was analyzed for non-protein nitrogen (22), and total nitrogen (21), and albumin and globulin separation was carried out with the Spinco model R electrophoresis system.

RESULTS AND DISCUSSION

The results of this 2 × 3 × 3 factorial experiment were subjected to analyses of variance (23). Exercise brought about significant reductions in total serum protein, albumin, globulin, and albumin-to-globulin ratio (table 1). In contrast with the observations of Yoshimura (14) on young men in a shorter trial, this reduction occured regardless of the dietary protein level. Albumin-to-globulin ratios decreased to the same level in exercised groups fed 15 and 3.75% casein (0.61 and 0.59, respectively). Groups fed the 3.75% casein diet had significantly lower ($P < 0.01$) total protein, albumin and albumin-to-globulin ratios than those fed the diet containing 7.5% casein. In keeping with the observations of Allison (13), feeding protein in excess of the requirement did not increase blood protein levels.

The difference in age between the two youngest groups did not result in changes in amounts of blood proteins, and hence it may be concluded that the increased total serum protein and reduced albumin-to-globulin ratios which was due to increased globulin level in the 12.5-month-old group was associated with preconditioning. Blood volume was not measured, hence it is not known to what extent blood volume changes affected globulin level. From this observation it appears that where long-term exercise is imposed, albumin-to-globulin ratios could give misleading results.

In agreement with the observations of Summers and Fisher (24) and of Mendes

TABLE 1

Effect of exercise, dietary protein level, and conditioning on total serum proteins, albumin, globulins, and albumin-to-globulin ratios of adult female rats [1]

Treatment group	No./group	Total serum protein	Serum albumin	Serum globulin	Albumin-to-globulin ratio
		g/100 ml	*g/100 ml*	*g/100 ml*	
No exercise	18	6.57[a]	2.65[a]	3.92[a]	0.68[a]
Excercise	18	6.00[b]	2.30[b]	3.70[b]	0.63[b]
15% Casein diet	12	6.44[a]	2.50[a]	3.94[a]	0.65[ab]
7.5% Casein diet	12	6.49[a]	2.67[a]	3.82[a]	0.70[b]
3.75% Casein diet	12	5.92[b]	2.25[b]	3.67[a]	0.62[a]
Not conditioned (10.5 months old)	12	6.08[a]	2.45[a]	3.63[a]	0.68[a]
Not conditioned (11.5 months old)	12	6.10[a]	2.48[a]	3.62[a]	0.68[a]
Preconditioned (12.5 months old)	12	6.68[b]	2.51[a]	4.17[b]	0.61[b]
SD of the general mean		0.42	0.24	0.30	0.07

[1] Means within a column and treatment group are not significantly different ($P < 0.05$) if followed by the same superscript.

and Waterlow (12) dietary protein level did not influence the amount of DNA in the liver (table 2), nor did it influence amount of RNA; but as observed by numerous other workers (12, 25, 26) there was a reduction in liver nitrogen with reduced dietary protein level.

Although this experiment supported the use of DNA as a basis for measuring changes in liver composition when protein level is varied, preconditioning resulted in a significant increase ($P < 0.05$) in liver DNA (table 2). Hence, where intermittent exercise is involved DNA ratios could not be used satisfactorily.

In contrast with the effect of preconditioning, a single period of exercise brought about a decrease ($P < 0.07$) in total liver DNA (table 2). As shown in table 3, this reduction occurred mainly in those groups which had not been preconditioned. These observations indicate that whereas a single exercise period inhibits liver DNA formation, intermittent periods of exercise stimulate its formation. It is possible that in the former case formation of polyploid nuclei is inhibited, whereas during the rest periods following exercise the rate of formation of polyploid nuclei was accelerated. However, since there was a parallel increase of both RNA and total liver nitrogen ($P < 0.01$) in the preconditioned group (table 2) but a significant reduction in RNA ($P < 0.05$) in all of the exercised groups regardless of previous exercise history, it appears that either an increase in size or number of liver cells was inducted by preconditioning. Since there was an apparent loss of these materials during exercise this increase presumably occurred in one of the post-exercise periods during preconditioning. These results show that liver DNA ratios cannot be used in experiments where exercise is one of the variables.

Dietary protein level did not significantly change the total amount of DNA in the gastrocnemius muscles in the hind legs, nor did it result in reduced amounts of RNA in these muscles (table 2). There was a slight but not statistically significant reduction in total muscle nitrogen with reduced dietary protein level.

Exercise brought about statistically significant increases in the total amounts of DNA, RNA and nitrogen in these muscles

TABLE 2

Effect of exercise, dietary protein level and conditioning on amounts of DNA, RNA and nitrogen in the liver, muscle and skin of adult female rats [1]

Treatment group	No./group	Liver			Muscle			Skin		
		DNA	RNA	Nitrogen	DNA	RNA	Nitrogen	DNA	RNA	Nitrogen
		mg/liver			*mg/both gastrocnemius muscles*			*mg/sample*		
No exercise	18	22.9[a]	93[a]	262[a]	4.0[a]	27.2[a]	111[a]	10.8[a]	32.6[a]	362[a]
Exercise	18	20.4[a]	81[b]	253[a]	4.5[b]	30.2[b]	124[b]	10.8[a]	34.2[a]	377[a]
15% Casein diet	12	21.5[a]	85[a]	274[a]	4.3[a]	29.2[a]	121[a]	10.5[a]	31.9[a]	341[a]
7.5% Casein diet	12	21.1[a]	92[a]	267[a]	4.4[a]	29.0[a]	117[a]	10.7[a]	34.4[a]	379[a]
3.75% Casein diet	12	22.5[a]	84[a]	231[b]	4.0[a]	27.9[a]	114[a]	11.2[a]	34.0[a]	389[a]
Not conditioned (10.5 months old)	12	20.3[a]	77[a]	244[a]	4.0[a]	27.8[a]	114[a]	10.3[a]	33.1[a]	360[a]
Not conditioned (11.5 months old)	12	20.2[a]	83[a]	230[a]	4.0[a]	27.7[a]	114[a]	10.9[a]	35.3[a]	387[a]
Preconditioned (12.5 months old)	12	24.5[b]	102[b]	297[b]	4.7[b]	30.6[b]	125[b]	11.1[a]	31.5[a]	357[a]
SD of the general mean		3.87	11.4	22.9	0.61	3.3	11.8	1.75	3.3	37.4

[1] Means within a column and treatment group are not significantly different ($P < 0.05$) if followed by the same superscript.

over the non-exercised controls (table 2) and preconditioning brought about significant ($P < 0.05$) increases in these components over the levels observed in either of the groups not conditioned. These effects of exercise and preconditioning were occasioned by statistically significant interactions between exercise and conditioning in the case of each component ($P < 0.05$ for RNA and nitrogen, $P < 0.01$ for DNA). In table 4 it is shown that exercise produced increased amounts of DNA, RNA and nitrogen in those exercised rats that had been preconditioned, and in only those preconditioned rats that were exercised. From these observations, either preconditioning did not induce any change in amounts of muscle components, or if induced, the change had disappeared from those preconditioned rats that were not exercised in this experiment. Although the increases in muscle nitrogen and RNA were anticipated as a result of hypertrophy, the marked increase in DNA was unexpected. For, as Joubert (27) concluded, growth of muscle tissue is generally accepted to occur by hyperplasia during prenatal life and exclusively by hypertrophy following birth. Therefore, it appears unlikely that the increased DNA is due to formation of new cells. Striated muscle cells are known to have several nuclei (28) and hence the increase in DNA could, by conjecture, be due to either an increase in the number of nuclei or formation of polyploid nuclei.

There was a statistically significant ($P < 0.05$) interaction between diet and conditioning in both RNA and nitrogen content of muscle (table 5). This inter-

TABLE 3

Effect of conditioning and exercise on liver DNA [1]

Group	No exercise	Exercise
	mg/liver	*mg/liver*
Not conditioned (10.5 months old)	21.9[bc]	18.7[c]
Not conditioned (11.5 months old)	22.3[abc]	18.1[c]
Preconditioned (12.5 months old)	24.6[a]	24.5[ab]

[1] Six rats per mean. Means are not significantly different ($P < 0.05$) if followed by the same superscript.

TABLE 4

Interaction of exercise and conditioning on muscle DNA, RNA and nitrogen in adult female rats

Group	No exercise			Exercise		
	DNA	RNA	Nitrogen	DNA	RNA	Nitrogen
	mg/both gastrocnemius muscles			*mg/both gastrocnemius muscles*		
Not conditioned (10.5 months old)	4.0 [1]	26.8	109	4.0	28.8	118 [2]
Not conditioned (11.5 months old)	4.1	27.7	114	3.9	27.7	115
Preconditioned (12.5 months old)	3.9	27.1	110	5.5 [3,4]	34.0 [2,4]	139 [2,4]

[1] Six rats per mean.
[2] Different from corresponding non-exercised group ($P < 0.05$).
[3] Different from corresponding non-exercised group ($P < 0.01$).
[4] Different from both corresponding groups of non-conditioned rats ($P < 0.05$).

TABLE 5

Interaction of diet and conditioning on muscle RNA and nitrogen in adult female rats

Group	RNA			Nitrogen		
	Casein, %			Casein, %		
	15.0	7.5	3.75	15.0	7.5	3.75
	mg/both gastrocnemius muscles			*mg/both gastrocnemius muscles*		
Not conditioned (10.5 months old)	27.1 [1]	29.3	25.1 [2]	117	117	107 [2]
Not conditioned (11.5 months old)	30.5	25.2 [2]	27.4	128	104 [2]	110
Preconditioned (12.5 months old)	27.9	32.7	31.1	118	131	126

[1] Four rats per mean.
[2] Different from corresponding preconditioned group ($P < 0.05$).

action resulted from a tendency for reduced muscle RNA and nitrogen content in non-conditioned rats when the two lower protein diets were fed, whereas in the preconditioned groups the levels of muscle RNA and nitrogen were maintained regardless of protein level in the diet.

Analyses of variance of DNA, RNA and nitrogen in skin samples showed that its composition was remarkably constant in that none of the treatments resulted in statistically significant effects on amounts of these components (table 2).

Total feed consumed during the 42-day period, expressed as therm intake/100 g of initial weight, was reduced by exercise (table 6). A similar observation was reported by Mayer (29) in rats subjected to strenuous exercise. Moreover, weight change was significantly reduced ($P < 0.01$) by exercise as a result of exercised rats not maintaining their initial weight, whereas the cage-idle rats gained markedly.

Preconditioning did not significantly influence energy intake or weight change (table 6). Rats fed the diet containing 15% casein had a significantly lower energy intake than rats fed either of the other 2 diets. Despite this lower intake they gained weight, whereas those rats fed the 3.75% casein diet could not maintain their weight even though they consumed more feed. The group fed the 7.5% casein diet did not differ in energy intake from those fed the 3.75% casein diet but their body weight increased to an extent equal to those fed the 15.0% casein diet.

In agreement with earlier observations (11, 12, 24) this experiment showed that DNA content of tissues is not affected by variation in dietary protein level. Exercise, however, particularly intermittent periods of exercise, resulted in significant changes in total DNA in liver and muscle which would rule out its use as a base for measuring other components. Moreover, intermittent periods of exercise increased blood globulin levels and hence the use of albumin-to-globulin ratios to compare exercised and unexercised rats is questionable.

The skin clearly does not contain reserve protein in a form which can be withdrawn during exercise or when low protein diets are fed. The loss of serum albumin as a consequence of exercise cannot be modified by changes in dietary protein level. The main benefit of feeding higher levels of protein to the exercised rats was that a greater increase in muscle RNA and nitrogen was permitted in those rats that had not been preconditioned.

The hypothesis that exercise might lead to a reduction in reserve protein therefore cannot be verified from the results of this experiment. Nevertheless, additional studies on the effects of exercise on the individual components of the labile nitrogen pool should be carried out to illuminate the effects of exercise when the protein intake

TABLE 6

Effect of exercise, dietary protein level and conditioning on energy intake and body weight change [1]

Treatment group	No./group	Energy intake/ 100 g initial wt	Final wt minus initial wt
		therms	*g*
No exercise	18	0.887[a]	+18[a]
Exercise	18	0.786[b]	− 5[b]
15% Casein diet	12	0.784[a]	+11[a]
7.5% Casein diet	12	0.855[b]	+12[a]
3.75% Casein diet	12	0.870[b]	− 4[b]
Not conditioned (10.5 months old)	12	0.861[a]	+ 9[a]
Not conditioned (11.5 months old)	12	0.852[a]	+ 8[a]
Preconditioned (12.5 months old)	12	0.797[a]	+ 2[a]
SD of the general mean		0.083	13.8

[1] Means within a column and treatment group are not significantly different ($P < 0.05$) if followed by the same superscript.

is at or below the estimated maintenance requirement.

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Utilization of Algae as a Protein Source for Humans [1,2]

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ABSTRACT Two experiments were conducted using algae as the principle source of nitrogen for human subjects. This represented approximately 90 to 95% of the total nitrogen intake. In the first experiment *Scenedesmus obliquus*, 7.1 g N/day, was fed as the whole green lyophilized algae. At the end of 5 days the mean nitrogen balance for 5 subjects was +0.20 g N/day. In the second experiment, using 5 different subjects, 2 levels of ethanol-extracted *Chlorella pyrenoidosa* were fed (6.0 and 10.0 g N/day), each for 10 days. The mean nitrogen balance for 6.0-g algae nitrogen was negative, 0.84 g N/day, whereas the mean nitrogen balance for 10.0-g algae nitrogen level was positive, 0.61 g N/day. High fecal excretion of nitrogen was characteristic of all levels of algae. As a result, the algae used in these studies had low apparent nitrogen digestibility, 68% for dried green *Scenedesmus obliquus* and 58% for ethanol extracted *Chlorella pyrenoidosa*. This apparent digestibility was the major contributing factor to positive or negative nitrogen balance. The second experiment demonstrated that human subjects were capable of consuming algae as the principle source of protein in the diet for a 20-day period without ill effect.

During the past several years, many unconventional foodstuffs have been investigated as potential food sources for man. One of these foodstuffs was algae which has received especial attention because of its possible use in closed ecological systems. Even though interest in algae is widespread, few studies have been conducted on the nutritional adequacy of this material for man (1–5). Earlier workers (3) have reported the "true digestibility" of decolorized *Chlorella* to be 83.0%. They reported that the residual smell and taste of the decolorized *Chlorella* were intolerable and impaired appetite. In later studies (4) "true digestibilities" of 75% for blanched algae and 86% for methanol-extracted algae were reported. In these 2 studies approximately 30 g of algae were fed per day. This supplied about one-third of the total protein. Only one study (5) reports feeding amounts of algae in excess of 35 g (dry weight) per day. Subjects tolerated amounts up to 100 g/day quite well, but acute toxicity symptoms developed at 200- and 500-g levels.

The present paper presents data from 2 separate nitrogen balance studies with human subjects, using an experimental diet in which algae was the principle source of protein nitrogen. Algae contributed 90 to 95% of the total dietary nitrogen intake.

EXPERIMENTAL

In the first experiment, 5 inmates of the Nebraska State Penitentiary were used as subjects. These men were 27 to 33 years of age and weighed from 68 to 82 kg. All of the men had been subjects in previous diet studies. They were selected for this experiment because information was available on their caloric and nutrient requirements and on the basis of their previous cooperation. Mixed amounts of 2 strains of *Scenedesmus obliquus* ATCC 11457 and 11477 were used in the first experiment. Each strain was cultured separately and grown autotrophically in the double strength medium of Chorney et al. (6). Cultures were grown under constant illumination and were continually aerated with a mixture of 95% air 5% CO_2. Cells were harvested by continuous centrifugation and then lyophilized. The dried algae was autoclaved at 121° for 30 minutes. Ethanol extraction was carried out in a large Soxhlet extractor for 48 hours. The extracted material was air dried. The proximate analyses of the 2 forms of algae are shown in table 1.

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TABLE 1

Chemical composition of algae samples [1]

	Scenedesmus obliquus, ATCC 11457, 11477 mixed		*Chlorella pyrenoidosa*, thermophillic strain 71105	
	Green lyophilized	48-hour-extracted	Green lyophilized	144-hour-extracted
	% dry wt	*% dry wt*	*% dry wt*	*% dry wt*
Moisture	3.7	2.6	5.8	6.2
Crude protein (N × 6.25)	48.2	58.7	56.5	69.2
Ether extract	6.8	0.8	2.2	1.5
Crude fiber	6.8	9.7	2.5	5.1
Carbohydrate (by difference)	27.3	19.4	25.8	9.7
Ash	7.2	8.8	7.2	8.3
Total	100	100	100	100
Calcium	0.36	0.44	0.07	0.09
Phosphorus	1.74	2.05	1.79	1.97
Isoleucine [2]			2.20	2.76
Leucine			4.41	6.40
Lysine			3.50	4.31
Methionine			0.81	1.18
Phenylalanine			2.65	3.24
Threonine			2.02	2.80
Valine			3.01	3.78

[1] Standard AOAC procedures (Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D. C.).

[2] All amino acid values were obtained microbiologically using *Streptococcus spp.* ATCC 8042, after acid hydrolysis.

A preliminary adjustment period of 5 days preceded a 5-day algae trial period using dried whole green *Scenedesmus obliquus* as the primary source of protein. The first trial period was followed by a second brief adjustment period, then a second 5-day algae trial period during which time ethanol-extracted *Scenedesmus obliquus* served as the primary source of protein. During the perliminary period subjects were fed 2500 to 3000 kcal/day. All subjects received 6.5 g of nitrogen as protein nitrogen in the form of a dietary beverage,[3] fortified with non-fat dry milk. The subjects also received canned fruits and instant coffee or tea. The total nitrogen intake varied then from 7.2 to 7.3 g depending on these supplements. A daily vitamin supplement [4] provided the following: (in milligrams) thiamine, 2; riboflavin, 2.5; ascorbic acid, 50.0; pyridoxine, 1; niacinamide, 20; pantothenic acid, 1; and vitamin B_{12}, 1 µg; vitamin A, 5000 IU; and vitamin D, 500 IU. A daily mineral supplement was not given. Daily intakes of calcium, phosphorus, sodium and potassium were estimated from analytical data available for algae and tabulated values for other dietary ingredients.[5] In experiment 1 the calculated average daily intakes during the preliminary period were: (in grams) calcium, 2.1; phosphorus, 1.3; sodium, 2.7; potassium, 3.6; for the algae period the intakes were calcium, 1.3; phosphorus, 1.9; sodium, 3.2; and potassium, 1.9. In experiment 2 the calculated average daily intakes were: (in grams) calcium, 0.9 to 1.7; phosphorus, 1.3 to 2.2; sodium, 4.4 to 7.7; and potassium, 0.4 to 1.1. In experiment 2 the range of the values reflects differences in intake of algae and caloric supplements. The values for sodium and potassium in experiment 2 do not include amounts present in the extracted algae, since analytical data on this material are not available. The sodium content of this material is estimated to be almost nil and the potassium content about 0.5 g. During the first algae experimental period, 92.5 g whole green lyophilized algae provided 7.4 g nitrogen/day. Supplemental nitrogen from fruits and coffee increased the levels to a total of 7.8 to 8.2 g/day. Caloric intake was maintained at the same level as in the preliminary period. For the first 4 days, algae was given in biscuit

[3] Metrecal, Edward Dalton Company, Evansville 12, Indiana.

[4] Miles Products, Division of Miles Laboratories, Inc., Elkhart, Indiana.

[5] Watt, B. C., and A. L. Merrill 1963 Composition of Foods — Raw, Processed, Prepared. Agriculture Handbook no. 8, Consumer and Food Economics Research Division, ARS, U.S. Department of Agriculture, Washington, D. C.

form only. On the fifth day part of the algae was incorporated into other foods in the diet. One-half of the algae (46 g) was added to the following foodstuffs in the amounts indicated: tomato juice, 10 g/473 ml; flavored gelatin,[6] 7 g/118 ml; canned plums, 5 g/100 g; pineapple drink, 18 g/708 ml; and beef bouillon, 6 g/236 ml. The algae biscuit formula was: (in grams) wheat starch,[7] 160; sugar, 20; baking powder, 14.4; salt, 2; butter oil, 100; algae, 92.5 and water, 80. This formula provided one day's supply of 12 biscuits per subject.

A second experiment was conducted using a commercial preparation of *Chlorella pyrenoidosa*, thermophillic strain 71105. This was grown in the medium described by Lubitz (7).[8] The entire sample was autoclaved for 15 minutes at 121°. This material was extracted for 144 hours with ethanol. The chemical analysis of this material is shown in table 1. Subjects were selected from staff and graduate students at the University of Nebraska. A group of 12 prospective subjects was fed 3 meals typical of those that they would have during the experimental period. Five subjects, 4 male and one female, were selected from this group. These subjects ranged in age from 24 to 35 years, and in weight from 55 to 97.5 kg. Algae nitrogen was fed at 2 levels, 6.0 and 10.0 g, each for a period of 10 days. These levels were equivalent to 54.2 and 90.3 g of extracted algae, respectively. Two of the subjects started with the diet at the 10.0-g level and three at the 6.0-g level. At the end of 10 days these levels were reversed. The first algae period was preceded by a 3-day depletion period during which time the subjects received 2.8 to 3.0 g of nitrogen per day. Caloric intake was maintained at approximately 45 kcal/kg body weight for the men and 40 kcal/kg for the female subject. Fat intake was adjusted to 40% of the total caloric intake.

The algae was fed primarily in the form of a more concentrated biscuit. This biscuit formula was: (in grams) wheat starch, 50; sugar, 55; salt, 0.5; butter, 40; algae, 90.3 and water, 60. This provided one day's supply of 4 biscuits per subject. To diminish further the bitter taste, the algae was first fried in butter. On one occasion during the first 10-day period and on 3 occasions during the second 10-day period one biscuit was replaced by an algae pizza. This pizza was essentially the same as the biscuit, but contained some added starch and seasoning, and was topped with tomato paste. Variable ingredients in diets consisted of extra calorie wafers, butter, jelly, coffee or tea, sugar, carbonated soft drink, roll candies and pickles. These were used to adjust the fat and caloric intake of the subjects. These ingredients allowed some selection by the subject, but once selection was established they were held constant. A sample of each ingredient used in the experiment was analyzed for nitrogen content by the Kjeldahl method.

In both experiments, total urine and fecal collections were made daily. Creatinine determinations were carried out daily on urine samples as a check on the completeness of individual collections. Carmine was used as a marker to indicate the beginning or ending of fecal collection periods.

RESULTS

The first experiment was designed to determine nitrogen utilization and acceptability of green and ethanol-extracted *Scenedesmus obliquus*. In determining the acceptability of this material it also seemed important to determine whether individuals would prefer to receive this material in a single concentrated form such as a biscuit or to have it distributed throughout the diet mixed with foods that might counteract the algae flavor. During the first 4 days, the subjects were able to ingest the diet containing green algae although they complained about the green color of the biscuits, the bitter taste of the whole algae, of upset stomach, and of a bloated feeling. Also of concern was algae halitosis, which made the subjects somewhat less acceptable to their compatriots. On the fifth day when algae was mixed with other foods, all of these complaints were greatly accentuated, resulting in nausea. Nitrogen balance results for the preliminary and first algae periods are presented in table 2. Mean nitrogen balance approached equi-

[6] Jello, General Foods, Inc., New York.
[7] Generously supplied by Hercules Powder Company, Harbor Beach, Michigan.
[8] Purchased from General Dynamics Corporation, Electric Boat Division, Groton, Connecticut.

TABLE 2

Nitrogen balance and apparent digestibility of algae diets

Treatment	Fecal N	N balance	Apparent N digestibility
	g/day	*g/day*	*%*
	Experiment 1		
Preliminary	0.75 ± 0.10 [1]	−0.19 ± 0.66 [1]	90
Green lyophilized *S. obliquus*	2.60 ± 0.31	+0.20 ± 0.46	68
	Experiment 2		
Extracted *C. pyrenoidosa*, low level	2.86 ± 0.23	−0.84 ± 0.30	57
Extracted *C. pyrenoidosa*, high level	4.35 ± 0.22	+0.61 ± 0.25	59

[1] SE of mean.

librium in both periods with the response being slightly improved in the period of algae feeding. However, one individual had an increased nitrogen excretion with the green algae diet.

The response to the ethanol-extracted material was rather disappointing. This material was tolerated less well than the fresh material, which is contrary to reports from other laboratories. This extraction process had removed almost all of the green color and the bitter taste. A faint-forage-like flavor remained. Four of the subjects became nauseated after completing one meal and were forced to discontinue the study. However, one subject completed the study period without any ill effect. Whether this unfavorable response was due to the extracted algae itself, or due to psychological or physiological factors from recent association with the fresh algae, is not known. It was concluded that algae was most acceptable when it was incorporated into one food in the diet. Incorporation of this green material in several foodstuffs aggravated any objections subjects might have to the algae.

A second experiment was conducted to obtain further information on the level of dietary algae nitrogen necessary to maintain a positive nitrogen balance. *Chlorella pyrenoidosa* thermophillic strain 71105 was used in this experiment. Sufficient material was available to feed algae nitrogen at 2 levels, 6.0 and 10.0 g. These levels were slightly lower and higher, respectively, than the level of algae nitrogen required in the first experiment to maintain nitrogen equilibrium.

A summary of the nitrogen balances of these subjects on 6.0 g of algae nitrogen is presented in table 2. One subject completed only the first 5 days at this level. Two days later he withdrew from the study. However, balance data indicated he was apparently in more positive nitrogen balance than any of the other subjects during this period. The remaining 4 subjects were in negative balance for the overall period. In order to have at least 5 subjects studied at the 10-g level of algae nitrogen, another subject was added to the experiment. He was given a 2-day depletion diet and then started to eat the diet providing 10 g nitrogen from algae. The summary of results of nitrogen balance data for the 10-g level are also presented in table 2. The mean balance for the 10-day period was positive, +0.62 g nitrogen compared with a negative balance, −0.84 g nitrogen, when subjects were fed 6.0 g nitrogen from algae. Four of the five subjects were in positive balance for this 10-day period. The acceptability of the algae diet in experiment 2 was markedly improved over that in experiment 1. In the second experiment no complaints of nausea, bloated feeling or bitter taste were attributed to the algae. Those complaints that were registered concerned the variable ingredients in the diet, that is, the extra calorie wafers, roll candy, jelly, etc., and the monotomy of the diet.

A high fecal excretion of nitrogen was characteristic of all levels of algae in both experiments. In experiment 1 the contrast in fecal nitrogen excretion between the preliminary period and the algae period is very marked, 0.75 g nitrogen/day, average, versus 2.60 g nitrogen/day, average. The variation in fecal excretion between individuals eating the algae diet was also

quite marked. Response to the algae diet indicated a mean apparent digestibility of 68% (range 59 to 77%) in contrast with a value of 90% (range 87 to 94%) for the diet fed during the preliminary period. High fecal nitrogen excretions were also characteristic of both levels of algae nitrogen used in the second experiment. The apparent nitrogen digestibility of the 6.0-g nitrogen diet was 58% (range 51 to 65%), and that of the 10.0-g nitrogen diet, 59% (range 54 to 66%). Again there was variation between individuals in fecal nitrogen excretion, particularly with the 10.0-g nitrogen level. The difference in apparent digestibility of the algae was the major contributing factor to a positive or negative nitrogen balance.

DISCUSSION

In these studies algae was fed as the primary source of protein to human subjects. The authors are unaware of previous studies in which algae has served as the primary source of protein for a period as long as 20 days. A major observation in the present experiments is that nitrogen balance can be maintained in human subjects using algae as the protein source. The data presented in table 2 suggest that 8.0 to 10.0 g of algae nitrogen would be sufficient. A second observation in the present studies is the low apparent nitrogen digestability of the algal species used. Over 30% of the nitrogen present in the diet in experiment 1, and over 40% in experiment 2 was apparently not absorbed from the digestive tract. This utilization would undoubtedly be improved through use of a processing technique to rupture the cell walls before the incorporation of algae in the diet.

The digestibility values presented in this paper are much lower than those reported by others (3, 4) for human subjects. In the present study a direct method was used to measure nitrogen digestibility, whereas in the previous studies an indirect method was used. Thus differences between the present study and prior studies may have reflected differences in methodology used. If algae is to be considered as a major source of dietary protein the direct method is preferred since this method more adequately reflects digestibility.

Two reasons for the poor digestibility of algae may be considered: 1) resistance of the algae cell wall to disruption by the normal digestive process, and 2) release of nitrogen from the cell in a form which cannot be digested or absorbed from the digestive tract.

Assuming complete disruption of the cell, the chemical form and anatomical location in the algal cell of the nitrogen-containing compounds may exert some influence on digestibility. Data on *Chlorella pyrenoidosa* (8) suggest that approximately 3.7% of the total protein of dry algae is in the cell wall. This percentage of the total protein would be increased after ethanol extraction. This protein is part of the continuous matrix and is probably resistant to attack by proteolytic enzymes in the digestive tract. If allowance was made in the nitrogen balance data for this material, the digestibility data would be somewhat improved. However in no way could this account for the 30 to 40% of non-absorbed nitrogen.

Another possible source of non-digestible nitrogen is the non-protein nitrogen fraction. This fraction represents 15 to 20% of the total nitrogen. Nitrogen-containing compounds in algae, other than amino acids, are chlorophyll, amides, purines and pyrimidines, and amino sugars. The chlorophyll content of *Chlorella pyrenoidosa* may vary from 3.3 to 6.6% of the total weight depending upon the light intensity under which it is grown (9, 10). It is conceivable that chlorophyll may account for 3 to 5% of the total nitrogen in algae. Ammonia or amide nitrogen accounts for 8.1 to 8.9% of the total nitrogen in this species (11). Nitrogen provided by glucosamine amounts to a much smaller fraction of the total, about 0.4% (8). Of these non-protein forms of nitrogen it appears most likely that only chlorophyll would affect digestibility, since the phytyl side chain would make this compound sufficiently insoluble to be poorly absorbed. If the cell wall protein and the chlorophyll are considered essentially inert, unabsorbable forms of nitrogen, the digestibility data for the non-extracted algae (exp. 1) are somewhat improved. In experiment 2, ethanol extraction would have removed a large portion of the chlorophyll. In this

case, the effect on digestibility would be less pronounced.

Even if these factors are considered, the value for fecal nitrogen excretion would still be very high, 25 to 30% of the total nitrogen. The explanations remaining are that the bulk proteins of algae are not digestible per se, or that an intact cell wall prevents or retards access of the digestive enzymes to the bulk proteins. The latter is supported by the presence of intact algae cell membranes in the feces of human subjects (12) and that increased destruction of the algal cell wall results in increased nitrogen utilization in rats (13). That nitrogen equilibrium was maintained in the present studies indicates that the nitrogen made available to the individual was well utilized.

A problem area in algae feeding has been that of palatability. The whole green algae has a bitter taste and a forage-like flavor. The latter appears to result in a definite after-taste when this product is consumed in foods. Ethanol extraction greatly alleviated these conditions. This is in agreement with the previous reports of other workers (3). In the present studies, treatment of the extracted algae by frying in butter before incorporation into foodstuffs appeared to further reduce these objectionable properties. Others (14) report the elimination of this problem by a photobleaching process. Such material remains to be tested in larger scale nutritional studies. It appears, however, from the present studies, and those of other workers, that the problem of palatability can be overcome.

A second problem of some concern has been that of possible toxicity of algal preparations. Previous workers (5) reported that gastrointestinal symptoms occurred when 100 g or more of algae were fed. Similar observations were made in experiment 1 using whole green *Scenedesmus obliquus* as the source of algae. That algae toxicity evidenced by gastrointestinal distress may be caused by bacterial contamination has been suggested (15). These workers, in a digestion trial with rats, compared sterile heterotrophic and autotrophic cultures of *Chlorella pyrenoidosa* with a contaminated culture of this algae. It has been pointed out (14) that these experiments (15) were not continued long enough to permit an evaluation of toxicity or differences in acceptability with sterile versus "contaminated" algae. Indeed, compositional changes resulting in difference in amounts of cell wall material as well as in carbohydrates and lipids may have explained the difference in digestibility between the 2 sterile forms. Furthermore, the sterile and contaminated autotrophic cultures were not grown under the same laboratory conditions so that compositional changes resulting from such factors as differences in illumination, age of cell at harvest and components of media may have been more prominent than the effect of bacterial contamination in explaining the difference in response to the 2 autotrophic cultures. Another possible cause of the gastrointestinal disturbances might be the presence of antibacterial substances reported present in these algal species (16–19).[9] Previous workers (5) reported that symptoms were most marked at the beginning of each new algae period, and that the subjects readapted to each level. A similar adaptation phenomenon was noted in experiment 1 of our studies. Part of this adaptation was undoubtedly due to taste. Part of it might also represent an adaptation or alteration of the microflora of the gastrointestinal tract. Whatever the explanation, gastrointestinal disturbances were not a problem in experiment 2 of the present studies. Either the processing treatment was sufficient to overcome these factors, or else the factors were not present in this species of algae.

Feeding algae to humans as a major dietary item has been a virtually unexplored field of investigation. Many questions remain to be answered. What effects do species and culture conditions have on digestibility? What processing conditions will yield the most acceptable and nutritious product? What is the availability of the vitamin and caloric content of algae? Can some intermediate food producer, plant or animal, be profitably inserted in the food chain between algae and man? These remain a challenge. As the present

[9] Levina, R. I. 1961 Antagonism between planktonic algae and microflora in biological ponds. In The Purification of Waste Waters in Biological Ponds. Minsk. Akad. Nauk USSR p. 136 (cited in Biol. Abstr., 42: 18828, 1963).

studies indicate that it is possible to consume algae as a major dietary component without ill effect for longer periods than previous studies have indicated, it should be possible to investigate these questions.

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Iron Deficiency in Rats:

CHANGES IN BODY AND ORGAN WEIGHTS, PLASMA PROTEINS, HEMOGLOBINS, MYOGLOBINS, AND CATALASE [1,2]

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ABSTRACT Changes in body and organ weights; plasma protein components; hemoglobins; skeletal and heart muscle myoglobins; and liver catalase were investigated in rats consuming a diet lacking in iron. Iron-deficient rats displayed gray rather than black fur, an early marked cardiomegaly, and a delayed massive splenomegaly. Total plasma protein concentration in deficient animals, 6.49 g/100 ml, was significantly greater than that of control animals, 5.58 g/100 ml. This difference was due to a higher level of β-globulins in the deficient groups, 2.75 g/100 ml as compared with 1.80 g/100 ml in the control group. Albumin levels in deficient animals, 2.65 g/100 ml, did not differ significantly from control animals, 2.45 g/100 ml. Myoglobin concentration in hind leg muscle did not change significantly during 12 weeks of dietary iron deprivation. In weanling rats, this myoglobin concentration was 0.71 ± 0.28 mg/100 mg N, and in deficient animals at 15 weeks of age it was 0.61 ± 0.10 mg/100 mg N. During this time, however, myoglobin concentration in skeletal muscle of pair-fed control animals increased to 1.90 ± 0.31 mg/100 mg N. Heart myoglobin concentration increased in both deficient and control animals. Because of the massive increase in size, total myoglobin content of deficient hearts was much greater than that of controls. Weanling rat hearts contained an average of 0.29 mg myoglobin; after 6 weeks on the diets hearts from deficient animals contained an average of 1.43 mg myoglobin, and hearts from pair-fed controls, an average of 0.53 mg. Catalase activity did not differ in control and deficient animals. Unit activity did not change significantly during the study, but as liver size increased with age, total catalase activity of liver increased also. Heterogeneity of hemoglobin and myoglobin was demonstrated by electrophoretic separation of 4 hemoglobin fractions and 2 myoglobin fractions. However, bands from deficient animals appeared similar to those of control animals.

Lowered blood hemoglobin as a result of lack of dietary iron is well documented, but the fate of the smaller iron compartments, such as myoglobin, the cytochrome enzymes and catalase, is less certain.

Hahn and Whipple (1) observed both myoglobin and iron-containing enzymes to be normal in iron-deficient dogs and suggested that tissue iron was "inviolable." On the other hand, Gubler and co-workers (2) noted cytochrome c and myoglobin levels to be severely reduced in iron-deficient pigs. The experiments of Beutler and Blaisdell (3) with iron-deficient rats indicated decreased levels of cytochrome c in liver and kidney and of cytochrome oxidase in kidney, with no change in catalase in liver or red cells. These later workers reported cases of iron deficiency in humans not accompanied by anemia and noted subjective improvement in other patients before an improvement in the hemoglobin level.

The present study was designed to investigate the progressive effects of dietary iron deficiency in the rat on myoglobin, catalase and plasma protein concentrations as related to changes in hemoglobin. In addition, the presence and possible change of multiple forms of hemoglobin and myoglobin under the influence of severe deprivation of iron were investigated, and changes in general appearance and sizes of livers, spleens, kidneys, and hearts were noted.

EXPERIMENTAL

Male weanling rats of the Long-Evans strain from the colony of the Nutritional Sciences department were used in the experiment. They were housed in individual

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galvanized iron cages equipped with bottles of distilled water. The iron-poor diet, fed ad libitum, was whole dry milk with 10 mg $CuSO_4 \cdot 5H_2O$, 250 mg $MnSO_4$, 1 mg KIO_3 and 1.8 g choline bitartrate added/kg of milk. The control diet contained in addition 300 mg $FeSO_4 \cdot 7H_2O$/kg and was fed to 2 groups. Animals in one group were pair-fed to the experimental group, receiving food 3 times weekly. The second control group was fed ad libitum. The deficient diet contained 6 ppm iron and the supplemented diet 66 ppm.

A complete vitamin mixture was given to all animals 3 times weekly in glass castor cups. The water-soluble vitamin mix contained: (in milligrams) thiamine·HCl, 84; riboflavin, 126; niacinamide, 945; Ca pantothenate, 630; pyridoxine·HCl, 147; folic acid, 21; biotin, 21; vitamin B_{12}, 0.63; and menadione, 21; dissolved in 1500 ml 5% ethanol. Two milliliters of this solution were given each time along with 3 drops of an oil solution containing 2 g vitamin A distillate (500,000 IU/g), 250 mg irradiated ergosterol (400,000 IU/g) and 5 g *dl*-α-tocopheryl acetate and 993 g cottonseed oil.

Animals were taken for analysis at weekly intervals for 6 weeks, then every other week for six additional weeks. A total of 113 animals was analyzed; five weanlings, five in each deficient and pair-fed control group and two in each ad libitum control group. They were anesthetized with intraperitoneal injections of pentobarbital sodium [3] at levels of 9 mg/100 g of body weight. The abdominal wall was opened, blood was withdrawn from the heart with a syringe coated with a 1% solution of heparin and transferred to test tubes containing 0.15 mg heparin/ml of blood as anticoagulant. Liver, kidneys, heart, and spleen were removed, the heart was washed in saline solution and all organs were blotted and weighed. The liver and heart were wrapped in individual water-impermeable packages and frozen immediately in dry ice. In addition the hind leg muscle was removed from the bone and frozen.

Hemoglobin concentration was determined as the metcyano derivative by the method of Collier (4) modified to use larger volumes of blood, i.e., 0.1 ml from control and 0.2 ml from deficient animals. Absorption was measured at 540 mμ in a Bausch and Lomb model 20 spectrophotometer. The method was standardized against the total iron determination of Wong (5).

Total plasma protein concentration was determined by the Biuret method (6) using the Bausch and Lomb spectrophotometer. The method was standardized using crystalline bovine plasma albumin.[4] For determination of individual protein components, plasma was separated from erythrocytes by centrifuging at 3000 rev/min for 20 minutes in a refrigerated centrifuge. The proteins were separated in a Spinco Model R paper electrophoresis chamber using barbiturate buffer, pH 8.6, ionic strength 0.075, and a current of 12 ma for 16 hours. Bands were stained with Bromphenol Blue and their density measured by a Spinco Model RB Analytrol; the percentage of each protein was calculated from the densitometer tracings.

Erythrocytes were washed 3 times with 0.9% NaCl, then frozen in small plastic test tubes. To remove hemoglobin, the cells were thawed, then lysed with an equal volume of water. A half volume of chloroform was added, the mixture shaken, then centrifuged, and the clear red supernatant decanted. Multiple forms of hemoglobin were separated by electrophoresis in columns of acrylamide gel using the Canalco Model 12 disc electrophoresis apparatus.[5]

Myoglobin concentration in heart and leg muscle was determined by the method of Brown (7). The frozen muscle was sliced with a scalpel and weighed. It was ground with sand in a mortar and pestle, then transferred to a large centrifuge tube with a minimum of cold water and refrigerated overnight. Following centrifuging for 30 minutes at 10,000 rev/min, the supernatant was decanted and measured. A one-milliliter sample of this extract was applied to a 1 × 6 cm column of DEAE cellulose equilibrated with 0.05 M tris buffer, pH 8.6. On elution with buffer,

[3] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.
[4] Armour Laboratories, Chicago.
[5] The procedure as outlined in newsletter no. 3 of the Canal Industrial Corporation, Bethesda, Maryland, on hemoglobin analysis was followed.

hemoglobin remains at or near the top of the column and myoglobin passes through. The concentration of the colored eluate was measured in a Cary model 11 spectrophotometer by recording the absorption of the Soret peak between 410 and 420 mμ. A standard absorbancy of $A_{1\%} = 80$ (at the peak) was used for calculating concentration.

Multiple forms of myoglobin were separated from a concentrated solution obtained by passing a water extract of muscle through a column using 0.1 M tris buffer. At this ionic strength the myoglobin band is less diffuse than at the lower molarity and can be recovered in a more concentrated eluate. The ionic strength of the muscle extract results in a movement of hemoglobin also, but the band is distinct and slower moving than that of myoglobin so the two can be separated.

The eluate was applied to cellulose acetate strips (Sepraphore III) in a Gelman electrophoresis chamber containing 0.1 M tris-EDTA-borate buffer, pH 8.6. A current of 250 volts was applied for 90 minutes and the strips stained with Amido Schwartz.

Catalase activity was determined in one- to 2-g samples of liver. Samples were homogenized with 5 ml 0.067 M phosphate buffer pH 6.8 in a glass tissue grinder held in a beaker of ice. The homogenate was transferred to a 25-ml graduated cylinder, refrigerated for one hour, then filtered through glass wool. The filtrate was diluted 1:50, then one milliliter was used to determine catalase activity by the peroxide method of Sumner and Dounce (8). During the iodine reaction the solution was placed under a stream of nitrogen. Titration was with 0.05 N sodium thiosulfate using paragon starch indicator for the end point. The formula for calculating units of activity was: $K = \frac{1}{t} \ln \frac{C_o}{C_t}$ in which C_o = hydrogen peroxide content at zero time and C_t = peroxide content at time t.

At the time of sampling for catalase activity or myoglobin, 300- to 500-mg samples of liver or muscle were removed for nitrogen analysis by the Kjeldahl method. Iron content of livers was determined from an aliquot of this same sample after digestion with sulfuric acid. The method of Sandell (9) was used except that 0.5% 2,2-bipyridyl was used for the color formation instead of ortho-phenanthroline.

RESULTS

General appearance. Deficient animals appeared different from the controls in several ways. The coat was more sparse and unkempt. The hair of the young deficient rats was softer than that of the controls, but was matted and harsh to the touch in older animals. A red scaly deposit was observed on the skin of some of the deficient animals, especially on the back.

The animals were all black or black and gray hooded. By the end of the first month a definite graying of the hair of the iron-deficient group was noticed. This observation has been reported elsewhere (10).

The two long inciser teeth of the deficient animals were pearly-white as compared with the brown-tinged ones of the controls. The paws of the deficient group were white in contrast with the pink paws of the controls. These changes were apparent by the end of the first week.

Body and organ weights. Weights given in tables 1–5 are those at autopsy and are the average of 5 animals for deficient and pair-fed control groups and 2 animals for the ad libitum control groups. By the twelfth week, iron-deficient animals had larger hearts, spleens and kidneys, but the rate of increase varied for each organ. Cardiomegaly was pronounced by the first week. By the sixth week of the study, the heart from any deficient animal was about twice the size of that from its pair-fed control, and this ratio was maintained for

TABLE 1

Body weights of iron-deficient, pair-fed control, and ad libitum control rats

Week	Deficient [1]	Pair-fed [1]	Ad libitum [2]
	g	*g*	*g*
0	57 ± 1	57 ± 1	57 ± 1
1	83 ± 3	83 ± 11	99 ± 7
2	104 ± 7	104 ± 5	116 ± 13
3	102 ± 23	124 ± 6	138 ± 11
4	106 ± 41	121 ± 29	173 ± 0
5	126 ± 28	149 ± 20	207 ± 5
6	128 ± 7	133 ± 30	262 ± 4
8	195 ± 30	199 ± 36	283 ± 4
10	238 ± 28	248 ± 38	294 ± 45
12	254 ± 45	286 ± 23	373 ± 18

[1] Mean from 5 animals and standard deviation.
[2] Mean from 2 animals (except five at zero week)

TABLE 2

Weight of hearts from iron-deficient, pair-fed control and ad libitum control rats

Week	Deficient [1]	Pair-fed [1]	Ad libitum [2]
	g	*g*	*g*
0	0.29 ± 0.05	0.29 ± 0.05	0.29 ± 0.05
1	0.47 ± 0.03	0.35 ± 0.04	0.37 ± 0.01
2	0.62 ± 0.11	0.38 ± 0.01	0.40 ± 0.05
3	0.74 ± 0.20	0.41 ± 0.02	0.51 ± 0.06
4	0.75 ± 0.10	0.39 ± 0.09	0.57 ± 0.03
5	0.89 ± 0.32	0.47 ± 0.05	0.62 ± 0.03
6	0.90 ± 0.17	0.43 ± 0.09	0.77 ± 0.01
8	1.09 ± 0.16	0.54 ± 0.07	0.79 ± 0.03
10	1.33 ± 0.08	0.66 ± 0.06	0.82 ± 0.14
12	1.41 ± 0.17	0.74 ± 0.11	0.96 ± 0.04

[1] Mean from 5 animals and standard deviation.
[2] Mean from 2 animals (except five at zero week).

TABLE 3

Weight of spleens from iron-deficient, pair-fed control and ad libitum control rats

Week	Deficient [1]	Pair-fed [1]	Ad libitum [2]
	g	*g*	*g*
0	0.32 ± 0.05	0.32 ± 0.05	0.32 ± 0.05
1	0.31 ± 0.06	0.37 ± 0.08	0.50 ± 0.04
2	0.40 ± 0.13	0.38 ± 0.07	0.42 ± 0.01
3	0.48 ± 0.06	0.37 ± 0.03	0.48 ± 0.00
4	0.81 ± 0.19	0.32 ± 0.09	0.54 ± 0.06
5	0.92 ± 0.29	0.41 ± 0.12	0.64 ± 0.12
6	1.31 ± 0.22	0.36 ± 0.10	0.59 ± 0.16
8	1.60 ± 0.20	0.42 ± 0.08	0.58 ± 0.10
10	2.10 ± 0.66	0.47 ± 0.12	0.61 ± 0.11
12	2.34 ± 0.51	0.61 ± 0.12	0.62 ± 0.01

[1] Mean from 5 animals and standard deviation.
[2] Two animals (except five at zero week).

the remaining 6 weeks. This increase in weight was accompanied by a decrease in color, the deficient hearts being much paler.

The increase in size of spleens did not start until the third week but was more pronounced than that of the hearts during the latter half of the experimental period. During the first 10 weeks, the size of spleens in the deficient animals increased some eightfold, from 0.32 g at weaning to 2.34 g at 12 weeks.

After the third week, kidneys were also larger in the deficient animals. Even at 12 weeks, however, the difference in size was not as marked as that of hearts or spleens, being only about one-half again as large: 2.36 g compared with 1.67 g for the pair-fed controls. The color of the kidneys in deficient animals was pale yellow, as contrasted with deep red in controls.

The size of the liver increased with increasing body weight in all animals and was not influenced by diet. The appearance of the liver, however, differed greatly in deficient and control animals. Control livers were dark red and tender in texture; livers from deficient animals were yellow-brown and tough when cut.

Plasma proteins. Total plasma protein concentration was higher in deficient animals than in pair-fed controls (table 6). The difference was apparently due to an increase in the β-globulin fraction. In table 7 these differences are expressed on a statistical basis. The mean plasma protein concentration in the deficient animals was 6.49 g/100 ml and in the control animals 5.58 g/100 ml. β-Globulin concentration in the deficient group was 2.74 g/100 ml and in the control group 1.80 g/100 ml. Both of these differences were highly significant ($P < 0.001$) but the small change in albumin concentration was not significant.

TABLE 4

Weight of kidneys from iron-deficient, pair-fed control and ad libitum control rats

Week	Deficient [1]	Pair-fed [1]	Ad libitum [2]
	g	*g*	*g*
0	0.70 ± 0.06	0.70 ± 0.06	0.70 ± 0.06
1	0.84 ± 0.18	0.95 ± 0.11	1.04 ± 0.03
2	1.17 ± 0.11	1.17 ± 0.08	1.12 ± 0.11
3	1.22 ± 0.21	1.03 ± 0.11	1.18 ± 0.08
4	1.31 ± 0.26	1.13 ± 0.28	1.38 ± 0.23
5	1.44 ± 0.37	1.28 ± 0.16	1.50 ± 0.01
6	1.54 ± 0.13	1.10 ± 0.16	1.74 ± 0.10
8	1.87 ± 0.26	1.35 ± 0.21	1.82 ± 0.15
10	1.97 ± 0.31	1.49 ± 0.18	1.95 ± 0.18
12	2.36 ± 0.26	1.67 ± 0.16	2.07 ± 0.16

[1] Mean from 5 animals and standard deviation.
[2] Two animals (except five at zero week).

TABLE 5

Weight of livers from iron-deficient, pair-fed control and ad libitum control rats

Week	Deficient [1]	Pair-fed [1]	Ad libitum [2]
	g	*g*	*g*
0	2.33 ± 0.09	2.33 ± 0.09	2.33 ± 0.09
1	2.85 ± 0.27	3.13 ± 0.75	4.22 ± 0.85
2	3.66 ± 0.37	4.76 ± 0.39	4.30 ± 0.30
3	3.63 ± 0.57	4.16 ± 0.45	5.69 ± 0.17
4	4.30 ± 1.60	4.59 ± 1.23	7.25 ± 0.10
5	4.47 ± 1.03	4.90 ± 0.72	8.07 ± 0.99
6	4.80 ± 0.53	4.05 ± 0.75	9.81 ± 0.01
8	6.49 ± 0.99	5.70 ± 0.95	9.31 ± 0.42
10	7.87 ± 0.88	6.34 ± 1.22	8.83 ± 1.20
12	8.09 ± 1.54	7.68 ± 0.68	11.59 ± 1.16

[1] Mean from 5 animals and standard deviation.
[2] Two animals (except five at zero week).

TABLE 6

Plasma protein concentration in iron-deficient and pair-fed control rats

Week	Diet	Total	β-Globulin	α-Globulin	Albumin
		g/100 ml [1]	*g/100 ml*	*g/100 ml*	*g/100 ml*
0		5.1 ± 0.5	1.3 ± 0.2	0.6 ± 0.1	3.2 ± 0.2
4	− Iron	6.3 ± 0.9	2.6 ± 0.4	1.4 ± 0.4	2.3 ± 0.9
	+ Iron	6.1 ± 0.9	2.0 ± 0.5	1.5 ± 0.4	2.6 ± 0.5
5	− Iron [2]	5.8 ± 1.4	2.4 ± 0.4	1.6 ± 0.2	1.8 ± 1.0
	+ Iron	5.4 ± 0.5	1.7 ± 0.2	1.2 ± 0.2	2.6 ± 0.7
6	− Iron	6.4 ± 1.1	2.5 ± 0.7	1.3 ± 0.1	2.6 ± 0.8
	+ Iron	5.1 ± 0.7	1.5 ± 0.2	0.9 ± 0.2	2.7 ± 0.5
8	− Iron	6.4 ± 0.7	2.6 ± 0.3	1.2 ± 0.1	2.5 ± 0.3
	+ Iron [3]	5.3 ± 0.2	1.5 ± 0.3	1.0 ± 0.3	2.8 ± 0.4
10	− Iron	7.4 ± 2.6	3.4 ± 0.8	1.0 ± 0.2	3.0 ± 0.7
	+ Iron	5.7 ± 0.3	1.9 ± 0.2	1.0 ± 0.3	2.8 ± 0.2
12	− Iron	6.4 ± 0.6	2.9 ± 0.4	1.2 ± 0.2	2.4 ± 0.2
	+ Iron	5.7 ± 0.5	2.1 ± 0.5	1.2 ± 0.2	2.5 ± 0.3

[1] Mean of 5 animals and standard deviation with exceptions noted in footnotes 2 and 3.
[2] Three animals.
[3] Four animals.

TABLE 7

Statistical analysis of plasma protein concentration in iron-deficient and pair-fed control rats

Plasma protein fraction	Concentration (A) − Iron	Concentration (B) + Iron	Difference A − B [1]	*t*	*P* [2]
	g/100 ml	*g/100 ml*			
Total	6.49	5.58	0.91 ± 0.24	3.81	< 0.001
β-Globulin	2.74	1.80	0.94 ± 0.15	6.36	< 0.001
Albumin	2.45	2.65	−0.20 ± 0.15	1.36	ns

[1] Average difference between paired means and standard error. Data from 27 pairs of animals, from 4 to 12 weeks postweaning.
[2] Students *t* test (27).

Hemoglobin. Hemoglobin levels are presented in table 8. In the deficient animals there was a rapid decrease to 4.5 g/100 ml after one week from the already low value at weaning, 7.2 g/100 ml. During this first week the level increased to 10.8 g/100 ml in pair-fed control and to 11.2 g/100 ml in ad libitum control animals. Changes after the first week were more gradual; levels dropped in the deficient animals to about 3 g/100 ml at 4 weeks and increased in the pair-fed controls to about 15 g/100 ml at 6 weeks. Changes thereafter were not significant. In general, hemoglobin concentration in the ad libitum controls was lower than that of the pair-fed group of the same age, especially after the fifth week.

Myoglobin. Myoglobin concentration in hind leg muscle of 3-week-old weanlings and of animals fed the deficient diet or pair-fed the control diet for 12 weeks is presented in table 9.

In the deficient animals, the myoglobin concentration of the leg muscle was the same at the end of the experimental period as at the beginning. Although the mean concentration of deficient muscle, 0.61 mg/100 mg nitrogen, was somewhat less

TABLE 8

Hemoglobin concentration in blood of iron-deficient, pair-fed control and ad libitum control rats

Week	Deficient [1]	Pair-fed [1]	Ad libitum [2]
	g/100 ml	*g/100 ml*	*g/100 ml*
0	7.2 ± 0.5	7.2 ± 0.5	7.2 ± 0.5
1	4.5 ± 0.7	10.8 ± 1.6	11.2 ± 0.7
2	4.6 ± 0.7	12.2 ± 0.6	11.7 ± 0.9
3	3.8 ± 0.8	13.6 ± 0.8	13.2 ± 0.5
4	3.2 ± 0.4	13.7 ± 0.8	13.7 ± 1.0
5	3.4 ± 0.4	14.4 ± 0.7	13.1 ± 0.5
6	3.2 ± 0.5	14.9 ± 0.9	14.1 ± 0.4
8	3.3 ± 0.3	15.4 ± 0.9	14.0 ± 0.0
10	3.4 ± 0.4	15.5 ± 0.2	15.3 ± 0.6
12	3.2 ± 0.6	15.0 ± 0.6	14.3 ± 0.2

[1] Mean from 5 animals and standard deviation.
[2] Two animals (except five at zero week).

TABLE 9

Myoglobin concentration in hind leg muscles of weanling, iron-deficient and pair-fed control rats

Week	Diet	Myoglobin [1]	
		mg/g wet tissue	*mg/100 mg nitrogen*
0		0.21 ± 0.08	0.71 ± 0.28
12	− Iron	0.21 ± 0.03	0.61 ± 0.10
12	+ Iron	0.64 ± 0.11	1.90 ± 0.31

[1] Mean from 5 animals and standard deviation.

than the 0.71 mg/100 mg nitrogen for the weanlings, this difference is not statistically significant because of the wide variation in the weanling values.

On the other hand, leg myoglobin concentration in the control group increased to 1.90 mg/100 mg nitrogen at 15 weeks of age, which is 3 times that of the deficient group of the same age. The nitrogen content of deficient and control muscle of the same age was similar; hence this difference in myoglobin concentration is apparent also when expressed on the basis of wet weight, namely 0.64 mg vs. 0.21 mg/g.

Table 10 illustrates the change of myoglobin concentration of the heart with age. Beginning at a level of 3.5 mg/100 mg nitrogen at 3 weeks, the myoglobin level at 15 weeks was increased to 8.2 mg in the unrestricted control and to 7.9 mg in the pair-fed control animals. The concentration increased more rapidly in the pair-fed group. This doubling of the myoglobin concentration in the heart during the 12 weeks of the experiment is similar to the 2.5-fold increase in concentration in the leg during the same period.

Myoglobin concentration in hearts from deficient animals was higher than that from pair-fed controls some weeks and lower in others, but differences were small. Data are not from individual animals but from 4 or 5 pooled hearts and cannot be analyzed statistically. However, the myoglobin concentration of the heart of the deficient animal was not decreased as much as in the leg muscle. The myoglobin concentration of the heart, in fact, increased while the animal ate an iron-poor diet up to twice the original value of 3.5 mg/100 mg nitrogen.

Since the heart of the deficient animal was larger than that of the control, the total heart myoglobin was greater in the deficient animal than the control for all weeks except the first, when the difference was small (table 11). For some weeks the total myoglobin content of the deficient hearts was more than twice that of the controls, e.g., at 6 weeks 0.53 mg in the control and 1.43 mg in the deficient animals.

Multiple hemoglobin and myoglobin. Figure 1 shows the result of electrophoretic separation of hemoglobin on acrylamide gel. The bands are not stained and the picture was taken immediately after completion of the electrophoresis run. Four bands are clearly visible. The hemoglobin is from the red cells of animals receiving the experimental diets for 12 weeks. The gel on the right shows separation of hemoglobin from a control animal compared with that of a deficient animal on the left. No differences between the two can be seen, either in the number or location of the bands or in their apparent concentra-

TABLE 10

Myoglobin concentration in hearts of iron-deficient, pair-fed control and ad libitum control rats

Week	Deficient [1]	Pair-fed control [1]	Ad libitum control [2]	Deficient [1]	Pair-fed control [1]	Ad libitum control [2]
	mg/g wet tissue			*mg/100 mg nitrogen*		
0	0.96	0.96	0.96	3.5	3.5	3.5
1	1.22	1.70	1.09	4.4	6.1	3.7
2	1.58	1.65		5.9	5.7	
3	1.29	1.75	1.27	4.6	6.1	4.3
4	2.07	1.70		7.2	5.6	
5	1.31	1.75	1.88		5.5	6.3
6	1.63	1.37		5.9	4.5	
8		2.36	1.73		7.7	5.8
10		2.06	1.97		7.8	6.5
12		2.33	2.42		7.9	8.2

[1] Five hearts pooled for the determination.
[2] Four hearts, weeks 1 to 6; 2 hearts, weeks 8 to 12.

TABLE 11

Total myoglobin content of hearts from iron-deficient, pair-fed control and ad libitum control rats

Week	Heart weight			Myoglobin content		
	Deficient [1]	Pair-fed [1]	Ad libitum [2]	Deficient [1]	Pair-fed [1]	Ad libitum [2]
	g	*g*	*g*	*mg*	*mg*	*mg*
0	0.30	0.30	0.30	0.29	0.29	0.29
1	0.44	0.34	0.39	0.54	0.58	0.42
2	0.60	0.37		0.95	0.61	
3	0.82	0.39	0.51	1.06	0.68	0.65
4	0.73	0.37		1.51	0.63	
5	0.86	0.43	0.67	1.13	0.75	1.26
6	0.88	0.39		1.43	0.53	
8		0.53	0.75		1.25	1.30
10		0.64	0.75		1.32	1.54
12		0.72	0.93		1.68	2.25

[1] Five hearts pooled for the determination.
[2] Four hearts, weeks 1 to 6; 2 hearts, weeks 8 to 12.

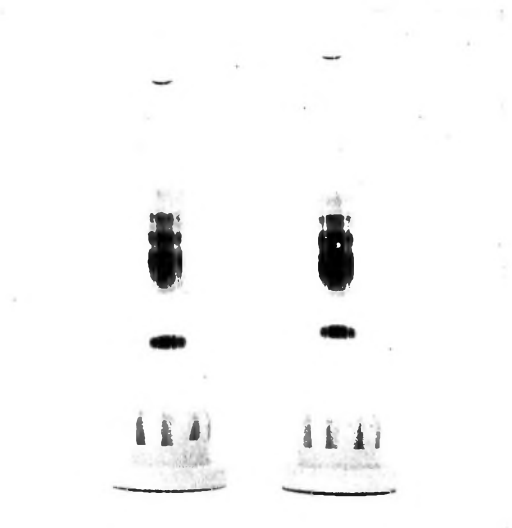

Fig. 1 Electropheretic separation on acrylamide gel of hemoglobin from blood of iron-deficient and pair-fed control rats. Column on the left from iron-deficient and on the right from pair-fed control animal. Bottom band is bromphenol blue tracking dye. Hemoglobin bands unstained.

tion. The hemoglobin concentration in the blood of the control animal was 15.6 g/100 ml and that of the deficient animal was 3.8 g/100 ml. Although the quantity of hemoglobin being produced was decreased fourfold, the types of hemoglobin apparently remained the same.

Electrophoresis on cellulose acetate strips of myoglobin from either deficient or control animals produced 2 components which appeared identical in location and similar in quantity. On acrylamide gel, myoglobin also separated into 2 bands, one of much greater quantity than the other.

Catalase. Catalase activity in deficient and control livers did not differ (table 12) and did not change as the animals grew older, remaining essentially the same whether related to the wet weight or nitrogen content of the liver or to the total body weight of the animal. The variation within any group was considerable, however.

Total catalase activity of the liver increased as liver size increased, but the size of the liver was related to body weight, and not to the presence or absence of iron in the diet.

Liver iron. Iron content of livers from pair-fed control animals from weeks 6 through 12 is shown in table 13. The concentration remained about the same during this time, averaging from 0.19 mg to 0.24 mg/g, but with increasing liver size, total liver iron content increased steadily with age, from 0.9 mg at 6 weeks to 1.6 mg at 12 weeks. Iron levels in livers from weanling or deficient animals were below the limit of detection of the method used.

TABLE 12

Catalase activity in livers from iron-deficient and pair-fed control rats

Week	Diet	Catalase activity [1]			
		units/g tissue	*units/g nitrogen*	*units/ liver*	*units/g body wt*
0		81 ± 15	2.49 ± 0.38	188 ± 41	3.26 ± 0.71
2	− Iron	95 ± 23	2.87 ± 0.62	344 ± 91	3.29 ± 0.76
	+ Iron	72 ± 12	2.34 ± 0.33	314 ± 39	2.77 ± 0.43
3	− Iron	80 ± 30	2.47 ± 0.88	291 ± 141	2.76 ± 0.95
	+ Iron	86 ± 23	2.59 ± 0.61	348 ± 79	2.99 ± 0.69
4	− Iron	78 ± 23	2.42 ± 0.66	313 ± 153	3.00 ± 0.79
	+ Iron	88 ± 10	2.53 ± 0.25	404 ± 156	3.24 ± 0.54
5	− Iron	83 ± 12	2.56 ± 0.39	370 ± 84	3.01 ± 0.59
	+ Iron	93 ± 27	2.68 ± 0.67	445 ± 136	2.99 ± 0.78
6	− Iron	83 ± 14	2.57 ± 0.34	392 ± 60	3.08 ± 0.47
	+ Iron	90 ± 8	2.42 ± 0.23	357 ± 65	2.73 ± 0.42
8	− Iron	99 ± 16	3.05 ± 0.48	642 ± 66	3.28 ± 0.40
	+ Iron	104 ± 20	2.98 ± 0.64	687 ± 354	3.33 ± 1.23
10	− Iron	97 ± 14	3.00 ± 0.43	750 ± 99	3.18 ± 1.02
	+ Iron	111 ± 22	2.97 ± 0.66	715 ± 245	2.83 ± 0.61
12	− Iron	109 ± 35	3.49 ± 1.13	949 ± 414	3.64 ± 1.15
	+ Iron	131 ± 28	3.73 ± 0.71	1013 ± 272	3.42 ± 0.70

[1] Mean of 5 animals and standard deviation.

TABLE 13

Iron content of livers from rats receiving a diet adequate in iron but limited in quantity (pair-fed to animals eating an iron-deficient diet)

Week of experiment [1]	Liver iron [2]	
	mg/g wet	*mg/liver*
6	0.24 ± 0.08	0.9 ± 0.4
8	0.19 ± 0.05	1.1 ± 0.2
10	0.23 ± 0.03	1.4 ± 0.2
12	0.21 ± 0.03	1.6 ± 0.3

[1] Dietary treatment begun with weanling animals, 3 weeks old.
[2] Mean of 5 animals and standard deviation.

DISCUSSION

The cause of the graying of the fur in iron-deficient animals is not known. Reviews of iron deficiency (11) do not mention this phenomenon although the bleaching of the incisors is indicated as a classic symptom of iron deficiency in the albino rat. The metal commonly associated with achromotricia is copper rather than iron (12), but changes in fur in mink and of feathers in chickens have been attributed to a dietary lack of iron. A cotton-fur effect noted in mink fed a fish diet was subsequently attributed to lack of iron in the diet (13). Davis and associates (14) reported a depigmentation from red to white in feathers of iron deficient chickens eating treated soybean protein diets. Wintrobe (15) mentions, without references, that early graying of hair is often observed in anemic women. Morgan and co-workers (16) reported graying in milk-fed rats that was partly remedied by iron and copper feeding.

Splenomegaly is not common in human nutritional anemia. The cause of the massive splenic enlargement observed in this experiment is not known. It may have been secondary to changes in the liver. The livers became hard and yellow early in the experiment, whereas massive splenic enlargement occurred only after the fifth week. Blaustein and Diggs (17) report that chronic congestive splenomegaly can result from portal hypertension caused by cirrhosis of the liver. The irregular size and shape of erythrocytes in severe iron deficiency makes them more susceptible to culling by the spleen, and this greater work may result in hyperplasia. The retention of a normal, dark red color by the spleens of iron-deficient animals noted in this study is not understood. Whether this color is due to the presence of ferritin, hemosiderin or hemoglobin, it is not expected that the spleen would sequester iron rather than release it in the face of drastically lowered blood hemoglobin levels.

Significant changes in the β-globulin fraction were observed in the present study. Since the diets used were relatively high in fat, it might be assumed that

there is an increase in blood cholesterol levels with an accompanying increase in those β-globulins used for transport of cholesterol. However, the control animals were pair-fed, and we therefore assume that the increase in β-globulins in deficient animals represents an increase in circulating transferrin. Although transferrin is considered to be a plasma protein, experiments with isotopic tracers have shown that more than 50% of the exchangeable pool of transferrin is extravascular (18), that is, on the surface of cells, in the intestinal mucosa and in the bone marrow. The role of transferrin in iron absorption is under dispute at the present time, as are other facets of iron absorption. Wheby and Jones (19) indicate that transferrin saturation does not influence absorption. They do not indicate, however, whether the amount of iron in the intestine influences the distribution of transferrin. Why the transferrin would leave the site of absorption in an iron-deficient animal and enter the vascular compartment, where it is not needed for iron transport, is not clear. Some protein needs to enter the bloodstream to maintain blood volume, or in the instance of maintained volume, to maintain osmotic pressure when the packed cell volume decreases. The response of utilizing a protein already formed rather than synthesizing more albumin, for instance, would represent remarkable physiological economy on the part of the animal.

The expected lowering of hemoglobin levels in deficient animals occurred, although the fact that the low levels of about 3 g/100 ml were reached after the diet had been consumed for 4 weeks was somewhat unexpected. The ability of the animal to increase the myoglobin concentration and content of the heart and to maintain the myoglobin concentration in growing leg muscle while consuming an iron-poor diet is in contrast with this immediate decrease in hemoglobin level.

Our data on myoglobin concentration in hearts is at variance with that reported previously. Gubler and associates (2), using iron-deficient pigs, observed that myoglobin concentration in the deficient heart was less than half that of the control heart, 1.4 mg vs. 3.0 mg/g. Since cardiac hypertrophy was only moderate, the total myoglobin content of the heart was still less than that of the control, namely 194 vs. 322 mg. However, these workers did find blood hemoglobin levels reduced from 13.2 to 3.2 g/100 ml and leg muscle myoglobin reduced from 0.44 mg to 0.12 mg/g. These values are similar to levels observed in this experiment in which hemoglobin was reduced from 15.0 to 3.2 g/100 ml and leg muscle myoglobin from 0.64 to 0.21 mg/g of wet tissue.

Poel (20) noted an increase in myoglobin concentration in the heart and a decrease in the leg in simulated altitude tests with rats after 152 days. Hearts were also grossly hypertrophied and the hemoglobin level higher than normal. Poel suggests, then, that the myoglobin content of muscle is determined by muscular activity rather than anoxia. The skeletal myoglobin change noted by Poel is opposite to that observed by Reynafarje (21) in native Peruvians who had lived at sea level or at high altitude all their lives. Reynafarje reported the myoglobin concentration in the altitude-adapted men, 7 mg/g, to be significantly higher than that in men who lived at sea level, 6 mg/g.

In the vitamin E-deficient guinea pig, myoglobin concentration was higher after the animals had been fed a deficient diet for 15 days, but lower after 30 days (22). At 30 days the levels in the gastrocnemius muscle were 1.14 mg/g in the deficient and 1.51 mg/g in the supplemented animals. In the red masseter muscle, the decrease was from 3.93 mg in the control to 3.14 mg/g in the deficient group.

On the other hand, severe folic acid deficiency in the guinea pig resulted in an increase in myoglobin concentration (23). Typical figures for the combined gastrocnemius and soleus muscles were 1.64 mg/g in the deficient and 1.12 mg/g in the control animals.

Although the myoglobin content changes under the influence of these various stresses, it is not the only physiological response occurring and the situations cannot be exactly equated with one another. The increase in hemoglobin in anoxic anoxia has been mentioned. Changes in blood dissimilar to those occurring in

iron deficiency in the other stresses mentioned are an increase in red cell fragility in vitamin E deficiency and leukopenia and macrocytic anemia in folic acid deficiency.

Of the three iron compartments studied, hemoglobin showed the greatest percentage difference between deficient animals and controls at the end of the experimental period. Myoglobin was substantially reduced in skeletal muscle, and catalase was not changed. (The total amount of iron present in catalase is insignificant compared with that in hemoglobin and myoglobin.) The limited experiments carried out on multiple hemoglobins and myoglobins give no evidence for a preferential alteration of any species.

A gradually increasing liver iron concentration, from 0.06 to 0.12 mg/g has been reported in 4- to 36-week-old rats eating a laboratory chow diet (24). Total liver iron also increased from 0.2 to 2.1 mg. Although iron concentration noted in the study reported here is higher, the total liver content is about the same for animals of the same age.

McCall et al. (25, 26) measured hemoglobin, tissue cytochrome c, plasma iron, and carcass iron in rats fed iron-poor diets similar to those used in our study, and suggested that when insufficient iron is available, the relative competitive power of some body component for iron may increase at the expense of others. These workers believe that the tissue concentration does not fall below a given minimal level, and that the animal grows within the limiting amount of iron available.

Overall results indicate that several adaptive changes in the metabolism of the rats enable them to survive in the face of extremely low hemoglobin levels resulting from lack of dietary iron during a period of rapid growth. These include maintenance of original skeletal myoglobin concentration; increase in heart muscle myoglobin concentration accompanied by massive increase in heart size; increase in β-globulins and hence total plasma protein concentration; and maintenance of heterogeneous forms of both hemoglobin and myoglobin.

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Vitamin B_{12} Distribution in Cow's Milk [1,2]

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ABSTRACT The amounts of free and bound vitamin B_{12} in cow's milk were determined microbiologically with the use of the test organism, *Lactobacillus leichmannii*. Bound vitamin B_{12} was released by autoclaving the samples in the presence of acid and cyanide. Approximately 95% of the total vitamin B_{12} in milk was in the bound form. Milk proteins were separated into gross fractions by isoelectric precipitation, coagulation with rennin, centrifugation, and salting-out with ammonium sulfate. Bound forms of the vitamin occurred in all protein fractions, although the whey proteins contained more than the casein proteins, based on the amount of bound vitamin B_{12} per mg of protein. The amount of bound vitamin B_{12} in the original milk was accounted for by analysis of the casein and whey protein fractions.

Attention has been given to the determination of the vitamin B_{12} content of milk from various species, to methods for releasing bound forms in milk, and to the binding of the vitamin by a highly purified protein prepared from sow's milk (1–5). The binding of vitamin B_{12} by intrinsic factor and other protein preparations has been reviewed by Glass (6). These studies showed that greater quantities of vitamin B_{12} were bound by sow's milk proteins than by the proteins from the cow or human (7). The present study was undertaken to determine the amount of bound vitamin B_{12} present in protein fractions prepared from cow's milk.

EXPERIMENTAL AND RESULTS

Methods for measuring the "free" and "bound" forms of vitamin B_{12} in milk and milk protein fractions. Vitamin B_{12} was measured by microbiological assay with *Lactobacillus leichmannii* ATCC 7830 as the test organism and the procedures described by Gregory (1), Scheid and Schweigert (8), USP XVI (9) and the AOAC (10). The amount of free vitamin B_{12} was determined in Seitz or membrane ultrafiltrates of the samples (table 1). Three methods of obtaining maximal release of the bound form of vitamin B_{12} were compared: a) autoclaving in the presence of HCl and cyanide; b) autoclaving in sodium acetate buffer with added cyanide, and c) digestion with papain. These procedures were carried out as described below, and the results are presented in table 1.

a) Twenty milliliters of milk were diluted with an equal volume of water and 0.1 N HCl was added until the pH was reduced to 4.6. Twenty drops of a 1% (w/v) NaCN solution were added and the mixture was autoclaved for 10 minutes at 115°. After cooling, the extract was diluted to 100 ml with water and filtered. Five milliliters of the filtrate were adjusted to pH 6.8 with 0.1 N NaOH and diluted with water to a predetermined volume for assay.

b) Twenty milliliters of 0.1 M sodium acetate buffer, pH 4.6, and 20 drops of a 1% (w/v) NaCN solution were added to a 20-ml sample of milk. The mixture was heated in steam for 30 minutes, cooled to room temperature, diluted to 100 ml, adjusted to pH 4.6 and filtered. Five milliliters of solution were adjusted to pH 6.8 and diluted to volume with water in preparation for the vitamin B_{12} assay.

c) For enzymatic digestion with papain, 20 ml of milk were mixed with 20 ml of 0.1 M sodium acetate buffer, pH 4.6, warmed to 60°, and one gram of powdered papain preparation in 10 ml water was added. The enzyme was activated by the

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[3] Research taken in part from a thesis submitted in partial fulfillment of the requirements of the M.S. degree in Food Science, 1964.

TABLE 1

Vitamin B_{12} content of pasteurized milk

	Total vitamin B_{12}			Free vitamin B_{12}	
	Treated with cyanide and HCl	Treated with cyanide and sodium acetate	Treated with papain	Membrane ultrafiltrate	Seitz ultrafiltrate
	mμg/ml milk	*mμg/ml milk*	*mμg/ml milk*	*mμg/ml milk*	*mμg/ml milk*
Average [1]	269	255	267	16	14
Range	210–357	180–294	255–303	8–28	4–17

[1] Average of 3 samples.

addition of 10 drops of a 1% (w/v) NaCN solution. The mixture was incubated for one hour at 60°, steamed for 10 minutes to inactivate the enzyme, made to pH 4.6, diluted to 100 ml with water and filtered. Five milliliters of solution were adjusted to pH 6.8 and diluted for assay.

In view of the results obtained and the simplified procedures involved, Seitz filtration prior to analysis for free vitamin B_{12} and autoclaving of the samples in the presence of HCl and cyanide prior to measurements of total vitamin B_{12} were the procedures adopted for subsequent experiments. In this report, bound vitamin B_{12} is defined as the difference between the total and free vitamin B_{12} when determined by microbiological assay.

Subsequent experiments showed that the free and total vitamin B_{12} content of raw milk, pasteurized milk, and pasteurized skim milk were similar.

Determination of the free and bound vitamin B_{12} in milk protein fractions and the methods used in the preparation of these fractions. Whole casein was obtained from skim milk by 3 procedures: a) ultracentrifugation, b) coagulation with rennin, and c) isoelectric precipitation at pH 4.6. The details of the procedures used are as follows:

a) Ten milliliters of 2 M $CaCl_2$ were added to 200 ml of skim milk and the solution centrifuged at 21,000 rev/min for 90 minutes in a type 21 rotor with a Spinco model L centrifuge. Whey was separated from the casein by decantation. The pelletized casein was resuspended in 250 ml of 0.085 M NaCl solution. One hundred milliliters of 1.5 M potassium oxalate along with sufficient oxalic acid to maintain a pH of 6.7 were added. The resulting calcium oxalate precipitate was removed by centrifuging at 2,000 rev/min.

TABLE 2

Vitamin B_{12} content of skim milk and its constituent casein and whey fractions

Exp. no.	Vitamin B_{12} content of unfractionated skim milk	Method of fractionation					
		Centrifugation		Coagulation with rennin		Isoelectric precipitation	
		Casein	Whey	Casein	Whey	Casein	Whey
	mμg/100 ml	*mμg/100 ml skim milk*		*mμg/100 ml skim milk*		*mμg/100 ml skim milk*	
				Total vitamin B_{12}			
1	332	128	120	156	139	147	103
2	350	156	—	156	102	217	131
3	332	125	—	147	171	176	127
4	350	140	139	146	140	187	147
Avg	341	137	130	151	138	182	127
		(40%) [1]	(38%)	(44%)	(41%)	(53%)	(37%)
				Free vitamin B_{12}			
1	21	2	10	12	11	50	14
2	26	6	13	5	14	—	18

[1] Percentage of vitamin B_{12} accounted for in fractions.

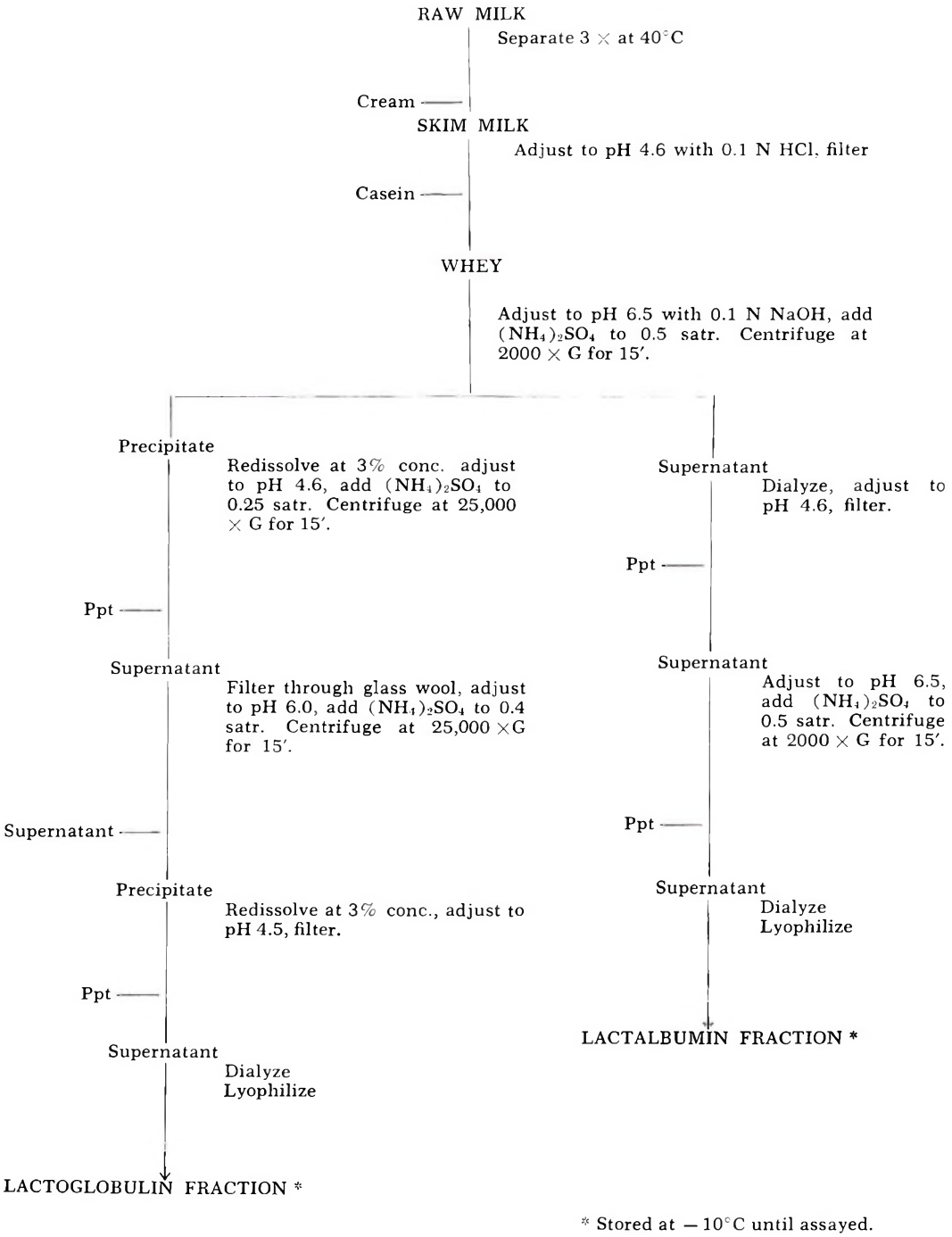

Fig. 1 Procedure for isolating lactoglobulin and lactalbumin fractions from cow's milk.

TABLE 3
Electrophoretic evaluation of lactalbumin and lactoglobulin fractions (Veronal; pH 8.6, Γ/2 = 0.1)

Fraction	Electrophoretic mobility (μ = × 10^{-5}, cm^2, v^{-1}, sec^{-1})			Proteins present [1]
	Peak	Ascending pattern	Descending pattern	
Experiment no. 1				
Lactalbumin	1	−4.12	−3.90	α-lactalbumin (75%)
	2	−5.58	−5.49	β-lactoglobulin (25%)
Lactoglobulin	1	−2.21	−2.0	immune globulin fraction (60%)
	2	−3.85	−3.65	α-lactalbumin (40%)
Experiment no. 2				
Lactalbumin	1	−3.25	−4.0	α-lactalbumin (74%)
	2	−4.17	−5.4	β-lactoglobulin (19%)
	3	−5.46	−7.2	blood serum albumin (7%)
Lactoglobulin	1	−2.30	−2.13	immune globulin fraction (73%)
	2	−4.54	−4.23	α-lactalbumin (27%)

[1] Identifications were based on data presented in the 1960 report of the Committee on Milk Protein Nomenclature and Methodology, American Dairy Science Association (13).

b) Approximately 2 ml of rennin (0.45 mg/ml) were added to 200 ml of skim milk in a water bath at 36 to 37° until coagulation was complete (30 minutes). The coagulum was dispersed and centrifuged at 2,000 rev/min for 30 minutes. Whey was separated from the casein pellet by decantation. The casein was washed with water and centrifuged at 2,000 rev/min for 30 minutes.

c) Fifty milliliters of water were added to 200 ml of skim milk and the pH of the mixture was adjusted to 4.6 with 1 N HCl. The mixture was stirred with a magnetic stirrer and centrifuged at 2,000 rev/min for 30 minutes. Whey was removed from the casein by decantation and the casein was washed with water and centrifuged at 2,000 rev/min for 30 minutes.

By these methods, about 20 to 30 g of moist casein and 170 to 180 ml of whey were obtained from 200 ml skim milk. The whey and washings from the casein were combined and treated as the whey fraction for these experiments.

TABLE 4
Vitamin B_{12} content of milk protein fractions

Fraction	No. of samples	Vitamin B_{12} content	
		Range	Avg
		μμg/mg protein	
Skim milk	6	150–209	186
Casein	5	109–183	126
Whey	9	274–495	380
Lactalbumin	16	231–482	363
Lactoglobulin	13	138–326	203

Total vitamin B_{12} was determined by grinding the casein samples with a mortar and pestle until the casein was in the form of fine particles. The ground casein was transferred to a 250-ml volumetric flask and made to 250 ml with water. Five milliliters of the diluted casein sample, as well as suitable portions of the whey samples, were assayed for vitamin B_{12} as described previously. The results obtained from these experiments are presented in table 2.

When these data are considered on the basis of concentration of vitamin B_{12} per mg of protein, vitamin B_{12} is more concentrated in the whey proteins. Consequently, the whey proteins were separated into lactoglobulin and lactalbumin fractions according to the scheme outlined in figure 1. The protein distribution in these fractions was assayed by free-boundary electrophoresis (table 3). The amount of vitamin B_{12} in the major milk proteins, expressed in micromicrograms per milligram of proteins, is listed in table 4.

DISCUSSION

The values for total vitamin B_{12} content of milk and skim milk reported herein are in good agreement with those obtained in other laboratories (11, 12). Extraction of vitamin B_{12} by autoclaving with cyanide and HCl, or with cyanide and NaAc, and digestion with activated papain proved to be satisfactory methods for the release of the protein-bound form of vitamin B_{12}. Recovery studies with added cyanocobalamin

resulted in the detection of from 95 to 101% of the original amount added.

Free vitamin B_{12} accounted for approximately 5% of the total vitamin B_{12} content of milk. Both methods, Seitz filtration and membrane ultrafiltration, gave 60 to 80% recoveries of cyanocobalamin added to milk. Gregory (1) showed that when cow's milk was extracted by cyanide or papain before ultrafiltration, 50 to 75% of the total vitamin B_{12} present in the milk could be recovered in the ultrafiltrate. Her explanation for this low recovery was that some substances present in regenerated cellulose tubing may combine with free vitamin B_{12}. The same phenomenon might account for the low recoveries observed in these studies.

The data in table 2 show that the whey samples from three different methods of casein preparation gave slight but probably nonsignificant differences in vitamin B_{12} content ranging from 103 to 171 mμg of total vitamin B_{12} in the whey prepared from 100 ml of skim milk. Also, these data show that vitamin B_{12} was approximately equally distributed between the casein and whey fractions. However, the free vitamin B_{12} in isoelectrically prepared casein showed an unusually high value. No rationale is apparent to explain this observation.

Seventy-eight to 95% of the bound vitamin B_{12} present in milk was accounted for in the casein and whey protein fractions. Of this, 90% of the vitamin present in the whey fraction was in the lactalbumin and lactoglobulin fractions. These results indicate that significant losses of the bound vitamin did not occur as a result of the fractionation procedures. As shown in table 4, the concentration of vitamin B_{12} per unit of protein was two to three times higher in the whey proteins than in the caseins. Also, it may be concluded that vitamin B_{12} is bound by all the milk protein fractions or that the specific protein-B_{12} complex was a contaminant in all protein fractions. The vitamin B_{12} content of the lactalbumin and lactoglobulin fractions varied with individual samples but was approximately equal.

It appears, therefore, that although some differences were observed in the bound vitamin B_{12} content of the major milk protein fractions, no unique or predominant binding by one fraction occurred. Experiments are now in progress to determine the magnitude of binding of added vitamin B_{12} by specific milk protein fractions and to ascertain whether specific peptides obtained from milk proteins have different binding capacities.

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Effects of Dietary Lipid and Diethylstilbestrol upon Liver Fatty Acids of Choline-deficient Rats [1]

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ABSTRACT The effects of various dietary lipids upon the composition of liver fatty acids in choline-supplemented and choline-deficient rats with and without diethylstilbestrol (DES) treatment, are presented and discussed. Changes in liver fatty acids of choline-deficient rats receiving different dietary lipids appear to be due to specific accumulation of triglycerides with their characteristic fatty acid compositions. In most cases DES treatment resulted in increases in liver monoenoic acids and decreases in stearic and arachidonic acids, particularly in the cholesterol ester fraction. However, these fatty acid effects appeared to be unrelated to the lipotropic action of DES.

Lipid accumulation in livers of choline-deficient rats is influenced by the type of dietary lipid (1), yet there is not a great deal of information concerning the effects of dietary lipids upon liver fatty acid composition in choline-deficiency. Even though it is known that estrogenic substances produce lipotropic effects in choline-deficient rats little is known of the influence, if any, of these substances upon liver fatty acid composition. This present work was an attempt to study these conditions and effects.

EXPERIMENTAL

Weanling, male albino rats (about 3 weeks old) from a stock colony were provided with tap water and experimental diets ad libitum. The basic diet was the same as reported previously (2) except that several different dietary lipids were used.[2] These lipids were selected for their varying content of saturated, monoenoic and dienoic fatty acids. The types and sources of the lipids are as follows: A, lard;[3] B, palm oil;[4] C, corn oil;[5] D, olive oil;[6] E, soybean oil;[7] F, safflower oil.[8] Vitamins and diethylstilbestrol[9] (10 ppm) were administered as reported (3). Five animals received each experimental diet for 28 days.

Liver lipids were extracted by the procedure of Hanson and Olley (4) and the percentages of total lipids determined gravimetrically. Total sterol was determined by the procedure of Pearson et al. (5) as further modified (6). Phospholipids were estimated by a method described by Sunderman et al. (7). Composite samples of some of the liver lipids were separated into sterol esters, triglycerides and phospholipids by the micromethod of Lis et al. (8).

For gas-liquid chromatographic analysis all lipids were saponified and the free acids methylated with a sulfuric acid-methanol (1%) reagent. The methyl esters were then determined by an instrument employing a thermal conductivity detector. The stainless steel column (213 cm × 0.6 cm) contained 15% ethyleneglycol-succinate on 60/80 mesh Chromosorb W-HMDS, and was operated at 187° with a helium flow rate of about 80 ml/min. Injector temperature was 250° and detector temperature was 220°. Each methyl ester preparation was run at the same sensitivity to

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[2] The diet consisted of the following: (%) soy protein (Drackett Assay Protein C-1, Archer-Daniels-Midland Co., Minneapolis), 20; sucrose, 68; salt mixture-U.S.P. XIV, 4; cellulose, 3; and lipid, 5. Vitamins: (mg/kg diet) thiamine·HCl, 5; riboflavin, 5; Ca pantothenate, 10; niacin, 50; pyridoxine, 10; *p*-aminobenzoic acid, 50; inositol, 250; biotin. 0.2; folic acid, 0.2; menadione, 1; choline (when present), 1500. Vitamin A alcohol, calciferol and tocopherol were dissolved in 50% ethanol and administered *per os*. Diethylstilbestrol (10 ppm) was added to the diets in 95% ethanol solution.

[3] Rex Brand, Cudahy Packing Company, Omaha, Nebraska.

[4] The Matheson Company, Inc., East Rutherford, New Jersey.

[5] Mazola, Corn Products Company, New York.

[6] Pompeian Olive Oil Corporation, Baltimore, Maryland.

[7] Crisco Oil, Procter and Gamble Company, Cincinnati, Ohio.

[8] Gratuitously supplied by Vegetable Oil Products Company, Inc., Wilmington, California.

[9] See footnote 2.

increase reproducibility. Relative peak areas were measured as heighth times width at half heighth. A magnifying lens with inscribed scale was used to measure the widths. After multiplying each peak area by the square root of the molecular weights of the component, weight percentage compositions were calculated. Quantitative results with National Heart Institute Fatty Acid Standards B and D agree with the stated composition data with a relative error less than 0.5% for both major and minor components.

Where applicable, data were tested for significance by means of analysis of variance (9). In all cases where significant results are discussed, $P < 0.05$.

RESULTS AND DISCUSSION

The content of liver lipids in rats receiving the various treatments was not influenced by the dietary lipids. Therefore, the values were combined and the average percentages of total liver lipids with standard deviations are as follows: choline-supplemented rats, 4.9 ± 0.4; choline-deficient rats, 9.7 ± 2.0; choline-supplemented and diethylstilbestrol (DES)-treated rats, 4.8 ± 0.3; choline-deficient and DES-treated rats, 6.1 ± 0.8. The values are expressed on a fresh-weight basis and show the expected results. Choline-deficiency increased liver lipids, and DES produced a lipotropic action in choline-deficient rats.

A. Fatty acid composition of total liver lipids

The fatty acid composition of the dietary lipids appears in table 1 and the fatty acid composition of total liver lipids in rats receiving the various dietary lipids is shown in table 2.

Choline-supplemented rats. Varying levels of dietary 16:0 had little influence upon its level in liver lipids. The percentage of liver 16:0 remained fairly constant over a dietary range of 8 to 46%. With the exception of rats receiving lard, liver 16:1 was higher than might be expected from its level in the dietary lipids. This would indicate that a proportion of liver 16:1 was of endogenous origin. Like 16:0, liver 18:0 was relatively unaffected by its level in the diet. Liver 18:1 was high in all cases but was highest when dietary 18:1 was the predominant dietary fatty acid. The percentage of liver 18:2 was strongly reflected by the proportion of this acid in the dietary lipid. It appeared that dietary lipids high in 18:1 but low in 18:2 (lard, palm oil and olive oil) caused decreased levels of liver 20:4.

Choline-deficient rats. The percentage of liver lipid 16:0 in choline-deficient rats was not significantly affected by the level of this acid in the dietary lipid. Changes in dietary lipids did not influence the relative percentage of 16:1 in liver lipids. Regardless of its percentage in dietary lipids, the percentage of 18:0 in liver lipids was always decreased during choline deficiency. On the other hand, liver lipid 18:1 was always increased during choline deficiency regardless of its level in the dietary lipids. The percentage of 18:2 in liver lipid was only increased when this acid was the predominant dietary fatty acid (soybean and safflower oils). Liver lipid 20:4 was always decreased during choline deficiency. It is clear that liver lipid accumulation is not due to simple accumulation of dietary lipids.

TABLE 1

Dietary lipid fatty acid composition

Fatty acids	(A) Lard	(B) Palm oil	(C) Corn oil	(D) Olive oil	(E) Soybean oil	(F) Safflower oil
	%	%	%	%	%	%
16:0	31	46	11	10	13	8
16:1	7	1	< 1	< 1	< 1	2
18:0	22	0	0	2	3	< 1
18:1	35	44	50	83	25	8
18:2	4	9	39	5	59	82
20:4	1	0	0	0	0	0

TABLE 2

Total liver lipid fatty acid composition

Fatty acid	With choline + DES [1]	Without choline + DES	With choline	Without choline	With choline + DES	Without choline + DES	With choline	Without choline	With choline + DES	Without choline + DES
	%	%	%	%	%	%	%	%	%	%
	(A) Lard			(B) Palm oil			(C) Corn oil			
16:0	30 ± 3 [2]	34 ± 2	30 ± 2	33 ± 2	27 ± 2	29 ± 2	26 ± 2	29 ± 3	27 ± 2	29 ± 2
16:1	7 ± 1	6 ± 1	5 ± 1	5 ± 1	10 ± 1	9 ± 1	4 ± 1	5 ± 1	10 ± 1	8 ± 0
18:0	17 ± 1	10 ± 2	16 ± 1	11 ± 1	10 ± 1	9 ± 1	16 ± 1	9 ± 1	12 ± 1	10 ± 1
18:1	31 ± 1	42 ± 3	35 ± 2	46 ± 3	40 ± 2	47 ± 3	24 ± 2	39 ± 3	32 ± 2	36 ± 3
18:2	8 ± 1	5 ± 1	7 ± 1	5 ± 1	9 ± 1	6 ± 1	14 ± 1	13 ± 2	14 ± 1	14 ± 1
20:4	7 ± 2	3 ± 1	7 ± 1	< 1	4 ± 1	< 1	16 ± 2	5 ± 1	5 ± 1	3 ± 1
	(D) Olive oil			(E) Soybean oil			(F) Safflower oil			
16:0	28 ± 2	31 ± 1	25 ± 1	29 ± 2	32 ± 3	33 ± 2	29 ± 3	30 ± 1	29 ± 2	32 ± 2	27 ± 2	30 ± 1
16:1	6 ± 1	5 ± 1	12 ± 1	7 ± 1	2 ± 0	2 ± 1	6 ± 1	5 ± 0	2 ± 0	2 ± 0	6 ± 1	4 ± 0
18:0	14 ± 1	9 ± 1	10 ± 1	9 ± 1	13 ± 2	7 ± 1	9 ± 1	8 ± 2	16 ± 1	9 ± 1	12 ± 0	10 ± 1
18:1	40 ± 3	50 ± 3	44 ± 1	49 ± 2	22 ± 1	30 ± 2	28 ± 2	31 ± 2	20 ± 2	24 ± 0	26 ± 1	27 ± 2
18:2	6 ± 1	4 ± 1	6 ± 1	4 ± 1	17 ± 1	21 ± 1	18 ± 1	19 ± 1	22 ± 1	29 ± 2	23 ± 2	25 ± 1
20:4	6 ± 1	1 ± 0	3 ± 1	2 ± 0	14 ± 2	7 ± 2	10 ± 1	7 ± 1	11 ± 1	4 ± 0	6 ± 1	4 ± 1

[1] Diethylstilbestrol.

[2] Average values ± SD.

Choline-supplemented and DES-treated rats. The most striking effect of DES treatment of choline-supplemented rats was the almost consistent increase in the percentages of the liver lipid monoenoic acids, 16:1 and 18:1. An exception was the rats fed a dietary lipid containing over 80% of 18:1 (olive oil). In these rats liver lipid 18:1 was already high and DES did not significantly increase this fatty acid. Accompanying the increases in these monoenoic acids were consistent decreases in liver lipid 18:0 and 20:4. Liver lipid 16:0 and 18:2 were not significantly affected.

Choline-deficient and DES-treated rats. The liver fatty acid composition of these rats appeared to reflect both the effects of choline-deficiency and DES treatment. Liver lipid 18:0 and 20:4 were decreased and 18:1 was increased as in both choline-deficient and DES-treated rats. Liver lipid 16:1 was increased as in DES treatment and liver lipid 18:2 was increased in those rats receiving dietary lipids containing the highest level of 18:2 as in choline-deficiency.

B. Fatty acid compositions of liver lipid fractions

Composite samples of the liver lipids of rats receiving lard, olive oil and safflower oil were separated into cholesterol esters, triglycerides and phospholipids and the fatty acid percentages of these lipids were determined. These values appear in table 3. Analysis of variance could not be performed upon these values, and therefore, only large differences or consistent differences throughout the groups of rats are discussed.

Choline-supplemented rats. Cholesterol esters. Lard contained more 16:0 and 18:0 than olive oil and safflower oil and the liver cholesterol esters in rats fed this lipid contained more 16:0 and 18:0 than esters of the other 2 groups. Olive oil contained over 80% 18:1 and the cholesterol esters of rats fed this lipid contained the highest level of 18:1. However, 18:1 was the predominant cholesterol ester in all groups regardless of its dietary level. Cholesterol ester 18:2 was also influenced by the quantity of dietary 18:2 since safflower oil produced the highest level of this cho-

TABLE 3

Fatty acid composition of liver lipid fractions

Fatty acid	(A) Lard				(D) Olive oil				(F) Safflower oil			
	With choline	Without choline	With choline + DES [1]	Without choline + DES	With choline	Without choline	With choline + DES	Without choline + DES	With choline	Without choline	With choline + DES	Without choline + DES
	%	%	%	%	%	%	%	%	%	%	%	%
Cholesterol esters												
16:0	24	23	17	19	18	17	16	16	17	16	13	16
16:1	9	13	19	19	10	10	21	17	10	8	19	16
18:0	19	12	< 1	< 1	8	7	< 1	< 1	5	5	< 1	< 1
18:1	36	41	61	57	58	60	58	60	38	38	51	47
18:2	6	6	3	5	6	6	5	7	22	27	17	21
20:4	6	5	< 1	< 1	< 1	< 1	< 1	< 1	8	6	< 1	< 1
Triglycerides												
16:0	33	34	33	33	34	34	31	33	34	35	30	32
16:1	8	9	10	11	8	8	12	11	8	6	10	10
18:0	2	2	1	2	2	2	2	1	4	3	3	2
18:1	54	53	55	52	53	54	54	54	26	26	33	34
18:2	3	2	1	2	3	2	1	1	28	30	24	22
20:4	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Phospholipids												
16:0	29	32	30	29	28	27	29	29	28	28	30	29
16:1	5	3	8	6	5	4	8	6	< 1	< 1	4	4
18:0	24	29	19	22	25	29	19	22	29	32	21	23
18:1	19	16	22	22	21	17	27	24	8	8	12	9
18:2	8	7	9	10	8	8	9	8	17	16	19	20
20:4	15	13	12	11	13	15	8	11	18	16	14	15

[1] Diethylstilbestrol.

lesterol ester. The presence of very high levels of dietary 18:1 (olive oil) caused a decrease in cholesterol ester 20:4.

Triglycerides. Liver triglyceride 16:0 was relatively unaffected by dietary levels of this acid. The triglycerides were characterized by low levels of 18:0 and only traces of 20:4. The level of 18:0 remained low even when the dietary lipid (lard) contained 22% of this acid. In the triglycerides of rats fed lard and olive oil, 18:1 made up over 50% of the total acids. Large quantities of dietary 18:2 (safflower oil) caused quite high levels of triglyceride 18:2 with a corresponding decrease in 18:1.

Phospholipids. As in triglycerides, the level of phospholipid 16:0 was unaffected by levels of dietary 16:0. In all groups the predominant acid in the phospholipids was 18:0 and its percentage was not influenced by its dietary level. The feeding of lipid containing high levels of 18:1 (olive oil) did not cause an increase in phospholipid 18:1. High levels of dietary 18:2 (safflower oil) did increase phospholipid 18:2, and this increase was accompanied by a decrease of 18:1 as in the triglycerides. The phospholipids contained appreciable quantities of 20:4 and the percentage of this acid was not influenced by the changes in dietary lipids.

Choline-deficient rats. Cholesterol esters. Lard, containing mainly 16:0, 18:0 and 18:1, produced no changes in 16:0, a decrease of 18:0 and an increase of 18:1 in the liver cholesterol ester of choline-deficient rats. Olive oil which contained high levels of 18:1 did not cause an increase in cholesterol ester 18:1 during choline-deficiency. However, the percentage of this acid in the cholesterol ester of choline-supplemented rats was high. High levels of dietary 18:2 (safflower oil) did not cause appreciable increase in cholesterol ester 18:2.

Triglycerides. Choline-deficiency did not appear to have any effect upon the fatty acid compositions of liver triglycerides in any of the 3 groups.

Phospholipids. There was a consistent, slight increase in liver phospholipid 18:0 during choline-deficiency in all 3 groups. There were no consistent or large changes in the percentages of the other fatty acids in this lipid fraction after choline-deficiency.

Choline-supplemented and DES-treated rats. Cholesterol esters. DES treatment almost doubled the percentage of liver cholesterol ester 16:1 regardless of the dietary lipid fed. Cholesterol ester 18:1 also increased in those rats fed lipids containing low or moderate levels of 18:1 (lard and safflower oil). In those rats fed lipid with a high content of 18:1 (olive oil), liver cholesterol ester 18:1 was not increased. However, this acid already contained about 60% of the cholesterol ester fatty acids in choline-supplemented rats. Accompanying the increases in 16:1 and 18:1, were consistent decreases in cholesterol esters 18:0 and 20:4.

Triglycerides. In all cases DES treatment caused a slight increase in liver triglyceride 16:1 and a slight decrease in 18:2. DES treatment did not increase triglyceride 18:1 in livers of those rats fed lard and safflower oil, but this acid was high in the triglycerides of the choline-supplemented rats. There was a definite increase in triglyceride 18:1 in livers of those rats fed safflower oil after DES treatment. The percentage of triglyceride 18:0 was low in the choline-supplemented rats and DES did not decrease it further.

Phospholipids. There were consistent, although small, increases in liver phospholipid 16:1 and 18:1 after DES treatment, and these were accompanied by consistent and small decreases in phospholipid 18:0 and 20:4.

Choline-deficient and DES-treated rats. There were indications that both choline deficiency and DES treatment were influencing the fatty acid composition of liver cholesterol esters and phospholipids in all 3 groups. Only the influence of DES was noted in the liver triglyceride fatty acids of choline-deficient rats since the composition of these acids was unaffected by choline deficiency.

It is well known that the increase of liver lipids in choline-deficient rats is mainly due to an accumulation of triglycerides. Data presented here indicate that choline-deficiency itself has no effect on the fatty acid composition of liver triglycerides. However, due to an accumulation of this fraction with its specific fatty acid

TABLE 4

Liver sterols [1]

Dietary lipid	Sterols			
	With choline	Without choline	With choline + DES [2]	Without choline + DES
	%	%	%	%
(A) Lard	0.22 ± 0.01 [3]	0.27 ± 0.05	0.52 ± 0.03	0.43 ± 0.01
(D) Olive oil	0.35 ± 0.03	0.43 ± 0.08	0.47 ± 0.05	0.58 ± 0.08
(F) Safflower oil	0.34 ± 0.06	0.39 ± 0.02	0.43 ± 0.01	0.43 ± 0.01

[1] Expressed as percentage of fresh weight.
[2] Diethylstilbestrol.
[3] SD.

composition, choline-deficiency should produce effects on the fatty acid composition of total liver lipids. Values in table 3 show that the liver triglycerides of choline-supplemented rats fed lard and olive oil contained a high level of 18:1 and low levels of 18:0 and 20:4. Furthermore, these proportions were not changed by choline-deficiency. Therefore, an accumulation of these triglycerides during choline-deficiency could cause an increase in the total liver lipid percentage of 18:1 and decreases in 18:0 and 20:4. Table 2 shows this to be true. The liver triglycerides of rats receiving safflower oil contained appreciable quantities of 18:1 and 18:2 and low quantities of 18:0 and 20:4. Accumulation of these triglycerides might cause increases in percentage of total liver lipid 18:1 and 18:2 and decreases in 18:0 and 20:4. Table 2 also indicates that this is true. However, the liver triglycerides in all 3 groups of choline-supplemented rats contained high levels of 16:0 and yet this acid was not significantly increased in the total liver lipids after choline-deficiency. This could be explained by the fact that phospholipids and triglycerides contain similar percentages of 16:0 (table 3). Therefore, an increase in one of these fractions would not necessarily increase the proportion of 16:0 in the total.

The DES treatment significantly increased liver cholesterol in choline-supplemented rats fed lard, olive oil and safflower oil (table 4). It is known that this increase in liver cholesterol is due to a specific increase in cholesterol esters (10). Furthermore, this study has shown that DES caused considerable increases of 16:1 and considerable decreases of 18:0 and 20:4 in these cholesterol esters (table 3). These effects, coupled with small but consistent increases of 16:1 in both triglycerides and phospholipids and decreases of 18:0 and 20:4 in phospholipids noted after DES treatment (table 3), could therefore mean increases of 16:1 and decreases of 18:0 and 20:4 in the total liver lipids of these 3 groups of choline-supplemented rats after DES treatment. Data in table 2 show this to be true.

Even though DES treatment produced specific effects upon the levels of certain fatty acids in rat livers, there did not appear to be any correlation between these effects and the lipotropic effect. Consequently DES does not cause decreased accumulation of liver lipid during choline-deficiency by decreasing the levels of specific fatty acids.

Liver lipid phospholipids were calculated in the livers of the rats receiving lard, olive oil and safflower oil but these values were not significantly influenced by the dietary lipids. Therefore, the values were combined and the average percentages of liver phospholipids with standard deviations are as follows: choline-supplemented rats, 2.6 ± 0.25; choline-deficient rats, 2.1 ± 0.50; choline-supplemented and DES treated rats, 2.3 ± 0.14; choline-deficient and DES treated rats 2.4 ± 0.20. It is apparent that the lipotropic action of DES is involved in a specific decrease, or inhibition in accumulation, of liver triglycerides.

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Dietary Phosphorus and Magnesium Deficiency in the Rat [1]

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ABSTRACT Calcium, phosphorus and magnesium balance was studied in weanling rats, using 3 dietary levels of magnesium (130, 260 and 1,000 ppm) at each of 3 levels of phosphorus (0.3, 0.5 and 1.0%). The effect of changes in dietary phosphorus on magnesium balance varied and was dependent on the dietary magnesium concentration; high intakes of phosphorus lowered the apparent absorption of magnesium when magnesium was ample, but improved absorption when magnesium was limiting. Similarly, high intakes of magnesium reduced the apparent absorption of phosphorus only when ample quantities of phosphate were consumed. Variations in magnesium intake caused a pattern of calcium absorption which closely resembled the pattern obtained for fecal phosphorus, implying a possible effect of magnesium on calcium phosphate solubility in the gut. Accumulation of calcium in the kidneys was aggravated by either an increase in phosphorus or a decrease in magnesium. Microscopic examination of the kidneys after staining with alizarin red S revealed urolithiasis alone when the accumulation of excess calcium was minimal, but both urolithiasis and nephrocalcinosis when considerable calcium was present. This evidence supports the observations of others that intratubular cast deposition is the primary lesion in magnesium deficiency and thus suggests that magnesium might be of value in the prevention or control of spontaneous urolithiasis.

An elevation of dietary phosphorus has been reported both to antagonize and to accelerate the signs of magnesium deficiency in the guinea pig (1–5), rat (5), chick (6) and dog (7). The mechanism of this effect, however, is not yet clear.

O'Dell et al. (4) studied the effect of high phosphorus intakes on magnesium balance in the quinea pig and concluded that excess (1.8%) phosphorus interfered with magnesium absorption. Tibbets and Aub (8) and Meintzer and Steenbock (9), however, observed phosphorus to be without effect on magnesium absorption in the human and rat, respectively, although the levels used were considerably lower than those of O'Dell's group. More recently, Forbes (10) has reported that an increase in dietary phosphorus from 0.19 to 0.50% actually improved magnesium absorption and balance although precipitating a magnesium deficiency. He attributed the deleterious effect of phosphorus in this instance to the faster rate of gain with the higher phosphorus intake.

The present study was designed to measure calcium, phosphorus and magnesium balance at several levels of phosphorus and magnesium intake to gain additional information on the interrelationship of these nutrients.

EXPERIMENTAL

Nine diets were prepared yielding 3 levels of phosphorus (0.3, 0.5 and 1.0%) at each of 3 levels of magnesium (130, 260 and 1,000 ppm). The calcium level was kept constant and averaged 0.65% of the diet. The basal diet (0.3% P, 130 ppm Mg) was composed of casein, 20%; sucrose, 62%; cottonseed oil,[4] 8.0%; cellulose,[5] 5.0%; mineral mixture,[6] 4.5%; vitamin mixture,[7] 0.4%; and choline chloride, 0.1%. Elevation of the phosphorus level from 0.3 to 0.5% was achieved by replacement of potassium carbonate in the basal mineral mix with the necessary amount of

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[1] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

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[4] Wesson Oil, Wesson Oil Sales Company, Fullerton, California.

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

[6] Jones-Foster salt mix (11) with $MgSO_4$ deleted and phosphorus content adjusted to the desired level by replacement of a portion of the KH_2PO_4 with K_2CO_3.

[7] Identical to that used by Leveille et al. (12).

KH_2PO_4. An additional 2.05 g of NaH_2PO_4 (anhydrous)/100 g diet replaced an equal weight of sucrose in the preparation of the 1.0% P diet. Magnesium was added when necessary as anhydrous magnesium sulfate with a corresponding reduction in sucrose. Thus, a relatively constant potassium level was maintained and the small variation in dietary sodium was assumed to be without significance on the questions under study.

A total of 108 weanling male rats of the Holtzman strain, weighing between 40 and 50 g each, were divided into 9 groups and allowed to consume the various rations for 2 weeks. They were then placed in metabolism cages for a 14-day period. Aliquots of the feces and urine and triplicate samples of each diet were then wet-ashed (13) and analyzed for calcium and magnesium using a slight modification of an EDTA-eriochrome black T method (14) and for phosphorus, with the method of Fiske and Subbarow (15). At the conclusion of the balance trial, the animals were killed and samples of kidney were fixed in 10% buffered formalin and stained with hematoxylin eosin and alizarin red S for histopathological examination. The remaining kidney tissue was dried to a constant weight, wet-ashed and also analyzed with the methods noted above. Aliquots of the serum collected at the time of killing were analyzed directly without prior ashing.

RESULTS

With a 20% protein diet, the lowest level of magnesium (130 ppm) was expected to cause visible signs of deficiency, that is, hyperemia, ulcerations of the skin, and reduced weight gain. The highest level of phosphorus was also expected to suppress weight gain at all levels of dietary magnesium. As expected, all groups fed a 130-ppm magnesium ration developed the characteristic visible magnesium deficiency syndrome within 2 weeks. No difference, however, could be detected in the severity or rate of appearance of the symptoms as a direct function of the dietary phosphorus. Weight gains (table 1) also followed the expected pattern with one exception. The gain of the rats fed 0.3% phosphorus was significantly less than that of the 0.5% group when magnesium was limiting (130 ppm), although no difference was noted when magnesium intakes were adequate (260 or 1,000 ppm). Presumably, this was a result of the diminished magnesium balance discussed in the next paragraph.

The mean cumulative balance of calcium, phosphorus and magnesium for all groups during the 14-day balance period is presented in table 2. The effect of variations in dietary phosphorus on magnesium balance is shown in figure 1. When dietary magnesium was present at a concentration of 130 ppm, each increase of phosphorus resulted in an increased apparent absorption of magnesium. With higher intakes of magnesium, however, apparent absorption of magnesium was significantly reduced with the 1.0% phosphorus diet as compared with levels of 0.3 or 0.5% phosphorus. Urinary magnesium excretion did not simply reflect the apparent absorption. Urinary magnesium loss appeared to be generally encouraged by the lowest phosphorus regimen. The absence of this trend at the 260-ppm plane of magnesium intake may well have been caused by small but physiologically significant variations in the actual phosphorus concentration of the various diets. Urinary magnesium content was not further reduced by the increase in

TABLE 1

Effect of the level of dietary phosphorus and magnesium on weight gain of weanling rats

Group no.	1	2	3	4	5	6	7	8	9
Dietary supplement:									
Phosphorus, %	0.3	0.5	1.0	0.3	0.5	1.0	0.3	0.5	1.0
Magnesium, ppm	130	130	130	260	260	260	1000	1000	1000
	g	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*
Days on experiment									
7	21.8	28.6	25.2	32.2	31.7	31.0	28.9	36.1	33.8
14	49.2	68.2	48.5	72.5	72.7	63.3	71.8	76.9	69.9
21	79.5	104.3	74.8	113.6	110.3	94.8	117.0	118.2	105.3
28	98.9	124.8	86.0	149.9	145.0	125.8	145.3	143.5	128.1

TABLE 2

Effect of the level of dietary phosphorus and magnesium on mineral balance in rats

Group no.	1	2	3	4	5	6	7	8	9
Dietary supplement:									
Phosphorus, %	0.3	0.5	1.0	0.3	0.5	1.0	0.3	0.5	1.0
Magnesium, ppm	130	130	130	260	260	260	1000	1000	1000
				Mean cumulative balance for 14 days					
Calcium									
Intake, mg	1064	1219	999	1214	1181	1018	910	942	957
Feces, mg	338	370	318	542	494	369	170	309	361
Feces, %	32	29	31	45	42	36	19	33	38
Urine, mg	40	3	3	21	4	4	74	8	11
Urine, %	4	0.2	0.3	2	0.3	0.4	8	1	1
Retention, mg	675	888	686	651	683	646	666	633	585
Retention, %	64	71	69	53	58	64	73	66	61
(mg/g gain)	13.6	15.7	18.3	8.4	9.5	10.3	9.1	9.5	10.1
Phosphorus									
Intake, mg	458	958	1590	609	1171	1857	469	797	1651
Feces, mg	61	132	150	157	181	234	44	128	254
Feces, %	14	14	9	26	15	13	9	16	15
Urine, mg	3	258	740	22	342	897	3	154	809
Urine, %	1	27	47	4	29	48	1	19	49
Retention, mg	387	568	700	430	648	726	422	515	588
Retention, %	85	59	44	71	55	39	90	65	36
(mg/g gain)	7.8	10.1	18.7	5.6	9.0	11.6	5.7	7.7	10.1
Magnesium									
Intake, mg	22.5	27.9	17.1	52.6	51.2	44.9	185.3	203.3	191.2
Feces, mg	24.5	20.4	11.3	28.2	23.8	30.6	46.9	55.4	81.7
Feces, %	109	73	66	54	46	68	25	27	43
Urine, mg	6.5	3.4	3.8	6.4	7.3	7.2	89.6	34.9	36.8
Urine, %	29	12	22	12	14	16	48	17	19
Retention, mg	−8.5	4.1	2.0	18.0	20.1	7.1	48.8	113.0	72.7
Retention, %	−38	15	12	34	39	16	26	55	38
(μg/g gain)	−171	72.4	53.3	232.5	278.0	113.6	663.9	1696.7	1249.4
Weight gain, 14 days, g	49.7	56.6	37.5	77.4	72.3	62.5	73.5	66.6	58.2
Ca/P ratio, retained/g gain	1.74	1.56	0.97	1.51	1.05	0.89	1.58	1.23	1.00

dietary phosphorus from 0.5 to 1.0% despite the diminished apparent absorption which accompanied this change at the higher magnesium intakes.

If increases in dietary inorganic phosphorus caused a reduced apparent absorption of magnesium because of the formation of relatively insoluble complexes, then it would also be expected to find greater quantities of fecal phosphorus following increases in dietary inorganic magnesium. This expected trend was observed (fig. 2) when phosphorus was in excess (1.0%) and to a lesser degree at the 0.5% plane. It is unexpected however, that an elevation of dietary magnesium from 260 to 1,000 ppm had no greater effect quantitatively than an elevation from 130 to 260. Moreover, the variations at the 0.3% plane followed a different pattern and were highly significant. These results suggest that interactions with other dietary constituents might be of considerable importance. The influence of magnesium on urinary phosphorus excretion also varied, dependent on the level of dietary phosphorus. No effect was observed with a 1.0% phosphorus ration. A trend towards greater phosphorus retention accompanied the highest magnesium intake at the other 2 planes of phosphorus, but a reduction of equal magnitude was also noted with the lowest magnesium level and 0.3% phosphorus. Fractionation of the fecal and urinary phosphorus into their organic and inorganic constituents might have been helpful in explaining these results.

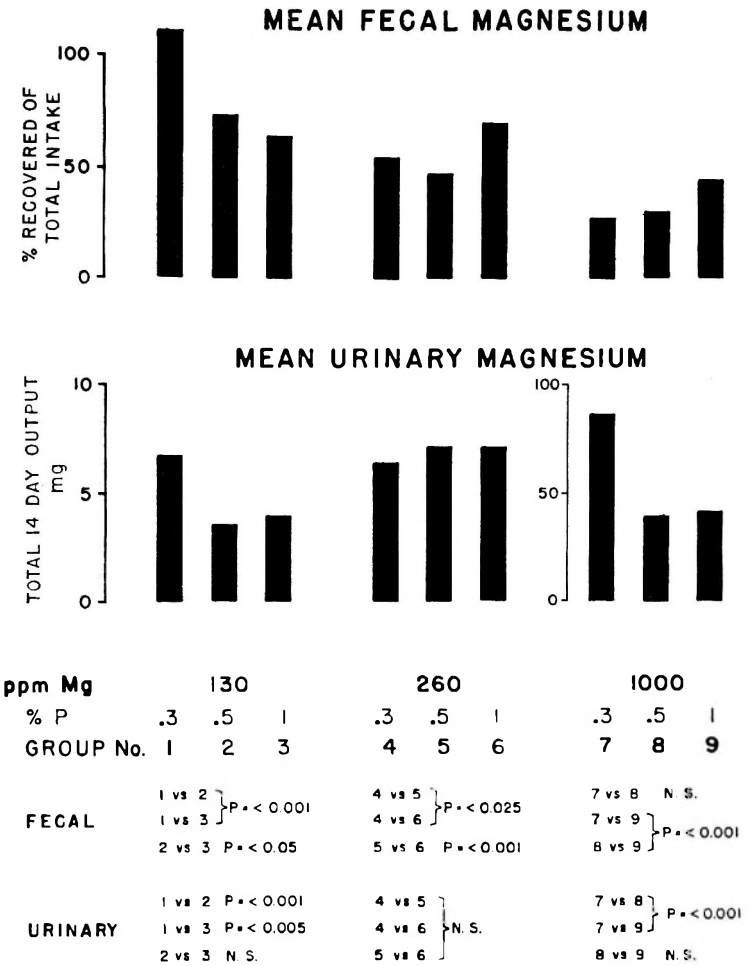

Fig. 1 Effect of variations in dietary phosphorus on magnesium balance.

The effect of dietary variations of both phosphorus and magnesium on calcium balance are also of interest. Calcium might have been expected to respond to modifications in the dietary phosphorus (fig. 3) in a manner similar to that observed for magnesium (fig. 1). The urinary excretory patterns were similar in that significantly larger quantities of calcium were excreted in the urine with the lowest phosphorus ration as compared with the higher levels. Fecal calcium also increased in response to elevations of dietary phosphorus when the dietary magnesium was high. Calcium apparent absorption, however, was improved by increases in dietary phosphorus at the 260–ppm plane of magnesium and unaffected at the 130-ppm magnesium level. Changes in dietary magnesium produced a pattern of fecal calcium (fig. 4) similar to that obtained for fecal phosphorus (fig. 2), an observation which implies that the concentration of magnesium in the gut may have an important effect on the solubility of calcium phosphate during absorption. The urinary excretory pattern showed a general tendency towards increased excretion of calcium with the highest magnesium intake at all levels of dietary phosphorus.

Analysis of the kidneys for total calcium (table 3) showed that at every level of magnesium, an increase in dietary phosphorus from 0.5 to 1.0% resulted in an

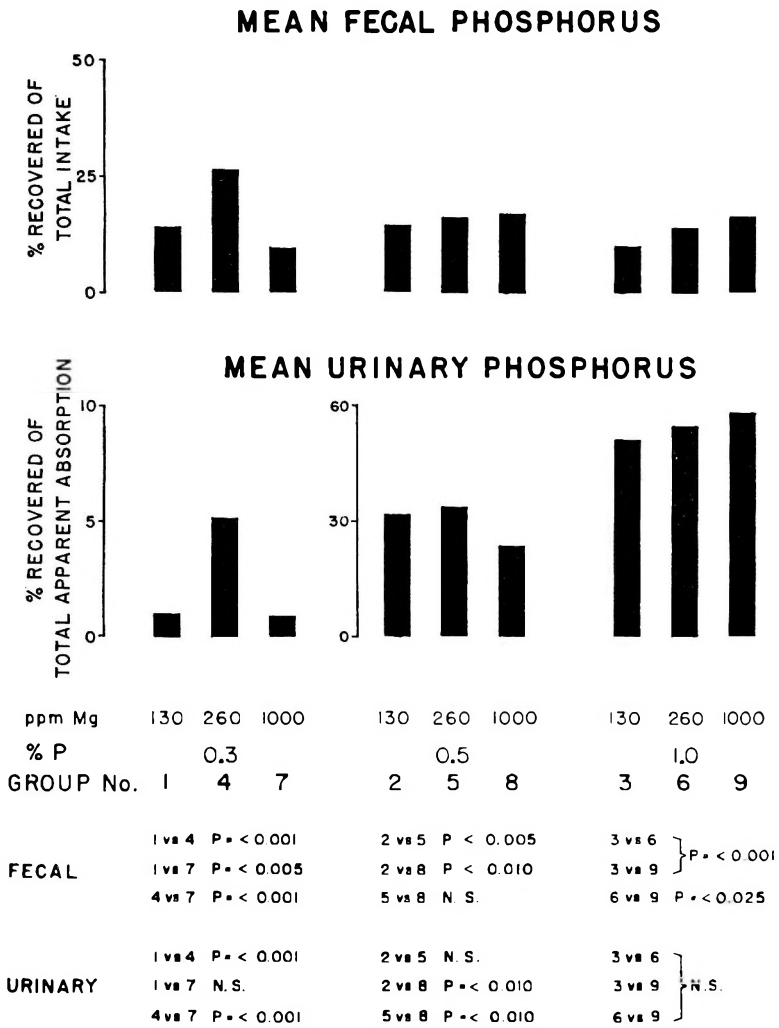

Fig. 2 Effect of variations in dietary magnesium on phosphorus balance.

increase in renal calcium. With the lowest magnesium intake, an increase in phosphorus from 0.3 to 0.5% also caused a marked increase in the quantity of kidney calcium. In particular, group 1 (130 ppm Mg, 0.3% P) had normal renal calcium values despite definite hyperemia and dermal lesions, whereas groups 6 (260 ppm Mg, 1.0% P) and 9 (1,000 ppm Mg, 1.0% P) were free of visible signs of magnesium deficiency, yet had large accumulations of renal calcium. Microscopic examination showed that those groups with a mean kidney calcium of less than 10 mg/100 g dry weight appeared normal. Both urolithiasis (intratubular calcified casts) and nephrocalcinosis (intracellular calcium deposition) were widespread in the kidneys of those animals from the 2 groups with a mean kidney calcium in excess of 1,000 mg/100 g dry weight. Urolithiasis alone was observed in the 2 groups with mean kidney calcium values between 10 and 1,000 mg/100 g dry weight.

DISCUSSION

Increases in dietary phosphorus have been reported to precipitate or aggravate magnesium deficiency in several species. The mechanism of this effect, however, is

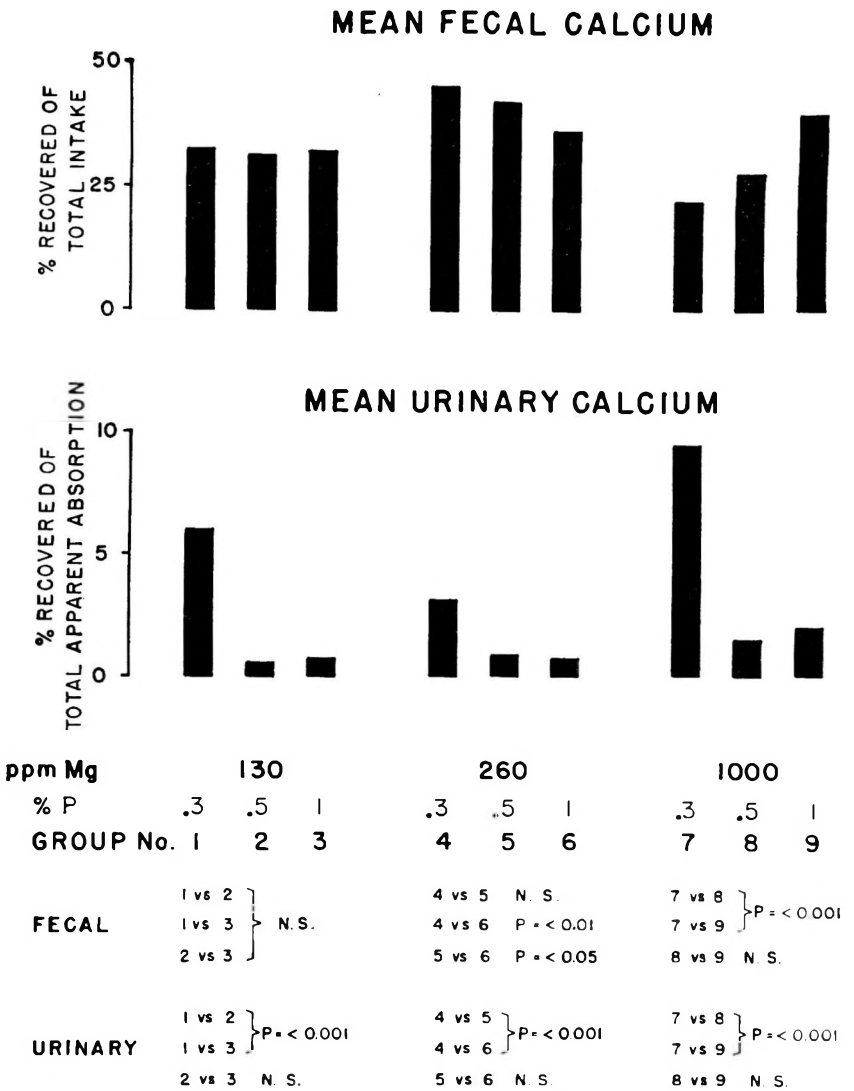

Fig. 3 Effect of variations in dietary phosphorus on calcium balance.

still in question. O'Dell et al. (4) compared the effect of increases in dietary phosphorus from 0.4 to 0.9 and 1.8% with 2 levels of magnesium (1,000 and 3,000 ppm) and concluded that a high content of dietary phosphorus contributes to the magnesium deficiency syndrome primarily by interfering with magnesium absorption and secondarily by maintaining a high blood phosphate level. This study confirms that dietary phosphate appears to interfere with magnesium absorption so long as the dietary magnesium content is relatively high. At lower levels of dietary magnesium, however, magnesium apparent absorption was actually enhanced by the higher phosphate intakes. Furthermore, no consistent elevation of serum inorganic phosphate was observed as a consequence of elevations of the dietary phosphate up to 1.0%. The importance of this mechanism at physiological levels, therefore, is questionable.

Forbes (10) has reported that increases in dietary phosphorus from 0.19 to 0.50% improved magnesium balance with diets

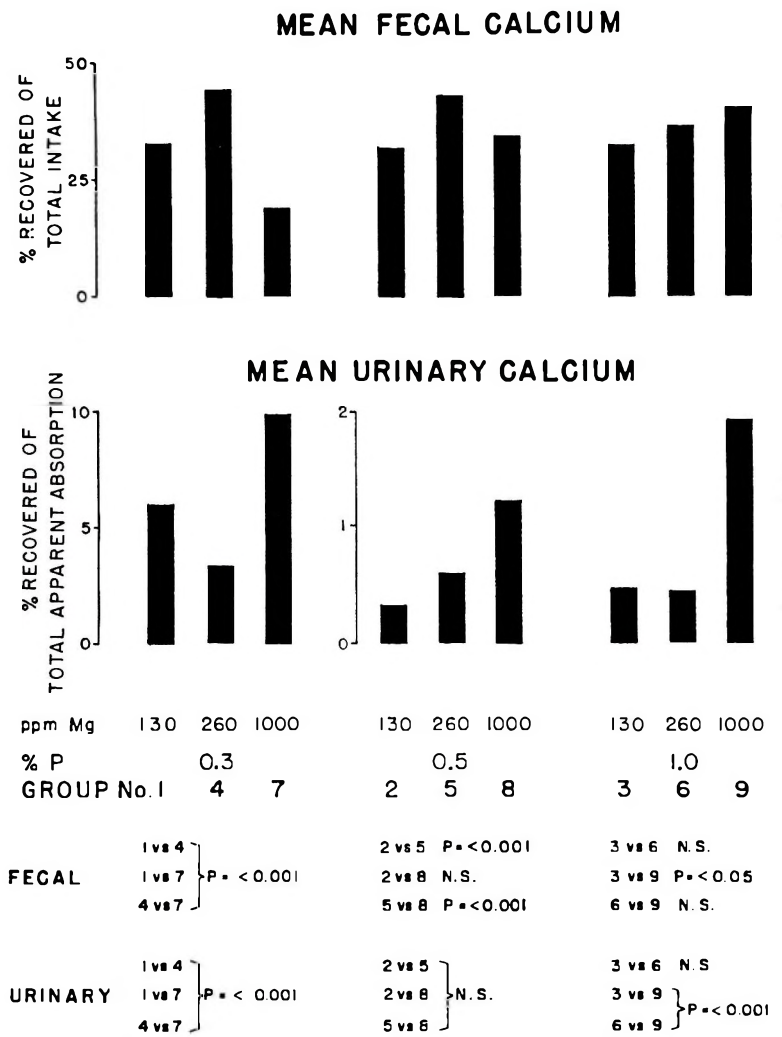

Fig. 4 Effect of variations in dietary magnesium on calcium balance.

TABLE 3

Effect of variation in level of dietary phosphorus and magnesium on tissue mineral composition

Group no.	1	2	3	4	5	6	7	8	9
Dietary supplement:									
Phosphorus, %	0.3	0.5	1.0	0.3	0.5	1.0	0.3	0.5	1.0
Magnesium, ppm	130	130	130	260	260	260	1000	1000	1000
				Mean content					
Kidney									
Calcium, mg/100 g dry tissue	1	120	2028	3	7	1679	0	4	72
Phosphorus, mg/100 g dry tissue	1174	1398	2149	1101	1386	2433	1373	1397	1450
Serum									
Ca, mg/100 ml	12.6	11.5	12.4	14.2	12.9	13.3	14.2	9.2	10.4
P, mg/100 ml	11.0	8.9	11.6	11.4	10.8	9.9	8.9	8.6	8.2
Mg, mg/100 ml	0.89	1.45	1.26	2.57	1.97	1.96	2.12	2.45	2.42

containing either 0.4 or 0.8% calcium and 142 or 420 ppm magnesium, while simultaneously aggrevating the external signs of magnesium deficiency. He attributed this apparent contradiction to the greater magnesium requirement associated with the faster rate of gain at the higher phosphorus intake. Our results are in agreement since an increase in dietary phosphorus from 0.3 to 0.5% was accompanied by an improvement in magnesium balance at the 130 and 260 ppm levels of magnesium. Furthermore, in this instance, the lowest phosphorus level was not limiting of itself and, as a consequence, no aggrevation of the external signs of magnesium deficiency were detected.

Kidney calcification is a well-known feature of magnesium deficiency in the rat. Orginally, it was thought to occur as a sequel of cellular death and tubular degeneration. Ko et al. (16) and Whang et al. (17), however, conducted careful histopathological studies and concluded that intratubular cast deposition precedes rather than follows cellular death. Ko's group proposed that magnesium might be necessary to prevent calcium phosphate precipitation within the tubule. These results support this concept in that only urolithiasis occurred when renal calcium accumulation and magnesium deficiency were minimal. Furthermore, urolithiasis is not an imperative consequence of magnesium deficiency; that is, it may not be observed when the phosphate intake is low, even when the other characteristic symptoms are evident. Thus, it appears that a critical dietary intake of magnesium is necessary to prevent urolithiasis and that the absolute amount necessary is directly dependent upon the phosphate load and possibly other factors as well. It further suggests that a magnesium imbalance may well be a key nutritional factor in spontaneous urolithiasis (18, 19). The mechanism whereby magnesium might promote solubilization of calcium phosphate in the urine is not known. Morris et al. (20), however, have recently presented strong evidence of a role for magnesium in the maintenance of the integrity of body mucopolysaccharides and, since a mucoid matrix is an essential component of all calculi, this is a logical area for further investigation.

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Nutritional Value of Haitian Cereal-Legume Blends [1]

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ABSTRACT The nutritional value of various beans indigenous to Haiti was evaluated in the rat, both as a sole source of protein and when blended with rice, sorghum, or corn. For the beans alone maximal PER and feed efficiency were observed at 15 to 18% dietary protein. Amino acid supplementation of bean protein confirmed, in general, the deficiencies predicted from chemical analysis, the conspicuous exception being failure of added isoleucine to stimulate growth. Blends of beans with cereals showed maximal PER and feed efficiency when beans constituted 20 to 30% of the mixture, the higher level being particularly advantageous with sorghum. Amino acid supplementation of the blends resulted in growth responses generally predicted from chemical analysis. The quality of the protein in most of the blends was significantly inferior to casein, but comparable to Incaparina. The ability of the blends to contribute to human nutrient requirements other than for protein and calories was evaluated by comparing composition with National Research Council recommendations.

Formulation of low-cost blends of leguminous and cereal foods in which the various components compensate each other's amino acid deficiencies is one of several useful approaches to combating the malnutrition which is characteristic of the emerging nations. The Institute of Nutrition for Central America and Panama (INCAP) pioneered practical use of this principle through its development of Incaparina (1). Both the cultural and economic practicality of the INCAP approach is now established by INCAP's success in introducing the blend through normal channels of trade in Central America and Northern South America. The public health significance of the INCAP blend is perhaps most clearly apparent from the fact that under present commercial conditions the cost of 454 g of protein as Incaparina approximately equivalent in quality to milk is about one-tenth the cost of 454 g of protein purchased as milk itself. In many areas of the world products of this type have a major role to play in alleviating the multiple nutritional stresses to which large segments of the population are subjected, particularly children of pre-school age.

In most ways, the Republic of Haiti presents a nutritional picture which is typical of technically underdeveloped nations. An extensive nutritional survey concentrating on the dominantly rural population was conducted by Sebrell et al. (2). Jelliffe and Jelliffe (3) simultaneously studied the nutritional status of pre-school children. These results are in general agreement with those of Grant and Groom (4) and other nutrition-related studies (5, 6).

Several characteristics of the Haitian situation led us to conclude that cereal-legume blends even simpler than Incaparina were essential to making progress in alleviating the nutritional stress on Haitian children. The fact that the Haitian economy is the most depressed in the Western Hemisphere places products even as inexpensive as Incaparina beyond the reach of the population groups most in need of improved nutrition. The fact that very little food in Haiti passes through anything resembling a wholesale channel limits the effectiveness of any manufactured food item. Because the bulk of the rural population is illiterate and extremely naive in its nutritional understanding, any blend to be encouraged through rural

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health centers, home economists, agricultural field workers, and governmental or missionary school programs must be extremely simple. For economic and cultural reasons the exclusive use of indigenous foods seems dictated. Three cereals provide the bulk of the calories in the Haitian diet, (rice, sorghum and corn) but in specific areas only one of these is often used. As a result, separate blends for each of the cereals are needed.

We have, therefore, set as objectives the development of a series of binary cereal-legume blends of indigenous foods having a level and quality of protein adequate to prevent clinical protein-calorie deficiency disease, but not necessarily adequate to support maximal growth and development of children or to correct the endemic vitamin and mineral deficiencies that are known to exist.

In developing those binary blends, reliable information was needed on the amino acid composition, total protein content, annual production, and cost of the staple foods potentially useful in blending. These data describing 86 samples including all varieties of all seed-crop foods indigenous to Haiti have been published elsewhere (7). The present data describe rat performance when fed blends were selected on the basis of chemical composition, cost, and annual production to be most likely to be useful in combating malnutrition in Haiti.

EXPERIMENTAL

All of the natural foods were samples collected in local Haitian markets (7) brought into the United States without sterilization. On arrival, seeds damaged by insect infestation or fungal infection were removed, and the samples were stored frozen. The refuse and surplus supplies were sterilized at 121° for one hour before being discarded to prevent introduction of new insect pests or plant pathogens. All beans were heated at 121° for 15 minutes in 2 volumes of water and then dried at 80° for 18 hours to destroy trypsin inhibitor and other toxins. These conditions were selected on the basis of the detailed study by Bressani et al. (8) of the effect of heat on the toxicity and nutritional value of taxonomically identical beans from Central America. The foods were ground to pass a 40-mesh screen before formulation into diets.

The results of proximate analyses (9, 10) are shown in table 1.

In all experiments except that carried out according to the method of Campbell (11) weanling male rats derived from the Sprague-Dawley strain were fed ad libitum for one week a stock diet containing: (in grams) cottonseed oil, 8; salt mixture USP XVI, 5; powdered cellulose,[3] 2.4; vitamin mixture, 1; vitamin-free casein, 15; sucrose, 63.6; and water, 5. The vitamin mixture contained: (in grams) Ca pantothenate, 2; niacin, 1; inositol, 1; thiamine ·HCl, 0.5; menadione, 0.4; riboflavin, 0.3; pyridoxine·HCl, 0.3; folic acid, 0.02; biotin, 0.01; vitamin B_{12}, 0.001; and sucrose to make 1000. At the end of this period the animals were divided by weight into groups of 6 rats each and fasted for 24 hours before being weighed. Water was available at all times. Vitamins A, D, and E were administered orally each week in cottonseed oil. Experimental periods are indicated in footnotes to the individual tables.

In the balance study feces were collected daily and frozen. Urine was collected in 6 N HCl under toluene and frozen daily.

All diets were equalized for fat, moisture, ash, and fiber at 8, 6.2, 5, and 6%, respectively, by appropriate addition of cottonseed oil, water, USP XVI mineral mixture, and powdered cellulose.[4] Protein levels were determined assuming protein = N × 6.25. Varying protein levels were introduced at the expense of sucrose. Diets were refrigerated, and fresh food was provided daily. Any uneaten diet was discarded.

The experiment carried out according to the method of Campbell (11) was conducted precisely as recommended despite reservations concerning the value of certain of the procedural details.

RESULTS

Table 1 shows the proximate analysis of the foods used here. In each instance the samples were pooled from the several

[3] Solka Floc, Brown Company, Berlin, New Hampshire.
[4] See footnote 3.

varieties grown in different areas of the country because no significant differences within varieties were noted in the earlier chemical studies (7).

Table 2 summarizes the PER's and feed efficiencies at several levels of dietary protein of the 3 beans that were indicated by amino acid composition and current production rates to be most promising for blending with cereals.

The results of nitrogen balance studies of the same 3 varieties of bean and of *Cajanus indicus* are shown in table 3. These evaluations were carried out at the levels of dietary protein indicated earlier to result in optimal PER.

Comparison of the amino acid composition of these beans with the estimates of Rama Rao et al. (12) of the requirements for the growing rat indicated methionine to be first limiting and isoleucine second limiting. In *C. indicus* the order of deficiency from most to least was tryptophan, isoleucine, valine, and methionine. Whether this was biologically true was assessed in the experiment summarized in table 4.

The extent to which a blend would contribute to solution of nutritional problems other than protein-calorie malnutrition is of importance also. In Haiti the most conspicuous clinical problems of inadequate vitamin intake involve riboflavin and vitamin A. Therefore it was determined whether the beans cooked long enough to accomplish the necessary detoxifications would retain enough riboflavin to represent a significant contribution to control of ariboflavinosis. Diets were prepared from the red, black, and lima bean samples of *Phaseolus vulgaris* to provide 18% dietary protein. In addition to the usual vitamin mixture, batches were prepared containing no riboflavin and one-half the usual amount of riboflavin. Diets like those described earlier were then prepared using each type of bean but having no added riboflavin, one-half the usual amount, and the usual amount. Over a 4-week period of ad libitum feeding no significant differences between growth rate or PER of weanling male rats were observed with the 9 diets.

TABLE 1

Proximate analysis of Haitian foods

Food	Protein	Ash	Moisture	Ether-extractable fraction	Crude fiber	N-free extract
	%	%	%	%	%	%
Phaseolus vulgaris, rouge [1]	23.2	3.4	6.7	1.6	6.8	58.3
Phaseolus vulgaris, noir	25.5	3.5	6.0	2.7	5.6	56.7
Phaseolus vulgaris, blanc	24.3	3.5	5.1	1.0	7.7	58.4
Phaseolus lunatus, *beurre*	24.8	3.5	6.2	2.5	6.3	56.7
Cajanus indicus, congo	21.9	2.8	5.9	2.5	8.1	58.8
Zea mays (corn)	11.1	1.2	11.9	5.3	1.8	68.7
Sorghum vulgare (sorghum)	10.6	0.6	12.0	1.3	0.2	75.3
Oryza sativa (rice)	8.1	0.3	11.2	0.7	0.3	79.4

[1] Rouge, noir, blanc, and beurre are common names referring to the red, black, white, and lima beans in Haiti. Pois congo refers to *C. indicus*.

TABLE 2

Effect of dietary protein level on PER and feed efficiency of Haitian beans

Protein source	Dietary protein level, % [1]									
	10		12		15		18		20	
	PER [2]	Eff [3]	PER	Eff	PER	Eff	PER	Eff	PER	Eff
Phaseolus vulgaris, rouge	0.69	0.08	0.83	0.12	1.14	0.21	1.16	0.25	0.92	0.23
Phaseolus vulgaris, noir	1.00	0.13	0.83	0.12	1.06	0.19	1.17	0.26	1.09	0.27
Phaseolus lunatus, beurre	0.90	0.11	0.96	0.14	1.34	0.25	0.98	0.21	1.02	0.25
Casein + 0.2% L-cystine	3.45	0.42	2.99	0.44	2.44	0.45	2.09	0.46	1.81	0.44

[1] Experimental period: 10 days.
[2] PER indicates protein efficiency ratio; all PER values adjusted to casein (unfortified) = 2.50.
[3] Feed efficiency in g of gain/g of diet consumed.

TABLE 3

Multiple assessments of nutritional value of Haitian bean proteins [1]

Parameter [2]	Dietary protein sources					
	None	10% Casein [3]	18% Pois rouge [4]	18% Pois noir	15% Pois beurre	15% Pois rouge + pois congo mix [5]
N-intake, g	0	1.33	2.14	2.34	1.94	1.97
Fecal N, g	0.06	0.14	0.74	0.80	0.63	0.69
Urinary N, g	0.10	0.25	0.77	0.79	0.68	0.61
Weight gain, g	−8	33.0	17.0	21.0	18.0	18.0
PER (casein = 2.5)	—	3.25	1.06	1.17	1.15	1.16
Feed efficiency	—	0.40	0.23	0.27	0.21	0.21
True digestibility	—	94.0	68.2	68.4	70.6	68.0
Net protein ratio	—	4.88	1.87	1.99	2.15	2.11
Protein retention efficiency	—	78.1	29.9	31.8	34.4	33.8
Biological value	—	88.0	54.0	57.0	58.0	62.0
Net protein utilization	—	0.83	0.37	0.39	0.41	0.42

[1] Experimental period: 7 days.
[2] Calculated values as described (11).
[3] Casein fortified with 0.2% L-cystine.
[4] Pois rouge, noir and beurre are common names referring to the red, black and lima beans in Haiti. Pois congo refers to *C. indicus*.
[5] One-half of the total protein in this mixture came from each of the 2 beans.

TABLE 4

Effect of amino acid supplementation on the nutritive value of Haitian beans [1]

Protein source	Supplement	Dietary protein level	PER [2] (casein + 2.5)	Feed efficiency [3]
		%		
Phaseolus vulgaris, rouge	none	18.0	0.98	0.21
	meth [4]	18.2	1.63	0.36
	isoleu	18.2	0.82	0.18
	meth + isoleu	18.4	1.51	0.33
Phaseolus vulgaris, noir	none	18.0	0.94	0.12
	meth	18.2	1.67	0.36
	isoleu	18.2	1.09	0.23
	meth + isoleu	18.4	1.49	0.33
Phaseolus lunatus, beurre	none	15.0	0.91	0.17
	meth	15.2	1.54	0.28
	isoleu	15.2	1.11	0.20
	meth + isoleu	15.4	1.83	0.34
Cajanus indicus, congo	none	15.0	1.47	0.27
	tryp	15.2	1.53	0.28
	tryp + isoleu	15.4	1.34	0.24
	tryp + val	15.4	1.67	0.30
	tryp + isoleu + val + meth	15.7	2.00	0.36
	isoleu + val + meth	15.5	1.45	0.27
Casein	cys	10.0	3.10	0.39

[1] Experimental period: 10 days.
[2] PER indicates protein efficiency ratio.
[3] Feed efficiency in g of gain/g of feed consumed.
[4] Supplementation with chromatographically pure L-isomers at 0.2% of the diet in all instances except 0.1% methionine in the *C. indicus* diets.

The effectiveness of the 4 beans as supplements at the 10, 20, and 30% levels to rice, corn, and sorghum was evaluated in terms of weight gains and PER with the results shown in table 5.

An indication was sought as to whether the amino acids predicted to be limiting in the blends from comparison of chemical data with the requirement of the rat were actually limiting in growth performance. Composition of the amino acid blends selected as representative of the more promising combinations was calculated. These values were compared with

TABLE 5

Weight gain and protein efficiency ratio (PER) of rats fed cereal-legume blends varying in composition [1]

Legume blended		Corn-bean diets [2]		Sorghum-bean diets		Rice-bean diets	
		Gain	PER [3]	Gain	PER	Gain	PER
	%	g		g		g	
None		17	1.40	6	0.53	27	2.38
Pois rouge [4]	10 [5]	26	1.78	17	1.22	34	2.60
Pois rouge	20	30	2.05	30	1.83	41	3.03
Pois rouge	30	30	1.98	30	2.13	36	2.90
Pois noir [4]	10	27	1.78	23	1.65	38	2.83
Pois noir	20	36	2.30	36	2.13	39	2.95
Pois noir	30	32	2.10	43	2.45	36	2.75
Pois blanc [4]	10	23	1.68	21	1.50	36	2.65
Pois blanc	20	28	1.83	35	2.25	39	2.80
Pois blanc	30	31	2.00	36	2.30	39	2.98
Pois beurre [4]	10	32	1.53	20	1.43	41	2.78
Pois beurre	20	36	2.28	33	1.98	42	2.90
Poie beurre	30	35	2.13	38	2.45	30	2.35

[1] Experimental period: 2 weeks.
[2] Corn and sorghum diets contained 8.3% crude protein. Rice diets contained 6.4% crude protein.
[3] All PER values adjusted to casein = 2.5.
[4] Rouge, noir, blanc, and beurre are common names referring to the red, black, white and lima beans in Haiti.
[5] Per cent represents the % by weight of beans in the bean-cereal mixture.

the estimates of Rama Rao et al. (12) of the requirements of growing rats. Diets were then prepared which were supplemented with chromatographically pure, allo-free, L-amino acids in amounts to raise them to 100% of Rama Rao's estimates of requirement. Growth and PER were then measured over a 2-week period, the results being summarized in table 6.

For purposes of comparing the nutritive value of these blends with that of similar preparations a final determination of PER was made following the procedure described by Campbell (11). These results are summarized in table 7. With all its limitations Campbell's procedure is sufficiently well-described that reproducibility of PER values from different laboratories should be good except for variability introduced by differences in the genetic line of the rats and in the environmental conditions in the animal room.

DISCUSSION

The usefulness of blends of this type remains to be determined in clinically controlled field tests in Haiti. With the data presented here, however, it appears warranted to proceed with testing the blends in humans because the major criteria for the blends have been met. A number of the blends (table 7) have PER values of 2.0 to 2.2 at the 10% protein level, and at the 15% protein levels under which actual field use is anticipated, the PER values are 2.0 to 2.3 with corn, 2.1 to 2.5 with sorghum, and 2.4 to 3.0 with rice (table 5). Total crude protein levels in blends containing 20% beans are approximately 13.7, 13.3, and 11.3% for corn, sorghum, and rice, respectively; at 30% beans the corresponding crude protein levels are 15.0, 14.7, and 12.9%. The cost of 20% bean blends and 30% bean blends compared with that of the 3 basic cereals alone has been estimated from the market data of Sebrell et al. (2). The cost of rice and beans being equivalent, blending does not add to the price of 11 cents/454 g. For sorghum a blend with 30% beans would cost about 8.9 cents/454 g compared with 8 cents/454 g for sorghum alone. Corn values fall in the range of 5 cents/454 g, and a corn-bean blend with 30% beans would cost about 6.8 cents/454 g. Market costs [5] as of January, 1965 were similar to those re-

[5] Current Haitian food prices supplied by Mlle. Gladys Dominique, Dietitian, Bureau of Nutrition, Department of Public Health, Republic of Haiti, Port-au-Prince.

TABLE 6

Effect of amino acid supplementation of cereal-legume blends [1]

Cereal-legume mixture	Supplementation	Gain	PER [2]
		g	
Corn-pois rouge (70:30) [3]	none	47	2.41
	0.28 meth [4]	58	2.60
	0.28 meth, 0.36 lys	42	2.35
	0.28 meth, 0.36 lys, 0.19 thre	66	2.69
	0.28 meth, 0.36 lys, 0.19 thre, 0.21 isoleu	43	2.38
	0.28 meth, 0.36 lys, 0.19 thre, 0.21 isoleu, 0.14 val	43	2.45
Sorghum-pois noir (70:30)	none	56	2.54
	0.30 meth	58	2.61
	0.30 meth, 0.40 lys	64	3.05
	0.30 meth, 0.40 lys, 0.21 thre	77	3.29
	0.30 meth, 0.40 lys, 0.21 thre, 0.21 isoleu	67	3.16
	0.30 meth, 0.40 lys, 0.21 thre, 0.21 isoleu, 0.13 val	69	3.33
	0.30 meth, 0.40 lys, 0.21 thre, 0.21 isoleu, 0.13 val, 0.07 tyro, 0.01 hist	72	3.41
Rice-pois rouge (80:20)	none	57	2.85
	0.25 meth	62	2.98
	0.25 meth, 0.24 thre	57	2.93
	0.25 meth, 0.24 thre, 0.41 lys	67	3.20
	0.25 meth, 0.24 thre, 0.41 lys, 0.22 isoleu	63	3.69
	0.25 meth, 0.24 thre, 0.41 lys, 0.22 isoleu, 0.09 tyro	70	3.68
	0.25 meth, 0.24 thre, 0.41 lys, 0.22 isoleu, 0.09 tyro 0.12 val, 0.08 leu, 0.03 hist	70	3.74
Corn-pois beurre (80:20)	none	52	2.50
	0.26 meth	51	2.56
	0.26 meth, 0.41 lys	46	2.49
	0.26 meth, 0.41 lys, 0.20 thre, 0.22 isoleu	49	2.94
	0.26 meth, 0.41 lys, 0.20 thre, 0.22 isoleu, 0.15 val, 0.05 tyro, 0.02 tryp	65	3.09
Sorghum-pois beurre (70:30)	none	50	2.44
	0.29 meth	50	2.47
	0.29 meth, 0.41 lys	77	3.27
	0.29 meth, 0.41 lys, 0.20 thre	65	3.18
	0.29 meth, 0.41 lys, 0.20 thre, 0.18 isoleu, 0.11 val, 0.06 tyro	66	3.28
Rice-pois beurre (80:20)	none	62	3.08
	0.25 meth	63	3.13
	0.25 meth, 0.24 thre	66	3.17
	0.25 meth, 0.24 thre, 0.39 lys	73	3.66
	0.25 meth, 0.24 thre, 0.39 lys, 0.21 isoleu, 0.07 tyro, 0.08 leu, 0.06 val, 0.02 hist	72	3.82

[1] Experimental period, 2 weeks.
[2] PER indicates protein efficiency ratio.
[3] Ratios are weight of cereal:weight of bean.
[4] Number preceding each amino acid supplement is the grams of amino acid added per 100 g of diet.

ported by Sebrell et al. (2) being as follows: pois blanc, 10; pois rouge, 14.5; pois beurre, 12; pois noir, 10; sorghum, 6; corn, 4; and rice, 10 cents (American) per 454 g. Prices fluctuate considerably with season and with crop yields, but in general this pattern is pertinent. In terms of protein quality, total protein, and cost, the 30% blends meet the first criteria sought. There may even be use for the 20% blends, particularly with corn and sorghum where the problem of low total protein is less acute than with rice.

Reasonable estimates of cereal consumption in Haiti were developed by Sebrell et al. (2) as 120 pounds [55 kg] of corn, 95 pounds [43 kg] of sorghum and millet, 23 pounds [10 kg] of rice, and 20 pounds [9 kg] of wheat per person per year. Considering the relatively large consumption of sorghum and its extremely low nutritional value (table 5), special attention to en-

TABLE 7

Growth performance and protein efficiency ratio (PER) of cereal-legume blends, Incaparina and casein determined as described by Campbell (11)

Cereal	Bean [1]	Bean in mixture	Protein [2] in diet	Avg PER [3]	Avg gain	Multiple range [4] test groupings
		%	%		g	
Sorghum	rouge	20	10.00	1.45	48.5	
Sorghum	blanc [5]	20	9.88	1.72	59.0	
Sorghum	blanc	30	10.31	1.77	63.6	
Corn	rouge	20	10.06	1.88	71.0	
Incaparina [6]	—	—	10.25	1.78	72.4	
Sorghum	rouge	30	10.13	2.02	72.8	
Sorghum	noir	20	9.81	1.85	74.2	
Rice	noir	30	9.63	2.04	76.1	
Rice	rouge	20	9.31	2.04	77.6	
Sorghum	noir	30	10.00	1.90	84.0	
Corn	noir	20	10.25	1.99	86.3	
Rice	rouge	30	9.94	2.12	86.8	
Rice	noir	20	9.38	2.19	88.7	
Corn	blanc	30	10.31	1.97	90.0	
Corn	rouge	30	10.13	2.12	94.0	
Rice	blanc	30	10.00	2.10	94.1	
Corn	blanc	20	9.81	2.04	95.8	
Corn	noir	30	10.19	2.09	95.9	
Rice	blanc	20	9.31	2.56	116.0	
Casein	—	—	10.00	2.50	131.9	

[1] Rouge, noir, and blanc are common names referring to the red, black and white beans in Haiti.
[2] Determined by Kjeldahl analysis of diets as fed.
[3] Adjusted to casein = 2.50.
[4] Bars connect values of mean gains that are not distinguishably different at $P = 0.05$ as described by Duncan (13).
[5] In this experiment only the pois blanc samples were lyophilized rather than dried at 80°.
[6] Incaparina generously supplied by Dr. Ricardo Bressani.

couraging use of sorghum-bean blends is warranted.

As in many other parts of the world, the nutritional problem in Haiti involves more than protein-calorie malnutrition. Riboflavin, vitamin A, and iodine deficiencies are prevalent, and it is probable that the intakes of iron and calcium are marginal (2). The extent to which increased uses of such blends would contribute to alleviating these nutritional stresses in addition to the protein stress is, therefore, of importance. In table 8 a general summary of the nutritional value of blends containing 30% beans and 70% of each of the three basic cereals is presented. Assuming that 75% of the daily caloric requirement of infants, children, and adults is supplied by the blends, the percentages of the NRC recommended dietary allowances (14) of other major nutrients have been calculated. Significant amounts of iron, thiamine, riboflavin, and niacin are present particularly in the corn and sorghum blends; but little contribution to the vitamin A and calcium needs is to be anticipated, particularly among the vulnerable pre-school age children.

Only in the experiment summarized in table 7 was the exact procedure of Campbell (11) used in determining PER's. Because the procedure carries the sanction of the Committee on Protein Malnutrition of the Food and Nutrition Board of the National Research Council, it can be expected to serve at least informally as a "standard" procedure. The principal procedural differences in the earlier experiments here were (a) use of protein levels other than 10%; (b) use of 6 rather than 10 animals per group; and (c) use of experimental periods of less than 28 days.

Two practical and two theoretical reasons led to our use of these modifications. From our own experience and the published reports of Bender and Doell (16), it is apparent that in general, 28-day PER values are lower than those at 10 days and 2 weeks, but the relative values remain the same. Using shorter periods and some-

TABLE 8

Nutrient contribution of cereal-legume blends [1] for people receiving 75% of their calories as blends [2]

	70% Rice + 30% bean					70% Corn + 30% bean					70% Sorghum + 30% bean				
	Infants	Children		Women [3]	Men	Infants	Children		Women	Men	Infants	Children		Women	Men
	1 year	1–3 years	3–6 years	18–35 years	18–35 years	1 year	1–3 years	3–6 years	18–35 years	18–35 years	1 year	1–3 years	3–6 years	18–35 years	18–35 years
Weight to provide 75% of total calories, g	193	315	419	585	838	194	317	421	590	842	203	331	440	616	880
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Protein	118	120	125	123	146	136	139	147	142	169	143	146	155	150	177
Calcium	8	11	15	20	29	3	8	11	15	20	10	14	20	28	40
Iron	70	118	126	113	252	97	159	170	157	340	126	206	219	205	438
Vitamin A	0	0	0	0	0	7	8	9	6	9	1	1	2	1	2
Thiamine	99	128	142	148	141	225	190	322	338	321	255	196	330	345	329
Riboflavin	23	29	34	40	33	42	51	54	59	64	47	58	61	65	72
Niacin	52	57	61	104	71	63	67	72	124	84	99	107	116	199	135
Ascorbic acid	0	0	0	0	0	0	0	0	0	0	7	7	8	9	11
Vitamin D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1] Nutrient content based on ICNND–INCAP data (15).
[2] Figures represent percentage of NRC dietary allowances (14).
[3] Non-reproductive.

what smaller groups of animals here permitted answers of satisfactory reliability from experiments approximately one-fourth as large. The resulting economy in time, equipment, labor, and materials is obvious.

Use of protein levels other than 10% is justified on 2 bases. Experimental variability is inherently minimal in the region of optimal response for any experimental system. For blends of the type studied here 15 to 18% protein is optimum in terms of PER. In addition an indication of possible behavior at the protein levels anticipated for field use was sought. Certainly Campbell's procedure has value as a detailed experimental protocol facilitating inter-laboratory comparisons of protein sources. Rigid adherence to its details in all PER measurements, however, is both unsound and beyond the stated intention of the Committe on Protein Malnutrition.

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Decrease in Appetite and Biochemical Changes in Amino Acid Imbalance in the Rat [1]

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ABSTRACT Rats fed imbalanced diets containing 10 and 15% of wheat gluten supplemented with lysine, to which a 3.1% mixture of essential amino acids lacking threonine was added, had lower "appetite quotient" values than control animals fed balanced diets. This confirms our assumption that decreased food intake is a primary effect of the imbalance. The plasma amino acid pattern produced by the imbalance was similar to that described in earlier experiments: the concentration of threonine, limiting amino acid of the diet decreased markedly, and the ratios between the concentration of other essential amino acids to threonine increased markedly. Animals fed the imbalanced diets showed an increased liver weight correlated with a high glycogen content, when expressed per 100 g of body weight. The liver lipid of imbalanced animals was decreased possibly as a consequence of the lipotropic effect of lysine and threonine that were ingested in amounts higher than needed for growth. These changes could be related to the mechanism responsible for food intake depression. The similarity between some of these observations and some of the changes occurring with kwashiorkor supports the theory that imbalance of amino acids in the diet plays an important role in the incidence of that disease.

Food intake of rats fed a diet in which there is an imbalance of amino acids decreases rapidly. Some earlier observations suggested that the decrease in food intake could be secondary to some change in amino acid metabolism producing a decrease in the efficiency of utilization of the limiting amino acid of the diet and, hence, a retarded growth rate (1, 2).

In previous studies concerning the effect of amino acid imbalance on food intake and preference (3) we observed that protein-depleted rats given a choice between an imbalanced and a protein-free diet, showed a preference for the protein-free diet after the third day, and rejected the imbalanced diet. Further experiments demonstrate that rats that have been previously starved or fed ad libitum showed a similar preference after various periods of time (4). The preference for the protein-free diet over the imbalanced diet cannot be attributed to any superiority in the nutritive value of the protein-free diet because it did not support growth, whereas the imbalanced diet did. These observations indicate that an amino acid imbalance may affect directly some basic mechanism regulating food intake.

If appetite is in some way affected directly by the diet, the food consumption of experimental animals measured as metabolizable energy would not be proportional to their maintenance requirements (5). On this basis Carpenter (6) established a method for calculating the food intake of ad libitum-fed rats, in terms of their body size, as a measure for the detection of differences in their appetite according to the diets they receive. He called this the "appetite quotient," representing the ratio of intake of metabolizable energy to body weight$^{0.88}$.

In the present investigation, we used Carpenter's method to determine the appetite quotient of rats fed imbalanced diets. The results showed that these values are significantly lower than those obtained in rats fed balanced diets; this confirms our assumption that food intake is directly affected by the dietary amino acid pattern.

Data are also presented to show the influence of imbalanced diets on body

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composition in protein-depleted and nondepleted rats. In previous experiments it was observed that changes in plasma amino acid pattern precede changes in appetite or occur at the same time (7, 8). As a working hypothesis it was assumed that this altered blood amino acid pattern triggers an appetite-depressing mechanism that causes the decrease in food intake (8, 9).

The results obtained in this study, indicate that concurrently with the variations in the plasma amino acid pattern, some changes occur in weight and composition of liver in rats fed imbalanced diets; these changes may be also related to the mechanism responsible for curtailing appetite.

EXPERIMENTAL

Groups of 6 male rats of the Wistar strain were used in these experiments.

Nondepleted rats. Rats weighing an average of 50 to 60 g were fed the experimental diets ad libitum for 2 weeks.

Protein-depleted rats. Animals weighing on the average 80 to 85 g were depleted of protein by feeding them a protein-free diet for 6 days. Rats losing 15 to 17 g were selected; they were fed the experimental diets ad libitum for 2 weeks.

The rats were housed in individual suspended cages with screen bottoms and water was offered ad libitum. In all the experiments, food intake was measured and recorded daily, and the animals were weighed twice a week. The appetite quotient was calculated according to Carpenter (6).

Collection of material

Plasma. At the end of the experimental periods, the rats were anesthesized with ether, and blood was withdrawn by heart puncture. The blood of each group was pooled and plasma separated from the heparinized blood by centrifugation. The samples of plasma were deproteinized with 10% of perchloric acid for the subsequent amino acid analysis.

Liver. After weighing the whole liver of exsanguinated rats a piece was placed in 10% perchloric acid and blended in a Virtis homogenizer; suitable aliquots of this suspension were used for protein determination. A large piece was frozen for subsequent lipid determination. A third piece weighing approximately one gram was placed in 30% KOH for glycogen determination.

Gastrocnemius muscle. The right gastrocnemius muscle, was weighed, placed in 10% perchloric acid and blended in a Virtis homogenizer. Aliquots of this suspension were used for protein determination.

Chemical analysis

Amino acids. Plasma amino acids were determined by the method of Levy as modified by Peraino and Harper (10).

Protein. Tissue protein was determined by the method of Marenzi et al. (11).

Lipid. Lipid content of liver was determined by the procedure described by Sidransky and Baba (12).

Glycogen. Liver glycogen was determined according to the method indicated by Hawk et al. (13).

Diets. Two different basal diets containing 10 and 15% of wheat gluten, respectively, were used in the experiments, as shown in table 1. In both diets the content of lysine was increased to 0.9%; therefore threonine, the next limiting amino acid of the wheat gluten remained as the first limiting amino acid of the diets. Supplemented diets were made by adding 0.1% of DL-threonine to the basal diets. Imbalanced diets were obtained by further addition to the supplemented diets of 3.1% of a mixture of essential amino acids lacking threonine.[2] The protein-free diet had the same composition as the basal diets, except that wheat gluten and lysine were replaced by dextrin.

RESULTS

The effect of the addition of DL-threonine, and DL-threonine plus the imbalanced amino acid mixture lacking threonine, to the basal diets is shown in table 2.

As expected, growth rate of nondepleted rats increased by the supplementation of both basal diets with threonine; groups 2 and 5 fed the supplemented diets had a higher intake and gained more weight, respectively, than groups 1 and 4 fed the

[2] Sanahuja, J. C., M. E. Rio and M. N. Lede 1964 Effect of amino acid imbalance on nutritive value of supplemented proteins. Federation Proc., 23: 878 (abstract).

basal diets. But the improvement obtained by the addition of DL-threonine was completely offset by the further addition of the amino acid mixture; rats of groups 3 and 6, fed the imbalanced diets, showed a considerable depression in food consumption and in growth rate compared with those fed the supplemented diets, their performance being similar to that observed in groups receiving the basal diets.

As the effect of the imbalance in nondepleted rats was markedly higher in animals fed diets containing 10% wheat gluten than in groups receiving diets with 15% wheat gluten, only the former diets

TABLE 1

Composition of diets

	A	B	C	D	E	F
	Basal	Supplemented	Imbalanced	Basal	Supplemented	Imbalanced
	%	%	%	%	%	%
Wheat gluten [1]	10	10	10	15	15	15
L-Lysine [2]	0.80	0.80	0.80	0.75	0.75	0.75
DL-Threonine	—	0.10	0.10	—	0.10	0.10
Amino acid mixture [3]	—	—	3.10	—	—	3.10
Minerals [4]	5	5	5	5	5	5
Vitamin mixture [4]	0.25	0.25	0.25	0.25	0.25	0.25
Choline chloride	0.15	0.15	0.15	0.15	0.15	0.15
Corn oil [5]	5	5	5	5	5	5
Dextrin [6]	78.80	78.70	75.60	73.85	73.75	70.65
Total protein content	9.18	9.28	12.38	12.57	12.67	15.77
Total threonine content	0.20	0.30	0.30	0.30	0.40	0.40

[1] Containing 13.4% N (83.8% of protein), 2% of threonine and 1% of lysine.
[2] As L-lysine·HCl.
[3] The amino acid mixture provided in % in the diet: L-arginine·HCl, 0.2; L-lysine·HCl, 0.4; DL-isoleucine, 0.4; L-leucine, 0.3; DL-methionine, 0.4; DL-phenylalanine, 0.2; L-histidine·HCl, 0.4; DL-tryptophan, 0.4; DL-valine, 0.4.
[4] Harper, A. E. (14).
[5] Fat-soluble vitamins were included in the corn oil (14).
[6] Moist cornstarch heated at 121° in an autoclave for 3 hours.

TABLE 2

Food intake, change in weight and "appetite quotient" of rats fed the experimental diets ad libitum for 2 weeks

Group	Diet	Food intake	Threonine intake	Wt gain	Appetite quotient [1]
		g/day	*mg/day*	*g*	
	Nondepleted				
1	A Basal (10% wheat gluten) [2]	8.3	16.6	15.0 ± 1.3 [3]	0.992 ± 0.020
2	B Supplemented (A + 0.1% DL-threonine)	13.6	40.8	36.8 ± 2.0 [5]	1.071 ± 0.020
3	C Imbalanced (B + 3.1% AA mix) [4]	7.0	21.0	19.5 ± 2.1	0.864 ± 0.027 [5]
4	D Basal (15% wheat gluten) [2]	11.2	33.6	42.3 ± 2.9	1.008 ± 0.020
5	E Supplemented (D + 0.1% DL-threonine)	14.0	56.0	59.0 ± 2.4 [5]	1.017 ± 0.027
6	F Imbalanced (E + 3.1% AA mix) [4]	11.0	44.0	48.6 ± 2.4	0.908 ± 0.014 [5]
	Protein-depleted				
7	A Basal (10% wheat gluten) [2]	10.8	21.6	17.5 ± 3.1	0.900 ± 0.020
8	B Supplemented (A + 0.1% DL-threonine)	15.5	46.5	54.8 ± 5.4 [5]	1.053 ± 0.023
9	C Imbalanced (B + 3.1% AA mix) [4]	10.5	31.5	23.8 ± 2.8	0.784 ± 0.025 [4]

[1] $Q = \dfrac{\text{Food intake} \times \text{metabolizable calories per gram}}{\int_0^T \text{Weight}^{0.88}\, d.t.}$

[2] Plus L-lysine, as L-lysine·HCl, up to 0.9% in the diet.
[3] Mean value ± SE of mean.
[4] For composition, see footnote 3, table 1.
[5] Highly significantly ($P < 0.01$) different from basal group mean.

were used in subsequent experiments with protein-depleted rats.

During 2 weeks, the protein-depleted rats of group 7, fed the basal diet, regained only the weight lost during the depletion period. Protein-depleted rats of group 8 receiving the supplemented diet had a much higher food intake and regained more than 3 times the weight lost during the protein depletion period. But here again this improvement was offset by the imbalance: the weight increase of rats fed the imbalanced diet (group 9) was poorer than that for group 8 and similar to that observed in rats fed the basal diet (group 7), even though the concentration of threonine was 50% higher in the imbalanced than in the basal diet. Table 2 also shows the appetite quotient values for all experimental groups. These values were similar in depleted or nondepleted rats consuming the balanced diets (basal or supplemented), but were significantly lower ($P < 0.01$) in rats receiving the imbalanced diets (groups 3, 6 and 9).

The results of analysis of amino acids in plasma of nondepleted rats after the experimental period of 2 weeks are shown in table 3. The plasma threonine concentration of rats fed the imbalanced diets (groups 3 and 6), decreased markedly, being lower at the end of the experiment than that for the groups fed the balanced diets (basal or supplemented), despite the fact that the threonine intake was higher in the imbalanced groups than in groups 1 and 4 receiving the basal diets (table 2). During the experiment the concentrations of other essential amino acids increased in the blood of animals fed imbalanced diets and after 2 weeks were higher than in rats fed the supplemented or basal diets.

TABLE 3

Plasma amino acid concentration of nondepleted rats fed the experimental diets ad libitum for 2 weeks (analysis from pooled blood of 6 rats)

	Group					
	1 (diet A) [1]	2 (diet B) [2]	3 (diet C) [3]	4 (diet D) [4]	5 (diet E) [5]	6 (diet F) [6]
	μmoles/100 ml plasma		*μmoles/100 ml plasma*		*μmoles/100 ml plasma*	
Threonine	6.0	10.0	3.0	6.0	24.0	3.0
Valine	25.0	23.5	40.0	28.5	43.0	51.5
Leucine + isoleucine	13.0	13.0	37.0	15.0	41.5	52.5
Lysine	19.0	24.0	47.0	59.0	43.0	90.0
Glutamic acid	18.0	27.0	28.0	116.0	57.0	102.5
Proline + OH proline	65.0	70.0		59.0	71.0	46.0
Glycine	21.5	17.0	20.5	56.0	39.5	43.5
Alanine	66.0	45.0	64.0	74.5	78.5	67.0
Serine	46.5	30.0	40.5	110.0	73.0	51.0

[1] Basal: 10% wheat gluten plus L-lysine, as L-lysine·HCl, up to 0.9% in the diet.
[2] Supplemented: diet A + 0.1% DL-threonine.
[3] Imbalanced: diet B + 3.1% amino acid mixture (for composition, see footnote 3, table 1).
[4] Basal: 15% wheat gluten, plus L-lysine, as L-lysine·HCl, up to 0.9% in the diet.
[5] Supplemented: diet D + 0.1% DL-threonine.
[6] Imbalanced: diet E + 3.1% amino acid mixture (for composition, see footnote 3, table 1).

TABLE 4

Ratio of selected indispensable amino acids to threonine in plasma of nondepleted rats fed the experimental diets ad libitum for 2 weeks

	Group					
	1 (diet A) [1]	2 (diet B) [2]	3 (diet C) [3]	4 (diet D) [4]	5 (diet E) [5]	6 (diet F) [6]
Valine/threonine	4.1	2.3	13.3	4.7	1.8	17.1
Leucine + isoleucine/threonine	2.1	1.3	10.6	2.5	1.7	17.5
Lysine/threonine	3.1	2.4	15.6	9.8	1.8	30.0

[1] Basal: 10% wheat gluten plus L-lysine, as L-lysine·HCl, up to 0.9% in the diet.
[2] Supplemented: diet A + 0.1% DL-threonine.
[3] Imbalanced: diet B + 3.1% amino acid mixture (for composition, see footnote 3, table 1).
[4] Basal: 15% wheat gluten, plus L-lysine, as L-lysine·HCl, up to 0.9% in the diet.
[5] Supplemented: diet D + 0.1% DL-threonine.
[6] Imbalanced: diet E + 3.1% amino acid mixture (for composition, see footnote 3, table 1).

TABLE 5

Weight and composition of liver and gastrocnemius muscle of rats fed the experimental diets ad libitum for 2 weeks

Group	Diet	Liver				Gastrocnemius muscle	
		Wet weight	Protein	Lipid	Glycogen	Wet weight	Protein
		g/100 g body wt	*g/100 g body wt*	*mg/100 g body wt*	*mg/100 g body wt*	*mg/100 g body wt*	*mg/100 g body wt*
		Nondepleted					
1	A Basal (10% wheat gluten) [1]	4.125 ± 0.020 [2]	712 ± 21	107 ± 10	339 ± 23	520 ± 15	107 ± 7
2	B Supplemented (A + 0.1% DL-threonine)	4.108 ± 0.080	710 ± 15	127 ± 10	309 ± 18	535 ± 13	103 ± 2
3	C Imbalanced (B + 3.1% AA mix) [3]	4.550 ± 0.100 [4]	760 ± 10 [5]	91 ± 5 [5]	449 ± 25 [4]	540 ± 25	108 ± 7
4	D Basal (15% wheat gluten) [1]	4.110 ± 0.100	720 ± 14	153 ± 3	263 ± 15	531 ± 17	104 ± 6
5	E Supplemented (D + 0.1% DL-threonine)	4.200 ± 0.090	720 ± 15	162 ± 10	295 ± 20	529 ± 16	103 ± 5
6	F Imbalanced (E + 3.1% AA mix) [3]	4.630 ± 0.120 [4]	780 ± 15 [5]	96 ± 9 [4]	440 ± 25 [4]	538 ± 20	105 ± 3
		Protein-depleted					
	O Time	4.600 ± 0.125	665 ± 15	350 ± 18	230 ± 17	525 ± 12	84 ± 5
7	A Basal (10% wheat gluten) [1]	4.680 ± 0.160	807 ± 25	405 ± 25	116 ± 10	575 ± 15	112 ± 7
8	B Supplemented (A + 0.1% DL-threonine)	4.205 ± 0.070	800 ± 15	275 ± 12 [6]	160 ± 18	562 ± 21	118 ± 7
9	C Imbalanced (B + 3.1% AA mix) [3]	4.700 ± 0.120 [4]	825 ± 19	258 ± 16 [6]	275 ± 16 [4]	595 ± 23	113 ± 2

[1] Plus L-lysine, as L-lysine·HCl, up to 0.9% in the diet.
[2] Mean value ± SE of mean.
[3] For composition, see footnote 3, table 1.
[4] Highly significantly ($P < 0.01$) different from supplemented group mean.
[5] Probably significantly (P between 0.01 and 0.05) different from supplemented group mean.
[6] Highly significantly ($P < 0.01$) different from basal group mean.

Of the dispensable amino acids the concentrations of glutamic acid and serine reached a high value in the animals fed the basal diet D containing 15% wheat gluten. In rats fed the imbalanced diets these changes resulted in substantial increases in the plasma ratios of various indispensable amino acids to threonine (table 4); these ratios had lower values and showed only little differences in rats fed the balanced diets.

Table 5 shows the changes in weight of liver and gastrocnemius muscle of protein-depleted and nondepleted rats expressed as grams per 100 grams of body weight after the experimental periods.

The mean wet weight of the liver in nondepleted rats was significantly elevated in groups 3 and 6, fed the imbalanced diets, compared with groups 2 and 5, respectively, receiving the supplemented diets. In contrast, no significant differences were observed between the liver weights of the animals fed the supplemented and the basal diets. Protein-depleted rats fed the basal and the imbalanced diets (groups 7 and 9) maintained their liver weight during the experiment at the higher level that was obtained at zero time after depletion; on the other hand, the liver weight of animals of group 8, fed the supplemented diet, reached a lower and more normal value after the experimental period than the weight at zero time.

The right gastrocnemius muscle weighed more or less the same in all the groups when expressed as grams per 100 grams of body weight.

The liver and gastrocnemius muscle composition, expressed in milligrams of constituent per total tissue per 100 g of body weight, is also shown in table 5. The liver protein content was slightly higher in nondepleted rats fed the imbalanced diets than in animals fed the supplemented diets, but no differences were observed in the liver of protein-depleted rats fed the experimental diets.

The gastrocnemius muscle protein was essentially similar in all the experimental groups of animals.

The liver lipid diminished in nondepleted rats fed the imbalanced diets (groups 3 and 6) and was lower than that of the animals fed the supplemented diets (groups 2 and 5), respectively. Protein-depleted rats fed the basal diet (group 7) showed only a slight difference in liver lipid content in respect to the zero time value, but the fat levels were decreased in groups 8 and 9 receiving the supplemented and the imbalanced diet, respectively.

The most important differences were observed in the liver glycogen content: nondepleted rats fed the imbalanced diets (groups 3 and 6) had a markedly higher glycogen content than the groups fed the balanced diets (supplemented or basal diets). The same picture was observed in depleted animals: the amount of glycogen in the liver of imbalanced rats (group 9) was significantly higher than in group 7, fed the basal diet and group 8, the supplemented one.

DISCUSSION

Appetite and amino acid imbalance. The concept of an appetite quotient developed by Carpenter (6), based on the studies of Hegsted and Heffenreffer (5), provides a practical method for determining whether the decreased appetite is a primary effect of the imbalance or whether it is only a consequence of a diminished nutritive value of the diet.

The appetite quotient values obtained in these experiments were similar for the groups fed the balanced diets (basal or supplemented) despite the differences in nutritive value, but in the groups fed the imbalanced diets the appetite quotient values were significantly lower ($P < 0.01$), indicating that the appetite of these rats was subnormal during the experimental period.

This suggests that the depression in food consumption showed by imbalanced animals would not be a consequence of the lower nutritive value of the imbalanced diet but of some specific physiological effects that affect directly the food intake.

These results may also explain the observations described in a previous study in which rats failed to discriminate between 2 balanced diets, one of higher nutritive value than the other, but always discriminating and refusing an imbalanced diet when it was offered simultaneously with a balanced diet.

The changes in the plasma amino acid pattern of nondepleted rats resemble those previously observed in animals fed imbalanced diets containing fibrin in which the altered amino acid pattern was associated with the depression in food intake (8). Here too, rats fed the imbalanced diets in which the appetite quotient was subnormal, showed a severely altered plasma amino acid pattern that resulted in very high values in the ratios of concentrations of several indispensable amino acids to threonine, the most limiting amino acid of the diet. In contrast, in animals fed balanced diets, in which the appetite quotient values were higher, the plasma pattern of amino acids was not altered.

Similar results were obtained in short-term experiments; plasma threonine concentration decreased markedly in rats fed the imbalanced diet C within the first 24 hours, when the decreased food intake became apparent.[3]

This relationship between appetite quotient and altered plasma amino acid pattern supports the assumption that the desire for food could be curtailed when the ratio of the plasma concentration of each indispensable amino acid to that of the one lacking in the amino acid mixture, reaches some critical level. This could be a part of a homeostatic mechanism tending to prevent the animal from ingesting an excessive amount of an imbalanced diet (8, 15). Krauss and Meyer (16) suggested that this regulating mechanism may be independent of the satiety centers of the hypothalamus.

Amino acid imbalance and liver composition. The most interesting of the changes observed in liver composition were those related to the content of glycogen, that reached nearly 10% of the liver weight of nondepleted rats fed the imbalanced diets. This high level of glycogen would account for the abnormally high liver weight in these animals since it is known that glycogen is stored with nearly twice its water weight.

In protein-depleted rats fed the imbalanced diets, even though the liver glycogen content was not as high as in nondepleted rats, the values obtained were also significantly higher than those for control animals fed the balanced diets.

Similar liver weight increase and its higher glycogen content was also observed in protein-depleted rats after 24 hours of being fed the imbalanced diet C.[3]

Sidransky and Faber (17) reported that the liver of rats force-fed a threonine-devoid diet contained high amounts of glycogen; these authors failed to observe an increased synthesis of liver lipid starting from the carbon chains of amino acids not used for protein synthesis. Since the liver lipid content was not increased in rats fed the imbalanced diets, the possible utilization of the excess of amino acids provided by the imbalanced mixture for the synthesis of glycogen cannot be disregarded.

These high levels of liver glycogen observed in rats fed the imbalanced diets could be associated with the depression in appetite; Anliker and Mayer (18) pointed out that in mice a relationship exists between feeding behavior and liver glycogen. This possibility, that would provide an alternative to the hypothesis that the depression in food intake may be the consequence of the altered plasma amino acid, appears worthy of further investigation.

The liver protein content showed only little difference between nondepleted or protein-depleted rats fed the imbalanced or balanced diets. Similar results were obtained in short-term studies:[4] the liver of protein-depleted rats pair-fed the imbalanced diet C and the supplemented diet B showed an identical increase in their total protein content within 24 hours. These observations agree with those of Kumta and Harper (19) and Spolter and Harper (20) indicating that the direct inhibition of protein synthesis could not be the primary cause for the reduced food intake. After the 2-week experiment, total body protein synthesis was diminished, since growth rate was depressed; but it is conceivable that this effect is secondary to the decrease in food intake.

The differences in liver lipid content could be related to the amounts of threonine and lysine ingested during the experimental period since it is known that

[3] Sanahuja, J. C., M. N. Lede and M. E. Sambucetti 1964 Appetite quotient and biochemical changes in rats fed imbalanced diets. Federation Proc., 23: 185 (abstract).

[4] See footnote 3.

these amino acids could exert a marked lipotropic action (21, 22); the higher requirements for the indispensable amino acids in nondepleted animals with a rapid growth rate (groups 2, 4 and 5) could produce deficiencies of threonine, the most limiting amino acid of the diet, or of lysine that would result in a higher liver fat content.

In nondepleted rats fed the imbalanced diets, the amounts of lysine and threonine ingested may be excessive for the requirements as a consequence of the slow growth rate due to the imbalance; this could explain the very low liver lipid content observed in groups 3 and 6.

The lipotropic effect of threonine may explain also the differences observed in the liver fat content of protein-depleted rats; the high lipid content at zero time, that resulted from the protein-free diet consumed during the protein-depletion period was greatly reduced in animals fed the diets supplemented with threonine (groups 8 and 9). In contrast, group 7, fed a diet not supplemented with threonine showed a high liver fat content, similar to that of protein-depleted animals (zero time).

Some of the results of this study are similar to those observed in kwashiorkor in which the decrease of appetite is a characteristic sign. Whitehead (23) reported an altered blood amino acid pattern in children suffering from severe kwashiorkor that is not observed in successfully treated cases. Stuart et al. (24) and Waterlow (25) have also observed that in infants with kwashiorkor, liver glycogen can reach values of 10 to 12% accounting for about one-half of the fat-free dry weight of the liver.

The similarity of these observations supports the assumption that the depression in food intake resulting from the consumption of poorly balanced proteins, as in the case of kwashiorkor, could be related to the imbalanced amino acid pattern of the diet (26).

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Absorption of Calcium and Phosphorus Along the Gastrointestinal Tract of the Laying Fowl as Influenced by Dietary Calcium and Egg Shell Formation [1]

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ABSTRACT The apparent absorption of calcium, phosphorus and total dry matter along the gastrointestinal tract of the laying hen, was followed using yttrium-91 as a non-absorbed tracer. Percentage calcium and phosphorus absorption appeared to be greater in the proximal parts of the intestine than in the distal parts. This difference was smaller for total dry matter. Percentage calcium absorption was not significantly influenced by its dietary level, nor did the latter influence the absorption of dry matter. Percentage absorption of phosphorus was, however, depressed by the higher dietary calcium level. Egg shell deposition was associated with increased calcium and to a smaller degree, phosphorus absorption. It did not influence dry matter absorption. A heavy endogenous phosphorus excretion was observed in the duodenum.

The identification of the sites of mineral absorption is important for understanding the mechanism involved in the mineral absorption. This problem was studied mainly by in vitro and in situ experimentation. The in vitro studies such as those of Schachter and associates (1, 2) provide information on the capacity of various intestinal segments to absorb calcium. However, the rate and percentage of calcium absorption may also depend on the rate of passage of food, which is quite variable in the various intestinal segments (3, 4). Therefore, in vitro methods can not measure the relative contribution of each segment to total calcium absorption in vivo. A different method for studying calcium absorption is the use of oral doses of ^{45}Ca or its injection into different parts of the intestine (5, 6). This method, however, measures mainly the outflux of calcium from the intestine and not the net absorption which is the difference between the outflux and the influx.

Chandler and Cragle (7) used ^{141}Ce as a non-absorbed tracer to study calcium and phosphorus absorption in calves. The ratio of calcium and phosphorus to ^{144}Ce at any point in the intestine measures the cumulative percentage absorption up to this point. Similarly, Bolton (8) used cellulose as a tracer for estimation of protein and available carbohydrate absorption along the tract of chickens. Marcus and Lengemann (3) reported that ^{91}Y was not absorbed in the rat.

Preliminary trials in this laboratory showed that this isotope was not absorbed by colostomized chickens. Following an oral dose of ^{91}Y mixed in some feed, 93% of the dose was detected in the feces voided during the first 24-hour period, and 3% in the following 24-hour period. No activity could be detected in the urine of these animals.

Therefore, ^{91}Y was used in the present trial to follow the absorption of calcium and phosphorus along the intestinal tract of laying hens. The possibility that dietary calcium and the presence of a calcifying shell in the uterus may modify the pattern of calcium and phosphorus absorption was also investigated.

EXPERIMENTAL

The experimental diets are shown in table 1. These diets were all-vegetable, of the practical type, and were prepared as follows: the calcium carbonate and the dicalcium phosphate portions of each ra-

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TABLE 1

Composition of the experimental diets

	Diet 1	Diet 2
	%	%
Yellow corn	30.00	30.00
Soybean oil meal (45% protein)	24.00	24.00
Soybean oil, refined	4.00	4.00
Vitamin mixture [1]	0.25	0.25
Mineral mixture [2]	0.30	0.30
DL-Methionine	0.10	0.10
Alfalfa meal, dehydrated	2.00	2.00
Calcium carbonate	3.50	7.50
Dicalcium phosphate	1.70	1.70
Milo	34.15	30.15
Calcium content, assayed, %	1.90	3.56
Phosphorus content, assayed, %	0.63	0.63

[1] Commercial premix, supplied per kg of diet: vitamin A, 10,000 IU; vitamin D_3, 1400 ICU; riboflavin, 3 mg; Ca pantothenate, 3 mg; niacin, 10 mg; choline chloride, 400 mg; vitamin B_{12}, 8 μg; butylated hydroxytoluene, 125 mg.

[2] Feed grade salt supplied in mg per kg of diet: Mn, 60; zinc, 50; iodine, 1.2; cobalt, 0.2; iron, 25; copper, 2.

tion were mixed well. A solution containing ^{91}Y was added to the mixture. After drying in an oven, the latter was passed 3 times through a thin-mesh wire screen. Count rates of samples were taken to assure proper mixing. The Ca-^{91}Y mixture was then added to the other pre-mixed dietary ingredients, all ground in a hammer mill. This was followed by thorough mixing. Concentration of ^{91}Y was approximately 25 μc/kg and 55 μc/kg in diets 1 and 2, which contained 1.90% (low Ca) and 3.56% (high Ca) calcium, respectively.

Forty White Leghorn laying hens, 16 months old, were selected for the experiment on the basis of previous egg production records. For a 1-week preliminary period they were fed a control diet identical to diet 2, but containing no radioactive supplement. At the end of this period they were divided into 2 lots receiving the respective labeled low calcium and high calcium diets. After 3 days of feeding the experimental diets, the birds were killed by dislocation of the neck in the following order. (a) Four birds of each lot were killed at 8 P.M. after the presence of a shelled egg in their uteri had been established. At this time calcification had already proceeded for 3 to 5 hours. Results from this group represent the absorption during the period of early calcification. (b) Four birds of each lot were killed immediately after oviposition. The time of killing ranged between 7 and 11:30 A.M. Since the egg is laid shortly after the end of shell calcification, results obtained with this group represent absorption during the period of late calcification. (c) Five birds of each lot were killed between 9 and 11 A.M. Those birds had not laid any eggs during the morning, but uncalcified egg was found either in the magnum or in the isthmus at the time of killing. Results from this group indicate the absorption of calcium and phosphorus when no shell is formed.

The gastrointestinal tract of each hen (excluding the esophagus and crop) was removed and separated into gizzard, duodenum, upper jejunum, lower jejunum, upper ileum, lower ileum and colon. The cecums were not taken for analysis. Meckel's diverticulum was arbitrarily taken as the point of demarcation between the jejunum and ileum, which were each divided into 2 parts of equal length. The contents of each segment were emptied into a crucible, oven-dried and ashed at 700°. The ash was then dissolved in 2 N hydrochloric acid. For ^{91}Y assay, an aliquot was dried on a glass planchet and counted in a gas-flow detector. Within the range of the weights of samples measured, there was no variation due to self absorption. Calcium was determined by EDTA titration as described previously (9) except that murexide was replaced by hydroxynaphthol blue.[2] Phosphorus was determined by the method of Gomori (10).

Results are expressed as ratios of calcium, phosphorus or dry matter to counts per minute (cpm) $\times 10^{-4}$ of ^{91}Y observed in the respective segment. This ratio decreases with progressive absorption of the test nutrient. The cumulative percentage absorption in any segment is given by

$$100 \times \left(1 - \frac{\text{nutrient}/^{91}Y \text{ in intestine}}{\text{nutrient}/^{91}Y \text{ in feed}}\right).$$

Because of the differences in the ^{91}Y content of the 2 diets, the appropriate corrections were made to bring the results to the same scale.

Analyses of variance of calcium, phosphorus and dry matter to ^{91}Y ratios in each intestinal segment were run according to Dixon and Massey (11). Pooled standard

[2] Mallinkrodt.

errors (n = 4) were calculated from the error term of the analysis of variance.

RESULTS

Since the trial was designed as a factorial of 2 calcium levels and 3 physiological states with respect to egg shell formation, the results are grouped according to the factorial variables.

With only one exception, there was no significant effect of any of the experimental variables on the dry-matter contents of the various intestinal segments. These were in milligrams as follows (average ± SE): gizzard, 3018 ± 254; duodenum, 671 ± 151; upper jejunum, 785 ± 85; lower jejunum, 1266 ± 333; upper ileum, 1075 ± 279; lower ileum, 845 ± 169; and colon, 268 ± 74.

The calcium, phosphorus and dry matter to ^{91}Y ratios in the various intestinal segments are shown in figures 1, 2, and 3 respectively. The probability levels for the effect of each factorial variable in any intestinal segment are shown in table 2. Following an increase from the gizzard to the duodenum, there was a marked decline

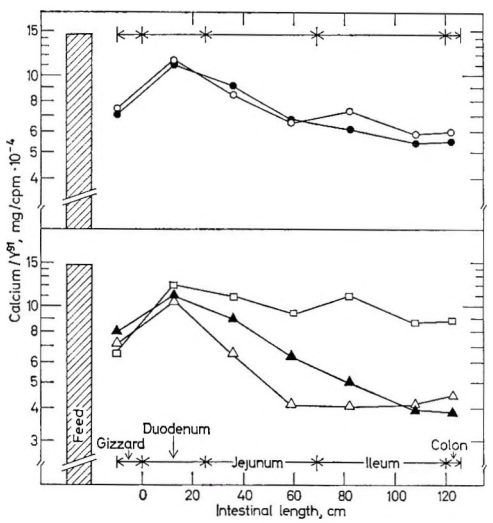

Fig. 1 Ca/^{91}Y ratio in gastrointestinal segments of laying hens fed ^{91}Y-labeled diets. Upper diagram, comparison between 1.90% calcium (●) and 3.56% calcium (○) diets. Lower diagram, comparison among periods of early egg shell calcification (△), late shell calcification (▲) and with no shell formation (□). Standard errors were as follows: gizzard, 0.90; duodenum, 1.24; upper jejunum, 0.85; lower jejunum, 1.09; upper ileum, 1.16; lower ileum, 0.81; colon, 1.09.

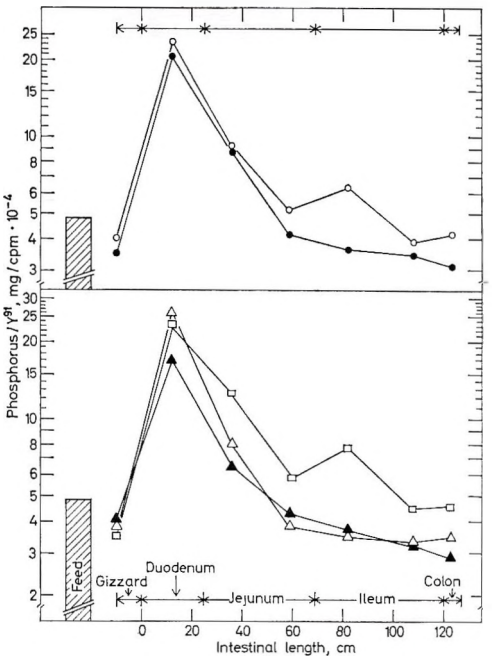

Fig. 2 P/^{91}Y ratio in gastrointestinal segments of laying hens fed ^{91}Y-labeled diets. Upper diagram, comparison between 1.90% calcium (●) and 3.56% calcium diets (○). Lower diagram, comparison among periods of early egg shell calcification (△), late shell calcification (▲) and of no shell formation (□). Standard errors are as follows: gizzard, 0.40; duodenum, 6.14; upper jejunum, 2.67; lower jejunum, 1.03; upper ileum, 1.27; lower ileum, 0.41; colon, 0.32.

in the Ca/^{91}Y ratio down to the lower jejunum with a smaller change in the following segments.

The average cumulative percentage absorption of calcium was 50.3% at the lower jejunum and 53.2% at the colon. The Ca/^{91}Y ratio in the duodenum was lower than that of feed, suggesting considerable absorption of calcium in this segment. In the high calcium hens with no shell present, the upper ileum showed an increase in the ratio, indicating net endogenous excretion of calcium. There was no significant effect of dietary calcium level on the percentage absorption of this mineral. Hence, the total amount of calcium absorbed was higher in the high calcium birds. Percentage calcium absorption was significantly greater during the 2 periods of shell calcification than when no forming shell was present. However, there was no

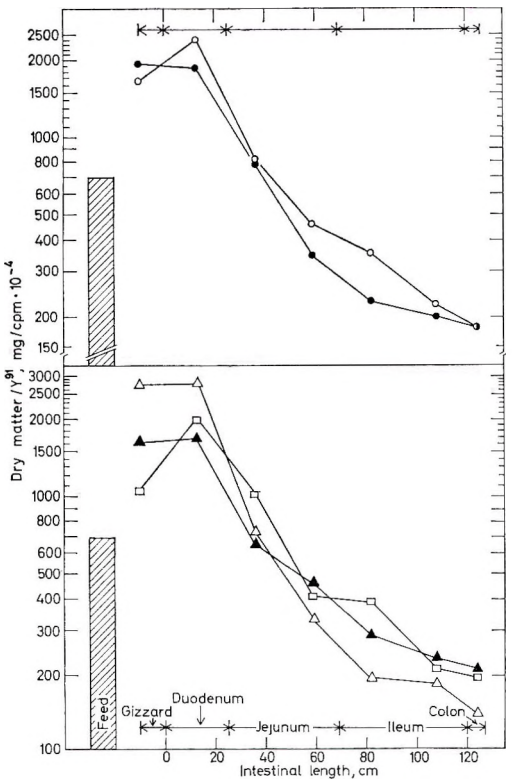

Fig. 3 Total dry matter/^{91}Y ratio in gastrointestinal segments of laying hens fed ^{91}Y-labeled diets. Upper diagram, comparison between 1.90% (●) and 3.56% (○) calcium diets. Lower diagram, comparison among periods of early egg shell calcification (△), late shell calcification (▲), and of no shell formation (□). Standard errors were as follows: gizzard, 479; duodenum, 370; upper jejunum, 278; lower jejunum, 84; upper ileum, 62; lower ileum, 26; colon, 18.

significant difference in calcium absorption between periods of early and late calcification. The former difference was observed along the entire intestine but did not reach significance in the duodenum. The calculated cumulative percentage of calcium absorption in the colon was 67 and 39%, during periods of calcification and no calcification, respectively.

Changes in the P/^{91}Y ratios (fig. 2) were similar to those observed for calcium, indicating that the main sites of phosphorus absorption are at the anterior intestine, i.e., jejunum. The P/^{91}Y ratio in the duodenum is much higher than that of feed, suggesting a large endogenous phosphorus excretion in this segment. Unlike calcium, the relative absorption of phosphorus was influenced by dietary calcium level; the high calcium level increased the P/^{91}Y ratio in the 3 lower portions of the intestine. During the 2 periods of egg shell calcification, there was a significant increase in phosphorus absorption in the lower portions of the intestine, although the same tendency was observed in the upper segments as well. There was also a significant interaction between dietary calcium and shell calcification on phosphorus cumulative absorption in the colon.

Dry matter/^{91}Y ratios in the various intestinal segments are shown in figure 3. The ratio was higher in the gizzard and duodenum than in feed. It then decreased at a progressively lower rate along the rest of the intestine. The calculated cumulative dry-matter absorption in the colon

TABLE 2

Levels of probability for the effects of the several factorial components for the various feed ingredients and intestinal segments [1]

Factorial variable	Dietary calcium			Diurnal variation (shell formation)			Interaction		
Parameter	Ca/Y	P/Y	Dm/Y [2]	Ca/Y	P/Y	Dm/Y	Ca/Y	P/Y	Dm/Y
Gizzard	ns	ns	ns	ns	ns	0.01	0.01	ns	ns
Duodenum	ns	ns	ns	ns	ns	ns	ns	ns	ns
Upper jejunum	ns	ns	ns	0.01	ns	ns	ns	ns	ns
Lower jejunum	ns	ns	ns	0.01	ns	ns	ns	ns	ns
Upper ileum	ns	0.01	0.05	0.01	0.01	0.05	ns	0.05	ns
Lower ileum	ns	ns	ns	0.01	0.05	ns	ns	ns	ns
Colon	ns	0.01	ns	0.01	0.01	0.01	ns	0.01	ns

[1] Probability levels calculated on the basis of 21 analyses of variance, each for one feed ingredient and intestinal segment.
[2] Dm = dry matter.

was about 73%, on the average. Although there is some tendency for dry matter to be better absorbed by the low calcium birds, this effect is only significant in one segment. There was also some, and at times significant, diurnal variation in dry matter/^{91}Y ratio. However, this variation appears small and inconsistent.

DISCUSSION

On the basis of the known functions of the gizzard it appears unlikely that large amounts of calcium and phosphorus could be absorbed in this organ, as appears to be suggested by the lower Ca and P/^{91}Y ratios compared with those of the feed. It also appears unlikely that very large quantities of dry matter are secreted in this organ as suggested by the very high dry matter/^{91}Y ratio. The examination of the gizzard contents revealed the presence of relatively large feed particles, mostly grains, with few smaller ones. Apparently, therefore, the gizzard retains the larger feed particles longer than the smaller ones and the solutes. Calcium and phosphorus which are mainly in solution in the gizzard (12) might be emptied more rapidly than the larger grain particles. On the other hand, ^{91}Y tends to be partially adsorbed on solid particles (3) even at a low pH such as that in the gizzard. This isotope will therefore be slower than calcium and phosphorus to leave the gizzard, but more rapid than the solids. This interpretation is also supported by a preliminary study in this laboratory with a single dose of ^{45}Ca and ^{91}Y and may explain the low Ca and P/^{91}Y ratios and the high dry matter/^{91}Y ratio in the gizzard.[3] In the steady state of feed passage from the gizzard it may be assumed that the latter passes the various feed components at constant rate. This assumption is supported by the dry-matter/^{91}Y ratios which show only slight variations that appear to be more of diurnal nature rather than related to shell deposition. There was also no significant difference in the nutrient/^{91}Y ratios in the duodenum, suggesting uniform emptying of yttrium, calcium, phosphorus and total solids from the gizzard.

In accordance with the observations of Marcus and Lengemann (13), calcium, phosphorus and yttrium are assumed to move at an equal rate along the small intestine. Preliminary results in this laboratory appear to support this assumption.[4] Therefore, nutrient/^{91}Y ratios appear to be suitable for calculation of cumulative absorption along the intestine. However, more evidence is necessary to clarify this point. Nevertheless, the comparison of the patterns of calcium and phosphorus absorption as influenced by dietary and diurnal variations, is apparently valid since it appears unlikely that the movement of ^{91}Y could be influenced either by dietary calcium or egg shell deposition. In this respect it should be recalled that the late-calcification hens and the no-calcification hens were killed at the same time of the day.

Those factors with respect to the rate of passage of the test nutrient relative to the tracer, demonstrate clearly the difficulties in the use of such tracer.

The pattern of dry matter/^{91}Y indicates absorption of dry matter along the entire intestine with a somewhat reduced rate at the posterior segments. The high dry-matter/^{91}Y ratio in the duodenum is probably due to secretion of digestive substances in this organ. The pattern in general is similar to that observed by Bolton (8) in chicks, for the absorption of protein and available carbohydrate.

Assuming the same passage time for calcium, phosphorus and yttrium along the intestine, the present results appear to indicate that the major portion of calcium and phosphorus absorption is located at the anterior parts of the intestine. It can be easily shown that calcium concentration in the total dry matter is actually increasing along the intestine and therefore could not be a factor in the reduced absorption in the posterior parts. The latter may be due either to the greater capacity of the anterior segments to absorb calcium (1, 2, 5, 14, 15) or to the reduced solubility of calcium due to increase in pH with the distance from the gizzard (12, 16).

Dietary calcium, at the levels used, did not influence the relative absorption of calcium, in agreement with previous balance studies (9). The absence of a calcifying

[3] Unpublished data, S. Hurwitz and A. Bar, 1965.
[4] See footnote 3.

shell was associated with a reduced rate of calcium absorption. This effect was identical for the high calcium and low calcium lots. The mechanism responsible for this variation is not clear, although it may be explained in terms of the greater need for calcium during periods of egg shell formation. It is accepted that absorption alone cannot supply all the calcium needed during egg shell formation and that bone reserves have to be utilized (17), even when birds are fed sufficient calcium. This conclusion is based on daily averages of calcium retention not considering any diurnal variations in calcium absorption. The greater calcium absorption during shell deposition, observed in this study, could mean that the importance of bone as a calcium reserve in birds fed sufficient calcium, is not as great as previously believed.

The absorption of phosphorus appears to be closely related to that of calcium, and is probably influenced by the latter. The depressing effect of high levels of dietary calcium on phosphorus absorption is well known (18). The significant increase in absorption of phosphorus during shell formation is not as striking as with calcium absorption, and appears to be secondary to the latter.

From the $P/^{91}Y$ ratios in the duodenum, it is apparent that large quantities of endogenous phosphorus are emptied into this segment. It may be calculated that the ratio of endogenous to dietary phosphorus in this segment is at least 4:1. This observation is consistent with the heavy ^{32}P secretion into the duodenum, observed in the laying hen by Shirley et al. (19).

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Mrs. M. Cotter.

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Proceedings of the Twenty-ninth Annual Meeting of the American Institute of Nutrition

SHELBURNE HOTEL, ATLANTIC CITY, NEW JERSEY
APRIL 9–14, 1965

COUNCIL MEETINGS

The Council of the American Institute of Nutrition met Thursday evening, April 8, and Friday morning and evening, April 9. The actions of the Council were presented at the Institute business meetings and are included in the report of those meetings.

SCIENTIFIC SESSIONS

A total of 306 abstracts of papers was submitted to the Institute; 9 of them were transferred to other societies; 50 were accepted from other societies, making a total of 347 papers programed by the Institute. These were arranged into 29 regular and 3 intersociety (atherosclerosis) sessions. In addition, two informal conferences were held, Poultry Nutrition and Ruminant Nutrition, and the following half-day symposia were presented:

1. Biological Availability of Different Forms of Vitamin A and E
2. Adolescent Obesity
3. Amino Acid Release, Transport and Metabolism
4. Nutritional Significance of the Non-Nutrient Components of Food
5. Nutrition in Relation to Certain Aspects of Lipid Metabolism

BUSINESS MEETINGS

Business meetings were held on Saturday, April 10 and Tuesday, April 13. Dr. R. W. Engel presided at both meetings.

I. *Minutes of 1964 meeting*

The minutes as published in the *Journal of Nutrition*, 83: 385, 1964, were approved.

II. *Elections*

The 609 ballots were counted by Drs. Dorothy Arata and Lewis R. Arrington. The following were elected:

President: O. L. Kline
President-elect: A. E. Schaefer
Secretary-elect: W. N. Pearson
Treasurer: W. A. Krehl
Councilor: E. L. R. Stokstad
Nominating Committee:
G. H. Beaton, Chairman
R. S. Harris
H. Linkswiler
G. Mann
R. J. Young

III. *Constitutional Amendments*

By over two-thirds of all votes cast, the following amendments to and changes in the constitution and bylaws were adopted:

A. By vote of 588 for to 8 against, the following addition was made to Article I:

> *Section 7. Emeritus Members.* A member in good standing for 25 years or upon reaching the age of 65 may apply to the Secretary for Emeritus Member status. Members who wish Emeritus status because of prolonged disability from work may also apply. Emeritus Members are exempt from dues but retain all other privileges of membership. They may subscribe to Federation and/or Institute publications at member rates.

B. By vote of 555 for to 36 against, the following addition was made to Article I:

> *Section 8. Supporting Members.* Members who voluntarily contribute $25 or more annually over and above regular assessment in support of the general activities of the Institute shall become Supporting Members.

C. By vote of 542 for to 47 against, the following addition was made to Article I:

> *Section 9. Sustaining Associates.* Any association, corporation, institution or individual desiring to support the Society may be invited by the President to become a Sustaining Associate.

D. By vote of 580 for to 15 against, Article III, Section 2, has been changed to read as follows:

> *Section 2. Council.* The President, Past-President, President-Elect, Secretary, Treasurer, Secretary-Treasurer of the Clinical Division, and three additional councilors, one of whom shall be elected annually to serve a term of three years, shall constitute a nine-member Board of Trustees and shall be known as the Council. The President-Elect shall be considered as a Vice-President and serve in the absence of the President.

E. By vote of 566 for to 19 against, Article IV, Section 1, has been changed to read as follows:

> *Section 1. Powers.* The general management of the Society during the intervals between meetings shall be vested in the Council, which shall regularly perform the ordinary duties of an executive committee and possess all the powers conferred upon the Board of Trustees of an educational institution chartered by the Education Department of the University of the State of New York. A permanent charter was issued to the American Institute of Nutrition under date of November 16, 1934. The Council may appoint and compensate an Executive Secretary who shall assist in carrying on the functions of the Society.

F. By vote of 586 for to 6 against, Article VI, Section 1, has been changed to read as follows:

> *Section 1. Dues.* The dues shall be an annual assessment which shall be determined by majority vote at the annual meetings, upon recommendation of the Council, plus the annual cost of a member subscription to one journal, as follows: A. Division members, when required to subscribe to the official journal of their division, shall not be obligated to subscribe to the official journal of the American Institute of Nutrition. B. All other members shall subscribe to the official journal of the Institute. Dues shall be paid by September 1. All members have the privilege of subscribing to any official journal of the Institute or its division at members' rates.

G. By vote of 572 for to 7 against, Paragraph 3 of the Constitution (below) is now *deleted* since it duplicates Article III, Section 2 (see D above):

> Paragraph 3. The management of the American Institute of Nutrition shall be vested in a council consisting of the President, President-Elect, Past-President, Secretary, Treasurer and three additional members.

IV. *Membership Status*

As of April 1, 1965, there were 999 members of the Institute: 891 active, 92 retired and 16 honorary. There was an increase of 61 members over last year. The Clinical Division had a total of 162 members.

Notice of the deaths of these members was received this year:

> William E. Abbott, December 27, 1963
> James B. Allison, September 25, 1964
> Hazel M. Hauck, April 23, 1964
> Martha Koehne (Charter Member), August 4, 1961
> Henry W. Loy, December 1, 1964
> Roe E. Remington, May 15, 1964
> V. P. Sydenstricker, December 12, 1964
> Martha F. Trulson, January 3, 1965
> Max Wachstein, January 15, 1965

The following resolution in honor of Dr. Allison was read:

> RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annual meeting, April 10, 1965, wishing to express its deep regret and sorrow at the passing of one of its most distinguished members, James Boyd Allison, place this statement in its minutes for permanent record.
>
> Dr. Allison served as President of the New York Academy of Sciences, he was a member of the editorial board of the Journal of Nutrition, Treasurer of the American Institute of Nutrition, and President-Elect of our society for this year. He served on important committees of the World Health Organization, the Food and Agriculture Organization, the American Medical Association, and other national and international organizations.
>
> He carried out research in many fields, including marine biology, kidney function, gravity shock, and cancer. However, his most important contributions were in the field of protein nutrition and metabolism, in which he was recognized as an international authority. He was also teacher, consultant, and administrator and at all he excelled.
>
> To everyone, everywhere, he was known as Jim Allison, for he was everyone's friend. He was patient and considerate in his dealings with others and modest about his own accomplishments. His death is a great loss to our Society and to his countless friends.

The following resolution in honor of Dr. Koehne was read:

RESOLVED: That the American Institute of Nutrition, assembled at Atlantic City, New Jersey, at its annual meeting, April 10, 1965, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of one of its Charter Members, Martha Koehne.

Dr. Martha Koehne was one of the distinguished women who received their doctorates under Professor Lafayette B. Mendel at Yale. Early in her career she was a member of the staff of Professor E. V. McCollum at the School of Hygiene and Public Health at Johns Hopkins University. She contributed much to the research of nutrition in relation to dental health when at the University of Michigan. She was teacher and administrator at the University of Washington, the University of Tennessee and for more than 15 years, chief nutritionist with the Ohio Department of Health. While at Ohio, Dr. Koehne played a major role in promoting the passage of legislation for the enrichment of flour.

V. *New Members*

The Membership Committee considered the qualifications of 77 nominees. The following 50 nominees recommended by the Committee were approved for membership at the business meeting:

NEW MEMBERS — 1965 *

Lilla Aftergood	Montague Lane (C)
Thomas A. Anderson	Hugh B. Lofland, Jr.
Lloyd W. Beck	Mary E. Lojkin
Walter D. Block	Paul Baker McCay
David F. Brown (C)	D. S. McLaren (C)
C. E. Butterworth, Jr. (C)	Walter Mertz
John Edward Canham (C)	James K. Miller
William Walter Carlton	Leonard V. Packett
Yet-Oy Chang	Albert M. Pearson
Herbert L. Chapman, Jr.	Karoly G. Pinter (C)
Albert J. Clawson	Clifford Julius Pollard
William J. Culley	Raymond Reiser
Indrajit D. Desai	George H. Reussner
William N. Garrett	Daniel Rudman (C)
J. A. Goyco-Daubon	Irving I. Rusoff
Barbara E. Gunning	Herta Spencer
James G. Hamilton	P. R. Sundaresan
Robert W. Harkins	Howard S. Teague
James Raymond Howes	André G. van Veen
Jin Soon Ju	Fernando E. Viteri (C)
Kung-Ying Tang Kao	Marjorie Grant Whiting
Fred Kern, Jr.	C. S. Wilson
D. Wei Cheng King	Herbert G. Windmueller
F. A. Klipstein (C)	Peter D. S. Wood
Lennart Krook	Paul Lee Wright

(C) Clinical Division.
* For institutional affiliations and addresses of new members, see the Federation *Directory of Members* to be published in the Fall of 1965.

Drs. Leonard A. Luhby, Irvin C. Plough, and Joseph J. Vitale, already members of the American Institute of Nutrition, were elected to membership in the American Society for Clinical Nutrition.

HONORARY MEMBERS

The following scientists were elected to Honorary membership:

Joachim Kühnau
Professor of Medicine
University of Hamburg, Germany

Conrado R. Pascual
Research Director
Food and Nutrition Research Center
The Philippines

VI. *Office of the Executive Secretariat*

In accord with the wishes of the society, as voted at the Annual Meetings in April, 1963 and 1964, the Institute has established a full-time office of the Secretariat at Beaumont House, 9650 Wisconsin Avenue, Bethesda, Maryland. Dr. James Waddell, formerly with E. I. duPont de Nemours and Company, was appointed Executive Secretary on a part-time basis.

The activities of the office will be in conformity with the broad objectives of such an office as adopted by the society and as published in the *Journal of Nutrition*, *83:* 385, 1964.

The office, with the advice and assistance of the Committee on Nutrition Research and Training, Food and Nutrition Board, National Academy of Sciences, National Research Council (NAS-NRC), and with the concurrence of the Council, has submitted a proposal to the National Institutes of Health (NIH) for the support of a project entitled "National Program to Promote Nutrition in Health." The objectives are to define better the scope of nutrition research, to identify those areas that are being neglected, to emphasize the need for increasing numbers of adequately trained personnel, to evaluate the current status of the science of nutrition and its accomplishments under its present support structures, and to review and identify current training programs and centers.

The application has been scheduled for NIH review in June 1965.

VII. *Treasurer's Report*

Dr. Douglas V. Frost presented the Treasurer's report which was approved. The Auditing Committee, Drs. Joseph H. Roe and Callie Mae Coons, reported that they had examined the books and records of the Treasurer and found them in order.

TREASURER'S REPORT

April 3, 1964 to April 1, 1965

Balance in checking account, April 3, 1964	$ 9,321.99
Citizens National Bank of Waukegan	6,194.02
First Western Savings and Loan	10,040.51
U. S. Series K Bond	500.00
	$26,056.52
	9,321.99

BEGINNING BALANCE IN CHECKING ACCOUNT

RECEIPTS:

AIN dues, 892 members	$ 4,460.00	
Federation rebate from 1964 annual meeting	4,335.26	
Wistar Institute for Editorial Office, *J. Nutrition*, 4 quarters at $2,425	9,700.00	
Wistar Institute, additional net subscriptions, 171 at 11.25	1,923.75	
Page Charge collections, *J. Nutrition*	7,670.26	
U. S. Series K Bond Interest	13.80	
Citizens National Bank, Interest	247.76	
Support for Award Winner Reception (Nutrition Foundation, Borden, Mead Johnson)	419.10	
Dues backpayments	39.50	
Canadian-U. S. Nutrition Conference (repayment for advance)	300.00	
	$29,109.43	
Overpayment from FASEB for AIN dues	365.00	
TOTAL RECEIPTS		29,474.43
Sub-total		38.796.42

DISBURSEMENTS:

Wistar Institute, subscriptions to *J. Nutrition* for 1963–64	2,190.00	
Barnes, R. H., for *J. Nutrition* Editorial Office	12,600.00	
Secretary's Office expense	1,157.88	
Office of Executive Secretary	4,000.00	
FASEB — Advance for management services	200.00	
Management services, collection 1.75/member	1,561.00	
Return on overpayment	365.00	
Coffee Lounge, Annual Meeting	186.68	
National Dairy Council	70.46	
Award Winner Reception	419.10	
Canadian-U. S. Nutrition Conference (advance)	300.00	
Printing Costs	248.78	
Miscellaneous	48.37	
Deposited (Allstate Savings and Loan)	10,000.00	
TOTAL DISBURSED		33,347.27
Balance on hand in checking account, April 1, 1965		$ 5,449.15

TOTAL ASSETS:

Bank Balance (Checking account) — April 1, 1965	5,449.15
First Western Savings and Loan	10,517.40
Allstate Savings and Loan	10,285.61
Citizens National Bank of Waukegan	6,194.02
U. S. Series K Bond	500.00
New April 1, 1965 TOTAL ASSETS BALANCE	$32,946.18

VIII. *Dues*

President Engel reported on the recommendation of the Council that the AIN annual dues be increased $2.00. He discussed the needs for an increase in dues because of the desire of the Council that the income from dues defray at least one-half of the cost of the recently established AIN Secretariat.

The motion was made, seconded and unanimously passed by the membership to increase the dues by $2.00.

IX. *Editor's Report — Journal of Nutrition*

The editor of the *Journal of Nutrition*, Dr. Richard H. Barnes, submitted his report for the calendar year 1964. Special attention was given to the reasons listed by the Editorial Board for rejecting manuscripts submitted in 1962 and 1963. The rate of acceptance and rejection of manuscripts for that period was also reviewed. The report and proposed budget were approved and are summarized below:

Editing and Publication Operations

	Calendar year		
	1962	1963	1964
Volumes published	76, 77,78	79, 80, 81	82,83, 84
Pages published (including papers, biographies, announcements, and proceedings)	1447	1436	1339
(Scientific papers only)	(1403)	(1383)	(1267)
Papers published (including 3 biographies)	209	204	191
Papers submitted	321	312	293
Papers rejected	85	113	112
Rejection rate (based on no. papers submitted)	26%	36%	38%
Supplements published	1	—	—
Letters (to the editor)			1
Operating Schedule			
Avg time from date of receipt of manuscript to mailing to Wistar:			
Avg no. days with reviewer	20	21.2	20.7
Avg no. days out for revision	17.9	24.8	22.6
Avg no. days in office, in mail or in unavoidable delay	32.6	34.9	29.2
Avg total days	70.5	80.9	72.5
Avg time from date of receipt of manuscript to mailing by Wistar:			
Avg no. months with Editorial Office	2.4	2.6	2.4
Avg no. months with Wistar Press	3.4	3.2	3.2
Avg *total months* from date of receipt to mailing of Journal	5.8	5.8	5.6

Summary of Finances in the Operation of the Editor's Office, *Journal of Nutrition*
July 1, 1964 — June 30, 1965

Balance brought forward	$(−115.29)
Receipts, AIN	12,000.00
Total receipts and balance available	11,884.71
Expenditures, partially estimated per schedule	11,284.71
Estimated balance July 1, 1965	$ 600.00

The Council approved the budget proposed by Dr. Barnes for the Editor's Office for the year starting July 1, 1965.

X. *Secretary's Report*

Secretary Mickelsen informed the membership of the following procedures approved by the Council during their April 1965 meetings:

Beginning next year, ballots for the election of officers and Nomination Committee members, and voting proposed constitutional amendments shall be received and counted by the Office of the Exeecutive Secretary. The ballots are to be turned over to the Tellers' Committee for verification and final count at the Spring Meeting.

The Executive Secretary may select a committee to assist in arranging the scientific program for the annual meeting. The travel expenses, if any, of this committee shall be borne by the Institute.

There has been a favorable reaction to the newly established *AIN Nutrition Notes* which fills a long-felt need. The newsletter is available to non-members at the subscription rate of $2.00 per year; it was also decided that the newsletter should be copyrighted. A vote of appreciation and thanks go to Dr. M. R. Spivey Fox, Editor, and J. William Boehne, Associate Editor, for their efforts in getting the newsletter started.

XI. *President's Report*

A. International Union of Nutrition Sciences:

1. Council approved the expenditures anticipated for the travel of Dr. Glenn King, U. S. representative to the IUNS Executive Council, to Helsinki. Dr. King, as a member of the Executive Council, will be involved in the final arrangements for the VIIth International Congress of Nutrition to be held in Hamburg. This meeting will also consider the means whereby the International Union of Nutrition Sciences can be elected to the International Congress of Scientific Unions. Dr. King has been proposed by the U. S. National Committee as the President of IUNS to succeed Dr. David Cuthbertson whose term expires shortly.

2. The Institute has made application to the National Institutes of Health and the National Science Foundation for funds ($25,000 each) to be used for defraying travel costs of U. S. scientists to the 1966 International Nutrition Congress. In addition, $15,000 remaining from the 1960 Congress will be added to these funds making approximately $65,000 to support travel grants. This $15,000 and the $500 (Dr. King's expenses) are to be taken out of the Pine Street Fund only when actually needed, to avoid any interest loss.

B. Dr. D. Mark Hegsted is the AIN Council representative to the annual meeting of the Nutrition Society of Canada, June 11 and 12.

C. President Engel announced the establishment of a new award, the Conrad A. Elvehjem Award for Public Service in Nutrition, sponsored by the Wisconsin Alumni Research Foundation. Invitation for nominations for this award appears at the end of these Proceedings.

XII. *Business Management*

On the recommendation of Mr. John Rice, Federation Business Manager, it was decided that the Institute's fiscal year be made to coincide with the calendar year beginning January 1, 1966.

A new agreement will be signed whereby all financial matters of AIN will be handled by the Federation Business Office.

The Council expressed appreciation to the Federation Business Office for its excellent handling of AIN business last year.

XIII. *Reports of Committees and Representatives*

A. *Standing Committee on Experimental Animal Nutrition:* G. F. Combs

The Committee was organized to emphasize the role that research workers in basic and applied experimental animal nutrition have played and will continue to play in the development of the science of nutrition and to arouse greater interest on the part of these scientists in the functions of AIN.

Standing Operating Procedure for the Committee has been approved by the AIN Council.

The activities of the Committee included the organization of one symposium and two conference programs at the 1965 annual meeting of AIN as follows:

a) "The Biological Availability of Different Forms of Vitamins A and E to Animals" chaired by L. D. Matterson. (Symposium)
b) "Sixth Annual Ruminant Nutrition Conference" chaired by R. S. Emory and R. L. Reid.
c) "Informal Poultry Nutrition Conference" chaired by H. M. Edwards, Jr., and F. H. Kratzer.

A sub-committee on Nutrition and Education, chaired by J. K. Loosli, has been formed. This committee proposed that, at the 1966 Federation Meetings, a symposium be held on formal nutrition education. In considering this proposal, the sub-committee established liaison with the Committee on Educational Policy in Agriculture, National Research Council. When this suggestion for a symposium was presented to the Council of the Institute, it was endorsed with the suggestion that the scope be expanded to include animal nutrition, clinical nutrition, dental nutrition and dietetics. The Symposium Committee is considering this as one of the regularly scheduled symposia for 1966.

The Committee is also considering the possibility of assembling a slide collection depicting nutritional deficiency diseases in experimental animals for use in nutrition instruction. Investigators having material which illustrates either gross or microscopic nutritional lesions suitable for inclusion in such a collection are requested to contact Dr. Gerald F. Combs, Department of Poultry Science, University of Maryland, College Park, Maryland.

B. *Finance Committee Report:* G. M. Briggs

This Committee was to assist the Council in matters of finance. From the time of its appointment in July until December, the Committee's major concern expressed to the Council was the urgent need for the establishment of an Executive Secretariat. We felt that by this means the Institute's financial structure would be benefitted more than by any other means.

To underwrite the costs of a Secretariat and to strengthen all activities of the Institute we have urged that some of the present surplus in the treasury be used, and that additional funds be obtained from such sources as a) a small increase in membership dues; b) requests for funds to the NIH and to private foundations; c) new classes of membership known as "supporting members" and "sustaining associate members"; d) a portion of the page charges from the *Journal of Nutrition* and of the income to the Institute from the Federation Meeting registration fees; e) increased advertising revenue from Institute journals; f) sale of a proposed collection of biographies from past issues of the *Journal of Nutrition;* g) the possible establishment of other journal(s); and h) other suggested means requiring approval of the Council and the membership.

The Finance Committee commends the Council under Dr. Engel's leadership for taking prompt action in the establishment of the Executive Secretariat, for the development of formal proposals to outside agencies for funds, and in taking other action necessary to finance the ever-increasing activities of the Institute. In the development of these matters by the Council the advice of the Finance Committee was sought at all times and we were kept informed of all progress. We believe that this has been a most important year for the future of the American Institute of Nutrition.

C. *Committee on Nutrition Training and Fellowships:* L. C. Norris

The following resolution was adapted by the membership at its meeting on April 13, 1965 and was sent to the Director of the National Institutes of Health on April 26, 1965:

RESOLUTION: WHEREAS the science of nutrition includes elements of physiology, biochemistry, pathology and related fields, it is also recognized as a distinct discipline in its own right as evidenced by the adherence of over one thousand scientists to its leading national scientific society, the American Institute of Nutrition, one of the Federation of American Societies for Experimental Biology and

WHEREAS the science of nutrition by its multidisciplinary nature is an important component in the study of diseases which are the concern of the National Institutes for Cancer, Heart, Dental Research, Child Health and Development, Arthritis and Metabolic Diseases, Mental Health, Neurological Diseases and Blindness, as well as the Institute of General Medical Sciences, and

WHEREAS the advancement of the science of nutrition deserves recognition of its significance in the training of investigators for the conduct of research to increase our understanding of normal and diseased states,

BE IT RESOLVED that the Institute of General Medical Sciences be urged to establish a training committee for Nutrition Training Grants to provide a continuing, consistent and effective procedure for the review and appraisal of applications for support of nutrition training.

D. *Ad hoc Committee on Nutrition Careers Brochure:* F. J. Stare

The revised career brochure was submitted by the Committee. The Council expressed appreciation to the Committee on the completion of the task and moved that the Committee be discharged.

The membership was urged to purchase the brochure in lots of 100 ($4.00 per 100) and to disseminate them through regular office mailings as a means of reaching interested groups or individuals.

E. *International Nutrition Committee:* W. N. Pearson

The Committee recently carried out a survey of the membership to determine their possible association with international nutrition programs. Of the approximately 1,000 questionnaires sent out, 407 were returned. Of those answering the questionnaire, 141 had been affiliated with foreign nutrition programs during the past 5 years, and 83 AIN members had actually worked in a foreign country. A large number of members (111) anticipated that they would participate in foreign nutrition programs during the next few years, and 258 members indicated that they would be interested in working in a foreign country. The Committee files now contain the names and current addresses of 625 foreign students trained in the U. S. by members of the AIN. Copies of this report are available from the Executive Secretary.

Dr. Pearson urged AIN membership participation in the VITA program (Volunteers for International Technical Assistance, Inc). The AIN Council appointed Dr. James Waddell as AIN's liaison with VITA. The program calls upon scientists to provide technical assistance for underdeveloped countries in one or more of the following ways: 1) act as area representative; 2) give general assistance (entertain students, collect technical books, etc.); 3) prepare state-of-the art reports on technical subjects; 4) participate in

research and development projects which do not conflict with one's full-time job; and 5) work up replies to specific technical questions.

F. *Report on International Biological Program:* G. K. Davis

As the result of the July 22–25, 1964 meetings, called under the auspices of the International Council of Scientific Unions in Paris, to consider the activation and implementation of an International Biological Program, a Special Committee for the International Biological Program (SCIBP) was established and charged with developing the various program sections.

A special working group has been set up consisting of representatives from the International Union of Nutrition Sciences, the section on Human Adaptability and the sections on Use and Management. This group is to prepare the definitive programs for nutrition.

The National Academy of Sciences has approved the establishment of a National Committee for the International Biological Program under its section on biology and agriculture.

G. *Publication Management Committee:* W. J. Darby

No meeting of this Committee was held during the year. The newly appointed Executive Secretary is to be the continuing Secretary to this Committee and should explore all means by which the journals of AIN can best serve the membership.

H. *Representative to the Food and Agriculture Organization:* B. S. Schweigert

FAO continues to be active in many areas of significance to nutritionists and has sponsored several important conferences and the publication of monographs on timely and important topics. Examples of recent publications include: specifications for the identity and purity of food additives and their toxicological evaluation; emulsifiers, stabilizers, bleaching and maturing agents; legumes in human nutrition; news bulletin of the Protein Advisory Group on the protein rich foods program and on aflotoxins (joint with WHO and UNICEF); contemporary views on world dairy education; and a biomonthly publication highlighting activities of the Freedom from Hunger Campaign.

We can look forward to expanded attention by FAO, other international agencies and national groups to coordinate agricultural production, nutrition and health, food processing and preservation, and population problems in their programs in the future.

I. *Representative to National Research Council Divisions and Boards:* G. F. Combs

Dr. A. Geoffrey Norman, Vice President for research at the University of Michigan, succeeds Dr. T. C. Byerly as chairman of the Division of Biology and Agriculture.

The National Academy of Sciences has established a National Committee for the International Biological Program under The Division of Biology and Agriculture. Dr. Roger Revelle will serve as chairman. Dr. George K. Davis, who is a member of this committee, also is international chairman of the section on Use and Management of Biological Resources, in which nutrition is a specific area. A working group in nutrition has been established to develop basic guide lines for projects in nutrition.

The Food and Nutrition Board (FNB), in its 25th year, sponsored an International Conference on Prevention of Malnutrition in the Pre-school Child which was held in the Academy, December 7–11 and a symposium on Significance of Plasma Free Amino Acid Levels for Evaluation of Protein Nutrition at Rutgers University, February 1–2 as a memorial to Dr. James B. Allison.

Recent publications of this board include:

NRC Pub. 1195, *"An Evaluation of Public Health Hazards from Microbiological Contamination of Foods"* and NRC Pub. 1225, *Food Habits Research; Problems of the 1960's."* NRC Pub. 398. *"The Use of Chemical Additives in Food Processing"* has been revised and is in press. Two other publications in preparation are *"The Role of Cereals in World Nutrition"* and *"Monograph on the Geographic Occurrence and Characteristics of Nutrition Diseases."*

The Food Protection Committee, FNB, has developed provisional specifications for approximately 400 food-grade chemicals and has issued 415 pages in loose-leaf segments to date. The final bound book defining 500 chemicals is expected to be published next year.

The Advisory Committee on Marine Protein Concentrates, FNB, has been requested by the Department of Interior to plan for the evaluation of the feasibility of introducing new marine protein products into commercial outlets.

At the April 2 meeting of the Food and Nutrition Board, Mr. Alan Berg, Assistant to the Director, Food for Peace, emphasized the intensified focus on nutrition in future programs for Food for Peace through the Agency for International Development (AID).

The Agricultural Board, Division of Biology and Agriculture, has produced the following revised reports:

1964 NAS Pub. 1192, *"Nutrient Requirements of Swine"*; 1964 NAS Pub. 1193, *"Nutrient Requirements of Sheep"*; and 1965 NAS Pub. 1232, *"Joint U. S.-Canadian Tables of Feed Composition."* In addition its Committee on Educational Policy in Agriculture has produced an illustrated brochure, "Threads of Life" for use by high school guidance counselors. Also the Institute of Laboratory Animal Resources has published standards for the breeding, care and management of laboratory dogs, cats and rabbits, augmenting the currently accepted standards on mice, rats, hamsters, and guinea pigs.

Proceedings of the annual meeting of the Agricultural Research Institute, October 12–13, 1964, are available. The central theme of this meeting was "Advancement of World Agriculture."

Proceedings of the Conference on Nutrition in Space and Related Waste Problems, April 27-30, 1964, are available (NASA, SP-70).

The National Committee, International Union of Nutrition Sciences, has obtained the support of the NAS for admission of IUNS into the International Council of Scientific Unions. This will greatly improve the opportunity for nutrition scientists to participate in international programs such as IBP.

J. *Report of the Federation Board Representative:* Grace A. Goldsmith

The proposed revisions of the Federation Constitution and Bylaws are still in the draft stage and have gone back to the Advisory Committee for review. AIN approved the draft as circulated by the Federation.

The Federation *Directory of Members* will hereafter include zip codes of the members.

Upon the recommendation of Dr. Maurice Visscher, the FASEB Representative on the National Advisory Committee to the Accreditation Board of the Animal Care Panel, the Federation will appoint a representative (and one alternate) to become a member of the newly formed American Council on Accreditation of Laboratory Animal Care. FASEB has approved a contribution of $1000 in support of this national accreditation program.

Exploration is continuing relative to the establishment of a Biology Joint Council. This Council is envisioned as a supra-organization which could be a spokesman for all of biology and provide an environment for the discussion of problems of mutual interest to its constituent organizations and a means for facilitating communication among all biologists. Further plans will be brought to the attention of members as they develop.

The following proposals for standardization of rules for the submission of abstracts (annual meeting) were considered but not accepted: a) A member may be responsible (responsible author or member) for only one paper on the program of each Society to which he belongs. b) A nonmember's name may appear only once in the program of the Federation.

Present Federation regulations limit any individual to present orally only one 10-minute paper. The following rule was adopted: Any Society may, at its discretion, elect to program for delivery only a fraction of the total number of papers submitted to it or transferred to it. Those abstracts not scheduled, under these circumstances, may then be published in the abstracts volume.

Dr. Milton Lee will retire as Executive Director of the Federation on July 1, 1965. He will continue to serve in an advisory capacity to the Chairman of the Federation Board until the time of his retirement at age 65. The Advisory Committee of the Federation has been asked to search for a replacement for Dr. Lee. Suggestions from the AIN Council were invited.

XIV. *Report of the Clinical Division:* J. F. Mueller

The fifth annual meeting of the Clinical Division of AIN will be held May 1, at the Seaside Hotel, Atlantic City, New Jersey. ASCN is initiating the McCollum Award, the first of which will be presented during the dinner meeting on May 1.

The McCollum Award is sponsored by the National Dairy Council and the ASCN and is presented for outstanding research in the field of clinical nutrition. The recognition consists of $1000 and a commemorative scroll.

The Norman Jolliffe Fellowship Award will be publicized and sponsored by the ASCN; the custodian of contributions will be the Nutrition Clinics Fund. The purpose of this Award is to stimulate an interest in clinical nutrition among medical students in the United States and to give financial support to those active in the teaching of clinical nutrition in American medical schools. The Fellowship carries a minimum of $4000 annually for the next five years.

XV. *Metric System Resolutions*

The following resolutions were adopted by the membership at its meeting on April 10, 1965:

WHEREAS the United States led the world with decimal currency and
WHEREAS Congress legalized metric weights and measures, July 28, 1866,
BE IT THEREFORE RESOLVED that the American Institute of Nutrition, at its Annual Meeting, April 10, 1965, recommends issuance of a metric centennial commemorative postage stamp on July 28, 1966.
WHEREAS more than 90% of the world's population now operates under the metric system, and whereas the *Journal of Nutrition, Poultry Science, Journal of Animal Science, Journal of Dairy Science, Food Chemicals Codex,* and publications of the National Academy of Sciences-National Research Council now use or will use metric weights and measures exclusively,
BE IT THEREFORE RESOLVED that the American Institute of Nutrition in its Annual Meeting, April 10, 1965, recommends passage of the bills now before Congress to study feasibility and practicability of conversion to the metric system of weights and measures for general use in the United States.
BE IT FURTHER RESOLVED that copies of this resolution be sent to committees concerned with metric conversion study bills S. 774, H.R. 2626, H.R. 38, and H.R. 1154 to achieve the above objective.

The first resolution was sent to the U. S. Postmaster General on May 7, 1965 with copies to the President of the Metric Association and Director of the Project for Metric Research.

The second resolution was sent to Senators Warren Magnuson, Norris Cotton and Clairborne Pell; Representatives G. P. Miller, Robert McClory and James Roosevelt; Drs. F. Seitz and A. G. Norman, NAS; Editor of *Science;* Secretary of Commerce; and Dr. D. F. Hornig, President's Science Advisory Committee.

XVI. *The Joint Canadian — U. S. Nutrition Conference:* Dr. H. H. Williams

The first Conference of Nutrition sponsored jointly by the Nutrition Society of Canada and the AIN was held at the Royal York Hotel in Toronto, Canada, on September 14 and 15, 1964. The total registration was 310 (248 active, 62 associate and graduate). Fifty-two scientific papers were presented (14 Canadian, 33 United States and 5 from other countries). Two hundred and seventy-six attended the dinner given by the Province of Ontario.

Total receipts were $4405 against $3730 in expenses. The small balance will be used to purchase reprints of the symposia presented at the meeting. The two symposia 1) Nutrition, Aging and Longevity, and 2) Development and Significance of Biochemical Lesions Due to Dietary Imbalance, will be published in the *Canadian Journal of Biochemistry* and the *Journal of the Canadian Medical Association.*

Although the cost for printing the abstracts and programs was over $1000, the Federation met approximately one-half this cost.

Sincere thanks were rendered Dr. Williams for his unstinting services on behalf of the Conference.

XVII. Future Federation meetings:
1966, April 11–16, Atlantic City
1967, April 16–21, Chicago
1968, April 7–12, Atlantic City

XVIII. The next Council meeting will be held on October 30, 1965 in Washington, D. C.

ANNUAL DINNER AND PRESENTATION OF FELLOWS AND AWARDS

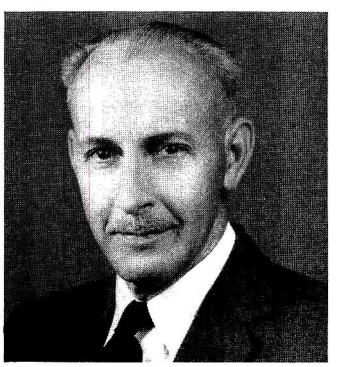
The annual banquet was held Monday, April 12, 1965 at the Shelburne Hotel, with 372 individuals attending. Dr. Engel presided.

Dr. James Waddell introduced the newly appointed Fellows, whose citations follow:

PAUL GYÖRGY — for a lifetime devoted to both experimental and applied nutrition; for basic contributions to our knowledge of pyridoxine, riboflavin, biotin, choline, and vitamin E; for studies on the prevention of hepatic injury by dietary means and on protein malnutrition. A physician for fifty years, he has applied his knowledge to the improvement of the nutrition of infants and children. He has received previously from this Society both the Borden Award and the Osborne and Mendel Award.

PAUL GYÖRGY

RAYMOND W. SWIFT — for his outstanding contributions to our knowledge of energy metabolism in animals and in man, and the effect of such factors as environment and nutrient balance on the efficiency of energy utilization. During his thirty-eight years of association with, and his fourteen years as director of, the group working with the Armsby respiration calorimeter he provided sound leadership as exemplified by his efforts in refining and improving feeding standards and in the nutritive evaluation of forages. A meticulous and resourceful investigator, a lucid writer and an inspiring teacher, he has greatly enhanced the status of the science of nutrition.



RAYMOND W. SWIFT

GENEVIEVE STEARNS — for a distinguished career as investigator and lecturer; a recognized authority on the mineral and protein metabolism of infants, children, adolescents, and adult women; on the vitamin requirements of infants and children; on the changes in body metabolism in diseases of bone. She served on the U. S. Committee to the World Health Organization Seminars on Infant Nutrition held in Leiden and Stockholm (1950). She received the AIN Borden Award (with P. C. Jeans) in 1946.

GENEVIEVE STEARNS

AMERICAN INSTITUTE OF NUTRITION

Founded April 11, 1933; Incorporated November 16, 1934; Member of Federation 1940

OFFICERS, 1965–1966

President: O. L. Kline, Food and Drug Administration, Washington, D. C.

President-Elect: A. E. Schaefer, Interdepartmental Committee on Nutrition for National Development, National Institutes of Health, Bethesda, Maryland.

Secretary: Olaf Mickelsen, Department of Foods and Nutrition, College of Home Economics, Michigan State University, East Lansing, Michigan (1966).

Treasurer: W. A. Krehl, Clinical Research Center, University of Iowa, Iowa City, Iowa (1968).

Councilors: D. M. Hegsted (1966), N. S. Scrimshaw (1967), E. L. R. Stokstad (1968).

COMMITTEES

Nominating Committee: G. H. Beaton, chairman; R. S. Harris, Hellen M. Linkswiler, G. V. Mann, R. J. Young.

Joint Committee on Biochemical Nomenclature (joint with American Society of Biological Chemists): S. R. Ames (1966), chairman; P. L. Harris (1966), H. H. Williams (1966).

Committee on Publication Management: W. J. Darby (1966), chairman; P. L. Harris (1966), J. K. Loosli (1966), D. M. Hegsted (1967), F. S. Doft (1967), James Waddell (continuing), secretary. Ex officio, Olaf Mickelsen, J. F. Mueller.

Nominating Committee for Mead Johnson Award: B. C. Johnson (1966), chairman; P. L. Day (1967), A. R. Kemmerer (1968).

Nominating Committee for Borden Award: L. D. Wright (1966), chairman; E. L. Hove (1967). P. H. Weswig (1968).

Nominating Committee for Osborne-Mendel Award: N. S. Scrimshaw (1966), chairman; M. O. Schultze (1967), Alex Black (1968).

Nominating Committee for Conrad A. Elvehjem Award for Public Service in Nutrition: B. S. Schweigert (1966), chairman; E. E. Howe (1967), F. W. Quackenbush (1968).

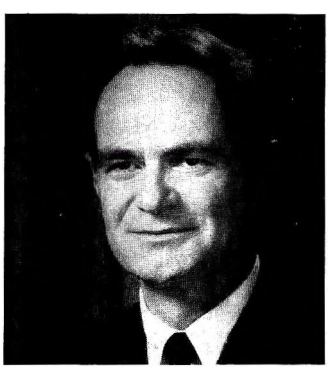
Mead Johnson Award for Research in Nutrition

The 1965 Mead Johnson and Company Award of $1000 and a scroll was awarded to Dr. John G. Bieri, Chief, Section on Nutritional Biochemistry, National Institute of Arthritis and Metabolic Diseases. It was presented for research on the utilization, metabolism, function and nutritional requirements of vitamin E in the chick, rat and man; the interrelationships of selenium, antioxidants, lipids, sulfur amino acids, and ubiquinone in the metabolism of vitamin E; and analytical methods for the measurement of tocopherols in animal tissues.

JOHN G. BIERI

Borden Award in Nutrition

The 1965 Borden Award of $1000 and a gold medal was presented to Dr. Alfred E. Harper, Professor of Nutrition, Massachusetts Institute of Technology. The award was presented for a series of investigations on the interrelationship of amino acids in nutrition. His contributions have advanced our knowledge of the concepts of amino acid imbalances, toxicities, and antagonisms — factors of importance in the prediction of the nutritive value of proteins. His recent observations that amino acid imbalance exerts its effect in large part by depressing the appetite and that this can be correlated with blood levels of amino acids are of particular significance. These investigations have contributed to a clearer understanding of the role played by amino acids in nutrition, particularly in populations where low levels of protein of poor quality are consumed.



ALFRED E. HARPER

Osborne and Mendel Award

The 1965 Osborne and Mendel Award of $1000 and a scroll was presented to Dr. David Mark Hegsted, Professor of Nutrition, Harvard School of Public Health. This was given for his research in the areas of mammalian calcium and protein requirements and for his studies of cholesterol metabolism. He has systematically investigated various methods of evaluating the role diet may play in the development of cardiovascular disease. He has combined a continuing interest in experimental nutrition while attempting also to improve the nutritional condition of man both in this country and abroad. During a distinguished academic career, he has trained many students who have become outstanding investigators in many parts of the world.

DAVID MARK HEGSTED

Fellows Committee: Charlotte M. Young (1966), chairman; W. D. Salmon (1966), S. L. Hansard (1967), P. E. Johnson (1967), W. H. Griffith (1968).

Public Information Committee: P. L. White (1966), chairman; M. S. Read (1966), A. D. Tillman (1966), L. J. Teply (1966), A. L. Forbes (1967), C. J. Ackerman (1967), Ruth L. Pike (1967), E. D. Wilson (1967). Ex officio, Olaf Mickelsen.

Committee on Honorary Memberships: Paul György, chairman; Grace A. Goldsmith, P. L. Day.

Auditing Committee: R. E. Hodges (1966), C. P. Berg (1966).

Symposium Committee: N. S. Scrimshaw, chairman; G. F. Combs, R. L. Jackson.

Ad hoc Committee on International Nutrition: W. N. Pearson, chairman; Guillermo Arroyave, C. F. Asenjo, K. W. King, W. C. Unglaub, C. W. Woodruff.

Ad hoc Committee on Nutrition Training and Fellowships: L. C. Norris, chairman; James McGinnis, co-chairman; C. O. Chichester, J. S. Dinning, W. H. Griffith, R. E. Hodges, O. N. Miller, Clara A. Storvick.

Ad hoc Committee on Recommended Constitutional Changes: A. E. Axelrod, chairman; G. M. Briggs, R. E. Hodges, R. E. Olson, Marion E. Swendseid.

Committee on Experimental Animal Nutrition: G. F. Combs, chairman; E. W. Crampton, F. W. Hill, F. H. Kratzer, J. K. Loosli, R. W. Luecke, L. D. Matterson, W. F. Pfander, R. L. Reed, R. W. Engel (ex officio).

Ad hoc Committee on Travel Funds to the 1966 Nutrition Congress: James Waddell, chairman; G. F. Combs, Dorothy Arata, A. E. Schaefer, E. L. R. Stokstad, O. L. Kline (ex officio).

Ad hoc Committee on Sustaining Associates: C. H. Krieger, chairman; W. R. Graham, H. S. Olcott, F. E. Shideman.

Membership Committee: G. P. Barron (1966), chairman; M. L. Scott (1967), K. W. King (1968), Dena C. Cederquist (1969), G. V. Mann (1970).

Finance Committee: H. H. Williams, chairman; D. V. Frost, P. E. Johnson, ex officio; W. A. Krehl, treasurer (1968).

U. S. National Committee, IUNS: G. F. Combs (1966), Charlotte M. Young (1966), O. L. Kline (1966), C. G. King (1967), W. J. Darby (1967), W. A. Krehl (1967), W. M. Beeson (1968), A. L. Forbes (1968), R. W. Engel (1968). Ex officio (non-voting) members: A. G. Norman, R. K. Cannan, Harrison Brown, E. C. Rowan.

REPRESENTATIVES

National Research Council Boards and Divisions: G. F. Combs (1968).

American Association for the Advancement of Science: R. L. Lyman (delegate), Ruth M. Leverton (alternate).

National Society for Medical Research: H. C. Spruth.

Food and Agriculture Organization: B. S. Schweigert (1966).

Federation Public Information Committee: P. L. White (1966).

Federation Proceedings Editorial Board: P. L. Harris (1967).

Federation Proceedings Translation Supplement: F. P. Stekol (1968).

Editorial Board
The Journal of Nutrition

R. H. Barnes, editor; H. H. Williams, associate editor; E. Neige Todhunter, biographical editor; John G. Bieri, George M. Briggs, Harry P. Broquist, George K. Davis, Samuel J. Fomon, R. M. Forbes, R. Gaurth Hansen, L. M. Henderson, F. W. Hill, Jules Hirsch, Ralph T. Holman, E. E. Howe, Gennard Matrone, Paul M. Newberne, Boyd L. O'Dell, H. E. Sauberlich, E. L. R. Stokstad and Clara A. Storvick.

Officers, American Society for Clinical Nutrition
(A division of the American Institute of Nutrition)

President, C. S. Davidson; President-Elect, R. E. Hodges; Past President, W. A. Krehl; Secretary-Treasurer, J. F. Mueller, Brooklyn-Cumberland Medical Center, 121 DeKalb Avenue, Brooklyn, New York; Councilors: T. B. Van Itallie, R. M. Kark, W. S. Hartroft.

Editorial Board
American Journal of Clinical Nutrition

W. A. Krehl, editor-in-chief; Robert E. Hodges, associate editor; Margaret J. Albrink, Ernest Beutler, George J. Gabuzda, D. Mark Hegsted, Robert Jackson, Francisco Grande, Sami A. Hashim, Robert E. Olson, Donald M. Watkin, Maurice E. Shils, Victor Herbert and Carroll M. Leevy.

JAMES WADDELL, *Executive Secretary*
American Institute of Nutrition

Invitation for Nominations for 1966 American Institute of Nutrition Awards

Nominations are requested for the 1966 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination. A bibliography and supporting letters are not to be submitted. (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1965*, to be considered for the 1966 awards.

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

Presentation of awards will be made at the annual dinner at the annual meeting.

1966 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. 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. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis
1965 – A. E. Harper

NOMINATING COMMITTEE:

L. D. WRIGHT, *Chairman*
E. L. HOVE
P. H. WESWIG

Send nominations to:

DR. L. D. WRIGHT
Graduate School of Nutrition
Savage Hall, Cornell University
Ithaca, New York

1966 Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
1964 – L. Emmett Holt, Jr.
1965 – D. M. Hegsted

NOMINATING COMMITTEE:

NEVIN SCRIMSHAW, *Chairman*
M. O. SCHULTZE
ALEX BLACK

Send nominations to:

DR. NEVIN SCRIMSHAW
Department of Nutrition and Food Science
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

1966 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 46th birthday during the calendar year in which the Award is given. Selection by the Jury of Award will be based primarily on a single outstanding piece of research in nutrition published in the year preceding the annual meeting or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hogan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
1952 – H. E. Sauberlich
1964 – J. S. Dinning
1965 – J. G. Bieri

NOMINATING COMMITTEE:

B. CONNOR JOHNSON, *Chairman*
P. L. DAY
A. R. KEMMERER

Send nominations to:

DR. B. CONNOR JOHNSON
University of Oklahoma
School of Medicine
Oklahoma City, Oklahoma 73104

1966 Conrad A. Elvehjem Award for Public Service in Nutrition

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

NOMINATING COMMITTEE:

B. S. SCHWEIGERT, *Chairman*
E. E. HOWE
F. W. QUACKENBUSH

Send nominations to:

DR. B. S. SCHWEIGERT, *Chairman*
Food Science Department
Michigan State University
East Lansing, Michigan 48823

Invitation for Nominations for 1966 American Institute of Nutrition Fellows

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

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

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

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

Fellows Committee:

CHARLOTTE M. YOUNG, *Chairman*
W. D. SALMON
S. L. HANSARD
PAUL E. JOHNSON
W. H. GRIFFITH

Send nominations to:

DR. CHARLOTTE M. YOUNG
Cornell University
Savage Hall
Ithaca, New York 14850

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

J. B. Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Henrik Dam (1964)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Wendell H. Griffith (1963)
Paul György (1965)
Albert G. Hogan (1959)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Helen T. Parsons (1961)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
Arthur H. Smith (1961)
Genevieve Stearns (1965)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)

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

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

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

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

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

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

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

Committee on Honorary Memberships:

PAUL GYÖRGY, *Chairman*
PAUL DAY
GRACE GOLDSMITH

Send nominations to:

DR. PAUL GYÖRGY
University of Pennsylvania
Pennsylvania General Hospital
Philadelphia, Pennsylvania 19104

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

Kunitaro Arimoto
W. R. Aykroyd
Frank B. Berry
Edward Jean Bigwood
Frank G. Boudreau
Robert C. Burgess
Harriette Chick
F. W. A. Clements
David P. Cuthbertson
Herbert M. Evans
Joachim Kühnau
Toshio Oiso
Lord John Boyd Orr
Conrado R. Pascual
V. N. Patwardhan
Emile F. Terroine
Eric John Underwood
Artturi I. Virtanen

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