Availability of Microbially Synthesized Thiamine in the Rat[1]

JOHN YUDKIN
Department of Nutrition, Queen Elizabeth College, University of London, London, England

ABSTRACT It has been shown that microbially synthesized thiamine is available to the rat after coprophagy. Thus, rats given a thiamine-free diet survived longer when kept directly on the floors of their cages, where they had easy access to their feces, than when kept on grids. The fecal excretion of thiamine, as determined by the thiochrome method, was about 2.5 μg daily for at least the first 3 weeks of depletion. The increased duration of survival of thiamine-deficient rats fed either the total daily output of feces, or graded amounts of thiamine, indicated that most if not all of the fecal thiamine was available for absorption.

Several substances, when included in the diet of the rat, are known to spare thiamine. This is true of some antibiotics such as penicillin (1) or of some carbohydrate derivatives such as sorbitol (2,3). The mechanism of the sparing action is a microbial synthesis of thiamine in the intestine, its excretion in the feces, and the ingestion of the feces by the rat. The prevention of coprophagy abolishes the sparing effect of penicillin (4) and of sorbitol (5).

There is also evidence that small amounts of thiamine are synthesized in the rat receiving no special dietary supplements. On the basis of experiments with S^{35}, Wostmann and Knight (6) confirm this synthesis, but claim that the synthesized thiamine is not available to the rat even if the animal is allowed to practice coprophagy. If these results are accepted, it would mean that there is a difference between the availability of microbially synthesized thiamine produced under the influence of antibiotics or carbohydrate derivatives, and that produced in the rat not receiving these dietary supplements. The investigation reported here does not confirm this difference in availability.

EXPERIMENTAL

Exp. 1. Effect of housing on grids on the duration of survival of thiamine-deficient rats. It is known that rats kept on grids are able to practice coprophagy (7). Nevertheless, such animals have more difficulty in securing their feces than animals housed directly on the floor of the cage. One would then expect that they are less easily able to obtain microbially synthesized thiamine excreted in the feces.

Twelve male albino rats, aged 30 days, were fed a purified diet, complete with vitamin supplements except for thiamine (table 1). They were divided into 2 groups by the usual littermate technique. Six of the rats were housed in individual cages with grids; the remainder were housed in similar cages from which the grids had been removed, and with sawdust on the floors.

TABLE 1
Purified diet[1]

Sucrose	600
Vitamin-free casein	200
Arachis oil	150
Salt mixture[2]	50

[1] Vitamin supplements: 1 kg diet contained: (in mg) choline, 1000; inositol, 220; niacin, 100; Ca-D-pantothenate, 100; p-aminobenzoic acid, 75; riboflavin, 30; pyridoxine, 8; folic acid, 1; biotin, 0.2; and cyanocobalamin, 0.1. In addition, each animal was given 1 mg α-tocopherol on one day weekly and, on another day, vitamin A, 120 IU; vitamin D, 20 IU; and vitamin K, 500 μg.

[2] Tricalcium phosphate, 400; potassium chloride, 250; sodium dihydrogen phosphate (anhydrous), 105; magnesium sulphate, 80; sodium chloride, 50; ferric citrate, 35; potassium iodide, 1; manganese sulphate, 0.2; sodium fluoride, 0.04.

Results. The animals on grids reached a lower maximal weight, developed polyneuritis earlier, and survived for a shorter

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TABLE 2

Growth and survival of thiamine-deficient rats in cages with or without grids[1]

Housing	Maximum wt	Duration of survival
	g	*days*
With grids	81 ± 3.3[2]	27 ± 2.0
Without	92 ± 5.0	42 ± 3.4

[1] Six rats in each group.
[2] SD of mean.

time, than the animals on the sawdust (table 2). The difference in the duration of survival in the 2 groups is significant at the 0.1% level.

Exp. 2. Thiamine content of feces and its availability. The thiamine content of feces of rats fed a thiamine-deficient diet was measured by the thiochrome method, and also by determining the increased survival time of rats fed such feces as the sole source of thiamine. At the same time, an approximate assessment was made of the availability of fecal thiamine to thiamine-deficient rats kept in gridless cages.

Seven littermate groups, each of 6 male albino rats 30 days old, were fed the thiamine-free diet previously described. All the rats were housed in individual cages, those from group 1 on sawdust and those from groups 2–7 on grids. Rats from groups 1, 2, and 3 received no supplements; those from groups 4, 5, 6 and 7 each received daily supplements of thiamine of 0.5 μg, 1 μg, 2 μg and 3 μg. Feces were collected from rats in group 2 and were deep-frozen for estimation of thiamine by the thiochrome method. Feces were also collected from each rat in group 3, and mixed with the food of the same rat the following day. Steps were taken to insure that all of the feces were consumed, by mixing them with only a little food each morning, and giving more food when the mixture was consumed. Rarely, during the first few days of the experiment, the mixture was not fully consumed by the end of the day; in these instances, the mixture was removed from the cage, kept in the refrigerator until the next day, and then mixed with the new lot of feces and some more food.

Results. As in experiment 1, the thiamine-deficient rats housed without grids survived somewhat longer than those housed with grids, 44 days compared with 33 days (table 3). Rats housed with grids but given 1 μg thiamine daily also survived for 44 days. If we assume that the rats housed without grids obtained a fairly constant amount of thiamine from their feces each day, it would appear that this was about 1 μg thiamine more than that obtained by the thiamine-deficient control group housed on grids.

By the same sort of calculation, the rats in group 3, fed each day the whole of their own feces from the previous day, and surviving for 63 days, appear to have been receiving a little over 2 μg thiamine daily more than the control group, since rats given 2 μg thiamine as a supplement survived 56 days. This amount of over 2 μg thiamine daily was very close to the amount actually observed in feces by chemical estimation (2.41 ± 0.22). The assays were carried out on feces voided on the 1st, 8th, 15th and 21st day of the experiment; since the values of about 2.4 μg were very similar, both for individual rats and for different times, it was

TABLE 3

Survival of thiamine-deficient rats in cages with or without grids, or fed feces or supplements of thiamine[1]

Group	Housing	Daily supplement	Duration of survival
			days
1	Without grids	0	44 ± 5.3[2]
2	With grids	0	33 ± 2.0
3	With grids	own feces	63 ± 9.2
4	With grids	0.5 μg vitamin B_1	40 ± 3.1
5	With grids	1 μg vitamin B_1	44 ± 3.1
6	With grids	2 μg vitamin B_1	56 ± 6.0
7	With grids	3 μg vitamin B_1	> 100

[1] Six rats in each group.
[2] SD of mean.

not considered worthwhile to carry out estimations on all the other fecal samples collected.

DISCUSSION

The delay in death due to thiamine deficiency in animals housed directly on the floor of the cages is most readily explained by the greater ease of obtaining their own thiamine-containing feces. Even though rats on grids can eat their own feces while being voided, it is likely that they get less of them than rats kept without grids. Our earlier experience, too, suggests that coprophagy is more difficult under these conditions. For example, it has been shown that the thiamine-sparing action of ascorbic acid and of starch ("starch refection") can be demonstrated in a higher proportion of rats if they are kept on sawdust than if they are kept on grids (8).[2]

The amount of fecal thiamine as determined by the increased survival-time of deficient rats is about the same as the amount determined by chemical means. Thus, if the thiamine exists in the cecum in a bound form as suggested by Wostmann and Knight (6) and is excreted as such, it is readily available to the rat once the feces have been consumed.

In a similar way to that reported here, in which the proportion of available thiamine was measured by comparing the growth of rats fed feces with those of rats fed graded amounts of thiamine, Barnes et al. (9) calculated that about 35% of the fecal thiamine was available to rats fed purified diets, with or without penicillin. In our experiments, the proportion of available thiamine was close to 100%. Although we do not know the reason for this difference, it is possible that it derives from differences in the total amount of thiamine excreted in the feces. In the experiments of Barnes and his colleagues, the rats fed the thiamine-free diet without penicillin appeared to be excreting about 12 μg thiamine daily. In our experiments, however, the rats excreted only about 2.5 μg daily, an amount more closely similar to that reported by other authors (10).

The increased survival time of deficient rats kept without grids is about that obtained by the addition of 1 μg thiamine daily to rats kept on grids. Since the daily fecal output of thiamine is about 2.4 μg, one might say that the rats kept without grids ate rather less than one-half of their feces each day. Observation of the rats' behavior, however, shows clearly that they are rather indifferent to their feces until they have begun to become deficient in thiamine; thereafter, they consume their feces with avidity (11). It is, then, more likely that rats kept without grids and fed the deficient diet got less than 1 μg thiamine daily for the first part of the experiment, and more than this in the latter part.

We believe that the experiments reported here show conclusively that small amounts of microbially synthesized thiamine are excreted in the feces of the rat, that in the conditions of our experiments this amounts to 2.4 μg daily both at the beginning of a thiamine-depletion period and during at least the next 3 weeks, and that this thiamine is almost entirely available to the rat after coprophagy, the amount depending on the amount of feces consumed.

It might be suggested that the longer survival of the rats kept on sawdust was due to the consumption of some of the sawdust. There is no thiamine in sawdust, but its consumption might have led to increased thiamine synthesis in the cecum. We have, however, shown that the inclusion of cellulose in the diet does not increase microbial synthesis of thiamine (8). Moreover, dietary cellulose causes considerable increase in the size of the cecum, whereas there was no increase in the rats kept on sawdust; it appears then that they do not eat any significant amount of sawdust.

It remains only to comment on the negative observations of Wostmann and Knight (6). In their experiments with rats, they fed, or injected into the cecum, $S^{35}O_4$. The animals were receiving one of two normal complete diets containing either 8.8 mg or 4.4 mg thiamine/kg. These workers showed that S^{35} was incorporated into thiamine mostly in the cecum. However, neither in rats kept in individual anti-coprophagy screen cages, nor in animals allowed access for 48 hours to their feces in conventional cages, was

[2] Morgan, T. B. 1960 Sorbitol refection in rats and mice. Thesis, University of London.

any labeled thiamine noted in the liver or heart.

The question then arises as to whether the conditions of the experiments would have allowed detectable amounts of radioactive thiamine to be incorporated into the tissues. The paper of Wostmann and Knight does not give sufficient information from which to calculate either the amount of thiamine which was consumed with the feces of the rats allowed to practice coprophagy, nor the proportion of this that was labeled. If we assume that the rats of Wostmann and Knight consumed something like 12 or 15 g of diet a day, they would have been getting between 50 μg and 150 μg unlabeled thiamine daily from this source. This is much higher than the daily requirements of about 10 μg daily. In these circumstances, it is unlikely that the rats without anti-coprophagy devices would have eaten a great deal of their feces. Moreover, Barnes et al. (12) have shown that rats eat very little of their feces for the first 2 or 3 days after the removal of anti-coprophagy cups. But even if the rats of Wostmann and Knight had eaten the complete output of feces, the author's assays of cecal thiamine as well as our direct assays of fecal thiamine suggest that the rats would have obtained less than 5 μg thiamine in the 2 days of the experiment. This would have been diluted with the very much larger amounts of thiamine in the diets.

We must ask whether, in these circumstances, tissues already replete with thiamine would show a sufficient turnover of the vitamin so that enough of the fecal thiamine, considerably diluted by dietary thiamine, would have been taken up to give a measurable uptake of the labeled thiamine that was present. Without knowing the answer to this question it remains possible that Wostmann and Knight's results were due to the conditions of their experiment. It is possible, that is, that a longer period of access by the rats to their feces, or easier access, or the feeding of a diet with less thiamine, might have led to the incorporation into the tissues of a measurable amount of the radioactive thiamine synthesized in the cecum.

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Effect of Iron Supplementation of Sow Rations on the Prevention of Baby Pig Anemia

C. H. CHANEY AND C. E. BARNHART
Department of Animal Science, University of Kentucky, Lexington, Kentucky

ABSTRACT In 3 experiments, 68 litters of pigs were used to test the effectiveness of ferrous fumarate in preventing iron deficiency anemia of baby pigs. This was approached by feeding their dams an iron-enriched ration of graded levels up to 1382 mg/kg. These iron-supplemented rations were fed for the entire gestation period, varying lengths of time during gestation and from farrowing. All of these treatments were continued until the pigs reached 22 days of age. No adverse effects were encountered by feeding these levels of iron for any of these periods. The results of the tissue analyses indicate that there was no increase in placental transfer of iron. However, it was found that the iron content of the sow's milk could be maintained at such levels to prevent anemia by feeding the sow high levels of iron at any of these periods.

Anemia in baby pigs occurs, almost without exception, if no supplemental iron is provided during the first few days after farrowing. A number of methods of administering iron have been used with varying degrees of success. McGowan and Crichton (1) demonstrated that iron oxide would increase hemoglobin levels of pigs by being fed to the sow during the lactation period. Later Buchanan (2) showed that supplementing the sow's ration with ferrous sulfate decreased the mortality rate of their litters. However, hemoglobin levels were below normal in these experiments for pigs at this age according to Dunne (3). Since some of the early work with pig anemia, a number of organic iron compounds have been synthesized that can possibly lend themselves to the use of preventing this nutritional deficiency. The purpose of the present experiment was to test the effectiveness of an organic iron compound in preventing anemia of baby pigs by supplementing the sow's ration at relatively high levels in an attempt to increase the placental iron transfer or to increase the iron content of the sow's milk, or both.

EXPERIMENTAL

Experiment 1. Twenty-eight purebred Hampshire gilts were allotted at the time of breeding to 4 ration treatments of supplemental iron. The level of iron added to ration treatments 1 through 4 was as follows: zero (basal ration), 346, 691, and 1382 mg of iron/kg of ration. Ferrous fumarate was the source of iron used in this experiment. Alfalfa pasture was provided each lot throughout the gestation period. Approximately 2.72 kg of the gestation ration were fed daily at 2 feedings. The sows were self-fed during the lactation period and were continued on the same level of iron. Creep feed was not available to any of the pigs in this experiment. The sows and their litters were confined to a concrete-floor house from the time of farrowing until the experiment was terminated. The gestation and lactation rations are presented in table 1.

Blood samples were collected from each pig at 1, 8, 15, and 22 days of age by an anterior vena cava puncture. Hemoglobin determinations were made by using a photo eletcric hemoglobin and glucose meter. Hematocrit determinations were made by drawing whole blood into heparinized capillary tubes, after which they were sealed and then centrifuged for 5 minutes at 11,400 rpm. Within a few hours after farrowing, one pig from each litter was killed to collect liver and spleen samples for laboratory analyses to determine whether any increase in fetal iron stores had occurred as a result of supplementing iron in the gestation ration. Milk samples were collected on the first day after

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TABLE 1

Composition of basal gestation and lactation rations with calculated chemical analyses

	Rations	
	Gestation[1]	Lactation[2]
	g/kg	*g/kg*
Ground yellow corn	49.0	58.8
Ground oats	25.0	20.0
50% Meat and bone scraps	5.0	5.0
50% Solvent soybean oil meal	5.0	8.0
17% Dehydrated alfalfa meal	15.0	5.0
Ground limestone	0.0	1.0
Steamed bone meal	1.0	1.0
Iodized salt	0.5	0.5
Trace minerals mix[3]	0.0	0.1
Vitamin supplement[4]	0.0	0.1
Vitamin A and D supplement[5]	0.55	0.55
Antibiotic[6]	0.0	0.5
Total	101.05	100.55

[1] The gestation ration contained the following by calculated analysis: protein, 14.72; fat, 3.78; fiber, 8.39; calcium, 0.79; and phosphorus, 0.57.

[2] The lactation ration contained the following by calculated analysis: protein, 14.91; fat, 3.75; fiber, 5.75; calcium, 1.12; and phosphorus, 0.58.

[3] The composition of one kilogram of the trace mineral mixture was: (in grams) manganese, 121.9 (minimum); iron, 95.9 (minimum); calcium, 94.8 (maximum); copper, 7.3 (minimum); zinc, 6.6 (minimum); iodine, 3.7 (minimum); and cobalt, 2.6 (minimum).

[4] One kilogram of the vitamin supplement supplied not less than 4,400 mg riboflavin, 8,800 mg pantothenic acid, 19,800 mg niacin, 22,000 mg choline chloride and not less than 132 mg folic acid.

[5] One kilogram of the vitamin A and D supplement contained not less than 9,988,000 USP units of vitamin A and not less than 1,248,500 units of vitamin D_2.

[6] The antibiotic furnished 22 g chlortetracycline·HCl/kg.

parturition and at 5-day intervals for the next 15 days. Milk let-down was affected by injecting 5 to 6 cm^3 of oxytoxin into the ham. Total iron was determined in both milk and tissue samples by using an AOAC (4) colorimetric procedure with slight modifications.

Experiment 2. Since anemia of baby pigs was prevented by supplementing iron to their dam's ration, this experiment was conducted to determine the time that iron should be added to the sow's ration. Forty-three purebred Hampshire sows were allotted to 5 ration treatments. The time intervals used in this experiment were as follows: 1) basal ration; 2) supplemental iron at farrowing; 3) through 5) supplemental iron at 2, 4, and 6 weeks before farrowing. Ferrous fumarate was the source of iron for all treatments and was fed at 1382-mg level/kg of ration for the gestation and lactation periods. Blood samples were collected at 15 and 22 days of age. The procedure for collecting blood and methods for determining hemoglobin and hematocrit values were the same as for the previous experiment. This experiment was terminated when the pigs reached 22 days of age.

Experiment 3. This experiment was conducted to test more thoroughly the manner in which the pigs were obtaining their iron for hemoglobin synthesis. Eight bred sows were randomly allotted to 2 ration treatments, 1) the basal ration, and 2) 1382 mg of iron/kg of ration in the form of ferrous fumarate. Two sows from each ration treatment had their litters removed from them at the time of farrowing. The other 2 sows suckled their litters in the normal fashion. The litters that were removed were kept in separate pens away from their dams. These pigs were provided water, but no creep feed. Every 2 hours the pigs were placed with their dams and were allowed to nurse. Before the pigs were placed with their dams, the sow was scrubbed down and the pen was thoroughly cleaned of all feed and feces. Litters were nursed one at a time and an attendant watched the pigs to keep them from any contact except to nurse. As soon as the pigs finished nursing, they were returned to their pens. This procedure was carried out for 22 days. The ration fed and type of housing were the same as in experiment 1. Blood samples were collected at 15 and 22 days of age of the pigs and were analyzed by the same procedures that were described in experiment 1.

RESULTS AND DISCUSSION

Although high levels of iron were fed throughout this experimental period, there were no signs of unpalatability, gastric irritations or symptoms of toxicity. The gilts farrowed without unusual difficulties and their litters were vigorous and of normal size. Apparently the milking ability of the gilts was normal because good growth was obtained by all litters in these 4 treatments. That the levels of iron used did not interfere with the metabolism of other nutrients during the gestation and lactation periods is indicated by the resulting normal litters.

TABLE 2

Results of bleeding of pigs in experiment 1

Treatment	No. sows	No. pigs	Age of pigs, days									
			1		8		15		22			
			Hemoglobin	Hematocrit	Hemoglobin	Hematocrit	Hemoglobin	Hematocrit	Hemoglobin	Hematocrit		
			g/100 ml	%	*g/100 ml*	%	*g/100 ml*	%	*g/100 ml*	%		
Basal (no iron)	3	21	6.7 ± 1.2[1]	26 ± 3.0	5.5 ± 1.3	26 ± 4.7	5.9 ± 0.8	30 ± 2.9				
Basal + 346 mg iron (ferrous fumarate)	4	31	8.0 ± 1.2	32 ± 5.0	7.7 ± 1.1	30 ± 4.2	7.3 ± 1.2	31 ± 4.7				
Basal + 691 mg iron (ferrous fumarate)	5	44	7.1 ± 1.3	31 ± 5.6	7.5 ± 1.3	31 ± 3.9	7.9 ± 1.5	33 ± 4.2	8.4 ± 1.9	35 ± 3.4		
Basal + 1382 mg iron	5	37	7.2 ± 1.2	29 ± 4.5	8.0 ± 0.8	35 ± 3.4	8.7 ± 1.2	36 ± 3.6	9.0 ± 1.4	37 ± 3.1		

[1] SD.

A summary of the data on the pigs is presented in table 2. The blood values were quite variable at the first bleeding. The hemoglobin determinations were from 6.7 to 8.0 g for treatments 1 and 2, respectively. The hematocrit readings also showed some variation at this time. At the second bleeding, treatments 1 and 2 had a lowered hemoglobin reading but the levels in treatments 3 and 4 had shown an increase of 0.4 and 0.8 g, respectively. The hemoglobin levels at 8 days of age were from 8.0 g for treatment 4, and 5.5 g for the control.

Approximately the same trend in blood values continued for the third bleeding with larger differences between treatments. The hemoglobin level in treatment 4 had increased to a level of 8.7 g which is considered normal according to Dunne (3) for pigs at this age. The blood determinations for the control treatment were 5.9 g hemoglobin and a 30% packed cell volume. The gilts receiving treatments 1 and 2 were not continued any further in this experiment because their pigs were showing an increased respiration rate and pale mucous membranes as well as the low blood values.

A further increase in blood values was noted by the pigs in both treatments 3 and 4 at the fourth bleeding. The hemoglobin levels were 8.4 and 9.0 g for treatments 3 and 4, respectively. The hematocrit levels also showed a further increase from the previous bleeding.

Results of the tissue analyses are presented in table 3. The iron content of the livers showed rather small differences between treatments. These values were from 84.4 to 96.8 mg/100 g of dry tissue for treatments 4 and 2, respectively. A similar variation was observed in the spleen analyses; however, these values were at a

TABLE 3

Iron content of liver and spleen tissue

Treatment	Liver	Spleen
	mg/100 g dry tissue	*mg/100 g dry tissue*
1	86.7 ± 22.3[1]	66.1 ± 9.1
2	96.8 ± 22.9	51.6 ± 6.3
3	95.9 ± 37.9	59.1 ± 18.8
4	84.4 ± 34.4	52.0 ± 5.0

[1] SD.

somewhat lower iron concentration. In both liver and spleen analyses, there was apparently very little relationship between these determinations and the blood values of the pigs in their respective treatments.

The milk analyses are presented in table 4. The results of the milk analyses showed that the quantity of iron supplied by the milk is somewhat lower than the amount needed for hemoglobin synthesis according to (Braude et al. (5), Venn et al. (6), Kotarbińska (7). However, the requirement for iron to incorporate into hemoglobin by these authors differs greatly. Braude et al. (5) states that the pig needs to retain 21 mg of iron/kg of weight gained. Furthermore, Venn et al. (6) calculated that the pig needs to retain 7 mg of iron/day to maintain the same proportion of iron in its body as that present at the time of farrowing. Kotarbińska (7) calculated the requirement to be 10 to 11 mg of iron/day.

At the first milking, the milk analyses showed very little difference between treatments, 222 to 267 μg of iron/100 ml of milk. A small downward trend was observed at the second milking in all treatments except the lot receiving the highest level of iron. Somewhat more inconsistent values were obtained at the third milking because a very marked decrease in the level of iron had occurred in the controls and the treatment receiving the lowest level of supplemented iron. However, the iron content of the milk in the 2 higher levels of supplemental iron was maintained more nearly at its colostric level. The iron content of the milk shows a very close relationship between the hemoglobin and hematocrit determinations of the pigs and their respective treatments. This illustrates that the level of iron of sow's milk can be maintained at sufficient concentration to prevent anemia in the suckling pig.

The result of experiment 2 is presented in table 5. The sows receiving the added iron in the gestation ration again did not show any signs of gastric disturbances or symptoms of toxicity during this period. Furthermore, the pigs farrowed by these sows were very thrifty and the litters were average or above in size and weight.

There was very little variation in blood values of pigs whose dams were receiving the supplemental iron when they reached 15 days of age. The hemoglobin levels were from 6.4 to 8.6 g for treatments 1 and 3, respectively. The hematocrit levels ranged from 25 to 36% for treatments 1 and 3, respectively. The litters in the control lot were showing signs of anemia in addition to the low hemoglobin and hematocrit readings observed.

At the second bleeding, when the pigs reached 22 days of age, all litters in the iron-supplemented groups showed a further increase in hemoglobin and hematocrit levels. The hemoglobin values ranged from 8.5 to 9.0 g which is considered normal according to the description given by Dunne (3). Symptoms of anemia were further exhibited by the pigs in the control lot whose hemoglobin levels had decreased to 5.7 g and the hematocrit to 24%.

The results of experiment 3 are presented in table 6. The pigs whose dams were fed the basal ration had symptoms of anemia that are described by Dunn (3) both in the pigs left with their dams and also in those that were separated from the sow except at the time of nursing.

In the iron-supplemented groups, both the pigs left with the sow and those that were separated from their dam did not show any symptoms of anemia as evi-

TABLE 4

Iron content of sow's milk

Treatment	12–18 Hours post-partum	5 Days post-partum	10 Days post-partum	15 Days post-partum
	μg/100 ml	*μg/100 ml*	*μg/100 ml*	*μg/100 ml*
1	230 ± 20.2[1]	213 ± 23.6	123 ± 13.0	127 ± 19.8
2	267 ± 85.0	173 ± 50.0	162 ± 0.0	131 ± 0.0
3	222 ± 27.0	216 ± 24.0	210 ± 17.0	233 ± 0.0
4	234 ± 46.0	244 ± 74.0	203 ± 65.0	194 ± 26.0

[1] SD.

TABLE 5

Results of bleeding of pigs in experiment 2

Treatment	No. sows	No. pigs	Age of pigs, days			
			15		22	
			Hemoglobin	Hematocrit	Hemoglobin	Hematocrit
			g/100 ml	*%*	*g/100 ml*	*%*
Basal (no iron)	9	71	6.4 ± 1.4[1]	23 ± 4.9	5.7 ± 1.5	24 ± 5.8
Basal + 1382 mg iron (ferrous fumarate) at farrowing	9	77	8.2 ± 1.3	33 ± 4.2	8.5 ± 1.4	35 ± 3.6
Basal + 1382 mg iron (ferrous fumarate) 2 weeks before farrowing	8	68	8.6 ± 1.0	35 ± 3.5	8.9 ± 1.2	36 ± 3.7
Basal + 1382 mg iron (ferrous fumarate) 4 weeks before farrowing	10	72	8.2 ± 1.1	34 ± 4.4	9.0 ± 1.2	35 ± 4.4
Basal + 1382 mg iron (ferrous fumarate) 6 weeks before farrowing	7	57	8.0 ± 1.1	33 ± 3.6	9.0 ± 1.3	38 ± 6.0

[1] SD.

TABLE 6

Results of bleeding of pigs in experiment 3

Treatment	No. sows	No. pigs	Age of pigs, days			
			15		22	
			Hemoglobin	Hematocrit	Hemoglobin	Hematocrit
			g/100 ml	*%*	*g/100 ml*	*%*
Pigs kept separate from sow entire period nursed at 2-hour intervals						
Basal (no iron)	2	21	6.3 ± 0.3[1]	27 ± 3.8	6.2 ± 0.8	26 ± 3.4
Basal + 1382 mg iron (ferrous fumarate)	2	18	8.0 ± 1.1	31 ± 3.8	9.0 ± 1.0	35 ± 3.3
Pigs left with sows entire suckling period						
Basal (no iron)	2	17	6.4 ± 1.0	27 ± 3.7	6.3 ± 0.9	25 ± 5.1
Basal + 1382 mg iron (ferrous fumarate)	2	17	8.8 ± 1.0	37 ± 4.0	9.2 ± 1.1	39 ± 3.4

[1] SD.

denced by the hemoglobin and hematocrit values. The hemoglobin and hematocrit values at the first and second bleeding were: 8.0 and 9.0 g, and 31 and 35%, respectively, for the pigs that were separated from the sow. For the litters raised under normal conditions, hemoglobin and hematocrit values at the first and second bleeding were: 8.8 and 9.2 g, and 37 and 39%, respectively. The observation of similar blood values in both of these iron-supplemented groups indicates that adequate iron was supplied by the milk to meet the requirement for hemoglobin synthesis of these pigs.

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Studies on Chloride Deficiency in Chicks

R. M. LEACH, JR. AND M. C. NESHEIM

U. S. Plant, Soil and Nutrition Laboratory, ARS, USDA, and Department of Poultry Husbandry, Cornell University, Ithaca, New York

ABSTRACT Chloride deficiency was produced by feeding young chicks a purified diet which contained 190 mg Cl/kg diet. Chicks fed this diet exhibited extremely poor growth rate, high mortality, hemoconcentration, dehydration and a reduced blood chloride. In addition, deficient chicks showed nervous symptoms which appeared to be characteristic for chloride deficiency. Supplementation of the basal diet with 1200 mg chloride resulted in optimal growth rate and prevented the occurrence of deficiency symptoms. Although excess sodium and potassium did not affect the growth rate of deficient chicks, these cations increased the incidence of mortality and nervous symptoms. Bromide (676 to 1352 mg/kg) added to the basal diet partially counteracted most of the symptoms of chloride deficiency except the nervous symptoms. Higher levels of bromide were of no additional value. Iodide (537 to 1074 mg/kg) depressed growth rate but did not interfere with the typical chloride growth response curve. However, the data on incidence of mortality and nervous symptoms suggested an interaction between iodide and chloride. Fluoride (268 mg/kg) had no effect on the course of chloride deficiency.

Although the physiological significance of the chloride ion has long been recognized, early attempts (1, 2) to produce a nutritional deficiency of this element were unsuccessful. Later experiments by Orent-Keiles and associates (3) provided nutritional evidence for the essentiality of the chloride ion. Rats fed a diet low in chloride had a retarded growth rate and symptomology different from that observed with a sodium-deficient diet. Greenberg and Cuthbertson (4) also noted reduced growth rate to be the primary symptom of chloride deficiency in the rat. Although deficient rats showed no clinical symptoms, it was possible to induce tetany in a few animals by means of auditory stimuli or mild galvanic shock. Deficient animals had reduced levels of blood chloride accompanied by increased amounts of carbon dioxide. Dietary carbonate or bicarbonate did not affect these blood changes. The almost complete disappearance of chloride from the urine of deficient rats indicated that these animals had a marked ability to conserve body chloride.

Scott et al. (5) have demonstrated that pheasant and quail require between 480 and 1100 mg chloride/kg diet for optimal growth rate. However, little is known about the chloride nutrition of the young chick. Attempts by Burns et al. (6) to produce a chloride deficiency in the young chick were unsuccessful. The authors concluded that the chloride requirement of the chick does not exceed 600 mg/kg of diet.

The studies presented in this report were conducted to 1) obtain information on the chloride requirement of the young chick; 2) study the symptoms of chloride deficiency; and 3) investigate the possible interaction between chloride, other halogens, and sodium and potassium.

EXPERIMENTAL

The composition of the basal diet used in this investigation is presented in table 1. This diet contained 195 mg chloride/kg diet by analysis (7). Washing the isolated soybean protein with demineralized water at its isoelectric point did not significantly reduce the chloride in the diet. Therefore, unwashed isolated soybean protein was used throughout this investigation. Chloride was added to the diet by the substitution of sodium chloride for sodium phosphate. Adjustments were made in other dietary mineral components so that the calcium, phosphorus, sodium and potassium levels were constant in all diets.

Triplicate lots of eight 2-day-old White Plymouth Rock male chicks were used in these experiments. The chicks were placed

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in electrically heated battery brooders with raised wire floors, and were supplied the experimental diets and demineralized water ad libitum. Data for body weight, feed consumption and symptoms were recorded at weekly intervals. The experimental period varied from 2 to 4 weeks as indicated for each experiment.

At the end of the experiment, 9 chicks/treatment were chosen at random for blood analysis. Blood was obtained by heart puncture, and heparin was used as an anticoagulant for all studies except where sodium and potassium were determined. Lithium oxalate was used as an anticoagulant, in those instances. The method of Whitehorn, described by Hawk et al. (8), was used for the determination of blood chloride.

Sodium and potassium were determined on blood plasma with a flame spectrophotometer. Blood hematocrit was determined by the micro-method using capillary tubes and an International Hematocrit Centrifuge and Hematocrit reader.[1]

TABLE 1

Composition of basal diet

	g/100 g
Glucose	58.32
Isolated soybean protein	27.00
Corn oil	3.00
Cellulose	3.00
Vitamin mixture[1]	1.38
Mineral mixture[2]	6.30
Glycine	0.30
DL-Methionine	0.70
Protein (N × 6.25), %	24.82
Metabolizable energy (ME), kcal/g	3.35

[1] Supplies the following per 100 g of diet: inositol, 25.0 mg; niacin, 5.0 mg; Ca pantothenate, 2.0 mg; pyridoxine·HCl, 0.45 mg; folic acid, 0.40 mg; menadione sodium bisulfite, 0.125 mg; biotin, 0.02 mg; vitamin B_{12}, 2.0 μg; α-tocopheryl acetate, 6.6 IU; vitamin A, 540 IU; vitamin D_3, 98 ICU; thiamine·HCl, 1.1 mg; riboflavin, 1.1 mg; and choline dihydrogen citrate, 380 mg.

[2] Supplies the following per 100 g of diet: $CaCO_3$, 2.80 g; $CaHPO_4 \cdot 2H_2O$, 0.34 g; KH_2PO_4, 1.39 g; $NaH_2PO_4 \cdot H_2O$, 1.44 g; $MgSO_4$, 0.25 g; $MnSO_4 \cdot H_2O$, 33.3 mg; $FeSO_4 \cdot 7H_2O$, 33.3 mg; KI, 0.26 mg; $CuSO_4 \cdot 5H_2O$, 1.67 mg; $CoSO_4 \cdot 7H_2O$, 0.20 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.83 mg; and $ZnCO_3$, 11.5 mg.

RESULTS

Preliminary studies showed that the basal diet (table 1) was very deficient in chloride for young chicks. The initial experiment in this study was designed to estimate the approximate dietary chloride requirement of the young chick. The results (table 2) indicate that 1500 mg of Cl/kg of diet were sufficient for maximal growth rate.

In addition to the poor growth rate of chicks fed chloride-deficient diets, a number of other consequences of the chloride deficiency were observed. Blood chloride levels were reduced and the chicks appeared to be dehydrated. High hematocrit values were also observed in the deficient chicks.

Characteristic nervous symptoms were observed in chicks fed the basal diet. When chicks were stimulated by a sharp noise or by handling, they pitched forward and extended their legs to the rear, and gave the appearance of being in a state of tetany. After 1 or 2 minutes, spontaneous recovery occurred, and another nervous spasm could not be in-

[1] Trade names and company names are included for the benefit of the reader and do not infer any endorsement or preferential treatment of the product listed by the U.S. Department of Agriculture.

TABLE 2

Effect of adding levels of chloride to a chloride-deficient diet

	Treatment	4-Week wt[1]	Mortality	Total blood chloride	Hematocrit
		g	*%*	*mEq/l*	*%*
1	Basal diet (table 1)	120	54	52	38
2	+ 500 mg Cl/kg	370	8	72	35
3	+ 1000 mg Cl/kg	546	8	77	33
4	+ 1500 mg Cl/kg	578	8	87	33
5	+ 2000 mg Cl/kg	578	0	91	31
6	+ 3000 mg Cl/kg	598	4	91	31

[1] Following analysis of variance, the following comparisons were made: Body weight: Treatment 1 *vs.* 2, 3, 4, 5, 6; treatment 2 *vs.* 3, 4, 5, 6; treatment 3 *vs.* 4, 5, 6; treatment 4 vs. 5, 6; treatment 5 vs. 6. Blood chloride: Treatment 1 *vs.* 2, 3, 4, 5, 6; treatment 2 *vs.* 3, 4, 5, 6; treatment 3 vs. 4, 5, 6; treatment 4 vs. 5, 6; treatment 5 vs. 6. Italicized comparisons were significant at $P < 0.05$.

duced for several minutes. The appearance of a chick showing the nervous symptoms is shown in figure 1. The nervous symptoms could be prevented by the addition of 500 mg chloride ion/kg to the basal diet, although this level was still below that needed for maximal growth rate.

The second experiment was conducted to determine the effects of relatively high or low dietary sodium and potassium levels on chloride deficiency. Previous studies had shown that tolerance to high levels of chloride was influenced by the sodium and potassium content of the diet (9). In the experiment shown in table 3, the sodium and potassium content of diets containing 500 mg Cl/kg was altered and the severity of the chloride deficiency was observed. The results show that altering the sodium and potassium levels of the diet in the presence of low dietary chloride had no effect on growth rate. However, the blood chloride level of chicks receiving the lowest dietary level of sodium and potassium was significantly higher than in chicks receiving diets with higher sodium and potassium levels. The chicks receiving the highest dietary levels of sodium and potassium showed high mortality and the nervous symptoms of chloride deficiency. These symptoms were not observed in the chicks receiving the other treatments at the same dietary chloride level. Previous studies (9) showed

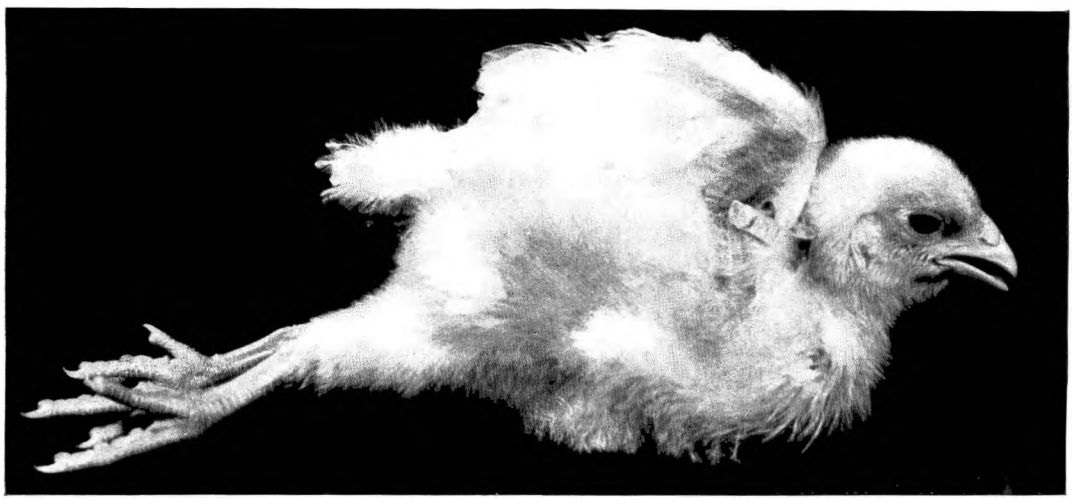

Fig. 1 Appearance of a chick showing the typical nervous symptoms of chloride deficiency.

TABLE 3

Effect of changing dietary sodium and potassium levels on chloride deficiency

Treatment	Avg wt, 26 days	Mortality	Nervous symptoms	Blood chloride[1]	Hematocrit
	g	*%*		*mEq/l*	*%*
Basal diet (table 1) (0.24% Na, 0.04% K)	141	42	+	47(a)	40
+500 mg Cl/kg	318	8	0	58(b)	33
+3000 mg Cl/kg	520	4	0	79(d)	26
+500 mg Cl/kg (0.14% Na, 0.24% K)	308	4	0	64(c)	31
+500 mg Cl/kg (0.64% Na, 1.08% K)	300	33	+	56(b)	33

[1] Analysis of variance of blood chloride data was carried out and treatment rankings were determined by Duncan's multiple range test (10). Values not followed by same letter are significantly different ($P < 0.05$).

that the high sodium and potassium levels used are not harmful when adequate chloride is present in the diet.

In addition to the effects of sodium and potassium, it appeared desirable to investigate the effects of other halides on chloride deficiency. The results of this experiment are presented in table 4. In this experiment, the basal diet was supplemented with sodium fluoride, sodium bromide and sodium iodide in amounts that were equimolar to 500 mg chloride/kg. The same levels of these halides were also fed in the presence of adequate chloride (3000 mg/kg). The results show that fluoride did not influence chick growth at either level of chloride. With the chloride-deficient diet, added bromide caused an increase in chick growth rate similar to that achieved by the corresponding level of chloride but did not prevent nervous symptoms. Bromide had no effect upon the growth rate of chicks receiving adequate amounts of chloride. The level of iodide fed severely depressed growth rate and increased the mortality of chicks receiving the basal diet. Chicks receiving iodide in the diet containing 3000 mg chloride/kg grew slowly and showed the typical nervous symptoms of chloride deficiency. These results suggested that iodide may have affected chloride metabolism.

In view of the positive results obtained with bromide supplementation of the chloride-deficient diet, another experiment was conducted to study the relationship between chloride and bromide in more detail. The design and results of this experiment are presented in table 5. Inspection of the data shows that supplementing the basal diet with increasing amounts of chloride resulted in a growth curve which reached a plateau at 1200 mg. Normal values for blood hematocrit and blood chloride were also obtained with this level of chloride. These results suggest that the total chloride requirement of the young chick is approximately 1400 mg/kg diet.

The results obtained with levels of bromide equimolar to the chloride levels indicate that this halide can only partially correct the symptoms of chloride deficiency. Although the 300 mg bromide ion/kg diet resulted in a growth response equal to that obtained with chloride, further increases in the bromide content of the diet were of little additional value. Furthermore, the 3 highest levels of bromide produced signs of toxicity. As in the previous experiment, bromide did not prevent the occurrence of the nervous symptoms associated with chloride deficiency. The combination of bromide and chloride gave results similar to those obtained with the individual halides. For example, the combination of levels equivalent to 300 mg of each halide was better than the 300 mg chloride but not equal to the 600 mg level of chloride. The chicks fed the combination of chloride and bromide equivalent to 3000 mg chloride/kg suffered from the toxic effects of high levels of bromide.

Total body water was determined by drying 9 carcasses each from several treatments of the above experiment. The treatments sampled and the percentages of body water obtained were as follows: 300 mg Cl, 63.2; 600 mg Cl, 63.9; 1500 mg Cl, 66.1; Br equivalent to 300 mg Cl, 62.6; and Br plus Cl equivalent to 600

TABLE 4

Effect of other halides on chloride deficiency

Treatment	Avg wt, 2 weeks	Mortality	Nervous symptoms
	g	%	
Basal diet (table 1)	113	21	+
+ 500 ppm Cl	169	8	0
+ 268 ppm F	113	29	+
+ 1127 ppm Br	164	8	+
+ 1789 ppm I	68	75	+
+ 3000 ppm Cl	224	4	0
+ 3000 ppm Cl + 268 ppm F	225	4	0
+ 3000 ppm Cl + 1127 ppm Br	232	0	0
+ 3000 ppm Cl + 1789 ppm I	126	8	+

TABLE 5

Effect of bromide on the chloride requirement of chicks

Level of Cl added to basal diet	Chloride				Bromide[1]				Chloride + bromide			
	Avg wt, 4 weeks	Mortality	Hematocrit[2]	Blood halide[2]	Avg wt, 4 weeks	Mortality	Hematocrit	Blood halide	Avg wt, 4 weeks	Mortality	Hematocrit	Blood halide
mg/kg	*g*	*%*	*%*	*mEq/l*	*g*	*%*	*%*	*mEq/l*	*g*	*%*	*%*	*mEq/l*
0	84	29	40a	45a	—	—	—	—	—	—	—	—
300	174	12	37ab	60b	177	33	36bc	57b	—	—	—	—
600	333	8	31de	72c	207	12	36bc	71cd	252[3]	21	33bcd	71cd
900	411	4	31de	78e	203	12	36bc	68c	291[4]	4	34bcd	76de
1200	442	12	28e	82e	193	46	—	—	—	—		
1500	438	12	29e	94f	194	42	—	—	—	—		
3000	435	4	28e	90f	142	92	—	—	351[3]	4		

[1] Levels of bromide fed equivalent on a molar basis to chloride levels given.

[2] Analysis of variance was carried out on the hematocrit and blood halide data. Treatment differences evaluated by Duncan's multiple range test (10). Values not followed by same letter are significantly different ($P < 0.05$).

[3] 1:1 molar ratios of Cl:Br equivalent to molar level of chloride shown.

[4] 1:2 molar ratios of Cl:Br equivalent to molar level of chloride shown.

mg Cl, 64.8. Although supplementing the chloride-deficient diet with bromide increased the body water of chicks, this increase in water content was not sufficient to account for the weight increase that resulted from bromide supplementation.

A previous experiment (table 4) suggested that iodide supplementation of the chloride-deficient diet would increase the severity of deficiency symptoms observed. The results of an experiment conducted to study in more detail the possible interaction between chloride and iodide are shown in table 6. A factorial arrangement of treatments was used for the 5 levels of chloride and 3 levels of iodide studied. The growth data furnished little evidence for an interaction between these 2 halides. Although iodide depressed growth at all levels of chloride feeding, iodide did not interfere with the typical response curve observed with increasing amounts of chloride. However, both levels of iodide increased the incidence of nervous symptoms and mortality on the chloride-deficient diets. In the chicks receiving diets containing 3000 and 6000 mg chloride/kg, nervous symptoms were still observed when either level of iodide was fed.

One additional experiment was conducted to determine effects of chloride deficiency on plasma sodium and potassium values. The data from this experiment are shown in table 7. In this experiment the growth rate and blood chloride levels were depressed by chloride deficiency, whereas blood hematocrit values and mortality were increased as in previous experiments. Chicks fed diets containing deficient levels of chloride had lower blood sodium and potassium values than chicks fed a diet containing adequate chloride. The reduction of plasma sodium and potassium values was roughly the same on a milliequivalent basis as the reduction in total blood chloride.

DISCUSSION

These experiments demonstrate the importance of chloride in the nutrition of the young chick. Chicks developed severe symptoms of chloride deficiency when fed a basal diet containing 190 mg chloride/kg diet. Supplementing this diet with 1200 mg chloride prevented the manifes-

TABLE 6

Effect of added iodide on responses to chloride

Level of Cl added to basal diet	0 ppm I			537 ppm I[1]			1074 ppm I[2]		
	Avg wt, 2 weeks	Mortality	Symptoms	Avg wt, 2 weeks	Mortality	Symptoms	Avg wt, 2 weeks	Mortality	Symptoms
mg/g	*g*	*%*	*%*	*g*	*%*	*%*	*g*	*%*	*%*
0	97	12	8	76	62	100	58	79	100
300	130	12	16	81	62	89	67	54	100
600	164	0	4	95	29	100	76	67	75
3000	224	0	0	177	0	12	131	25	39
6000	224	0	0	171	4	4	126	0	4

[1] 537 ppm I equivalent to 150 ppm Cl.
[2] 1074 ppm I equivalent to 300 ppm Cl.

TABLE 7

Effect of dietary chloride on blood sodium and potassium

Amount of Cl added to basal diet	Avg wt, 4 weeks	Mortality	Hematocrit	Blood chloride	Plasma[1] Na	Plasma[1] K
mg/kg	*g*	*%*	*%*	*mEq/l*	*mEq/l*	*mEq/l*
0	95	33	39	53	118	5.4
300	220	12	36	61	118	4.8
600	344	4	34	77	122	5.2
3000	539	0	30	95	152	6.9

[1] By analysis of variance, the values for 3000 mg/kg chloride are significantly higher ($P < 0.05$) than values for other treatments.

tation of the deficiency symptoms and resulted in optimal growth rate.

Some of the symptoms of chloride deficiency, such as dehydration and hemoconcentration, could be anticipated on the basis of the physiological importance of chloride in the maintenance of water and ionic balances. However, the nervous symptoms accompanying this deficiency are more difficult to explain. It is possible that these nervous symptoms are a form of tetany commonly associated with an alkalosis (10).

The results obtained with the other elements are difficult to interpret. For instance, excess sodium and potassium had no effect on growth rate, but did influence mortality and the occurrence of nervous symptoms in chicks receiving 500 mg chloride/kg diet. Possibly the low levels of sodium and potassium did not produce a growth improvement on the chloride-deficient diet even though blood chloride was raised, because the levels of sodium and potassium used were very close to the minimal dietary requirement for these elements.

Bromide was partially effective in counteracting most of the symptoms of chloride deficiency except the nervous symptoms. Possibly the greatest effect of the bromide was to partially replace chloride in the maintenance of water and ionic balance in body fluids. The nervous symptoms were not affected by bromide, and these may be a reflection of a more specific chloride function. Toxic amounts of iodide produced nervous symptoms similar to those of chloride deficiency even in the presence of high levels of dietary chloride without affecting the typical chloride growth response curve. Although the physiological relationship between chloride, sodium, potassium, bromide and iodide is not worked out in detail, these elements could serve as valuable tools in research on the nervous symptoms of chloride deficiency.

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Cardiovascular Lesions in Experimental Copper Deficiency in Chickens[1,2]

W. W. CARLTON[3] AND WILSON HENDERSON
Department of Veterinary Microbiology, Pathology and Public Health, School of Veterinary Science and Medicine, Purdue University, Lafayette, Indiana

ABSTRACT The cardiovascular lesions in young chickens deprived of copper were studied in male broiler chickens fed a milk-sucrose diet from one day of age. Over 50% of the birds died of arterial rupture after having been fed the test diet for 4 to 6 weeks. Gross lesions included massive abdominal hemorrhage and hemopericardium. Degenerative lesions in the tunica media were observed most consistently in the abdominal aorta, but were also present in sections of the thoracic aorta, brachiocephalic artery, carotid artery, sciatic artery, and small visceral arteries. In addition to aneurysm formation and spontaneous rupture, lesions obseved were disruption, fragmentation, and loss of elastic fibers, intramural hemorrhage, medial fibrosis, and deposition of an amorphous eosinophilic material in the proliferating connective tissue. The intramural hemorrhage appeared to be primary to the development of dissecting aneurysms, but was secondary to the degenerative alterations in the tunica media. The hemorrhage appeared to arise from the unsupported vasa vasorum by diapedesis or rhexis. Serial sections through rupture sites demonstrated that rupture of the medial and adventitial tunics preceded breakdown of the intimal tunic. Supplementation of the basal diet with ascorbic acid did not reduce the incidence of arterial rupture.

Elvehjem and Hart (1) established that copper was essential for hemoglobin synthesis in the chick. Their observations have been confirmed by Hill and Matrone (2) who produced anemia in young chickens fed a copper-deficient diet containing adequate amounts of iron. A broader physiological role for copper has been suggested by the alterations observed in the bones of dogs (3) and swine (4). Bone lesions in both swine and dogs resembled those observed in a deficiency of ascorbic acid. O'Dell et al. (5) reported that chicks fed a diet deficient in copper died from internal hemorrhage with rupture of major blood vessels. More recently, Shields et al. (6) described cardiovascular lesions in copper-deficient swine.

The work presented in this report was undertaken to investigate further pathological changes in the chick associated with copper deficiency. Ascorbic acid was added to the copper-deficient diet because of its known role in the maintenance of connective tissue and because of reported similarities between the bone lesions of copper deficiency and scorbutus.

MATERIALS AND METHODS

Ten experiments were completed with one-day-old cross-bred male broiler chicks obtained from commercial hatcheries. The birds were randomly distributed into lots of 14 to 20 each. They were housed in electrically heated battery brooders coated with a plastic spray paint.[4] Feeding troughs were heavily coated with the same material. Feed and water were supplied ad libitum. Glass-distilled water was provided in flint glass pars equipped with plastic fountain bases.

The basal diet had the following composition: (in per cent) dried whole milk, 57; sucrose, 34.5; soybean oil, 2.0; glycine, 1.5; L-arginine·HCl, 1.0; DL-methionine, 0.5; choline chloride, 0.2; reagent grade dicalcium phosphate, 1.0; and sodium chloride, 0.5.

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[2] Based on a portion of a thesis submitted by the senior author to the Graduate School, Purdue University, in partial fulfillment of the requirements for the Ph.D. degree.

[3] Present address: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge 39, Massachusetts.

[4] Krylon, Krylon, Inc., Norristown, Pa.

The vitamin mixtures contained the following vitamins: (mg/kg of diet) menadione, 22; α-tocopherol, 22; thiamine, 8.8; riboflavin, 8.8; pyridoxine, 8.8; Ca pantothenate, 27.0; niacin, 44.0; inositol, 440.0; folacin, 17.6; cobalamin, 0.044; biotin, 0.44; vitamin A, 4499 USP units; and vitamin D, 374 IC units.

The mineral mixture contained: (mg/kg of diet) manganese, 100; iron, 50; and iodine, 24. The basal diet supplied, on the average, 0.7 ppm of copper determined by atomic absorption spectrophotometry. The basal diet was supplemented to contain all the necessary vitamins, minerals, and amino acids necessary for chick growth. Copper as reagent grade copper sulfate was added at a concentration of 8 mg/kg to the basal diet to provide a control group. Ascorbic acid was added to the copper-deficient diet at the level of 5 g/kg of diet.

Tissues of histopathologic study were collected from birds dying of arterial rupture and from birds killed at the termination of the experimental period of 60 days. Representative sections of heart, thoracic aorta, abdominal aorta, spleen, proventriculus and associated arteries, and the muscle mass of the upper femur with the associated sciatic artery were collected at necropsy. The tissues were fixed in neutral formalin or Bouin's fluid, paraffin-embedded, sectioned at 5 to 7 μ and stained by various techniques. Staining methods used included hematoxylin and eosin, Gomori's iron stain (7), Verhoeff's or Hart's elastic stain (7), Masson's trichrome stain (7) and Movat's pentachrome stain (8).

RESULTS

Many birds died suddenly while consuming the test diet with or without ascorbic acid. The principal gross lesion was massive internal hemorrhage characterized by large clots of blood over the liver and intestine with clots of blood among the loops of the intestines. Often the region of fatal hemorrhage was localized in an area of the aorta at the level of the adrenal glands. Usually there was an adhering clot in this area, but gross rupture of the aorta was not demonstrated. These results differ from the observations of Shields et al. (6) who reported rupture only in the thoracic aorta of copper-deficient swine. Dilatation and loss of normal contour of the heart were noted in many deficient birds.

When aortic lesions could not be demonstrated, the hemorrhage could often be traced to rupture of one of the small arteries of the abdominal viscera. In many cases adhering clots were localized around the hilus of the spleen. In a number of birds large suffusion hemorrhages covered the serosa of the proventriculus and ventriculus. In addition to the rupture sites described above, a third site of fatal hemorrhage was the aorta at the base of the heart. This was accompanied by massive hemopericardium. Large blotchy hemorrhages were observed in some birds over the medial muscles of the thigh. These hemorrhages often extended into the proximal muscle mass of the upper tibia.

The incidence of arterial rupture varied among the experiments. Three experiments representative of the 10 completed are summarized in table 1. The growth obtained with the diets for the treatment groups in experiments 1, 2, and 3 are summarized in table 2. In the trials listed, the incidence of arterial rupture ranged from 45.0 to 61.9% of the birds on the experiment. The addition of ascorbic acid did not reduce total mortality or the number of birds dying of arterial rupture. There was no significant difference in survival time of copper-deficient birds whether or not ascorbic acid was included in the diet.

Changes in arterial microstructure were noted in several locations. The most common site of severe lesions was the abdominal aorta at the level of the adrenal gland. This region of the vessel represents a transitional zone between the highly elastic thoracic aorta and the more muscular abdominal aorta. Microscopic lesions were observed in sections from the thoracic aorta, carotid, and brachiocephalic arteries, the larger visceral arteries, the sciatic artery, and branches of the pulmonary arteries. Mild degenerative lesions were also observed in sections of the arteries of deficient birds dying without gross evidence of vascular rupture and in the arteries of birds fed the deficient diet and killed at the termination of an experiment.

TABLE 1

Incidence of vascular rupture

	Experiment 1			Experiment 2			Experiment 3		
	Diets			Diets			Diets		
	Control	Copper-deficient	Copper-deficient + vitamin C[1]	Control	Copper-deficient	Copper-deficient + vitamin C[1]	Control	Copper-deficient	Copper-deficient + vitamin C[2]
No. of birds	24	20	22	15	21	21	11	18	18
Total mortality	2	16	17	5	14	17	0	14	15
Arterial rupture	0	9	10	0	9	13	—	9	8
% Rupture/ total birds		45.0	45.4	—	42.8	61.9	—	50.0	44.4
% Rupture/ total mortality		56.2	58.8	—	64.3	76.5	—	64.2	53.3
Average age (days) to rupture		46.4 (41–58)[3]	39.5 (25–47)		38.9 (34–41)	35.1 (27–41)		39.0 (31–49)	33.0 (27–46)

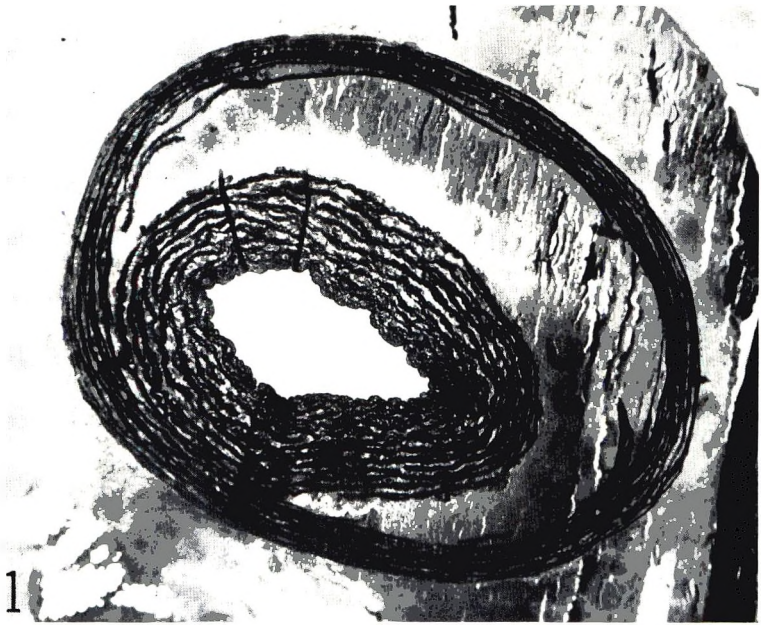
[1] Added at level of 5 g/kg of diet.
[2] Added at level of 2 g/kg of diet.
[3] Range of ages.

TABLE 2

Average weights

Week	Experiment 1			Experiment 2			Experiment 3		
	Diets			Diets			Diets		
	Control	Copper-deficient	Copper-deficient + vitamin C[1]	Control	Copper-deficient	Copper-deficient + vitamin C	Control	Copper-deficient	Copper-deficient + vitamin C[2]
	g	*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*
Starting wt	54.3	60.4	52.5	58.9	58.8	52.6	54.4	53.0	56.2
2	128.1	142.2	94.6	161.8	114.1	107.0	148.7	96.4	106.1
4	188.5	222.9	136.5	267.7	197.1	175.3	290.9	137.8	173.6

[1] Added at level of 5 g/kg of diet.
[2] Added at level of 2 g/kg of diet.



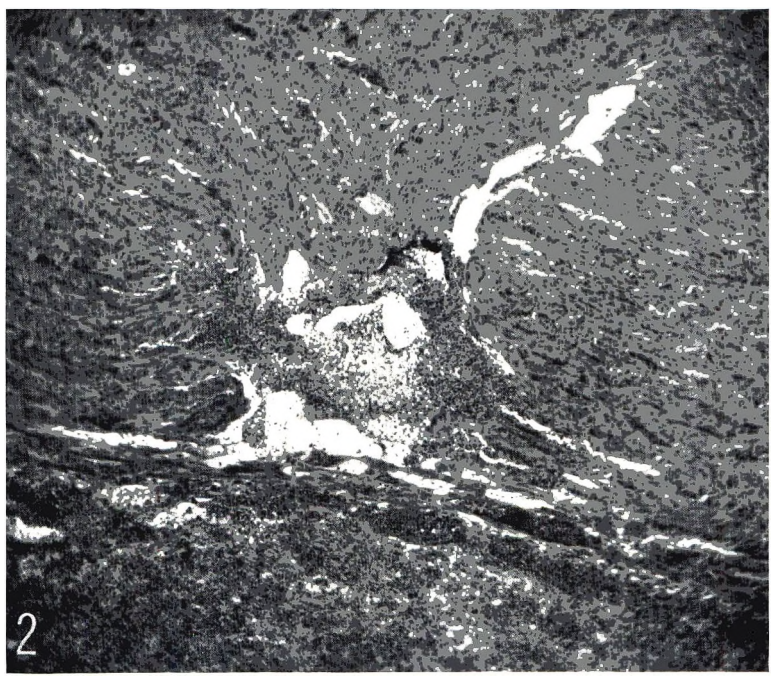


Fig. 1 Thoracic aorta. Dissecting aneuryism. Movat's pentachrome. × 10.

Fig. 2 Thoracic aorta. Intramural hemorrhage with rupture of the lamellae of the tunica media. H & E × 40.

Lesions in the thoracic aorta consisted of hemorrhage in the tunica media, with separation and rarefaction of arterial tissue, especially at the edges of the hemorrhage (fig. 2). Dilatation of the vasa vasorum was marked. Dissecting aneurysms were observed in sections of the thoracic aorta (fig. 1). Within the area of intramural hemorrhage fragmentation of the elastic fibers was evident. Portions of the thoracic aorta contained areas in which the bands of smooth muscle and elastic fibers were replaced by fibrous connective tissue (fig. 5).

In the visceral arteries and the abdominal aorta, the lesions could be divided into 3 types. In the mildest form, the alterations consisted of fragmentation and loss of elastic fibers. An increase in the fibrous connective tissue of the media was evident in sections stained by the pentachrome and trichrome methods (fig. 7). Connective tissue proliferation resulted in an uneven thickening of the vessel wall and an irregular vessel lumen (fig. 5). Moreover, the normal circular lamellae of the media was replaced by whorls of collagenous and smooth muscle fibers that projected into the lumen of the vessel (fig. 4). These projections were covered by endothelium and appeared to have arisen in the media.

A second type of change consisted of extensive fibrosis that resulted in a greatly thickened artery (fig. 7). Within the fibrous connective tissue there were numerous deposits of an amorphous eosinophilic material negative for iron and indistinctly stained by Hart's method for elastic tissue. Hemosiderin granules were evident immediately outside of the fibrotic areas.

The third type of change observed was one in which there was no evidence of repair. Fragmentation and loss of elastic fibers occurred throughout the media and in some areas the media was separated from the external elastic layer by hemorrhage. Portions of the media had apparently been sloughed into the lumen of the vessel (fig. 6). Rupture of these weakened arteries was demonstrated histologically.

Organizing intramural hemorrhage, observed in sections of the sciatic artery, filled the space between the external elastic layer and the media and mechanically displaced the media eccentrically to the periphery of the vessel (fig. 3). Special stains demonstrated fibrosis and hemosiderin in the area of hemorrhage. Extensive fibrosis between the tunica media and the external elastic layer was observed in some sections of the sciatic artery.

DISCUSSION

Although aortic rupture and aneurysm formation occur in mammals and turkeys with moderate frequency, they appear to be rare in chickens. Siller (9) reported 2 cases of aortic rupture in mature hens in which medial changes in the aorta consisted of primary degenerative changes in the smooth muscle with subsequent alterations in the connective tissue and elastic fibers. The lesions in the medial tunic of arteries of copper-deficient birds differ in several respects from those described by Siller. In mildly affected vessels only the elastic fibers of the media were involved in the degenerative process. Smooth muscle fibers appeared normal, although noticeable changes had already appeared in the elastic lamellae. Although Siller (9) considered smooth muscle degeneration to be primary in his cases, it appears certain that degeneration of elastic fibers and elastinolysis were of primary importance in the evolution of the medial lesions in copper-deficient chickens.

Among the diseases affecting the human thoracic arota, the idiopathic cystic medial necrosis of Erdheim is now being recognized more frequently as an important cause of aneurysms and rupture. Larsen (10) listed the medial changes in all his cases as idiopathic medial degeneration. In this disease, as it affects the human aorta, there is gradually developing necrosis and disappearance of elastic and muscle fibers in the media without inflammatory reaction. Development of the lesion results in the formation of cleft-like cysts filled with mucoid material and, later, fibroblastic proliferation is common (11). The medial lesions of arteries of copper-deficient chickens closely resemble those of Erdheim necrosis. However, it was never possible to demonstrate histologically medial cysts in the test birds.

Spontaneous aortic rupture and aortic aneurysms in turkeys have been described

Fig. 3 Splenic artery. Massive hemorrhage between the tunica media and the external elastic layer eccentrically displacing the medial tunic. Movat's pentachrome. × 10.

Fig. 4 Abdominal aorta. Fibrosis in the tunica media producing polypoid projections into the vessel lumen. Movat's pentachrome. × 40.

Fig. 5 Abdominal aorta. Fibrosis in the tunica media accompanied by loss of elastic and muscle fibers with an irregular thickening of the medial tunic. Movat's pentachrome. × 10.

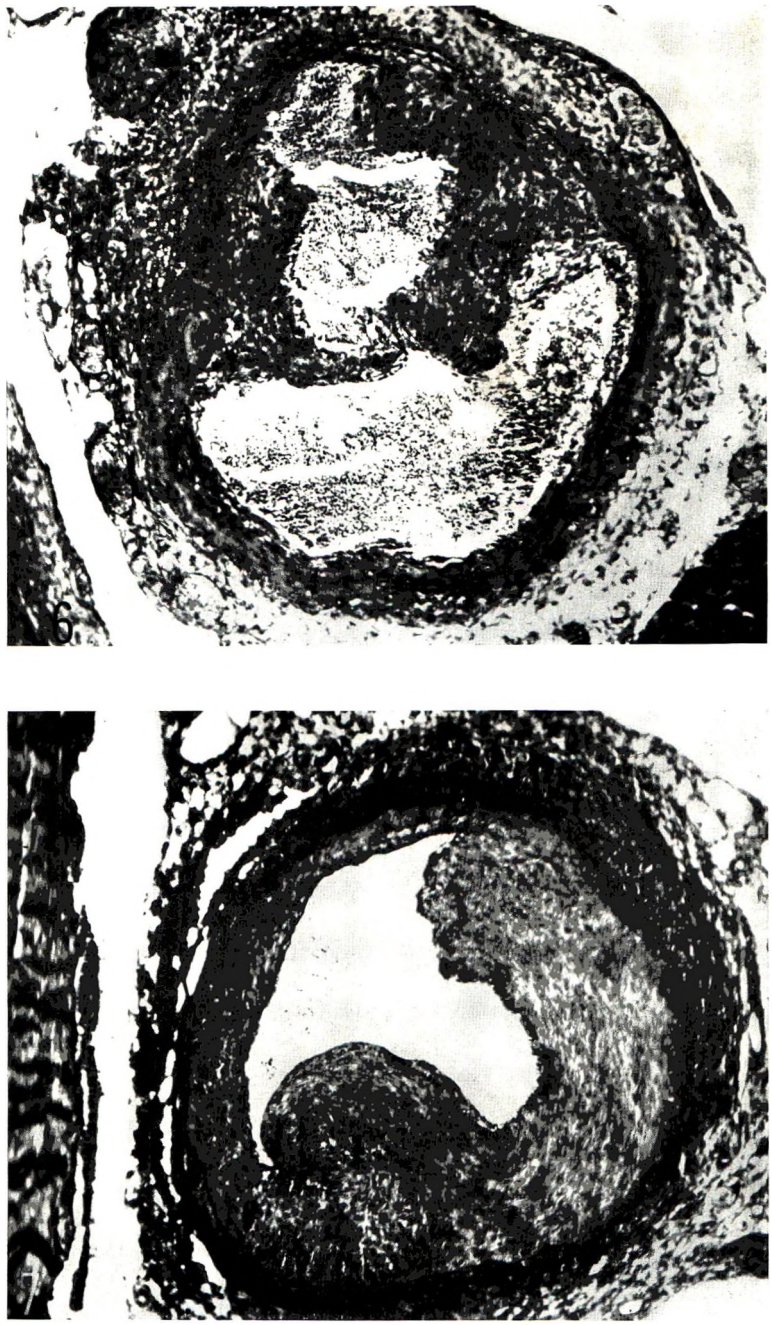


Fig. 6 Visceral artery. Portions of the vessel wall have been lost and adjacent sections of the tunica media are elevated by dissecting hemorrhage. Masson's trichrome. × 10.

Fig. 7 Visceral artery. Thinning of a portion of the wall accompanied by fibrosis and elevation of the remaining tunica media. Movat's pentachrome. × 10.

in several reports (12–14). These reports have indicated that aneurysms and rupture are secondary to the development of atherosclerotic plaques in the abdominal aorta. These lesions have been associated with diets that result in rapid growth. Neither rapid growth nor atherosclerosis appeared to be significant in the development of aneurysms and ruptures in the present study. Atherosclerotic plaques were not observed in deficient or control birds. Thus, although the whole milk diet that was used contained higher amounts of fat than commercial poultry rations, diet composition, other than copper content, appeared to have no etiological significance in the development of arterial lesions in the present study.

A number of other workers have repeated the experiments of Ponseti and Baird (15) who produced dissecting aneurysms and rupture in rats by feeding *Lathyrus odoratus*. The lesions in the tunica media have been described as alterations in the ground substance, elastinolysis, and fibroblastic proliferations at the rupture site. Bachhuber and Lalich (16) observed intramural hemorrhage and stated that connective tissue proliferation was an early manifestation of healing. Walker and Wirtschafter (17) noted that, although dissecting aneurysms were observed only in the aorta, lysis of elastic fibers and hyperplasia of fibroblasts were observed in the pulmonary and coronary arteries. Aortic rupture has been produced in turkeys fed the toxic agent, β-aminopropionitrile, of *Lathyrus odoratus* (18, 19). In copper deficiency, as in lathyrism, the elastic fibers appear to be quantitatively decreased in number and size. Moreover, in both conditions the fibers also show generalized disintegration and resorption with interruption of continuity.

The immediate cause of the ruptures in copper-deficient birds appears to result from weakening of the vessel wall following the severe and widespread degeneration. This is especially prominent in those areas in which fibroblastic proliferation is minimal. Gore (20) has emphasized the importance of medial degeneration and intramural hemorrhage in the pathogenesis of dissecting aneurysm and rupture of the human aorta. It appears that the intramural hemorrhages are primary to the development of dissecting aneurysms in arteries of copper-deficient chickens, but the intramural hemorrhage appeared to be secondary to the degenerative alterations in the media. Apparently the origin of the blood was from the unsupported vasa vasorum with the blood reaching the medial tissues from the vasa by diapedesis or rhexis. Local anoxia in the areas of foci of stagnant blood undoubtedly increases the severity of the medial degeneration. Vasa vasorum trapped within the spreading medial necrosis are especially vulnerable and it appears likely that rhexis of these vessels further increases the hemorrhages and dissection of the lamellae of the artery. These observations are supported by the general absence of intimal lesions as well as the failure to demonstrate atheromatous plaques covering the intima. Furthermore, interrupted serial sections through rupture sites demonstrated clearly that rupture of the medial and adventitial tunics precedes breakdown of the intimal tissue.

Support for the theory that degeneration and rhexis of vasa vasorum is the source of the medial hemorrhage comes from observations made on sections of small visceral arteries. Hemorrhages separating the media from the external elastic layer were observed in many sections. Intimal lesions were absent in these areas and interrupted serial sections failed to demonstrate a pathway through the media to the vessel lumen. In addition, many of the intramural hemorrhages were undergoing organization. This suggested that, although intramural hemorrhage is etiologically significant in the formation of dissecting aneurysms, it does not invariably result in dissection. Organization of medial hemorrhage was observed in sections of arteries from birds that had died of rupture of vessels far removed from the site of organizing hemorrhage.

In the arteries of copper-deficient chickens, degenerative changes in the tunica media with elastinolysis was primary with secondary alterations in the vasa vasorum resulting in medial hemorrhage. Subsequent spread of the hemorrhage within the media resulted in the formation of a dissecting aneurysm. Through the dis-

secting process the intima was reached and, finally, the vessel lumen. Rupture with fatal hemorrhage followed. If the sequence proceeds no further than elastinolysis and medial hemorrhage, the fibrous connective tissue proliferation must be interpreted as a manifestation of the healing response to the medial injury.

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Influence of Low, Intermediate and High Levels of Dietary Protein on Heat Production of Rats[1]

E. W. HARTSOOK AND T. V. HERSHBERGER
Department of Animal Industry and Nutrition,
The Pennsylvania State University,
University Park, Pennsylvania

ABSTRACT An experiment was performed in which 28 young, growing male albino rats were fed diets based upon casein, glucose monohydrate and lard plus corn oil as the protein, carbohydrate and fat sources, respectively. The 4 diets, essentially isocaloric and containing approximately 8, 25, 41 and 57% protein, were fed in equal quantities within each of 7 quadruplicates of animals for a 53-day period with total collection of excreta. Carcasses of experimental animals, diets and excreta were analyzed for nitrogen and energy. Weight gain, feed efficiency and the N, energy and dry matter of the weight gain were at maximal values at the 25% level of dietary protein. The N of feces and urine increased with increasing percentage of dietary protein. Digested energy tended to remain constant, but metabolized energy decreased with increasing percentage of dietary protein above 25% protein. The specific dynamic effect of the food and the total heat production (gross energy of food minus the sum of energy of excreta and energy of the weight gain) of the animals decreased as the dietary protein increased from 8 to 25% and then increased as the dietary protein increased from 25 to 57%. The compatibility of these results with the general principle that the optimal overall utilization of a diet will occur when all required nutrients are at optimal levels was pointed out. The applicability of the general observations to the interpretation of results of similar studies involving human subjects was suggested.

Rubner (1), in his classical studies on the dynamic effects of foods as contributors to the total heat production of animals, measured the specific dynamic effect (SDE) as the increment of heat produced subsequent to feeding a fasted animal a measured quantity of food. He assumed that the dynamic effect of a specific food nutrient (protein, fat, or carbohydrate) fed singly was indentical to its effect as a part of a complete diet. Forbes and associates (2) recognized that use of fasting metabolism as a base line for measuring SDE of food nutrients was to be criticized since heat production determined under these conditions is in error "by the amount by which the metabolizable energy of the test nutrients serves to diminish the dynamic effect of the body nutrients catabolized." Forbes and Swift (3) studied the associative dynamic effects of protein, carbohydrate and fat, using heat production at energy equilibrium as the base value, and concluded that the dynamic effects of diets are not the additive dynamic effects of their specific food nutrients.

In a series of studies on the effect of level of protein (casein, whole egg, or beef muscle) upon the efficiency of utilization of protein and energy, the Pennsylvania workers (4–9) used the "body balance technique" (10) to determine the heat production of weanling and mature albino rats. The technique involved equalized feeding of isocaloric diets to animals for a 10-week period, collection of all excreta and analysis of the excreta and animal carcasses for protein and energy at the completion of the experimental period. Heat production was obtained by subtracting the energy of excreta and of body weight gain from the gross energy of the food. Throughout the range of dietary protein studied (6 to 45%) heat production decreased as dietary protein increased, although at the upper levels of dietary protein, the decrements in heat production appeared to be non-significant. To the writers' knowledge, the "body balance tech-

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nique" has not been used to determine whether heat production continues to decrease at protein levels above 45%. It would not appear likely that the SDE of a diet would continue to decrease if its protein content were increased through its optimal level and to its theoretical maximum. Such a situation would not be consistent with the concept that optimal utilization of food nutrients is exhibited by diets most nearly balanced for meeting the animal's nutrient requirements. According to Mitchell (11), if a protein-deficient diet is optimal in all other nutrients, the diet becomes optimum for meeting both protein and energy needs as the protein content is increased to its optimal level. Addition of protein beyond this point results in poorer overall utilization. Optimal energy utilization implies minimal SDE, since the relationship between the two is reciprocal (4, p. 473).

Evidence that the SDE of diets tends to increase at high levels of dietary protein was supplied by Hamilton (12). This worker, using the Haldane (13) method and rats as the experimental animals measured the SDE of diets containing 4 to 54% protein. In these classical studies, it was found that the SDE of the diet decreased with each increase in protein to 18%, remained constant in diets containing 18 to 30% protein, and then increased again in diets containing more than 42% protein.

More recently, Yoshida et al. (14) reported that, in rats fed ad libitum diets of varying protein-to-calorie ratios, the total gain and gain/calorie increased gradually until the level of 60 mg protein/kcal was reached. Unfortunately, higher levels of protein were not investigated. From studies of somewhat similar type, Seidler et al. (15) concluded that rats fed ad libitum "diets containing 50% of their calories as casein had superior rates of gain and utilization of crude calories as compared with rats fed diets containing either 20 or 80% of their calories as casein."

The work to be described was performed to determine whether the SDE (and the less definitive indicator of SDE, heat production) continues to decrease or tends to increase as the level of dietary protein is increased to levels which clearly exceed levels of optimal[2] protein or energy utilization, or both. The "body balance technique" was chosen for this work because, in our opinion, it remains, though laborious and time-consuming, the best one available capable of rendering a decisive answer to the question.

EXPERIMENTAL

Twenty-eight male albino rats of the Wistar strain ranging in weight from 59 to 91 g were allotted to 7 groups of 4 rats each on the basis of body weight and litter. Each group of rats served as a replicate of 4 experimental treatments and the food intake within each group was limited to that of the animal consuming the least. Rat 1 of each replicate received diet 1, rat 2 received diet 2, and so on. The composition of the experimental diets (containing casein as the protein source, glucose monohydrate[3] as the carbohydrate source and lard and corn oil as the fat sources) appears in table 1. The diets were designed to be isocaloric with the protein content and the protein-to-calorie ratio[4] ranging from 8.0 to 56.7% and from 17.8 to 121.9, respectively. Distilled water was given ad libitum. The rats were weighed at weekly intervals. The animals were housed in metabolism cages in a room maintained at 26 ± 2°C and urine and feces were quantitatively collected for a 53-day period. At regular intervals the individual urine composites (preserved by making them approximately 0.15 N with added sulfuric acid) were filtered, made to volume and aliquots composited for analysis. Individual fecal collections were composited and allowed to air-dry in loosely stoppered sample bottles. At the end of the experimental period the animals were killed with ether, the contents of the gastrointestinal tract removed and discarded and the carcasses were stored frozen for analysis. To estimate the carcass composition of the experimental animals at the beginning of the experiment, 5 male rats of similar age, weight and parentage

[2] The optimal level of a nutrient, as defined by the authors in this paper, is that level that will yield maximal gain of energy under conditions of equalized feeding of isocaloric diets adequate in all other known nutrients.

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

[4] Milligrams of protein (N × 6.25)/kcal.

TABLE 1

Composition of experimental diets

	Diet no.			
	1	2	3	4
	%	%	%	%
Casein[1]	9.54	29.82	50.09	70.36
Glucose monohydrate[2]	64.82	50.09	35.36	20.64
Lard	16.64	11.09	5.55	0
Corn oil[3]	2.00	2.00	2.00	2.00
Cellulose[4]	2.00	2.00	2.00	2.00
B-vitamin mix[5]	0.50	0.50	0.50	0.50
Choline chloride, 25% mix	0.50	0.50	0.50	0.50
Mineral mix[6]	4.00	4.00	4.00	4.00
By analysis:				
Protein,%[7]	8.05	24.97	41.11	56.66
Energy, kcal/g[8]	4.514	4.561	4.605	4.647
Protein, mg/kcal	17.83	54.75	89.27	121.93

[1] Vitamin-free, containing 83.85% protein (N × 6.25).
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] Oleum Percomorphum (Mead Johnson and Company, Evansville, Indiana) and α-tocopheryl acetate added to the corn oil so that the diet contained 2000 IU vitamin A; 283 IU vitamin D, and 10 mg vitamin E/100 g.
[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[5] The vitamin mix had the following composition: thiamine·HCl, 500 mg; riboflavin, 600 mg; pyridoxine·HCl, 300 mg; Ca pantothenate, 3 g; niacin, 6 g; folic acid, 100 mg; menadione, 100 mg; vitamin B_{12}, 5 mg; biotin, 10 mg; and glucose monohydrate (Cerelose), to make 500 g total.
[6] Jones and Foster (16).
[7] Percentage of N × 6.25.
[8] Progressive decreases in analytical energy values may be due to losses of lard during mixing.

were similarly killed and stored for later analysis.

Fecal composites were ground in a micro-Wiley mill and analyzed for total nitrogen by the macro-Kjeldahl (17) method and for energy in an Emerson adiabatic bomb calorimeter (18). Urine composites were analyzed by the methods cited for total N and energy (using a small amount of corn oil as a primer) after removal of moisture by evaporation at 65°C in an oven having forced-air circulation. Animal carcasses were also analyzed by the methods previously cited after being prepared for sampling by the method recently described by Hartsook and Hershberger (19). Moisture content was taken as the weight lost during 30 hours' heating under vacuum at 68°C.

The statistical significance of differences between treatment means was determined by Tukey's test as modified by Hartley (20, p. 251). Analyses were made of variances resulting from treatments and the use of quadruplicates of animals, and the error variances were used to calculate sequential differences between treatment means required for significance. Bartlett's test (20, pp 285–89) was used to determine homogeneity of variances. In cases where such homogeneity was lacking the variance of the treatment with nonhomogeneous variance was excluded from the pooled error variance. Comparison of the treatment mean having nonhomogeneous variance with other treatment means was accomplished by using the pooled variance of the means being tested. In this report the terms "significant" or "significantly" are understood to mean statistical significance.

RESULTS AND DISCUSSION

In table 2 appear the mean values (7 observations) for quantities of food consumed, gains of body weight, gain per gram of food consumed, and the gains in nitrogen (N), energy and dry matter for

TABLE 2

Food eaten, gain/food, and amount and composition of body gains

	Treatment means[1]			
Diet no.	1	2	3	4
Protein in diet, %	8.05	24.97	41.11	56.66
Food consumed, g	508.4 ± 16.4[2]	506.8 ± 16.8	506.7 ± 16.8	506.7 ± 16.8
Live weight, g:				
Initial	74.6 ± 8.5	75.4 ± 6.3	75.4 ± 7.6	75.8 ± 8.7
Final	170.0 ± 9.4	239.1 ± 12.8	231.3 ± 13.9	207.9 ± 7.2
Gain, g[3]	92.4 ± 11.6	159.8 ± 11.4	150.9 ± 12.4	127.3 ± 11.1
Gain/feed, g/g	0.186 ± 0.024	0.316 ± 0.025	0.298 ± 0.023	0.251 ± 0.018
Components of gain:				
Nitrogen, g	2.97 ± 0.35	5.90 ± 0.36	5.60 ± 0.51	4.84 ± 0.51
Energy, kcal	316.3 ± 61.0	451.4 ± 42.2	411.0 ± 47.8	334.2 ± 46.6
Dry matter, g	43.6 ± 6.6	66.4 ± 5.4	61.9 ± 5.2	50.7 ± 4.2

[1] Mean ± SD.
[2] Mean values underlined do not differ significantly from one another (i.e., $P > 0.05$).
[3] Empty weight basis. Contents of gastrointestinal tract removed.

the 53-day experimental period. Statistical evaluations of differences between treatment means for each criterion of response (column 1) have been made and are indicated directly in the table. Means which do not differ significantly ($P > 0.05$) from one another are underlined; all other possible comparisons between means for each criterion (i.e., between 2 non-underlined means or between an underlined and a non-underlined mean) have differences which are significant at $P = 0.05$. Almost all differences between means are significant at $P = 0.01$, but in the interest of simplicity, only the 0.05% level of P is indicated. The same notation is also used in tables 3 and 4. In general, the data presented in table 2 agree well with those reported previously by workers at this Station (4–9) and are not at variance with those of Hamilton (21). The body weight gain increased up to approximately 25% of dietary protein and then decreased as the percentage of dietary protein was further increased. The same general pattern held true for gains of N, energy, and dry matter.

Table 3 presents data on the utilization of dietary N. Here, as in previous work at this Station (4–9), the fecal N and urinary N increased as the food N increased, with the increments being of similar magnitude with each increment in food N. The percentage of digested N (apparent) at the 8% level of protein intake was much lower than those of the remaining levels of protein intake, due no doubt to the much greater proportion of the fecal N accounted for by the metabolic N in the former instance.

The recovery of N of the food as N of excreta and N of body gain averaged 98.3% and is in excellent agreement with a similar value (98.3%) given by Forbes et al. (4). The loss of N represents experimental error, a slight loss by vaporization of ammonia from excreta, but mainly the N contained in shed hair. Such hair was not collected in the previously cited work at this Station and to obtain approximately comparable results, was not collected in the present work. It has been observed that, using the methods employed by Hartsook and Mitchell (22), based on data by Mitchell (23), rats weighing approximately 142 g (the mean weight of animals midway in this experiment) lost approximately 0.44 g of N as shed hair

TABLE 3

Distribution of food nitrogen at differing dietary protein levels

	Treatment means[1]			
Diet no.	1	2	3	4
Protein in diet, %	8.05	24.97	41.11	56.66
Nitrogen:				
Food, g	6.59 ± 0.21	20.25 ± 0.67	33.33 ± 1.16	45.94 ± 1.52
Feces, g[2]	0.55 ± 0.08	0.77 ± 0.11	1.07 ± 0.11	1.38 ± 0.32
Digested, g[3]	6.04 ± 0.20	19.48 ± 0.61	32.26 ± 1.06	44.56 ± 1.36
% of food N	91.7 ± 1.1	96.2 ± 0.5	96.8 ± 0.3[4]	97.0 ± 0.6
Urine, g	2.97 ± 0.21	13.07 ± 0.59	26.09 ± 0.75	39.24 ± 1.03
% of food N	45.0 ± 2.4	64.5 ± 1.5	78.3 ± 0.9	85.4 ± 1.3
Gain of N, g	2.97 ± 0.35	5.90 ± 0.36	5.60 ± 0.51	4.84 ± 0.51
% of food N	45.1 ± 4.6	29.2 ± 1.7	16.8 ± 1.3	10.5 ± 0.8

[1] Mean ± SD.
[2] Includes endogenous nitrogen.
[3] Apparent.
[4] Mean values underlined do not differ significantly from one another (i.e., $P > 0.05$).

TABLE 4

Distribution of food energy at differing dietary protein levels

	Treatment means[1]			
Diet no.	1	2	3	4
Protein in diet, %	8.05	24.97	41.11	56.66
Energy in kcal:				
Food	2294.9 ± 74.0	2311.5 ± 76.6	2333.4 ± 77.5	2354.7 ± 78.2
Feces	224.5 ± 33.3	137.9 ± 13.1	110.9 ± 4.6[2]	110.3 ± 10.8
Digested	2070.4 ± 54.7	2173.6 ± 68.4	2222.5 ± 73.0	2244.4 ± 70.8
Urine	23.4 ± 7.6	92.6 ± 13.7	166.2 ± 6.0	229.3 ± 10.8
Metabolized	2047.0 ± 51.5	2081.0 ± 61.4	2056.3 ± 68.0	2015.1 ± 69.3
Body gain	316.3 ± 61.0	451.4 ± 42.2	411.0 ± 47.8	334.2 ± 46.6
Heat production	1730.7 ± 77.0	1629.6 ± 69.9	1645.3 ± 96.7	1680.9 ± 85.7
Basal metabolism[3]	751.8 ± 19.8	929.1 ± 21.4	901.1 ± 34.7	848.6 ± 20.6
Heat production minus basal metabolic rate	978.9 ± 84.1	700.5 ± 76.9	744.2 ± 97.2	832.3 ± 84.0
Percentage:				
(Heat production − basal metabolic rate)/metabolized energy × 100	47.8 ± 3.6	33.7 ± 3.0	36.2 ± 3.8	41.3 ± 2.9

[1] Mean ± SD.
[2] Mean values underlined do not differ significantly from one another (i.e., $P > 0.05$).
[3] Calculated using the equation $Q = 72W^{0.75}$, where Q = BMR in kcal/day and W = weight in kg, according to Kleiber (28).

during a 53-day period. When this value is included with average values for N excreted and N of body gain, the average percentage recovery of dietary N becomes 100.1.

Miller and Payne (24, 25) have developed equations for predicting net protein utilization (NPU) values of diets when the "chemical score" of the protein and the extent of its contribution to the total energy of the diet [% protein calories (% PC)] are known. The authors (24, 25) assumed that the regression line of NPU on % PC decreases linearly as % PC increases and extrapolates to NPU = 0 at a value of % PC = 54 for most proteins. Njaa (26) showed that the latter assumption is untenable. Later, Morrison et al. (27) showed that if NPU is plotted against log % PC rather than against % PC the plot is linear and that an extrapolated value of NPU= 0 is not attained. Data of the present work are in agreement with those of Morrison et al. (27), because when NPU (% of food N appearing as carcass N) is plotted against log % PC there is no apparent departure from linearity through the range of % PC of 8 to 72.

Table 4 presents data on the utilization of dietary energy. The fecal energy decreased with each increment of dietary protein; however, the decrease for the increment of protein represented by diets 3 and 4 was small numerically and nonsignificant. The same general trend was observed in the previous work at this Station involving casein (4, 5) or whole egg (9) as the source of dietary protein. Urinary energy increased in a regular fashion with each increment of dietary protein — a response found in the previous work at this Station regardless of the source of dietary protein (4–9). Metabolized energy, a resultant of the increasing urinary excretion and decreasing fecal excretion of energy within the imposed condition of equal energy intake, increased, passed through a maximum at approximately 25% dietary protein and then decreased. In the previous work at this Station (4–8) over a dietary protein range of approximately 10 to 45%, the curvilinear relationship of metabolized energy to dietary protein was not evident. In the present work the maximal value of metabolized energy at approximately 25% dietary protein is significantly larger than values at 8 and 41%; furthermore, when the slight variations in food energy are rectified by calculating metabolized energy/food energy, the pattern of (or significance of) the response is not altered. The heat production, numerically equal to metabolized energy minus energy of body gain, represents the sum of the heat produced by voluntary activity, the heat increment (SDE) of the food and heat of the basal metabolism (BMR). The heat production significantly decreased as the dietary protein increased from 8 to 25%, showed no significant change from 25 to 41% dietary protein, and then increased significantly from 41 to 57% dietary protein. Previous reports of work at this Station (4–9) covered a range of dietary protein extending only from approximately 6 to 45% and showed decreasing heat production as the dietary protein percentage was increased, albeit at the upper limits of the range of protein content the successive decrements of heat production became progressively so small as to be of doubtful significance. The present work, however, by extending the range of dietary protein to 57% demonstrates clearly that as the percentage of dietary protein is increased beyond approximately 25%, the heat production steadily increases. However, the area of decrease to and increase from a minimal heat production as the dietary protein proceeds through the range 8 to 57% is of saucer-like shape rather than that of a youthful valley with precipitous sides.

One component, the BMR, making up the heat production is not of equal magnitude for each experimental treatment. The BMR is dependent upon body size and at the end of the 53-day experimental period the animals on the several treatments differed in weight to significant extents. The same condition existed in the previous work at this Station (4–9), but was not a factor in the work of Hamilton (12). Careful estimates of the BMR of the experimental animals were made by using the mean weekly body weight of each rat in the equation of Kleiber (28) to calculate the weekly BMR, then summing the weekly values for the entire

53-day experimental period to obtain the values given in table 4.[5] The values of heat production minus basal metabolic rate (HP – BMR) then represent the remaining increments of heat production—the heat expended in voluntary activity and the heat of the SDE. This value, in the opinion of the authors, is a better one for evaluating the effect of the level of dietary protein on the heat production of the animal, because the penalty imposed on the diet producing the greater growth (and the resulting larger maintenance requirement for energy) has been deleted. That the advantage of making such a correction was recognized previously at this Station is evidenced by the reporting in 1942 (30) of a method for correcting experimentally determined heat production values of cattle for changes in live weight occurring during the experimental period. The values of HP – BMR illustrate in a striking fashion the decrease of this fraction of the heat production to a minimum at approximately 25% dietary protein and the increase in heat production as the percentage of dietary protein is further increased. To render values of HP – BMR more nearly comparable within this experiment and between this experiment and previous similar ones at this Station, values of (HP – BMR)/ME are given in the table. Similar values were calculated for the earlier experiments done at this Station (4, 5) in which casein served as the source of dietary protein. All of such values have been plotted in figure 1. The solid line in figure 1 is a curve, drawn by visual inspection, connecting the observed mean values of the present work. The dashed line describes, in the authors' opinions, a probable relationship of the variables within the indicated range of the independent variable.

Thus, it appears that the relationship of the amount of heat production associated with the metabolism of the food (SDE) to the protein content of the diet can be adequately described by a smooth curve that is, in general, shaped concave upward. The minimal heat production would be realized at the optimal level of dietary protein (approximately 20 to 30% for the growing rat) and heat production would increase as dietary protein content would be lowered from or increased above the optimal range. There is every reason to believe that the validity of this relationship should generally hold true throughout the animal kingdom. Certainly, the data of Hamilton (12) with the rat support this concept. Considering this relationship and the data of figure 1, some of the general conclusions of Swift et al. (31, 32) should be discussed and evaluated. In the cited work at this Station male university students received conventional-type diets either high or low in protein (34 vs. 122 g protein/day in one study; 38 and 129 g protein/day in another), which contained 3111 to 3700 kcal/day. In general, the high protein diets exhibited considerably higher heat production than the low protein diets. The cited authors indicated that, in their opinions, such results were not in harmony with results of the rat work done previously at the same location [(4–9), and cited earlier in this paper]. The high levels of protein fed in the human work

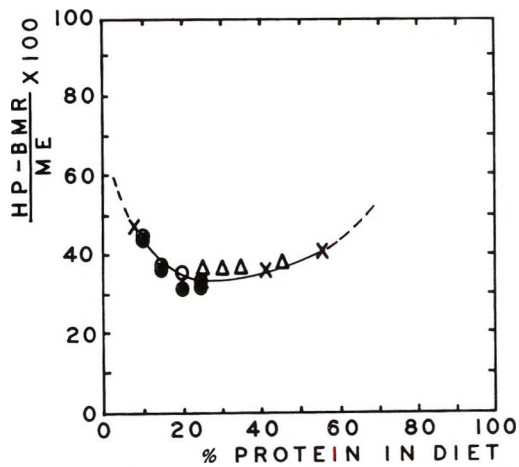

Fig. 1 Effect of level of dietary protein upon the percentage of metabolized energy accounted for by the heat production minus the heat of basal metabolism in young, growing, male albino rats. Data presented as follows: ● reference (4), experiment 1; ○ reference (4), experiment 2; △ reference (5); and × the present work.

[5] Forbes et al. (4) found no difference between treatments (10, 15, 20 and 25% dietary protein) for values of basal metabolic rate of young rats measured between the seventeenth and twenty-third or between the twenty-fourth and thirtieth hours after feeding. No appreciable differences in basal metabolic rate of mature rats receiving high and low protein diets are evident in the data of Black (29).

(122 and 129 g protein/day to subjects weighing around 68 to 70 kg) are in excess [6] of the recommended dietary allowance of the National Research Council (33). Therefore, they should be considered as well advanced along the portion of the curve (fig. 1) representing percentages of dietary protein in excess of the optimal level. It thus appears reasonable to conclude that the results with humans are, in fact, in harmony with the past and present results with rats.

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Effect of Egg White Diets on Calcium Metabolism in the Rat[1]

GEORGE W. ENGSTROM AND HECTOR F. DELUCA
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

ABSTRACT Increasing the egg white protein content from 18 to 36% in low phosphorus diets of rats resulted in an increased renal excretion of calcium, a decreased calcium balance and a decreased ash content of bone. The latter effects appeared only when egg white was used as the protein source. However, this effect was no longer evident with an egg white preparation from which conalbumin, lysozyme, and ovomucoid had been removed. On the other hand the increased renal excretion of calcium could be demonstrated when blood fibrin or the partially purified egg white were used as the protein source especially when vitamin D was fed. The increased renal loss of calcium in this case could be accounted for by an increased intake or absorption or both.

For many years it has been known that the protein component of the diet plays an important role in the absorption and utilization of calcium. In early work it was clearly demonstrated that diets lacking in protein resulted in greatly reduced calcium absorption and bone mineral deposition (1–4). As might be expected, diets deficient in one or more of the essential amino acids also resulted in poor calcium utilization (5–9) and in some cases, marked osteoporosis (10).

Conversely, it was clearly demonstrated that an elevated protein intake increased calcium absorption, utilization, and mineralization of bone (11–14).[2] It has been shown, however, that this is not true of all protein sources (15). Wasserman et al. (16) later reported that lysine and arginine markedly stimulated calcium absorption.

While studying calcium metabolism with low phosphorus diets, it was noted in this laboratory that increasing the egg white content of the diet from 18 to 36% resulted in reduced femur ash and elevated urinary calcium. This paper describes in detail the effect of dietary protein, especially egg white on the metabolism of calcium in rats.

METHODS

Thirty-day-old male rats of the Holtzman strain, weighing 75 to 85 g were used in all experiments. All rats were fed essentially the basal diet of Bellin and Steenbock (17) which contained: (in per cent) D-glucose monohydrate,[3] 67; protein, 18; cottonseed oil,[4] 10; roughage,[5] 3; calcium and phosphorus-free salts, 2 (17) and vitamins. The calcium- and phosphorus-free salts contained: (in per cent) KCl, 57.5; NaCl, 20.9; $MgSO_4$, 17.9; $FeSO_4 \cdot 7H_2O$, 3.22; NaF, 0.113; $CuSO_4$, 0.078; $MnSO_4$, 0.04; $K_2Al_2(SO_4)_2$, 0.018; KI, 0.010; $CoCl_2 \cdot 6H_2O$, 0.004 and $Na_3AsO_4 \cdot 12H_2O$, 0.0017. The water-soluble vitamin mixture supplied 28 mg calcium pantothenate, 20 mg niacin, 5 mg each of thiamine, riboflavin and pyridoxine, 0.2 mg folic acid, 0.1 mg biotin, 0.02 mg of vitamin B_{12} and 500 mg choline/kg of diet. In addition the rats received orally a fat-soluble vitamin mixture dissolved in cottonseed oil[6] which supplied 70 μg of β-carotene, 875 μg of α-tocopherol and 105 μg of menadione to each rat/week. Where designated, 75 IU of crystalline vitamin D_2 (calciferol) in 0.1 ml of cottonseed oil were given orally to each rat every 3 days.

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[2] Greenberg, R. M., M. G. Barry and J. Mayer 1952 Federation Proc., *11*: 444 (abstract).

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

[4] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

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

[6] Wesson Oil.

The desired levels of calcium and phosphorus were achieved by the addition of $CaCO_3$ or an equimolar mixture of KH_2PO_4 and K_2HPO_4, or both, at the expense of D-glucose monohydrate. In some experiments bovine blood fibrin,[7] vitamin-free casein[8] plus 0.2% L-cystine or purified egg white replaced whole egg white[9] as the protein source. The adequate calcium and phosphorus diet was non-rachitogenic (0.45% Ca and 0.3% P), whereas the low phosphorus diet (0.45% Ca and 0.02 % P) and the low calcium, low phosphorus diet (0.02% Ca and 0.02% P) were strongly rachitogenic (18). It is worthy of mention that there were 54 to 56 mg ash/femur in all rats at the beginning of the experiments described.

In each experiment the rats were divided into 4 groups: group 1 received an 18% protein diet; group 2, 18% protein plus vitamin D; group 3, 36% protein; and group 4, 36% protein plus vitamin D. The number of animals used per group in each of the 5 experiments was 4, 8, 8, 7, and 4, respectively. They were housed individually in hanging wire cages during the first 3-day period and given food and distilled water ad libitum. The animals were then placed in wire metabolism cages and were transferred to separate feeding cages for 3 one-hour periods daily during which time they had access to unlimited quantities of food. Feces and urine collections were made during the seventh through twelfth days of the experiment unless otherwise indicated. The urine was collected under a layer of toluene and the feces were retained on wire screens attached to the bottom of the cage. The funnels and screens were washed thoroughly and the washings were added to the urine samples. Urine and feces excreted during the feeding periods were collected on a pad of filter paper placed beneath the feeding cage. The filter paper was leached with dilute HCl and the leachings added to the urine samples. Urine, feces, and diet samples were then ashed with concentrated nitric and perchloric acid for analysis. At the end of each experiment the rats were killed by a sharp blow on the head followed by decapitation.

Femurs were dissected from the carcasses, freed of adhering tissue, extracted with alcohol for 24 hours and then with ether for 24 hours. They were ashed for 12 hours at 640°C in a muffle furnace. The rachitic state of the rats fed each of the diets was estimated by silver nitrate staining of sectioned radii. Calcium was determined by permanganate titration according to the method of Wang (19).

The partially purified egg white was prepared as follows. Egg whites from 360 eggs were separated from the yolks and mixed with an equal volume of distilled water. Ammonium sulfate was added to half saturation to precipitate the globulins and ovomucin, then the pH adjusted to about 4.6 with glacial acetic acid and more ammonium sulfate added to turbidity to precipitate the ovalbumin. All precipitated material was removed by centrifugation and the supernatant fluid containing conalbumin, ovomucoid and lysozyme was discarded. The precipitate was resuspended in a minimum of distilled water and dialyzed against water. After dialysis the material was steamed for 3 hours, dried, and used as the protein source where indicated. The method of statistical analysis was a calculation of the standard error of the mean ($S\bar{x}$) according to the formula

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

RESULTS

Previous reports of the effect of dietary protein on calcium metabolism have dealt primarily with cases in which the protein was either poor in quantity or quality. Furthermore no complete studies of overall calcium metabolism have been reported. By utilizing the low phosphorus diet of Cramer and Steenbock (20), in which net mineral accumulation in bone is kept at a minimum even in the presence of vitamin D, it appears likely that greater insight into the sites of action of dietary protein on calcium metabolism could be obtained.

[7] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[8] General Biochemicals, Incorporated, Chagrin Falls, Ohio.
[9] Stein Hall and Company, Inc., New York City, New York.

In the first series of experiments (table 1) it was clearly demonstrated that increasing egg white protein from 18 to 36% of the low phosphorus diet increased calcium intake and absorption whether vitamin D was present or not. Unexpectedly, however, this was associated with a greater negative calcium balance and a decrease in femur ash content. This discrepancy was fully accounted for by an increased loss of calcium in the urine.

The unusual effect of 36% egg white protein on femur ash content merited further study. To minimize the complication of dietary calcium, the previous experiment was repeated with the low calcium-low phosphorus diet. The results in table 2 show conclusively that the increased renal excretion of calcium with the 36% egg white diet could not be accounted for by increased intestinal absorption. A greater negative calcium balance was observed in the rats fed 36% egg white and again, as before, there was a marked decrease in the mineral content of bone. The results presented in table 3 demonstrate that the effect of egg white on demineralization of bone and calcium balance are unique to this protein source. Clearly, when 36% bovine blood fibrin was substituted for egg white in the low phosphorus diets, no decrease in the ash content of bone occurred. However, the increased dietary fibrin resulted in a greater loss of calcium in the urine.

Increasing the casein content from 18 to 36% in an adequate calcium and phosphorus diet resulted in an increased intake and, consequently, absorption of calcium and an increased accumulation of femur ash (table 4). No significant increase in urinary calcium occurred in this case, presumably because of the presence of sufficient dietary phosphorus for normal mineralization of bone to occur, thus minimizing the loss of calcium in the urine.

TABLE 1

Effect of steamed egg white protein and vitamin D on calcium metabolism of rats fed the low phosphorus rachitogenic diet[1]

Dietary protein	Vitamin D	Calcium intake	Urinary calcium	Fecal calcium	Net absorption	Calcium balance	Femur ash
%		*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg*
18	—	281 ± 10.2[2]	104 ± 3.7	224 ± 12.0	55 ± 4.9	−47 ± 8.7	43.1 ± 2.0
36	—	295 ± 7.8	153 ± 3.2	210 ± 8.4	85 ± 7.1	−68 ± 5.8	37.0 ± 0.6
18	+	215 ± 7.4	101 ± 5.4	89 ± 4.0	124 ± 5.1	+24 ± 9.1	60.0 ± 2.1
36	+	244 ± 8.0	161 ± 10.5	75 ± 3.1	169 ± 10.6	+10 ± 4.0	47.9 ± 1.3

[1] Powdered egg white was obtained from Stein, Hall and Company, Inc., New York, as "Egg Albumin" but includes all of the egg white proteins.
[2] The values are averages of 4 rats/group ± SE of mean.

TABLE 2

Calcium balance of rats fed a low calcium, low phosphorus rachitogenic diet with varying amounts of steamed egg white as the protein source[1]

Dietary protein	Vitamin D	Calcium intake	Urinary calcium	Fecal calcium	Net absorption	Calcium balance	Femur ash
%		*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg*
18	—	10 ± 0.1[2]	66 ± 3.4	12 ± 0.5	− 2 ± 0.5	− 68 ± 3.5	40.9 ± 1.0
36	—	12 ± 0.1	86 ± 2.9	9 ± 0.6	+ 3 ± 0.7	− 83 ± 2.9	33.4 ± 1.2
18	+	9 ± 0.1	65 ± 4.3	1 ± 0.3	+ 8 ± 0.3	− 57 ± 4.2	51.1 ± 0.8
36	+	12 ± 0.1	110 ± 3.5	2 ± 0.4	+10 ± 0.3	−100 ± 3.7	38.5 ± 1.3

[1] Powdered egg white was obtained from Stein, Hall and Company, Inc., New York, as "Egg Albumin" but includes all of the egg white proteins.
[2] The values are averages of 8 rats/group ± SE of mean.

Finally it could be demonstrated that this unique property of egg white in decreasing femur ash content was absent from a partially purified egg white preparation (table 5). Nevertheless, 36% of this protein preparation increased the renal excretion of calcium with the animals receiving vitamin D.

DISCUSSION

The results reported in this paper demonstrate that relative to 18%, 36% of egg white in low phosphorus diets will increase the intake or absorption of calcium, or both; urinary excretion of calcium; and decrease the ash content of bone. The former 2 effects are produced with other proteins as well, whereas the latter is unique with egg white. Furthermore this property is not noted with an egg white preparation from which the conalbumin, ovomucoid, and lysozyme have been removed. Lower femur ash values and reduced calcium balance were always ob-

TABLE 3

Effect of blood fibrin protein on calcium metabolism of rats fed the low phosphorus rachitogenic diet[1]

Dietary protein	Vitamin D	Calcium intake	Urinary calcium	Fecal calcium	Net absorption	Calcium balance	Femur ash
%		*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg*
18	—	288 ± 11.4[2]	103 ± 6.4	174 ± 11.7	114 ± 6.8	+11 ± 10.1	51.8 ± 1.1
36	—	299 ± 10.8	144 ± 6.9	174 ± 7.8	125 ± 6.7	−19 ± 5.8	49.4 ± 1.2
18	+	179 ± 7.9	69 ± 6.5	70 ± 7.2	109 ± 6.7	+36 ± 3.5	61.6 ± 1.5
36	+	216 ± 4.3	93 ± 8.5	89 ± 6.1	126 ± 5.3	+33 ± 6.0	61.2 ± 1.8

[1] Bovine blood fibrin was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2] The values are averages of 8 rats/group ± SE of mean.

TABLE 4

Calcium metabolism of rats fed a diet adequate in calcium and phosphorus with casein as the protein source[1]

Dietary protein	Vitamin D	Calcium intake	Urinary calcium	Fecal calcium	Net absorption	Calcium balance	Femur ash
%		*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg*
18	—	208 ± 10[2]	37 ± 5.6	105 ± 5.6	102 ± 8.0	+ 65 ± 8.8	86.7 ± 1.9
36	—	254 ± 10	34 ± 4.8	131 ± 13.6	125 ± 14.7	+ 92 ± 13.1	98.0 ± 1.7
18	+	352 ± 11	38 ± 2.3	91 ± 5.1	261 ± 8.0	+222 ± 7.4	109.0 ± 1.6
36	+	373 ± 15	43 ± 1.9	65 ± 5.1	302 ± 10.9	+259 ± 12.4	118.2 ± 1.9

[1] "Vitamin Free" Test Casein, General Biochemicals, Chagrin Falls, Ohio.
[2] The values are averages of 7 rats/group ± SE of mean.

TABLE 5

Lack of effect of purified egg white protein on calcium balance and femur ash of rats fed a low phosphorus diet[1]

Dietary protein	Vitamin D	Calcium intake	Urinary calcium	Fecal calcium	Net absorption	Calcium balance	Femur ash
%		*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg/rat/ 6 days*	*mg*
18	—	198 ± 2.4	82 ± 6.5	106 ± 4.7	92 ± 3.1	+11 ± 11.2	48.7 ± 0.9
36	—	194 ± 3.4	87 ± 8.7	88 ± 5.5	106 ± 4.3	+18 ± 8.4	47.7 ± 0.1
18	+	166 ± 2.1	68 ± 6.2	59 ± 4.7	107 ± 5.0	+42 ± 8.7	62.1 ± 2.5
36	+	146 ± 8.0	90 ± 1.0	27 ± 4.8	119 ± 8.9	+35 ± 7.6	62.7 ± 2.1

[1] The values are averages of 4 animals/group ± SE of mean.

served in rats fed low phosphorus egg white diets as compared with the corresponding blood fibrin diets even when the protein content of the diets was maintained at 18% (see tables 1 and 3). These results suggest that whole steamed egg white contains some material which decreases bone mineral content and that this factor is associated with the conalbumin-ovomucoid-lysozyme fraction. The nature of such a factor is unknown at the present time.

There is little possibility that the reported effect of egg white on bone has any practical significance. The diets used in these experiments were low phosphorus diets which are rarely encountered under natural conditions. In addition, the large intakes of egg white protein reported here are not likely to occur especially in combination with low phosphorus diets.

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Effect of Certain Factors on Nitrogen Retention and Lysine Requirements of Adult Human Subjects

IV. TOTAL NITROGEN INTAKE[1]

HELEN E. CLARK, MARY ALICE KENNEY,[2] ALICE F. GOODWIN, KAMLA GOYAL AND EDWIN T. MERTZ

Purdue University Agricultural Experiment Station, School of Home Economics Department of Foods and Nutrition, and Department of Biochemistry, Lafayette, Indiana

ABSTRACT The influence of 12, 9, 6 and 4.5 g of dietary nitrogen on nitrogen retention of young men and women was investigated. The basal diet supplied 4.5 g of nitrogen daily and contained 159 g of white wheat flour, 21 g of cornmeal and purified essential amino acids. A mixture of glycine, glutamic acid and diammonium citrate was added as necessary to increase total nitrogen intake to 6, 9 or 12 g. Mean balances in 2 experiments were significantly higher ($P < 0.01$, < 0.05) with either 9 or 6 g of nitrogen than with 4.5 g when 700 or 900 mg of lysine were administered but did not differ in another experiment when 1500 mg of lysine were provided. Intakes of 12 or 9 g of nitrogen maintained equilibrium in all subjects but smaller amounts were adequate only for certain individuals. There was no interaction between dietary nitrogen and lysine.

When minimal requirements of men and women for the essential amino acids were being established, nitrogenous supplements were always provided (1, 2) to permit the synthesis of nonessential amino acids. This approach was justified because total nitrogen intakes did not exceed those in self-selected diets, and also because all foods contain relatively high concentrations of nonessential amino acids. The amounts and sources of nitrogen consumed by individuals and by population groups differ widely, however. Evidence now available concerning the influence on nitrogen retention of variable nitrogen intakes has been obtained under diverse conditions and is not in agreement. As little as 3.5 g of nitrogen maintained equilibrium in men whose intake was reduced stepwise while purified essential amino acids were held constant (3). The mean nitrogen balance of women who consumed either purified amino acids or corn shifted from slightly positive to distinctly negative when nitrogen was decreased from 10 to 6 g (4). Changes in nitrogen retention of young men and women were negligible when dietary nitrogen was increased from 6.5 to 10 g by adding nonessential amino acids to a diet containing whole egg (5), but negative balances occurred more frequently in older men when they consumed 15 instead of 7 g of nitrogen (6).

PROCEDURE

The present investigation was designed to study the influence on nitrogen retention of 12, 9, 6 and 4.5 g of dietary nitrogen. The general procedure was similar to that in earlier experiments (7, 8).

Subjects. Healthy graduate students or upperclassmen between 20 and 28 years of age participated, as shown in tables 1 and 2. Subject MR served in 2 experiments, others in one.

The basal diet. All subjects consumed daily 159 g of white wheat flour, 21 g of yellow cornmeal, a few low-nitrogen foods and a mixture of purified essential amino acids. Carbohydrate and fat were adjusted to maintain a constant caloric intake for each individual (tables 1, 2), with which subjects maintained weight or made small

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[2] Present address: Food and Nutrition Department, Iowa State University, Ames, Iowa.

TABLE 1

Mean daily nitrogen balances of subjects in experiment 1 who consumed 12, 9 and 6 g of nitrogen and 900 mg of lysine

Subject[1]	Mean wt	Height	Calories	Fecal N	N balance with N intake of			Creatinine N
					12.00	9.00	6.00	
	kg	*cm*	*kcal*	*g*	*g*	*g*	*g*	*g*
WB	77.0	186	3500	0.86	0.23	0.47	0.22	0.57
DC	73.2	188	3650	0.78	0.09	0.12	−0.19	0.66
RDL	80.5	176	3700	0.79	0.41	0.52	0.41	0.62
RP	68.8	178	3500	0.69	0.01	0	−0.14	0.64
Mean					0.19	0.28	0.07	

[1] All were men.

TABLE 2

Mean daily nitrogen balances of subjects who consumed 9.0, 6.0 and 4.5 g of nitrogen

Subject[1]	Mean wt	Height	Calories	Fecal N	N balance with N intake of			Creatinine N
					9.00	6.00	4.50	
	kg	*cm*	*kcal*	*g*	*g*	*g*	*g*	*g*
				Experiment 2: 700 mg of lysine				
NH	62.6	168	2300	0.59	0.28	0.20	0.06	0.36
JI	69.9	173	3200	1.04	0.06	−0.19	−0.63	0.64
MR	81.8	180	2850	0.66	0.41	0.42	0.11	0.53
PS	67.6	183	3450	0.85	0.21	0.30	0.04	0.51
JY	67.4	185	3400	0.63	0.39	0.35	0.01	0.55
Mean					0.27	0.22	−0.08	
				Experiment 3: 900 mg of lysine				
BC	69.0	183	3200	0.77	0.26	0.19	−0.26	0.64
RM	82.6	182	3500	0.88	0.35	0.02	−0.11	0.72
DP	74.5	187	3450	0.69	0.47	0.09	−0.19	0.62
MR	85.5	180	2700	0.64	0.02	0.34	−0.10	0.55
Mean					0.27	0.16	−0.17	
				Experiment 4: 1500 mg of lysine				
MA	61.4	172	2450	0.63	0.50	0.59	0.10	0.41
JB	67.7	174	3350	0.81	0.10	−0.06	−0.09	0.60
SM	78.6	173	3200	0.67	0.46	0.71	0.64	0.71
DR	68.4	178	3200	0.63	0.28	0.55	0.41	0.63
TS	80.4	180	3300	0.76	0.06	0	−0.10	0.60
Mean					0.28	0.36	0.19	

[1] All were men except NH, MR and MA.

gains except RDL who lost 0.5 kg in one period. Supplements of vitamins[3] and minerals were given.

The cereals provided at least 40% of all essential amino acids consumed. Appropriate quantities of L-isomers of essential amino acids were added so that all except lysine were comparable to those in 20 g of egg protein. Daily intakes were: (in grams) isoleucine, 1.60; leucine, 1.84; methionine, 0.82; cystine, 0.48; phenylalanine, 1.26; tyrosine, 0.90; threonine, 0.98; tryptophan, 0.30; and valine, 1.46. In addition, 1.28 g of arginine and 0.42 g of histidine were included. The cereals also provided: (in grams) alanine, 0.65; aspartic acid, 0.90; glycine, 0.63; glutamic acid, 5.62; proline, 2.06; and serine, 0.92.

Dietary nitrogen. Three quantities of nitrogen were tested in each of 4 experiments as stated in table 3. Cereals supplied 3.27 g of nitrogen, other foods, 0.30 g, and the essential amino acid mix-

[3] The authors acknowledge Litrison vitamin capsules generously supplied by Hoffman LaRoche, Inc., Nutley, New Jersey.

TABLE 3

Total intakes of nitrogen and lysine

Experimental period	Experiment			
	1	2	3	4
	Nitrogen, g			
1	12.0	9.0	9.0	9.0
2	9.0	6.0	4.5	6.0
3	6.0	4.5	6.0	4.5
	Lysine, mg			
1,2,3	900	700	900	1500

ture 0.89 g. The basal diet consumed by all subjects therefore contained 4.5 g of nitrogen. It was fed without supplementation in one period during experiments 2, 3 and 4. Appropriate quantities of a supplement composed of glycine, glutamic acid and diammonium citrate were included in other periods to provide total intakes of 12, 9 or 6 g. The effectiveness of this supplement in which each component contributes one third of the nitrogen has been demonstrated in human subjects (9).

Lysine. It was important to avoid a deficit of lysine and at the same time to maintain an intake near the minimal requirements of the subjects. Between 400 and 800 mg of lysine maintained equilibrium in men who consumed purified amino acids (1). When 9 g of nitrogen and a basal diet similar to that used in the present series were provided, requirements of women varied from 300 to 700 mg and of most men from 400 to 900 mg (8). Subsequently 700 mg of lysine in one experiment and 950 mg in another produced increasingly positive balances for 30 successive days (10).

The amounts of lysine predicted (8) to meet minimal requirements of all participants in experiments 1, 2 and 3 were, respectively, 900, 700 and 900 mg (table 3). In experiment 4 all subjects consumed 1500 mg of lysine so that all essential amino acids were in the amounts found in 20 g of egg protein. The basal diet contained 450 mg of lysine, and the necessary amounts of L-lysine monohydrochloride were administered to provide a total intake of 700, 900 or 1500 mg of lysine/day. Any diet that contains as little as 4.5 or 6 g of nitrogen is unlikely to provide large amounts of lysine unless it is supplemented with that amino acid.

Plan of experiment. An initial 12-day adjustment period was provided during which the highest level of nitrogen to be tested was given, i.e., 12 g in experiment 1 and 9 g in all others. Lysine intake was 1500 mg as in 20 g of egg protein. The initial period was extended to 15 days to permit adjustment to 900 or 700 mg of lysine if these were to be tested subsequently.

Three 7-day experimental periods with intervening 5-day adjustment intervals followed the adjustment period. The relative stability of urinary nitrogen in the experimental periods indicated that satisfactory adjustment had occurred.

Analyses. Total nitrogen was determined by a macro-Kjeldahl method in daily collections of urine and in fecal material pooled for each period. Lysine in baked foods was assayed microbiologically (7). Alpha-amino nitrogen was determined by a photometric ninhydrin method (11), urea by the urease method (12) and uric acid by the method of Benedict and Franke using the uric acid solution of Folin (13). Creatinine was measured by a modification of the Folin-Wu procedure. Analysis of variance and the Duncan new multiple range test[4] at the 5% level were used to detect whether differences between treatments were significant.

RESULTS

Data pertaining to nitrogen retention in experiment 1 are presented in table 1 and for experiments 2, 3 and 4 in table 2. All balances were positive unless designated otherwise.

The nitrogenous supplement was well absorbed since fecal nitrogen values did not differ significantly as a result of treatment in any experiment. Because individuals differed markedly from each other, a mean value was used in calculating nitrogen balances of each subject that represented fecal nitrogen of that individual in all periods (tables 1, 2). Men excreted on the average 0.77 and women 0.63 g of fecal nitrogen.

[4] Duncan, D. B. 1955 Multiple range and multiple F test. Biometrics, *11*: 1.

Nitrogen retention of subjects who consumed 12, 9 and 6 g of nitrogen. Daily intakes of 12, 9 and 6 g were compared in experiment 1 because the earlier observation that men weighing 70 kg or more attained equilibrium more slowly than did women or smaller men suggested that 9 g of nitrogen might not be adequate for some individuals. Mean daily nitrogen balances in experiment 1 were 0.19, 0.28 and 0.07 g, respectively, when the men consumed 12, 9 and 6 g of nitrogen and 900 mg of lysine (table 1). Balances resulting from 9 g of dietary nitrogen were significantly higher ($P < 0.05$) than from 6 g, but 12 g did not differ significantly from either 9 or 6 g. The hypothesis that nitrogen retention of the men would be improved by increasing nitrogen intake to 12 g was not supported. Based on a mean body weight of 75 kg, they ingested 0.16, 0.12 and 0.08 g of nitrogen/kg in successive periods, equivalent to 1.0, 0.75 and 0.5 g of protein. All subjects maintained equilibrium when consuming either 12 or 9 g of nitrogen, but subjects DC and RP were in negative balance when they ingested 6 g. These men consistently excreted more urinary nitrogen than did subjects WB and RDL.

Subject MCM, a woman weighing 56 kg, was given simultaneously a comparable diet containing 600 mg of lysine and 2400 kcal. Balances resulting from nitrogen intakes of 9, 6 and 4.5 g were 0.29, 0.17 and −0.12 g, respectively. Only 9, 6 and 4.5 g of nitrogen were compared in subsequent experiments since 12 g were not superior to 9 g and 4.5 g did not induce a strongly negative balance in this subject.

Nitrogen retention of subjects who consumed 9, 6 and 4.5 g of nitrogen. Mean daily nitrogen balances of subjects in experiment 2 who consumed 9, 6 and 4.5 g of nitrogen in descending order of magnitude were 0.27, 0.22 and −0.08 g, respectively (table 2). Balances associated with 9 and 6 g of dietary nitrogen did not differ from each other but were significantly higher ($P < 0.01$) than with 4.5 g. Nitrogen retention of all individuals was improved by the addition to the basal diet of as little as 1.5 g of supplementary nitrogen. All subjects maintained equilibrium at all levels of nitrogen intake except JI whose balance was only slightly positive with 9.0 g of nitrogen. His retention was significantly lower ($P < 0.05$) than that of other subjects in the same experiment, largely due to unusually high fecal nitrogen.

In experiment 3, subjects consumed 9, 4.5 and 6 g of nitrogen in that sequence. Because nitrogen retention tends to improve with time (10), it seemed possible that testing 4.5 g of nitrogen in the final period as in experiment 2 would yield different results than if it were tested in another phase of an experiment. The preceding adjustment interval was extended because 4.5 g immediately followed 9 g instead of 6 g. Mean balances were 0.27, −0.17 and 0.16 g, respectively, when 9, 4.5 and 6 g of nitrogen were tested. As in experiment 2, balances resulting from intakes of 9 and 6 g did not differ from each other, but were significantly higher ($P < 0.05$) than when 4.5 g were provided. All subjects retained nitrogen when consuming either 9 or 6 g of nitrogen whereas all were in negative balance with 4.5 g. Nitrogen balances of individuals did not differ significantly from each other.

In experiment 4, mean balances were 0.28, 0.36 and 0.19 g, respectively, in response to 9, 6 and 4.5 g of nitrogen. In contrast with experiments in which minimal amounts of lysine were fed, differences related to treatment were not significant when 1500 mg of lysine were fed. All subjects maintained equilibrium throughout the experiment except JB with 6 g and both JB and TS with 4.5 g. Balances of JB and TS were significantly lower ($P < 0.05$) than those of other subjects when all treatments were considered.

When data pertaining to the 3 experiments in which subjects consumed 9, 6 and 4.5 g were analyzed together, the difference in nitrogen retention due to dietary nitrogen was highly significant ($P < 0.01$) and that due to lysine was significant ($P < 0.05$). There was no interaction between dietary nitrogen and lysine. Mean nitrogen balances of subjects who consumed 9 g of nitrogen in different experiments were almost identical (0.27, 0.27, 0.28 g) and all individuals were in equilibrium. Mean balances were positive (0.22, 0.16, 0.36 g) when 6 g of nitrogen were

provided and all but two subjects were in equilibrium. Mean balances were −0.08, −0.17 and +0.19 g, respectively, when 700, 900 and 1500 mg of lysine were given with 4.5 g of nitrogen. Only one-half of the subjects who consumed 4.5 g of nitrogen maintained equilibrium regardless of lysine intake.

Relation between dietary nitrogen and urinary nitrogen. Alterations in dietary nitrogen were followed promptly by changes in total urinary nitrogen. A typical equation expressing regression of urinary nitrogen (Y) on dietary nitrogen (X) was: $Y = 0.932\ X - 0.449$. Slopes and elevations of regression lines were similar in all experiments, and correlation coefficients were between +0.94 and +0.99.

Partition of urinary nitrogen. Distribution of end products in the urine was studied when 9, 6 and 4.5 g of nitrogen were consumed. Figures which represent the average of the mean daily excretion values of all subjects who participated in each experiment are presented in table 4.

A typical equation for regression of urea nitrogen (Y) on dietary nitrogen (X) was: $Y = 0.879\ X - 1.532$, ($r = +0.99$). A corresponding equation for the regression of urea nitrogen (Y) on total urinary nitrogen (X) was: $Y = 0.963\ X - 1.283$ ($r = +0.99$). Differences between individuals were observed, but variability in nitrogen retention could not be attributed entirely to urea.

TABLE 4

Mean daily excretion of certain constituents of the urine

Total nitrogen		Urea	α-Amino N
Dietary	Urinary		
g	*g*	*g*	*g*
	Experiment 2		
9.0	7.98	6.52	0.40
6.0	5.03	3.78	0.30
4.5	3.73	2.58	0.24
	Experiment 3		
9.0	7.99	6.39	0.38
6.0	5.10	3.65	0.32
4.5	3.95	2.49	0.29
	Experiment 4		
9.0	8.02	6.53	0.37
6.0	4.94	3.47	0.29
4.5	3.65	2.23	0.25

Differences in free α-amino nitrogen in the urine as a result of modifications in dietary nitrogen were highly significant ($P < 0.01$) in experiments 2 and 4, and all treatments differed from each other. Differences in experiment 3 were significant ($P < 0.05$), 9 g being different from 6 and 4.5 g. One third of the supplementary nitrogen in the present series was provided by glycine which directly influences free α-amino nitrogen excretion whereas glutamic acid and diammonium citrate do not (9). Differences between subjects were highly significant ($P < 0.01$) in all experiments.

A typical equation showing regression of free α-amino nitrogen (Y) on dietary nitrogen (X) was: $Y = 0.021\ X + 0.194$, and the corresponding regression of free α-amino nitrogen on urinary nitrogen was $Y = 0.024\ X + 0.192$. Alpha-amino nitrogen comprised on the average 4.7, 6.0 and 6.9%, respectively, of total urinary nitrogen when 9, 6 and 4.5 g of nitrogen were consumed.

No definite influence of dietary nitrogen on urinary creatinine was discernible. Creatinine contained 6.5, 9.5 and 12.6%, respectively, of urinary nitrogen when nitrogen intakes were 9, 6 and 4.5 g. The direct relationship between muscular mass and creatinine excretion and the relative constancy of the latter regardless of nitrogen intake may be an important factor in determining whether or not individuals maintain equilibrium when dietary nitrogen is severely restricted. Women eliminated from 0.37 to 0.55 g of nitrogen as creatinine and men from 0.51 to 0.72 g.

Urinary ammonia varied from 0.23 to 0.30 g/day and was not influenced consistently by dietary nitrogen. Uric acid nitrogen ranged from 0.06 to 0.08 g/day.

DISCUSSION

The effectiveness of the basal diet in maintaining nitrogen equilibrium must be considered before the influence of variable quantities of dietary nitrogen can be evaluated. A diet that supplied 4.5 g of nitrogen and contained amounts of essential amino acids comparable to those in 20 g of egg protein, except that 700, 900 or 1500 mg of lysine were included, permitted equilibrium in only one-half of the subjects. Two

subjects were in negative balance even with 1500 mg of lysine. The amounts of tryptophan and sulfur-containing amino acids in 20 g of egg protein are only slightly above minimal requirements (1), whereas other amino acids are nearly twice minimal needs. Quantitative relationships among essential amino acids in the basal diet thus differed from those provided by Rose and Wixom (3) who reported that men attained equilibrium with 3.5 g of nitrogen when twice the required amounts of all essential amino acids were consumed.

Nitrogen retention characteristic of the basal diet containing 4.5 g of nitrogen was improved in all subjects when 1.5 g of nitrogen were added as glycine, glutamic acid and diammonium citrate without modifying the essential amino acids. This observation suggests that essential amino acids may have been deaminated or diverted into other metabolic pathways so rapidly when only the basal diet was fed that the quantities of tryptophan, methionine or lysine (except in experiment 4) available for tissue synthesis were inadequate. Although the difference in retention related to intakes of 9 or 6 g was not statistically significant, a few subjects showed distinct improvement and all subjects attained equilibrium only with 9 or 12 g. That none of the subjects in experiment 1 retained more nitrogen when they consumed 12 instead of 9 g suggests that the maximal sparing action of supplementary nitrogen for this particular combination of essential amino acids was achieved with 9 g of dietary nitrogen.

Data obtained in this series of experiments indicate clearly that total dietary nitrogen influences nitrogen retention when the quantity of supplementary nitrogen is altered without concomitant changes in essential amino acids. This is in general agreement with observations of Linkswiler et al. (4). Different responses might be expected if total nitrogen were modified by increasing the intake of both essential and nonessential amino acids, or by increasing the intake of essential amino acids at the expense of the supplementary nitrogen source, or by altering the balance among essential amino acids. No attempt was made in the series reported herein to study the influence on nitrogen retention of different quantities of essential amino acids but the effect of varying the intakes of amino acids that are limiting in flour is currently under investigation. In a comprehensive study of supplementation of cereal proteins with amino acids, Bressani et al. (14) concluded that the response of the children was due principally to a combined lysine and tryptophan supplement and that the amount of lysine per gram of nitrogen that must be added did not vary significantly with protein intake. Harper (15) stated that the level of threonine required to prevent growth depression in young rats consuming a diet unbalanced in respect to essential amino acids was unaffected by the level of protein in the original diet.

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Amino Acid Balance in the Adult: High Nitrogen — Low Tryptophan Diets[1]

HANS FISHER, M. K. BRUSH, R. SHAPIRO, J. P. H. WESSELS, C. D. BERDANIER, P. GRIMINGER AND E. R. SOSTMAN
Nutrition Laboratories, Rutgers — The State University, New Brunswick, New Jersey

ABSTRACT Studies were carried out with adult roosters and with male college students to ascertain the influence of increasing the nitrogen (N) intake of diets suboptimal with respect to an essential amino acid (tryptophan), on N utilization. In experiments with adult male chickens, N retention was either the same or greater when the total amino acid intake was doubled, whereas the level of tryptophan remained unchanged and suboptimal. In one study with human subjects, doubling the N intake, while tryptophan remained suboptimal, resulted in a significant increase in N retention, when the period during which the N intake was doubled followed a period of negative N balance. In a second experiment, the high N — low tryptophan diet was given immediately following a period of strong N retention. In this instance, subjects remained in N equilibrium; they did not retain as much N as they had done previously. In contrast with studies with growing rats and chicks, in all experiments with adult chickens or man, no impaired retention of N as a result of increasing the N intake without a concomitant increase in the limiting amino acid was observed. A tryptophan intake of 200 mg/day was found to adequately meet the requirements of the nine subjects studied in these experiments.

The importance of amino acid balance in protein nutrition has been emphasized by Harper and associates (1, 2) working with the rat, and by Fisher and co-workers (3–5) using the chicken. Both groups observed a growth depression when a protein or amino acid supplement was added to a diet marginal in an essential amino acid without a concomitant increase in the quantity of the limiting amino acid. They also noted that the growth depression occurred only under dietary conditions that permitted relatively rapid growth of the experimental animals. It was, therefore, pertinent to investigate amino acid imbalance in adult animals and man under conditions of increased nitrogen (N) intake in the presence of a limiting amino acid.

GENERAL PROCEDURES

Studies with chickens. Five Leghorn males, over 12 months of age, were used for each dietary regimen. They were housed in individual metabolism cages and were given the experimental diets on the basis of 26 g/kg body weight/day. The diet supplied: (in per cent) glucose, 15.0; corn oil, 12.0; dextrin, 5.0; fiber, 4.0; mineral mix,[2] 3.0; antacid,[3] 1.0; choline chloride (70% concentrate), 0.3; vitamins,[2] 0.25; amino acids, variable; and corn starch, to 100. The amino acids, with the exception of tryptophan, were provided at levels adequate to meet the maintenance requirements (6), as follows: L-arginine·HCl, 0.56; L-lysine·HCl, 0.14; L-leucine, 0.48; DL-isoleucine,[4] 0.56; DL-valine, 0.23; DL-threonine, 0.56; DL-methionine, 0.27; L-phenylalanine, 0.10; L-cystine, 0.16; L-tyrosine, 0.13; L-glutamic acid, 0.57; and glycine 1.10% of the diet. When the N intake was doubled, the level of all amino acids was doubled to provide 9.76% of the amino acid mixture instead of the 4.88% provided in the low N diets.

All birds were given a protein-free diet for 10 days until N excretion had reached the endogenous level. They were then fed the experimental diets for 7 days, and three 48-hour excreta collections were made beginning with the second day.

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[2] For composition, see Summers and Fisher (13).

[3] Gelusil, Warner-Chilcott Pharmaceutical Co., Morris Plains, New Jersey.

[4] D-Allo, L-isoleucine.

The excreta were homogenized, made up to volume and N determined on an AutoAnalyzer by a modification of the method proposed by Ferrari (7).

Studies with man. Four, in one instance and, in the other, 5 male college students were selected for each of the 2 studies to be described. They were given a medical examination prior to their selection for participation. The subjects were weighed every morning before breakfast and their calorie intake adjusted so that their body weights were maintained within plus or minus 1 kg. The average calorie intake of the subjects in these studies was 2700 kcal/day.

The basic diet was low in N and provided only 13 mg of tryptophan.[5] The individual food items included were: margarine, jam, orange drink,[6] coffee, sugar, grapefruit or orange sections, lettuce, salad dressing, special low-N biscuits and cookies and a vitamin and mineral supplement.[7] The main protein source was salt-free hydrolyzed casein[8] which was given to the subjects dissolved in lemonade. For the high-protein diets gelatin was added to the hydrolyzed casein to supply approximately one-half the total N intake. Additional tryptophan was given in gelatin capsules as L-tryptophan.

Daily collections and analyses were made for urine, and the fecal collections were pooled for a 4- or 5-day period with carmine given as a marker at the beginning and end of the collection period. Urinary N was determined directly with the AutoAnalyzer. Fecal N was determined by Kjeldahl digestion in flasks, followed by the colorimetric determination of ammonium in the AutoAnalyzer (7). Urinary creatinine was determined by the Folin-Wu method (8).

In the second study blood was taken from the subjects on the sixth day of each dietary treatment. The plasma was analyzed for amino acid and urea N, the former by the method of Danielson, the latter by the method of Karr, both as described by Hawk et al. (8). Plasma protein was determined by the Biuret reaction adapted for use with the AutoAnalyzer.

EXPERIMENTAL PROCEDURES AND RESULTS

Studies with chickens. Two experiments were carried out with adult roosters after feeding the birds a protein-free diet until an endogenous N excretion had been reached (7 days). They were then given a diet which provided optimal amounts for maintenance of all essential amino acids (6) except for tryptophan, at a total daily N intake of 140 mg/kg/body weight (approximately equivalent to the endogenous N excretion). Tryptophan was provided at one-third the optimal maintenance level (6). After the birds had been fed this diet for 7 days, they were given double the amount of N (by doubling the amino acid mixture) so that the diet now provided 280 mg N/kg/day, with tryptophan remaining at its original, suboptimal level.

All the food offered to the birds was consumed and the results (table 1) show no impairment in N retention for the birds in either study. In study 2 they retained significantly more N with the high N — low tryptophan diet than they did on the low N — low tryptophan diet. Thus, under conditions which had caused a decreased food intake and consequent depressed weight gain in young birds, no impairment could be shown in adult birds during a short-term feeding period.

TABLE 1

Effect of doubling the amino acid intake of adult chickens, given a tryptophan-low diet, on nitrogen balance

Study	Daily intake		Nitrogen balance
	Nitrogen[1]	Tryptophan	
	mg/kg	*mg/kg*	*mg/kg/day*
1	140	3.5	18.7 ± 3.9[2]
	280	3.5	24.1 ± 4.8
2	140	3.5	6.2 ± 2.1
	280	3.5	40.3 ± 12.6

[1] Supplied as amino acids; with the exception of tryptophan which was supplied at one-third the maintenance requirement, the essential amino acids were provided in amounts adequate to meet the maintenance requirement.

[2] Mean value ± SE for three 48-hour collections with 5 birds.

[5] By microbiological assay.

[6] Tang Orange Drink, General Foods Company, White Plains, New York.

[7] Therogran M Tablets, E. R. Squibb and Sons Laboratories, New Brunswick, New Jersey.

[8] Hy-Case, Sheffield Chemical Company, Norwich, New York.

Studies with man. The first study, involving 5 subjects and three 7-day periods, is outlined in table 2. The subjects were given approximately 9 g of N, provided mainly as hydrolyzed casein. During period A they were given an adequate intake of tryptophan (213 mg); this was reduced during period B (to 113 mg) without change in total N intake. During period C the suboptimal level of tryptophan (113 mg) was maintained, but the N intake was doubled through the addition to the diet of 10 g N provided by gelatin.

The N utilization data (table 2) indicate that the men were in N equilibrium during period A and in negative N balance during period B, when the diet was suboptimal with respect to tryptophan, but were again in positive N balance during period C, when the diet, although suboptimal with respect to tryptophan, supplied double the amount of N. Thus, during the short-term periods investigated, no impaired N utilization occurred. We have no explanation for the decrease in creatinine with time during this experiment.

Although in this experiment a period of negative N balance (period B) preceded the period during which the subjects consumed the high N diet (period C), the order of experimental regimens was reversed in the next experiment. Period A of the second experiment (table 3) represented a standardizing period with a relatively high intake of good quality proteins from mixed foods.[9] The tryptophan content of this diet was also high (approximately 1 g). The period with the well-balanced diet was immediately followed by period B during which the subjects consumed the same low tryptophan — high N diet given during period C of the previous experiment. Period B was followed by the removal of gelatin from the diet without changing the tryptophan con-

[9] The following foods were provided: eggs, orange juice, biscuits, margarine, heavy cream, fat-free ham, cheddar cheese, wholewheat bread, sliced peaches, skim milk, cookies, fat-free beef, carrots, potatoes, white bread, lettuce, vanilla ice cream.

TABLE 2

Effect of doubling the protein intake of tryptophan-low diets on nitrogen balance in adult human subjects

Period	Daily intake		Nitrogen balance	Urinary creatinine
	Nitrogen[1]	Tryptophan		
	g	*mg*	*g/day*	*g/day*
A	9.34	213	0.58 ± 0.24[2]	1.97 ± 0.09
B	9.34	113	−1.10 ± 0.16	1.83 ± 0.16
C	19.20	113	2.91 ± 0.43	1.62 ± 0.15

[1] The dietary N was derived as follows: Period A, 8.40 g from hydrolyzed casein, and 0.94 g from miscellaneous food items (see text); period B, same as A; period C, 8.40 g from hydrolyzed casein, 10.0 g from gelatin, and 0.80 g from miscellaneous food (see text).
[2] Mean value ± SE for four 24-hour collections with 5 adult male subjects.

TABLE 3

Effect of doubling the protein intake of tryptophan-low diets on nitrogen balance and plasma constituents of adult human subjects

Period	Daily intake		Nitrogen balance	Urinary creatinine	Plasma		
	Nitrogen[1]	Tryptophan			Urea nitrogen	Amino acid nitrogen	Protein
	g	*mg*	*g/day*	*g/day*	*mg/100 ml*	*mg/100 ml*	*g/100 ml*
A	16.65	1104[2]	3.05 ± 0.21[3]	Not det.	15.0 ± 1.6	5.5 ± 0.3	6.1
B	19.58	113	0.22 ± 0.42	1.74 ± 0.11	19.1 ± 1.3	6.2 ± 0.1	6.5 ± 0.1
C	10.12	113	0.20 ± 0.26	1.57 ± 0.13	13.2 ± 0.8	5.9 ± 0.2	6.2 ± 0.02
D	10.12	213	1.27 ± 0.70	1.52 ± 0.16	14.5 ± 0.7	5.8 ± 0.5	6.8 ± 0.03

[1] The dietary N was derived as follows: Period A, from mixed foods (see text); period B, 9.0 g from hydrolyzed casein, 10.0 g from gelatin and 0.58 g from miscellaneous foods (see text); periods C and D, 9.0 g from hydrolyzed casein and 1.12 g from miscellaneous foods (see text).
[2] Approximated by calculation using the tables of Orr and Watt (14).
[3] Mean value ± SE for four 24-hour collections with 4 adult male subjects.

tent (period C) and, finally, by the addition of sufficient tryptophan to the low N diet (period D). Again, all periods were of 7 days duration.

The subjects were in strong positive balance (table 3) during the standardizing period (period A), but also maintained N equilibrium during the high N-low tryptophan period (period B). In comparing period C with period B, again there was no impairment in N utilization as a result of doubling the N intake. The subjects, however, were not in the same high positive N balance when the high N — low tryptophan period followed a period of strong N retention, as they were in the previous experiment when it followed a period of negative N balance. During period D, when tryptophan was once again adequate, the subjects immediately increased their N retention

There were no significant changes in urinary creatinine values, but there were changes in plasma urea and amino acid N associated with diet changes. The values for both measurements increased significantly ($P < 0.01$) during period B when the subjects received the high N— low tryptophan diet as compared with period A, and decreased again during period C, upon removal from the diet of 10 g gelatin. Since the levels of urea and amino acid N were essentially the same during periods A, C and D, even though the protein intake was appreciably higher during period A, the higher values during period B suggest that a not insignificant amount of non-protein N was being retained.

DISCUSSION

All 3 studies reported indicate that an increase in protein or amino acids to a diet limiting in an essential amino acid does not lead to impaired N retention in adults, an observation contrary to the growth depression observed when similar rations were given to young rats or chicks. These results may also be viewed in terms of Mitchell's postulate for amino acid requirements (9). He suggested that the amino acid requirements of adult animals for maintenance constitute a "particulate" requirement, allowing for the utilization of individual amino acids even in the complete absence of other essential amino acids. This is in sharp contrast with an "aggregate" requirement which emphasizes the importance of the simultaneous presence of all essential amino acids for growth purposes.

It is interesting to compare the results obtained in the first experiment with human subjects (table 2) with those obtained by Shapiro and Fisher (10) who studied the repletion of protein stores in adult birds. These authors observed that non-essential amino acids could be utilized effectively in repleting the protein stores of previously depleted adult chickens. Similarly, in the present study, our subjects showed strong N retention on a high N — low tryptophan diet, following a week of negative N balance. The same was also true in the present study with adult roosters (table 1).

We believe the inability to produce an amino acid imbalance in the adult to be related, in part, to this phenomenon. Thus, the protein stores of the animal may serve as a cushion either to accommodate excess dietary amino acids or to furnish amino acids to balance the imbalanced mixture. Allison has suggested (11) that during short periods an individual as a whole might be in positive N balance even though some body tissues are in negative balance.

These observations also corroborate our earlier work (5) in which it was shown that an imbalance did not interfere with utilization of N in the growing bird provided that the absolute intake of the limiting amino acid was unaffected.

The observed elevations for blood urea and amino acid N for the last experiment during the period on the high N — low tryptophan diet do not suggest that the N retained was necessarily in the form of protein. These observations may again be adequately restated in terms of Mitchell's "particulate" requirement concept.

The present study, as well as several preliminary trials in which the protein and tryptophan levels for this study were delineated, indicated that the tryptophan requirement of our subjects was in the range indicated by Rose (12), namely between 150 and 250 mg/day. For the 9 subjects in this study and for several

others in preliminary trials, the tryptophan requirement was always satisfied at a level below 200 mg, and in many cases not exceeding 150 mg.

The difference between the results obtained in the first and second human studies confirms the importance of equilibrating the physiological protein status of the subjects before an experiment of this type is undertaken. The prior state of depletion in subjects with protein- and amino acid-inadequate diets, altered the response to the subsequent dietary regimen. The altered response in the 2 human studies suggests that the results obtained in the rooster study also reflect the body protein status. The greater N retention with the higher N intake should therefore not be interpreted as a sparing effect on tryptophan.

It is reassuring that a high protein intake in diets limiting with respect to an essential amino acid does not interfere with N retention when the variations in food intake patterns experienced by much of the world's population are considered.

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Evaluation of Protein in Middle Eastern Diets[1]

I. ALMOND (*Prunus amygdalus*)

J. W. COWAN, Z. I. SABRY, F. J. RINNU AND J. A. CAMPBELL[2]
Division of Food Technology and Nutrition, American University of Beirut, Beirut, Lebanon

ABSTRACT The nutritive value of sweet almond (*Prunus amygdalus*), which is used in feeding infants in Lebanon, was studied from the aspects of protein quality and the possible presence of toxic factors. Weanling rats fed diets containing 20% almond oil or almond protein did not show any effects which would indicate that either the oil or protein fraction contained toxic substances. The quality of almond protein as determined by net protein ratio (NPR) and protein efficiency ratio (PER) was very poor as compared with casein. The addition of 4 limiting amino acids to almond protein diets resulted in values comparable to those of casein diets. The most limiting amino acid was found to be methionine followed by lysine, threonine and tryptophan. The supplementation of wheat or rice with almond improved protein quality only to a limited degree. It was concluded that, if fed to children, almond should be properly supplemented with other protein-rich foods to help overcome the amino acid deficiencies.

The occurrence of protein malnutrition syndromes, especially among children, has been reported from a number of Middle Eastern countries (1–6). Dietary surveys carried out in the area (5–7)[3] indicate that, in a large segment of the population, about two-thirds of the total caloric intake is derived from cereals and only about 5% from animal protein.

In the Middle East, few nutritional studies have been carried out, and information is lacking, concerning the nutritive value of dietary protein and the factors which may affect protein quality, such as amino acid availability and the presence of toxic substances. Such information is needed not only for individual protein foods but also for the numerous mixtures of these foods existing in different dietary patterns in the area (1, 7, 8). The studies to be reported in this series are being undertaken to obtain information concerning 1) the nutritive value of protein foods indigenous to the Middle East; 2) the supplementary effect of various protein foods in Middle Eastern dietary patterns; 3) the modification of existing mixtures of protein foods for optimal supplementary effect; 4) the formulation of new and acceptable protein mixtures for infants and pre-school children; and 5) the general improvement of diets in the Middle East.

A survey among groups of low income urban families in Lebanon has indicated the existence of a strong belief that sweet almond has a high nutritive value.[4] It has been observed that almond is used in several forms to supplement the diet of lactating mothers and of pre-weanling, weanling and post-weanling infants. In addition, almonds or almond preparations are used extensively as pacifiers and as laxatives for infants less than one year of age. Although almond is normally considered a condiment, its use as an infant food and galactogogue suggests that it may be consumed in relatively large amounts.

Toxic materials such as cyanogenic amygdalins have been detected in certain varieties of sweet almond (9, 10). In biological studies, Morgan et al. (11) observed poor growth in rats fed vitamin A-deficient diets containing 10 and 20% almond oil. It was not possible to determine, however, whether the growth de-

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[2] Permanent address: Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.
[3] Chopra, S. J., unpublished data.
[4] Harfouche, J. K., unpublished data.

pression was due to the vitamin deficiency of the almond oil or to the presence of toxic substances. Ujsaghy (12) fed "almond milk" to healthy infants and concluded from his results that the almond preparation could be used effectively as a substitute for human milk. The validity of this recommendation may be questioned, however, since the retention of nitrogen from the "almond milk" was only 38% as compared with 77.7% for human milk. Morgan et al. (11) fed mice a diet containing 17.2% protein from almond and reported that the protein efficiency ratio (PER) was low (0.63). Mitchell and Beadles (13) and Hall and coworkers (14), using as criteria biological value and PER, respectively, also observed that the quality of almond protein was low compared with that of beef. No attempt was made in these studies, however, to establish whether the low values were attributable to amino acid imbalance, toxicity or a combination of both factors.

The present study was undertaken to investigate the nutritive value of Lebanese-grown sweet almond from the aspects of protein quality and the possible presence of toxic substances.

EXPERIMENTAL

Proximate analyses were carried out using methods described by the AOAC (15).

The fatty acids were determined as methyl esters on a Pye argon chromatograph using a 112-cm column of 10% ethylene glycol adipate polyester on 100 to 120 mesh Celite[5] at a temperature of 180°C.

For the biological assays, male, albino, weanling rats of the Sprague-Dawley strain[6] were housed individually in screen-bottom cages in an air conditioned room held at 22° ± 1°C. Food and water were supplied ad libitum.

With the exception of the experiment in which the growth response to almond oil was investigated, the percentage composition of the basal diet was corn starch, 80; USP XIV salt mixture, 4; non-nutritive cellulose, 5;[7] vitamin mixture, 1;[8] vegetable oil, 10 (16). The basal diet for the oil studies differed in that it contained 70% corn starch and 20% oil. Test proteins were added to the required level at the expense of corn starch. The almonds used in the diets were shelled, coarsely ground and subjected to continuous extraction with light petroleum ether or ethyl ether for 24 hours. The defatted material was then reground in a Wiley mill to a fine (60-mesh) powder which contained 45 to 50% protein. This second grinding step eliminated most of the peels since they did not pass through the screen.

Four-week assays were carried out to compare the growth response to almond oil with the response to other vegetable oils. Groups of rats received isocaloric casein diets containing 20% casein, added at the expense of corn starch, and 20% oil. The oils used, in addition to almond oil, were corn, sesame, olive, peanut and cottonseed.

The possible toxicity of almond was tested by a method suggested by Campbell[9] in which the growth of animals fed the test food at 10 and 20% protein levels for 4 weeks was compared with that of animals fed similar levels of protein from casein.

The procedure of Chapman et al. (16) was used to determine protein efficiency ratio. Weanling rats were allocated in a randomized design to the test diets containing 10% protein. A non-protein control group was maintained so that net protein ratio (NPR) data could be calculated at 7 days from the same animals according to the procedure of Bender and Doell (17).

RESULTS AND DISCUSSION

The proximate analysis of a Lebanese-grown variety of sweet almond is presented in table 1. These data show that almond is rich in fat and protein.

The fatty acid pattern of the almond oil (table 2) indicates that the C_{18} mono- and di- unsaturated fatty acids are the most prevalent. None of the longer chain acids, which have been shown to be toxic (18), were present.

[5] Diatomaceous silica filter aid, Johns Manville, Manville, New Jersey.
[6] Obtained from Animal Suppliers (London) Ltd., London, England.
[7] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[8] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland.
[9] Campbell, J. A. 1961 A critical appraisal of methods for evaluation of proteins in foods. Nutrition Document R. 10 add. 37 WHO/FAO/UNICEF-PAG, United Nations, New York.

TABLE 1

Proximate analysis of a Lebanese-grown variety of sweet almond

	%
Moisture	4.1
Protein	19.4
Ether extract	60.5
Ash	3.4
Crude fiber	2.3
Nitrogen-free extract	10.3

TABLE 2

Fatty acid content of almond oil

Fatty acid	%
Myristic	trace
Palmitic	5.2
Palmitoleic	0.6
Stearic	1.5
Oleic	74.3
Linoleic	18.4
Linolenic	trace
Arachidic	trace

The 4-week growth curves presented in figure 1 show the response of rats to casein diets containing high levels of oil from corn, sesame, peanut, olive, cottonseed and almond. These data demonstrate that the nutritive value of almond oil is similar to that of the other commonly used oils.

The comparison of growth of animals fed casein or almond at 10 and 20% levels of protein as a test for possible toxicity is presented in figure 2. The slope of the line showing the increase in weight gain with the increase in protein level for almond (6.1) is essentially the same as that of the casein line (6.3). These results indicate that the poor growth observed at the lower level of almond protein was not due to the presence of toxic substances but rather to amino acid imbalance. Thus, increasing the protein level appears to have increased the levels of the limiting amino acids and promoted better growth. Almond diets were then supplemented with the limiting amino acids to determine whether the growth depression effect could be overcome.

The content of the indispensable amino acids in almond protein is presented in table 3. Lysine, threonine and methionine were the most limiting amino acids followed by tryptophan and isoleucine.

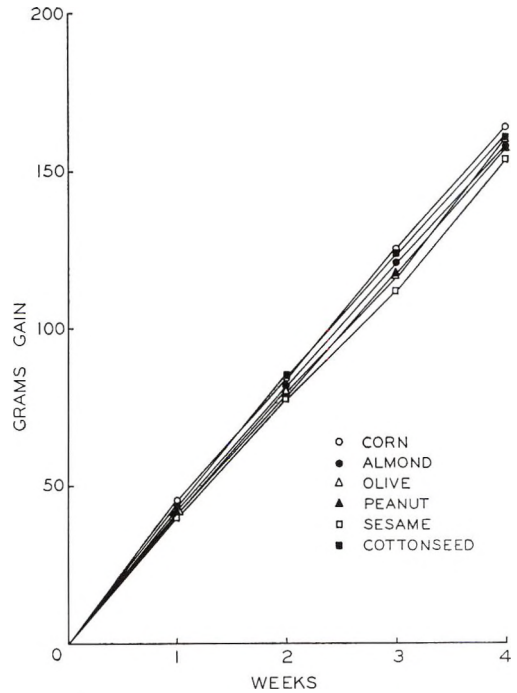

Fig. 1 Increase in weight of rats fed 20% oil from almond and other vegetable sources in a complete casein diet. The oil fraction of each diet contributed 43% of the total calories. Each curve represents the average of 10 animals.

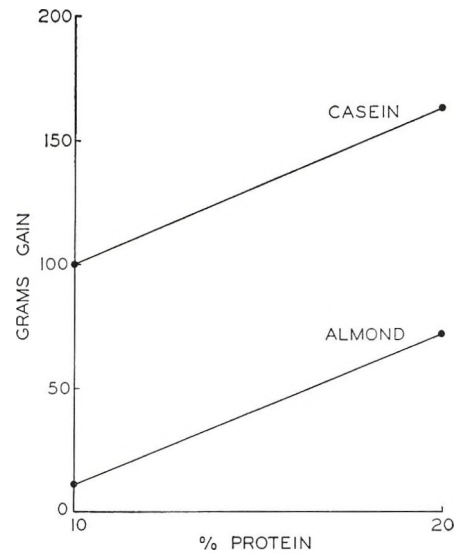

Fig. 2 Difference in 28-day gains of 2 groups of rats fed 10 and 20% almond protein, respectively, compared with gain differential of 2 comparable groups receiving 10 and 20% protein from casein. Each point represents the average of 10 animals.

TABLE 3

Supplementation levels of essential amino acids to 10% almond protein diets

Amino acid	Requirement of growing rat[1]	Contribution of almond[2]	Portion of requirement contributed by almond	Supplementation level
	g/kg diet	*g/kg diet*	*%*	*g/kg diet*
L-Tryptophan	1.1	0.8	73	0.4
L-Methionine + L-cystine[3]	4.9	2.8	57	2.0
L-Threonine	5.1	2.7	53	2.4
L-Lysine	9.0	2.6	30	6.4
L-Isoleucine	5.5	4.0	73	0
L-Valine	5.6	5.0	90	0
L-Leucine	6.9	6.5	94	0
L-Phenylalanine + L-tyrosine	7.2	7.9	109	0

[1] Rao et al. (20).
[2] Based on values taken from Orr and Watt (21).
[3] Supplemented with L-methionine only.

These predictions based on amino acid analysis were generally confirmed by the biological assays as shown in table 4. The supplementation of almond protein with lysine, methionine, threonine and tryptophan resulted in increasing NPR from 2.73 to 5.22 and PER from 0.32 to 2.44. The deletion of any one amino acid from the supplement resulted in NPR and PER values which indicated that methionine was most limiting followed by lysine and threonine. Although the order of limitation, established biologically, was not in direct agreement with the chemical data, the deletion of any one of the three amino acids resulted in very poor growth, indicating a severe deficiency of these amino acids. The deletion of tryptophan alone brought about only slight reduction in growth.

The data in table 3 show that isoleucine was as limiting as tryptophan. Since, however, the addition to almond protein diets of all limiting amino acids other than tryptophan resulted in growth comparable to that of casein (table 4), and since isoleucine was not available at the time of the experiment, the effect of supplementation with this amino acid was not tested.

The PER values for almond protein (1.61 and 1.62) reported by Hall et al. (14) are considerably higher than the value of 0.39 obtained in the experiment summarized in table 4. The highest value observed in other experiments performed in this laboratory was 0.68. The variation in results observed might be explained either by the difference in methods used or the variety of almond tested. In the present work, 21-day-old male rats were fed 10% protein diets ad libitum, whereas Hall et al. used older animals of both sexes, and restricted feeding of 12% protein diets. This higher level of protein would increase the content of limiting amino acids and thus promote better growth. Also, restricted feeding and the use of older animals tend to decrease the differences between PER values of good and poor quality proteins (16).

Since the present investigation was begun, Girard et al. (19) reported that the addition of both lysine and methionine improved the net protein ratio and the net protein utilization of 10% almond protein diets, whereas no effect was observed when either amino acid was added alone. These results are in direct agreement with the data in table 4 concerning lysine and methionine. In the present study, however, threonine was also found to be limiting and to a lesser degree, tryptophan.

Mixtures of almond and cooked wheat and of almond and cooked rice are commonly eaten in Lebanon as dishes called *snunyeh* and *mughli*, respectively. Data in table 4 show that the addition of almond to wheat or rice had only a limited effect on protein quality. Although the values for the mixtures were significantly higher than the value for almond alone, the resultant protein mixtures were still of relatively poor quality compared with casein or with almond supplemented with amino acids. These results were to be ex-

TABLE 4

Effect of supplementation on the nutritive value of almond protein

	Level and source of protein in diet[1]	Avg gain 7 days	Avg feed intake 7 days	NPR[2]	Avg gain 28 days	Avg feed intake 28 days	PER[3] 28 days	PER corrected
		g	*g*		*g*	*g*		
1	10% casein	23	59	5.80 ± 0.50[4]	99	324	3.05 ± 0.29	2.50
2	10% almond	−1	37	2.73 ± 0.67	7	168	0.39 ± 0.24	0.32
3	10% almond + methionine, lysine, tryptophan, threonine[5]	23	67	5.22 ± 0.81	114	381	2.98 ± 0.17	2.44
4	Same as 3 less methionine	−4	33	1.91 ± 0.80	4	139	0.22 ± 0.15	0.18
5	Same as 3 less lysine	3	46	3.06 ± 0.73	25	244	1.01 ± 0.25	0.83
6	Same as 3 less threonine	3	40	3.47 ± 0.71	38	232	1.54 ± 0.33	1.26
7	Same as 3 less tryptophan	14	60	4.22 ± 0.64	88	332	2.57 ± 0.22	2.12
8	5% almond + 5% wheat	4	46	3.35 ± 0.57	22	235	1.00 ± 0.24	0.82
9	5% almond + 5% rice	6	47	3.60 ± 0.62	37	274	1.35 ± 0.20	1.11

[1] Ten rats/diet.
[2] Indicates net protein ratio.
[3] Indicates protein efficiency ratio.
[4] SD.
[5] See table 3 for supplementation levels.

pected since both wheat and rice are relatively low in lysine and threonine which are limiting in almond.

No essential differences were observed in the trends indicated by the 2 methods of biological evaluation. Both NPR and PER assays were equally sensitive to the deletion of any one of the amino acids used for supplementation, and both methods show the same degree and order of limitation of the 4 amino acids. There was close agreement also in the relative values obtained by the 2 assay methods for the mixtures containing wheat and rice.

The experimental data presented here indicate that almond can be of little value in supporting growth unless the inherent amino acid limitations are overcome by proper supplementation with other protein foods. For example, supplementation with sesame, a good source of methionine and with chickpea or lentil, good sources of lysine and threonine would improve the quality of almond considerably. However, the acceptance of such mixtures is doubtful within the existing dietary patterns in the Middle East.

Although almond is grown in relatively large amounts in Lebanon and in other countries of the Middle East, it is by no means an inexpensive food item. For this reason and because almond protein is of poor quality, it is questionable that the use of almond as infant food should be encouraged. There appear to be other better foods which can be used for supplying protein to infants.

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Free and Total Cholesterol in Human Blood Fractions[1,2]

BETTY E. HAWTHORNE, ELVEDA SMITH
AND JOSEPHINE O. PESCADOR[3]
Foods and Nutrition Department, Oregon State University, Corvallis, Oregon

ABSTRACT The micromodification of the method of Sperry and Webb by Galloway and co-workers has been adapted to the determination of cholesterol in red cells and white cells plus platelets separated by differential centrifugation from 0.4 ml of blood. Using these procedures, free and total cholesterol were determined in the serum and cell fractions isolated from the blood of 33 subjects, men and women, ranging in age from 22 to 91 years. Values for total cholesterol range from 122 to 338 mg/100 ml in sera, from 82 to 151 mg/100 ml in red cells, and from 536 to 5124 mg/100 g in the white cells plus platelets. The concentrations of cholesterol in the white cells plus platelets are higher than values previously reported. The amount of free cholesterol in white cells plus platelets varies widely among individuals, whereas cholesterol present in the free form in sera and red cells is quite constant from person to person. White cell analyses may prove valuable in investigations of lipid metabolism in humans.

Cholesterol is commonly determined in the serum fraction of blood. Few direct measurements in human blood cells have been reported. Most of these have been analyses of red cells.

Brun (1) made a comprehensive study of the cholesterol content of red blood cells in man and summarized data previously reported for normal adults. He noted that the total cholesterol found varied widely, ranging from 85 to 211 mg/100 ml or 100 g of cells. In his own study of 25 men and 25 women in normal health, total cholesterol ranged only from 125 to 150 mg/100 ml, was almost entirely in the free form, showed no relation to age and varied insignificantly diurnally or over a month's period. The range reported by Brun is generally accepted as "normal" (2–4). Several authors (5–8) have reported similar concentrations. Erickson and co-workers (9), however, studied 16 children and found values lower than those reported by Brun. Concentrations of total cholesterol ranged from 100 to 129 mg/100 g cells and up to 30% was esterified.

Data on the cholesterol content of white cells plus platelets, the "buffy layer," in both normal and leukemic subjects were provided by Boyd in a series of studies from 1933 to 1936 (10–12). Concentrations of free and total cholesterol ranged from 70 to 450 and 110 to 495 mg/100 g of cells, respectively. He reported a mean of approximately 300 mg of total cholesterol/100 g of cells for 8 normal women. This value is quoted as "average" (4).

Platelets were analyzed by Erickson and co-workers (13). They reported the free and total cholesterol content of platelets as 3 and 4%, respectively, of the dry weight or 18 and 25% of the total lipids. Woodside and Kocholaty (14) and Troup et al. (15) more recently reported that human platelets contain 12.5 and 19%, respectively, of their total lipids as cholesterol, all non-esterified.

This paper describes the procedures developed in our laboratory for determining cholesterol in blood cells separated from small samples of blood and reports the concentrations of free and total cholesterol in the sera, red cells and white cells plus platelets from 33 subjects.

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[2] Portions of the data are taken from theses presented by Josephine O. Pescador and Elveda Smith to the Graduate School of Oregon State University in partial fulfillment of the requirements for the M.S. degree, June, 1959, and June, 1961, respectively.

[3] Present address: Youngstown Hospital Association, Youngstown, Ohio.

EXPERIMENTAL

Subjects. Blood samples were obtained after overnight fasting from 19 women and 13 men ranging in age from 22 to 91 years. Subjects were selected who were generally healthy and who represented a wide range of ages. One additional male subject who was hospitalized, XX, was included. We used an opportunity to analyze a blood sample obtained the day following a coronary occlusion.

A few subjects had some known organic disorder. Others were taking regular medication. Among the women: subject WM, 28 years, was taking thyroid and an appetite depressant; GB, 33, used aspirin for migraine headaches; IM, 40, had familial hypercholesterolemia and had been following a fat-restricted diet for several years; MW, 61, suffered rheumatoid arthritis controlled by cortisone, aspirin and other drugs; CA, 61, ingested hormones and medication for allergy; and GT, 66, was taking thyroid. Among the men: subject VC, 45, had been following a calorie- and fat-restricted diet for a year; and HH, 75, had chronic atherosclerosis and had suffered a coronary occlusion 16 years earlier. Subject HH was taking several prescribed drugs including 150 to 200 mg of niacin daily.

Isolation of blood fractions. Free-flowing blood was collected from the finger-tip after a quick cut with a Bard-Parker blade. Approximately 0.3 ml of blood was allowed to clot and the serum was isolated by centrifugation. The red cell and white cell plus platelet fractions were isolated from 20 to 25 one-tenth-ml aliquots of fresh whole blood by differential centrifugation as described by Bessey and coworkers (16, 17). Ample amounts of red cells were available. To double the amount of white cells plus platelets per sample, two-thirds of the supernatant solution containing the suspended cells from 2 tubes of blood and oxalate, 0.1: 0.5 ml, were combined before the cells were isolated. Separate samples were prepared for the determinations of total and free cholesterol, allowing 5 to 6 replications for each.

Analysis of serum. Sera were analyzed for free and total cholesterol by the procedures of Galloway et al. (18), a micro-modification of the method of Sperry and Webb (19). The reagents used are the same as listed by Sperry and Webb.

Forty-microliter aliquots of serum were delivered into 0.4 ml of acetone-ethanol in 1-ml volumetric flasks. Extraction was aided by mixing with a buzzer [4] and heating. After adjustment of the volume to 1 ml and centrifugation at 0°C, triplicate 100-µl aliquots of the extract were measured for analysis of total cholesterol and duplicate 200-µl aliquots, for analysis of free cholesterol. Five microliters of 33% potassium hydroxide were added to the total cholesterol aliquots and the tubes were heated for 30 minutes at 38°C for saponification. After cooling the contents of the tubes to room temperature, acetone-ethanol was added to bring the volume to 200 µl. Using phenolphthalein as an indicator, 10% acetic acid was added to bring the contents just to excess acidity. Following the addition of 100 µl of digitonin solution, the contents were mixed and the tubes were stoppered and stored overnight at room temperature. To the 200-µl aliquots for free cholesterol, approximately 3 µl of 10% acetic acid and 100 µl of digitonin solution were added. The contents were mixed and the tubes were stoppered and stored overnight. Triplicate aliquots of 100 and 200 µl of acetone-ethanol were treated identically to total and free cholesterol aliquots for blank determinations.

The next day the cholesterol digitonide precipitates were washed with acetone-ether and with ether and then dried for 30 minutes at 105 to 110°C. Fifty microliters of glacial acetic acid were added to each tube while hot. After cooling, 100 µl of Liebermann-Burchard reagent were added to each tube in a timed sequence and the tubes were placed in a 28°C water bath in a dark cabinet for color development. Exactly 30 minutes after the addition of the color reagent, optical densities were measured at a wave length of 635 mµ. Cholesterol concentrations were calculated by reference to determinations of standard cholesterol solutions and by appropriate adjustments for dilutions.

Analysis of red cells. In adapting the procedures for determining cholesterol in

[4] Lowry et al. (21).

serum to the analysis of red cells, changes were made to insure complete and consistent extraction of cholesterol: 1) 40 µl of cells were laked in 80 µl of redistilled water and then frozen; 2) 40 µl of thawed laked samples were extracted with 0.7 rather than 0.4 ml of acetone-ethanol; and 3) heating was eliminated. Occasionally, slightly higher values were obtained for free than for total cholesterol as has been reported by others (1, 20). Most consistent values resulted from 2 further changes in procedure: 1) aliquots of organic solvent extract for total cholesterol analyses were increased to 200 µl (twice the amount used for analyses of serum), thereby increasing final spectrophotometric density readings; and 2) the concentration of the 5 µl of potassium hydroxide used for saponification was decreased from approximately 33 to 16%, i.e., a decrease to one-fourth of the original alkalinity. Values of total cholesterol increased with decreasing alkalinity. The use of an 8% potassium hydroxide solution, however, yielded values similar to those obtained with the 16% concentration. The decrease in alkalinity required an appropriate adjustment in the amount of acid used for neutralizing the aliquots after saponification.

Analysis of white cells plus platelets. Samples of white cells plus platelets were thinly distributed over the bottom of the tube as suggested by Bessey and coworkers (16), laked in 30 µl of redistilled water and then frozen. After thawing, lipids were extracted by slowly adding 500 µl of acetone-ethanol while the contents of the tube were kept in motion by holding the tube against a buzzer.[5] The mixture was centrifuged at 0°C. The supernatant solution was sucked off with a pipet and transferred to a 1-ml volumetric flask. An additional 500 µl of solvent were added for a second extraction. The mixture was boiled briefly by placing the tube in hot water for 6 to 10 seconds, centrifuged as before and the supernatant solution was combined with the first extract. Acetone-ethanol was added to bring the solvent to volume.

The small amounts of cholesterol in the samples of white cells plus platelets necessitated adjustments in procedures to yield a sufficient density of color from the final Liebermann-Burchard reaction. The sample size was doubled in isolation as stated above. The amount of cholesterol in the organic solvent aliquots was increased by measuring 375-µl aliquots of extract, evaporating and re-extracting with 100 or 200 µl of acetone-ethanol for total or free cholesterol determinations, respectively. Finally, the density of color produced was doubled by dissolving the isolated cholesterol in 25 µl instead of 50 µl of glacial acetic acid and by decreasing the volume of color reagent proportionately.

It was not practical to determine the size of sample by weighing or measuring directly the volume of the packed white cells plus platelets. Therefore, the indirect method of estimating sample size by the determination of total phosphorus was used. Sixty-one micromoles of total phosphorus were equated to 1 g of white cells plus platelets (17). We determined total phosphorus by determining "organic solvent-soluble phosphorus" in duplicate 100-µl aliquots of the organic solvent extract plus "organic solvent-insoluble phosphorus" in the cell residue after the extract was removed. The modification of the Fiske-Subbarow method for phosphorus developed by Lowry et al. (21) was adapted. Ashing mixture 1, for "organic solvent-soluble phosphorus," contained 3.25 ml of 70% perchloric acid mixed with 25 ml of 20 N sulfuric acid and diluted to 100 ml with redistilled water. Ashing mixture 2, for "organic solvent-insoluble phosphorus," contained 6.5 ml of 70% perchloric acid mixed with 12.5 ml of 20 N sulfuric acid diluted to 50 ml with redistilled water. The ascorbic acid-molybdate color reagent was prepared as described by Lowry and co-workers (21). The adequacy of the ashing mixtures for the respective fractions of white cell plus platelet samples up to 0.5 mg wet weight was demonstrated by complete clearing of samples at the end of the ashing period. The sensitivity of the reaction was verified over the range of phosphorus concentrations measured, i.e., 0.0003 to 0.0080 µM.

[5] See footnote 4.

RESULTS AND DISCUSSION

The concentrations of free and total cholesterol in the serum, red cells and white cells plus platelets of fasting blood of 33 subjects are presented in table 1. The subjects are divided by sex and listed in order of increasing age. The serum values are means of duplicate samples which differed from each other by no more than 10% for free and 5% for total cholesterol. The values obtained for red cells are means of 4 determinations, 2 on each of 2 laked samples. Each value for white cells plus platelets is the mean of 5 or 6 separate cell aliquots.

Most of the subjects taking medications did not have cholesterol concentrations in any fraction which differed notably from other subjects. Subject HH, for his age and condition, had a low concentration of cholesterol in serum, 139 mg/100 ml. This may have resulted from the niacin therapy (22). In addition, HH had the lowest red cell cholesterol, whereas the concentration of cholesterol in white cells plus platelets, 2794 mg/100 g, was the second highest measured.

The high concentration in the white cells plus platelets of hospitalized subject XX, 5124 mg/100 g, was almost twice that of any other subject, whereas concentrations of cholesterol in neither his serum nor red cells were unusually high, 205 and 133 mg/100 ml, respectively.

The amounts of cholesterol observed in the sera were similar to those frequently reported in the literature. Total cholesterol ranged from 121 to 338 mg/100 ml with the percentage in the free form ranging from 25 to 32, except for one subject. For HD, a man 31 years of age, 39% was in the free form.

The concentrations of cholesterol in the red cells were generally lower than most reported. Total cholesterol ranged from 82 to 151 mg/100 ml. Only 5 of the subjects had concentrations between 125 and 150 mg/100 ml, the "normal" range of Brun (1), whereas more than a third ranged between 90 and 97. Most was observed in the free form as has been reported by others (1, 5–8). No relationship to sex or age was apparent either in total cholesterol or in the proportion present in the free form.

The lower concentrations of cholesterol noted in the red cells in this study may be the result in part at least of differences in the methods of extraction and cholesterol analysis used. Although several methods have involved heating (1, 5, 8), we extracted with vigorous mixing at room temperature which minimized the problem of color contamination and eliminated the necessity to decolorize. Our extraction procedure was verified by finding no significant amounts of cholesterol in successive second and third extractions, even with boiling. That different methods for the determination of cholesterol yield significantly different values is recognized (1, 6). In 2 recent comparative studies (23, 24), values determined for referee sera by the method of Schoenheimer and Sperry or its modifications (18, 19) averaged 15 to 25% lower than those found by the methods (25–27) which were used by some investigators of cholesterol in red cells (6–8).

The reproducibility of analyses of red cells for the subjects in this study is indicated by the calculated standard errors (table 1), which ranged from 1 to 5% of the mean values for each individual.

The total cholesterol concentrations in the white cells plus platelets of these subjects (omitting hospitalized subject XX), 536 to 2794 mg/100 g, are approximately 5 times higher than the range of values previously reported by Boyd (10–12; see also 3, 4). The lowest values are similar to means reported for platelets by Erickson et al. (13), if their data reported on dry weight of samples are calculated to an estimated wet weight. In our study, concentrations are based on an indirect estimate of the mass of white cells plus platelets, whereas Boyd (10) used a weighed aliquot of the "buffy layer" separated from a venous sample. The methods of cholesterol analysis also differed; Boyd determined cholesterol by a manometric measurement of the oxidized digitonide.

Among the subjects in the present study no consistent relationship was noted between either sex or age and the amounts of total or free cholesterol in the white cells plus platelets. In contrast with the limited individual differences in the proportions of free cholesterol in serum and

TABLE 1

Free and total cholesterol in three blood fractions

Subject	Sex	Age	Height	Weight	Serum cholesterol[1]			Red cell cholesterol			White cell plus platelet cholesterol		
					Total	Free	Free: total	Total	Free	Free: total	Total	Free	Free: total
		years	*cm*	*kg*	*mg/100 ml*		%	*mg/100 ml*	*mg/100 ml*	%	*mg/100 g*	*mg/100 g*	%
DS	F	22	165	58	122	31	25	90 ± 2[2]	100 ± 2[2]	112	1167 ± 16[2]	448 ± 18[2]	38
SM	F	25	155	50	201	57	29	130 ± 3	127 ± 4	98	767 ± 37	434 ± 56	57
ES	F	26	157	53	155	45	29	97 ± 2	87 ± 5	90	964 ± 45	736 ± 19	76
WM[3]	F	28	173	97	210	51	25	106 ± 1	97 ± 2	92	806 ± 41	614 ± 26	76
JP	F	32	152	50	200	55	27	113 ± 2	109 ± 2	96	851 ± 41	404 ± 64	47
EB	F	33	165	63	152	48	32	113 ± 2	107 ± 2	95	1169 ± 23	526 ± 32	45
GB[3]	F	33	157	59	209	62	30	141 ± 2	142 ± 2	101	669 ± 39	407 ± 27	61
IM[3]	F	40	160	63	244	69	28	121 ± 1	111 ± 3	92	1866 ± 68	623 ± 36	33
BH	F	40	175	69	158	48	30	94 ± 1	92 ± 2	98	1011 ± 31	392 ± 27	39
ME	F	44	163	64	169	48	28	96 ± 5	93 ± 2	97	1158 ± 85	391 ± 52	34
ML	F	51	168	69	218	54	25	97 ± 1	102 ± 1	106	1087 ± 46	577 ± 43	53
CS	F	54	175	75	142	43	30	97 ± 3	92 ± 2	96	1128 ± 61	824 ± 22	73
MS	F	60	168	80	141	37	26	115 ± 1	101 ± 3	88	536 ± 15	434 ± 20	81
MW[3]	F	61	163	52	176	56	32	113 ± 1	111 ± 2	98	1078 ± 50	445 ± 31	41
CA[3]	F	61	173	68	289	86	30	93 ± 3	88 ± 2	94	890 ± 45	525 ± 39	59
AG	F	66	168	68	259	75	29	114 ± 2	107 ± 3	94	1065 ± 74	622 ± 66	58
GT[3]	F	66	165	62	214	65	30	125 ± 3	119 ± 3	95	1110 ± 64	620 ± 28	56
MH	F	71	180	70	206	52	25	94 ± 1	93 ± 3	99	2214 ± 115	1452 ± 220	66
IT	F	91	150	45	193	54	28	97 ± 2	111 ± 4	114	1271 ± 182	560 ± 66	44
WP	M	22	201	98	143	45	32	97 ± 1	97 ± 2	100	1422 ± 92	671 ± 49	47
WC	M	29	180	88	121	34	28	113 ± 1	121 ± 1	107	1590 ± 95	629 ± 8	40
HD	M	31	173	76	131	51	39	93 ± 3	94 ± 1	101	1232 ± 32	576 ± 21	47
SB	M	38	178	75	161	49	30	91 ± 1	87 ± 3	96	1006 ± 46	970 ± 41	96
EV	M	40	175	66	257	73	28	97 ± 2	102 ± 4	105	1116 ± 29	617 ± 45	55
WF	M	41	180	93	191	62	32	87 ± 1	77 ± 1	89	1114 ± 66	727 ± 14	65
GE	M	44	175	63	338	94	28	88 ± 2	93 ± 4	106	1587 ± 48	608 ± 34	38
VC[3]	M	45	191	81	255	78	31	151 ± 2	150 ± 3	99	1093 ± 46	713 ± 61	65
RM	M	48	188	83	192	56	29	103 ± 3	110 ± 1	108	943 ± 40	912 ± 64	97
AE	M	48	178	65	190	59	31	93 ± 1	88 ± 2	94	1096 ± 40	734 ± 64	67
PH	M	54	183	86	199	56	28	122 ± 4	125 ± 4	102	1242 ± 28	643 ± 20	52
XX[4]	M	55			205	63	31	133 ± 2	133 ± 3	100	5124 ± 168	1196 ± 132	23
TS	M	69	173	79	231	52	23	104 ± 1	89 ± 3	86	1080 ± 83	504 ± 60	49
HH[3]	M	75	185	70	139	42	30	82 ± 1	78 ± 1	95	2794 ± 135	1651 ± 75	59

[1] Values are means of duplicate samples which differed from each other by no more than 10% for free and 5% for total cholesterol.

[2] SE of mean.

[3] These subjects had some disorder or were taking regular medication; see text for details.

[4] Subject hospitalized; blood sample obtained day following coronary occlusion.

red cells, the content of free cholesterol in white cells plus platelets varied from 23 to 97%.

Replicate analyses of the samples of white cells plus platelets varied considerably as indicated by the standard errors calculated (table 1). This is a common problem in microanalyses of this fraction of blood (16). In this procedure variation is increased because 3 separate determinations are involved in each analysis, i.e., cholesterol, "organic solvent-soluble phosphorus" and "organic solvent-insoluble phosphorus." Although the size of the standard error limits the significance of differences between absolute values determined by these procedures, the magnitude of the cholesterol concentration is not in doubt.

The degree of reproducibility of the procedure for white cells plus platelets is illustrated by total cholesterol values, arranged from lowest to highest, determined in 10 aliquots isolated from one blood sample: 939, 940, 969, 985, 987, 1030, 1066, 1079, 1183 and 1228 mg/100 g. Further, a series of aliquots of white cells plus platelets were isolated from a single venous sample and then laked and frozen for control analyses. Total cholesterol determined in duplicate at intervals over a 2-month period, ranged from 863 to 1132 mg/100 g with a mean for 12 day's determinations of 1013 ± 25.[6]

The high concentrations of cholesterol observed in white cells plus platelets in this study are of particular interest because of the cholesterol concentrations determined simultaneously in the serum and red cells separated from the same samples of blood. The higher amounts of cholesterol observed in the white cells plus platelets are comparable to higher concentrations of other constituents in this fraction, e.g., ascorbic acid and riboflavin (16, 17). Among our subjects there was no consistent relationship between the amounts of cholesterol in white cells plus platelets and those in serum or red cells. Among the women, the lowest and highest values for total cholesterol in white cells plus platelets, 536 and 2214 mg/100 g, were noted in subjects MS, age 60, and MH, age 71, respectively. Their concentrations of cholesterol in serum were 141 and 206 and in red cells, 115 and 94 mg/100 ml. And subject CA, age 61, with the highest concentration of cholesterol in serum, 289 mg/100 ml, had only 890 mg/100 g of white cells plus platelets and 93 mg/100 ml of red cells. Among the men, approximately 1600 mg of cholesterol/100 g of white cells plus platelets were the highest concentrations found, other than those of subjects HH and XX discussed above. These values were associated with 121 and 338 mg of cholesterol/100 ml of serum and 113 and 88 mg/100 ml of red cells in subjects WC, 29 years, and GE, 44 years, respectively.

The functions of cholesterol in blood, as in other tissues, are not well defined. Kritchevsky (28) speculated that free cholesterol in serum plays an integral metabolic role as a substrate for the structure of cells and for steroid synthesis, whereas the esterified cholesterol functions as part of the fatty acid transport mechanism. In the red cells, cholesterol is generally assumed to serve primarily in the structure of the cell wall. The roles of cholesterol in leucocytes and platelets are not known. Evidence has been presented (29–32) that the leucocytes are active in lipid synthesis and that the rate of synthesis increases in phagocytosis (33) and with increasing cell age (34).

The proportion of cholesterol observed in the free form in tissues has been related to function. Bloor and co-workers (35, 36) reported that with an increase of cell activity in any tissue the amount of free cholesterol increases, whereas with lowered activity, degeneration or retrogression of a tissue or organ, a decrease of free and an increase in esterified cholesterol occurs. The high and relatively constant proportion of free cholesterol observed in the red cell has been attributed to its active structural role (5, 6, 8). The significance of the variable amounts of free cholesterol observed in the white cells plus platelets is not known, but may indicate metabolic functions varying in degree from person to person or time to time. Boyd reported increases of as much as 200% in concentrations of free cholesterol

[6] SE of mean.

and phospholipids in human leucocytes in normal recovery from surgery or fever, whereas when recovery was not normal decreases to as low as one-half normal values occurred (37, 38). Lipid composition was not correlated with variations in total white cell counts, but free cholesterol and phospholipids were reported to be roughly proportional to the number of neutrophils (12).

In this study the higher concentrations of cholesterol observed in the white cells plus platelets compared with those in serum or red cells and the wide individual variation in the amounts noted in the free form indicate the desirability of increased use of leucocytes as a tool in human metabolic studies of cholesterol and other lipids. Since the metabolic mechanisms of leucocytes are similar to those of cells of other active tissues (39), changes in the concentration of cholesterol in leucocytes may be indicative of changes occurring in cells of other less accessible tissues. The procedures presented in this paper can be used for the simultaneous measurement of cholesterol in serum, red cells and white cells plus platelets separated from small samples of blood.

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Studies in Infantile Malnutrition

II. EFFECT OF PROTEIN AND CALORIE INTAKE ON WEIGHT GAIN[1]

GEORGE G. GRAHAM, ANGEL CORDANO AND JUAN M. BAERTL
British American Hospital, Lima, Peru

ABSTRACT The effect of different levels of protein and calorie intake on the weight gain of severely malnourished infants 6 to 30 months of age was studied in relation to the weight deficit. In the most severely underweight, 175 kcal/kg/day were necessary to establish an adequate rate of gain; intakes of more than 2.0 g of protein/kg/day were apparently superior to lower levels. In the less severely malnourished, progressively decreasing caloric intakes were necessary and 2.0 g of protein/kg/day or even less, evenly distributed throughout all feedings, were not inferior to higher levels, particularly if the source was milk. In the least severely undernourished or those well advanced in their recovery, a good mixture of vegetable proteins or a mixture of wheat and fish proteins appeared to be as efficacious as milk. These results were similar in marasmic infants or in cases of marasmic kwashiorkor after diuresis — confirming previous suggestions that there is no proven advantage to levels of protein intake above the usual and that on the other hand caloric requirements are directly proportional to the severity of the malnutrition. The practical implications of this study in the planning of feeding programs are discussed.

During the past decade there has been a growing awarness of the frequency with which severe protein deficiency occurs in the pre-school children of all the underdeveloped countries of the world. The epidemiology and the clinical characteristics of kwashiorkor have received a great deal of attention and notable efforts are being made to develop low-cost, high-protein foods. In certain areas it has been pointed out that protein deficiency in an isolated form is rare and that most commonly it accompanies or is even secondary to severe caloric deficiency (1); as a result, the expressions "marasmic kwashiorkor" and "protein-calorie malnutrition" are being used ever more frequently. In Peru (2), and we suspect in most other countries, severe infantile marasmus is far more common than kwashiorkor and is responsible in great part for the very high infant mortality; at the same time, varying degrees of caloric undernutrition play an important role in determining the physical and mental capabilities of the population.

In our experience kwashiorkor, or much more commonly marasmic kwashiorkor, represents an acute insult superimposed on a more or less prolonged state of undernutrition which has followed an initial period of relatively satisfactory breast feeding in the first year of life (2). Consequently it is seen late in the first year of life or during the second year, while the much more frequent marasmus, the result of prolonged and often life-long starvation, occurs principally in the first year. A very high initial mortality is the rule in kwashiorkor but once the initial danger is overcome, recovery tends to proceed satisfactorily and its rate depends to a great extent on the degree of caloric deprivation which preceded it and which influences the caloric requirements and the ease or difficulty with which they can be met.

Because of the emphasis put on protein deficiency as the major cause of infantile malnutrition, it is unfortunately the rule rather than the exception to see severe marasmus being treated with very high protein intakes and grossly inadequate caloric intakes. At the same time, inordinately high levels of dietary protein have been recommended for the treatment of kwashiorkor and are being used in many areas. During the initial stages of treat-

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ment patients with kwashiorkor do have the ability to retain a very high proportion of ingested nitrogen despite caloric intakes which subsequently prove inadequate to maintain body weight and adequate nitrogen retentions. After this initial stage, however, they behave exactly as all other infants, well nourished or not: if caloric needs are not met, protein is used as a source of energy, and growth will not be promoted. Waterlow and Wills (3) have emphasized the relatively low protein requirements and the high caloric needs of malnourished infants, and Montgomery (4) has documented the much increased basal energy requirements of the recovering malnourished infant.

It is a point of great practical importance to know what levels of protein and calories are to be recommended for the treatment and prevention of undernutrition. Hansen (5) has emphasized the three most important criteria to be used in judging the adequacy of the diet from this point of view: weight gain, nitrogen balance and levels of serum protein. In most experimental animals growth can be accelerated consecutively by raising the level of protein in the diet, but in normal infants (6) and in our experience with infants recovering from severe malnutrition, once a minimal protein requirement is met, growth is almost exclusively dependent on total caloric intake. Dean (7) was not able to show any correlation between protein intake and weight gain in children recovering from kwashiorkor. The more chronic and severe the malnutrition, the higher the caloric ingestion must be before satisfactory rates of growth are obtained. Weight gain is not synonymous with growth but is one important aspect of it and during short periods of observation is the only one easily measured. In the present paper we will consider it alone as an index of adequacy, but subsequently we will describe the changes in nitrogen balance and serum proteins resulting from different levels of protein and calories in the diet. We have made an effort to determine the minimal requirements of protein and calories for satisfactory weight gain in infants with marasmus and marasmic kwashiorkor and also to determine whether for a given level of caloric intake there is any apparent advantage to an additional intake of protein over and above the minimum.

METHODS

During the past 27 months 50 severely malnourished infants were admitted to our metabolic ward for treatment and study. Attention was initially given to their acute electrolyte imbalance and to the detection and treatment of apparent or inapparent septicemia, pyelonephritis and other important infections. As soon as possible after admission, dilute feedings of a modified cow's milk preparation[2] were started and increased to tolerance as rapidly as possible. Diets were recalculated daily to yield pre-established quantities of calories and protein per kilogram of body weight. In the majority of cases, a level of 2.0 g of protein and 75 kcal/kg/day was rapidly reached. When this failed to produce satisfactory gain in weight, calories were increased by adding cottonseed oil and cane sugar, maintaining the same relative proportion of carbohydrate and fat as in the original preparation.

In certain cases, when hypoalbuminemia was present or developed, with or without apparent edema, the protein intake was increased by substituting milk or casein[3] for oil and sugar. The caloric intake ranged from 75 to 175 kcal/kg/day. In some of the older infants the protein was intentionally reduced to 1.5 g/kg/day and in some of the smaller infants, to see whether weight gain could be accelerated without any increase in caloric ingestion, it was made as high as 5.0 g/kg/day. In a number of patients the protein intake was 3.0 g/kg/day during the entire period of study; as a result most of the data for intakes of over 2.0 g of protein correspond to intakes of this level.

In a number of cases, after a steady rate of gain in weight had been established, the source of protein was changed to a mixture of approximately equal parts of wheat and fish proteins (8) or to a mixture of vegetable proteins based on cottonseed flour (9), maintaining the same level of protein ingestion. In a few cases, one of

[2] Similac with iron, Ross Laboratories, Columbus, Ohio.

[3] Casec, Mead Johnson International, Evansville, Indiana.

these mixtures was used from the onset of treatment. Once a satisfactory or excessive rate of gain was established, or if the patient began to refuse feedings, the caloric intake was decreased accordingly. Thus, the higher caloric intakes were given most commonly during the middle of the observation period and the lower intakes at the beginning or end of hospitalization. Nitrogen balance studies were made at selected intervals and will be the subject of another report, as will be the serial serum protein values.

Weight gain for each change in the diet has been calculated by dividing the grams gained (or lost) by the duration in days of the period. We have excluded from this report all periods in which the serum albumin was less than 3.0 g/100 ml, as below this level there is some likelihood that changes in weight might be due in part to changes in water and sodium balance. Thus, by definition, nearly all periods of edema are excluded from consideration, most particularly the initial periods of the patients with kwashiorkor. Also, when there had been a significant apparent infection, the corresponding period was excluded. Only those patients between 6 and 30 months of age are included in the present evaluation. Patients with kwashiorkor on admission were included once their serum albumin had reached a level of 3.0 g/100 ml. Only 25 of our patients had one or more carefully controlled dietary periods satisfying the above requirements.

For purposes of comparison our patients have been classified as to their degree of malnutrition. This has been estimated on the basis of weight, calculating a "developmental quotient" for the beginning of each period: the actual weight was converted to the age in months to which it corresponded on a curve for healthy Peruvian children of the same racial background (10). This "weight age" divided by the chronolgic age and multiplied by 100 was taken as the "developmental quotient." Body weight reflects acute as well as chronic nutritional deficits whereas height is influenced to any degree by chronic deficits only. Because nearly all our patients were notably short in stature and because this measure changed slightly or not at all during the individual dietary periods, we have not taken it into account in our calculation. The patients have been classified into 4 categories according to their "developmental quotient" at the beginning of each dietary period: group 1, less than 10%; group 2, 10 to 29%; group 3, 30 to 49% and group 4, 50% or more. The corresponding quotient for height was seldom over 50%; thus when patients reached this quotient for weight they usually were obese. According to a patient's status at the beginning of each dietary period, any one patient could be in any or all of the 4 categories at different times.

RESULTS

Table 1 summarizes the changes in weight resulting from different caloric intakes in each nutritional category. The figures in parentheses indicate the number of days of observation which went in to make the average figure given for a particular change in weight and represent the pooling of the results of one or more patients. A direct relationship between caloric intake and weight gain is evident in each category, as well as between nutritional

TABLE 1

Effect of nutritional state on weight gain at different levels of caloric intake

		Intake in kcal/kg/day				
		75	100	125–130	150	175
Group	$\frac{\text{Wt age}}{\text{Age}} \times 100$[1]	Average weight gain[2]				
		g/day	*g/day*	*g/day*	*g/day*	*g/day*
1	Less than 10	−13(30)	6 (49)	10 (79)	16(153)	30(250)
2	10–29	−14(33)	10(192)	22(495)	29(374)	39(253)
3	30–49	−14(10)	11 (13)	30(319)	39(295)	—
4	50 or more	5(82)	19(119)	34(248)	55 (90)	—

[1] "Developmental quotient" at beginning of period; see Methods section for explanation.
[2] Figures in parentheses show number of days of observation.

state and weight gain for each level of caloric intake: the greater the weight deficit, the less the weight gained at any given level of caloric intake. If we assume that approximately 30 g daily is a satisfactory weight gain for patients of this age recovering from severe malnutrition, we can see that for those whose developmental quotient was less than 10% (group 1), 175 kcal/kg/day were necessary to achieve this rate of gain, whereas for those with a quotient of 10 to 29% (group 2), only 150 kcal were needed. For those with quotients of 30 to 49% and 50% or better, 125 to 130 kcal/kg/day were adequate.

In table 2 the weight changes for each nutritional category have been separated for 2 different levels of protein intake: 2.0 or less, and over 2.0 g of protein/kg/day. In the most severely malnourished (group 1), for each level of caloric intake producing a gain in weight, there is an apparent advantage to the higher level of protein intake, suggesting that 2.0 g of protein/kg/day does not meet the minimal requirement. In group 2, those with developmental quotients of 10 to 29%, the same advantage appears to prevail. In the 2 higher categories there is no apparent advantage to the higher intake of protein.

Since a significant percentage of the figures for intakes of 2.0 g or less of protein are accounted for by periods with the wheat-fish or vegetable mixtures as the source of protein, in table 3 the figures have been further subdivided as to the source of protein. Since no infants with developmental quotients of less than 10% were fed these sources of protein, the lowest category in this table is that of group 2 and here, at the lower caloric levels, there appears to be an advantage to milk which is not evident in the other categories and which accounts for the apparent disadvantage of the lower protein intake in table 2. The fact that this difference is not evident at the higher levels of caloric intake suggests that these proteins were utilized more efficiently when additional calories were provided.

DISCUSSION

The above results indicate that in malnourished infants 6 to 30 months of age, once a minimal protein and calorie requirement is met, weight gain depends on the caloric intake. In the most severely undernourished, receiving from 100 to 175 kcal/kg/day, there is a decided advantage to protein intakes of over 2.0 g/kg/day, suggesting that this figure is below the minimal protein requirement for infants with weight ages which are less than 10% of their chronologic age. For infants with developmental quotients for weight of 10 to 29%, 2.0 g of protein/kg/day is apparently adequate if the source of protein is of a high quality, such as cow's milk. For infants 6 to 30 months of age

TABLE 2

Effect of protein intake and nutritional state on weight gain at different levels of caloric intake

			Intake in kcal/kg/day				
			75	100	125–130	150	175
Group	$\frac{\text{Wt age}}{\text{Age}} \times 100$[1]	Protein intake	Average weight gain[2]				
		g/kg/day	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*
1	Less than 10	2.0 or less	−12(27)	−6 (18)	5 (21)	11 (98)	28(228)
		over 2.0	−23 (3)	12 (37)	12 (58)	24 (55)	44 (22)
2	10–29	2.0 or less	−14(33)	3 (65)	20(201)	28(314)	35(164)
		over 2.0	—	13(127)	24(294)	31 (60)	47 (89)
3	30–49	2.0 or less	−26 (6)	11 (13)	29(235)	40(194)	—
		over 2.0	3 (4)	—	33 (84)	37(101)	—
4	50 or more	2.0 or less	5(82)	19(119)	36(217)	55 (90)	—
		over 2.0	—	—	23 (31)	—	—

[1] "Developmental quotient" at beginning of period; see Methods section for explanation.
[2] Figures in parentheses show number of days of observation.

TABLE 3

Effect of protein source, protein intake and nutritional state on weight gain at different levels of caloric intake

Group	Wt age / Age × 100[1]	Protein source	Protein intake	Intake in kcal/kg/day							
				75		100		125–130		150	175
				Average weight gain[2]							
			g/kg/day	*g/day*		*g/day*		*g/day*		*g/day*	*g/day*
2	10–29	Vegetable or mixed	2.0 or less	−18(10)		−7 (18)		8 (54)		29(123)	34 (29)
		Cow's milk	2.0 or less	−13(23)		7 (47)		24(147)		27(191)	35(135)
		Cow's milk	over 2.0	—		13(127)		24(294)		31 (60)	47 (89)
3	30–49	Vegetable or mixed	2.0 or less	—		—		26(135)		39(128)	—
		Cow's milk	2.0 or less	−26 (6)		11 (13)		33(100)		43 (66)	—
		Cow's milk	over 2.0	3 (4)		—		33 (84)		37(101)	—
4	50 or more	Vegetable or mixed	2.0 or less	10(37)		21 (67)		37(193)		55 (90)	—
		Cow's milk	2.0 or less	1(45)		17 (52)		32 (24)		—	—
		Cow's milk	over 2.0	—		—		23 (31)		—	—

[1] "Developmental quotient" at beginning of period; see Methods section for explanation.
[2] Figures in parentheses show number of days of observation.

with lesser degrees of malnutrition an intake of 2.0 g of protein/kg/day is apparently adequate whether the source is milk, equal parts of wheat and fish protein, or a good vegetable mixture. Below the age of 6 months the requirements are somewhat higher and above the age of 30 months they are lower, as is to be expected. Actually, this dietary level of protein has proven satisfactory even in the early stages of treatment of kwashiorkor, provided the edema-free weight, a true reflection of the underlying nutritional state, does not put the infant in the lowest category of developmental quotient for weight, which is frequently the case in infants we have studied. These, once diuresis has occurred, are just as devoid of subcutaneous fat and have just as severe wasting of muscle as most of the marasmic infants and consequently have similar protein and calorie requirements.

In infants with kwashiorkor and obvious edema, if the vegetable or wheat-fish protein source was given from the beginning of treatment, there was a long delay in the correction of the hypoalbuminemia and simultaneously a delay in the clearing of edema. Paradoxically, however, these infants were able to gain weight, retain apparently adequate amounts of nitrogen and improve in their general condition while maintaining serum albumin values at approximately 2.0 g/100 ml, a level at which edema is reported to occur easily. If they are subequently given milk as the source of protein, they will retain a much higher percentage of absorbed nitrogen for a period of 10 to 14 days and very rapidly correct the hypoalbuminemia. Scrimshaw et al. (11) have reported lower serum protein levels in children fed their vegetable mixture for prolonged periods than in children of similar ages receiving milk. We have noted similar values, with the lower total serum proteins being accounted for by a lower serum albumin. This suggests a definite advantage for milk in the regeneration and maintenance of serum albumins which is not evident for other body proteins.

The protein requirements reported here are very similar to the average minimal requirements given by the FAO for children of this age (12). We did not attempt

in each child to determine the lowest ingestion of protein that could maintain an adequate rate of weight gain; if we had, it is likely that this figure would have been even closer to the FAO values. In brief, it appears that the protein requirements of malnourished infants are very close to, if not identical to, those of normal infants of the same age, or rather of the same age as the "developmental age" of our patients.

In the economy of an individual, a family, a hospital, or an entire community, it is important to know how much of the limited resources available should be spent on protein to assure normal growth of infants and children. If a given level of intake can promote satisfactory recovery in the severely malnourished, as demonstrated by growth at a normal or accelerated rate, and at the same time maintain normal serum protein values, it is logical to assume that this same level can prevent malnutrition if caloric and other needs are met. In these studies the protein ingestion was evenly distributed throughout all the food taken, and this in turn throughout the greater part of the day, probably making the utilization of protein as efficient as possible. The same is true of the FAO minimal requirements for normal infants and children cited previously. In any feeding program, when a protein supplement is given only once during the day, and no account is taken of the total caloric intake during the entire day, it is likely that this protein will not be used efficiently if there is a co-existing caloric deficit. We believe it is important to take this into account in formulating food supplements. If these are of a high protein content and are given only once during the day, they very likely will not prove as effective as another food with an ample caloric content and a normal protein content which might more readily be consumed throughout the day, as is the case with cow's milk. There is no evidence for the existence of protein "stores" over and above those in the liver and plasma usually considered as such, which might be achieved through intakes of protein higher than the minimum necessary for growth, or which might help an individual cope with periods of low protein intake or increased catabolism or decreased anabolism, or both, during periods of significant infection.

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Glutathione and the Antithyrotoxic Effect of Methionine[1]

L. W. CHARKEY AND D. F. HOUGHAM
Department of Chemistry, Colorado State University, Fort Collins, Colorado

ABSTRACT Glutathione (GSH) has been measured in blood and liver of white Leghorn cockerels subjected to deleterious dietary levels of iodinated casein and desiccated thyroid as thyroactive stressors, in diets deficient and sufficient with respect to methionine. GSH levels, as measured by the method of Kay and Murfitt, were decreased in both blood and liver by each of the thyroactive materials, and increased in both blood and liver by methionine. Methionine also has counteracted the thyroactive materials in terms of growth and feed utilization efficiency (decreased by iodocasein or thyroid). Since thyroxine uncouples oxidative phosphorylation in mitochondria, and glutathione reportedly counteracts thyroxine in this action, the present observations are construed as evidence for an antithyrotoxic action of methionine. The possibility of a similar action on the part of other nutrients is considered. These studies, while not disproving the existence of the postulated unidentified antithyrotoxic factor (ATF), render its existence open to question.

As reported previously (1) dietary DL-methionine can counteract in chicks the deleterious effects of 0.05% dietary iodocasein[2] on growth, feed utilization efficiency and oxygen consumption in vivo. Further studies have disclosed that methionine bears a different relation to 2,4-dinitrophenol (DNP), a non-thyroactive stressor, than it does to iodocasein. On the basis of these observations it has been suggested that methionine has an antithyrotoxic effect in chicks (2).

Thyroxine, a stimulant of oxidative metabolism, also is well established as an uncoupler of oxidative phosphorylation in cell mitochondria (3–5). Such uncoupling could well account for the loss of metabolic efficiency caused by iodocasein, since thyroxine is derived by digestion of iodocasein (6, 7).

Park et al. (8) have reported that glutathione (GSH) reversed the uncoupling of oxidative phosphorylation caused by thyroxine in vitro, but not that caused by DNP. Since in our studies methionine had counteracted all effects of iodocasein in vivo, and since methionine is metabolically a precursor of GSH, it seemed pertinent to determine how tissue levels of GSH are affected by dietary administration of thyroactive stressors and by dietary level of methionine.

METHODS

All experiments reported herein were conducted in one-day-old Single Comb White Leghorn cockerel chicks from commercial hatcheries of well known standard inbred lines. The basal diet was the same methionine-deficient practical-type diet (MS-1) for all experiments. This diet consisted, in per cent, of yellow corn meal, 66.0; soybean meal (50% protein), 13.5; dehydrated alfalfa meal (17% protein), 5.88; dried brewer's yeast, 5.00; gelatin, 4.00; corn oil, 4.00; steamed bone meal, 1.70; limestone, 1.00; DL-phenylalanine,[3] 0.214; DL-tryptophan, 0.041; iodized NaCl, 0.500; KCl, 0.200; $MgSO_4$, 0.242; and vitamins and trace minerals in milligrams per kilogram of diet as follows: $MnSO_4$, 50; $FeSO_4$, 20; $CuSO_4$, 2.0; $ZnCl_2$, 0.2; $CoCl_2$, 0.2; pyridoxine·HCl, 2.50; folic acid, 0.50; biotin, 0.10; and vitamin B_{12}, 0.025.

Experimental groups were selected, after a 2-day stabilizing period with corn meal, by a systematic body weight-equalization procedure, and placed in individually heated, wire mesh-bottom, all-metal pens

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[2] Protamone, Cerophyl Laboratories, Kansas City, Missouri.
[3] We are indebted to the Dow Chemical Company, Midland, Michigan, for certain amino acids for diet supplementation.

in batteries housed in an air conditioned, constant-temperature chick room. They were fed and watered ad libitum at all times.

Experimental criteria were body weight gain and feed consumption recorded weekly, oxygen consumption in vivo, and blood and liver content of GSH. The GSH determinations were made by the method of Kay and Murfitt (9) as adapted by us for application to liver as well as to blood, and to large numbers of samples within the time limitations given in the original procedure. This was largely a matter of scheduling reagent additions and final readings.

Kay and Murfitt in developing their procedure studied possible interference by glycine, methionine, cysteine, several other amino acids and several sequestering agents. A number of these had observable effects. But the authors were able to provide evidence based on ultraviolet absorption spectra that responses to deproteinized blood were specific for GSH. Three possibly interfering compounds not mentioned by Kay and Murfitt are homocysteine, oxidized glutathione (GSSG) and cystathionine. For the purposes of the present studies it was necessary to know that these were not exerting any large effect in the analytical system. Although analytical refinements will not be described in this report, these 3 substances have been tested. They were added separately and to the deproteinized supernatants of both blood and liver at levels equivalent to 2, 5 and 10 mg of each/100 ml blood (or 100 g liver). These levels are in excess of what might be expected for any of them except GSSG. In this range GSSG was without observable effect. Homocysteine resembled cysteine in these tests, which corroborated those of Kay and Murfitt on cysteine, in that both led to a reduced absorption. Cystathionine also led to slightly reduced absorption, observable only at the highest level (10 mg) used.

It thus appears that the effect of increasing the level of any of these compounds could only be a reduction in observed analytical values. In all probability any dietary treatment leading to higher tissue contents of GSH would lead also to higher tissue content of its biosynthetic precursors, homocysteine, cystathionine and cysteine. This would minimize, rather than account for, the differences in GSH values with treatments reported in the present paper. The method of Kay and Murfitt was described by them only in application to blood. Here it has been applied as well directly to trichloroacetic acid supernatants of chick liver. Care was taken to use the same trichloroacetic acid-to-protein ratio in deproteinization of both liver and blood.

RESULTS

Systematic measurements of GSH have been made on blood and liver samples from 5 different feeding trials, data from which are the substance of this report. Since 3 of the 5 involved the same dietary treatments, data from these 3 are collected, as averages for each treatment for each sampling date, in table 1.

The data show beyond reasonable doubt that (a) dietary methionine, with or without iodocasein, increased GSH in both liver and blood; (b) dietary iodocasein, with simultaneous methionine deficiency, decreased GSH in both liver and blood; (c) dietary iodocasein, with methionine sufficiency, decreased GSH in blood, but not in liver.

The levels of iodocasein and methionine used in the foregoing were based on previous experience with both in Leghorn chicks. The 0.05% level of iodocasein had been fully demonstrated to be deleterious in terms of reduced growth and feed efficiency, and increased oxygen consumption accompanying reduced efficiency. The 0.362% level of supplemental DL-methionine is considered plentiful to superabundant, since similar responses subsequently have been obtained with slightly more than one-half that amount.

Data on growth, feed utilization efficiency, liver percentage of body weight, and in some cases oxygen consumption were recorded in the present experiments but are not reported in detail for the experiments with iodocasein. Space is limited, and effects on these criteria in the same animal on the same dietary regimens have been the subjects of earlier publications (1, 2). In keeping with that earlier experience, iodocasein in the pres-

TABLE 1

Average glutathione (GSH) content of blood and liver of cockerels as affected by dietary iodocasein and DL-*methionine*

Exp. no.	Age of chicks	Blood				Liver			
		Basal	Iodocasein, 0.05%	Methionine, 0.36%	Iodocasein, 0.05% + Methionine, 0.36%	Basal	Iodocasein, 0.05%	Methionine, 0.36%	Iodocasein, 0.05% + Methionine, 0.36%
	days	*mg GSH/100 ml*		*mg GSH/100 ml*		*mg GSH/100 g*		*mg GSH/100 g*	
MS–4	10	57.8 (8)[1]	54.1 (7)	64.4 (6)	56.3 (5)	60.2 (8)	53.2 (7)	80.4 (7)	72.7 (7)
MS–5	13	64.6 (7)	54.0 (6)	59.5 (7)	59.8 (7)	55.4 (6)	53.8 (7)	71.4 (8)	68.9 (8)
	20	43.8 (7)	43.7 (8)	48.6 (7)	44.8 (8)	61.6 (8)	66.2 (8)	100.2 (8)	83.0 (7)
	27	62.0 (8)	56.8 (8)	64.1 (8)	71.6 (7)	77.2 (8)	76.4 (8)	128.1 (8)	151.2 (8)
	34	64.1 (8)	62.4 (7)	74.9 (8)	64.9 (8)	87.8 (8)	66.7 (8)	127.9 (8)	116.5 (8)
	41	53.8 (8)	51.6 (8)	51.8 (8)	63.5 (8)				
	48	67.1 (6)	57.5 (8)	60.1 (8)	64.6 (7)	97.4 (6)	65.3 (8)	128.2 (8)	85.1 (8)
MS–6	9	59.2 (6)	55.6 (6)	70.9 (6)	65.0 (6)	64.3 (5)	60.2 (6)	99.5 (6)	83.4 (6)
	23	41.4 (6)	40.2 (6)	44.8 (6)	40.0 (6)	63.0 (6)	58.7 (6)	131.7 (6)	129.6 (6)
	37	46.8 (6)	36.4 (6)	63.9 (6)	48.3 (6)	56.2 (6)	65.6 (6)	151.7 (6)	143.2 (6)
	51	38.3 (6)	35.0 (6)	51.7 (6)	42.1 (6)	68.5 (6)	40.0 (6)	132.6 (5)	152.9 (6)
Avg, all experiments		54.4(76)	49.8(76)	59.5(76)	56.4(74)	69.2(67)	60.6(70)	115.2(70)	108.6(70)

t tests for significance of mean differences between overall averages above ($t_{0.05} = 1.99$; $t_{0.01} = 2.64$).

Comparison	Observed *t*	
	Blood	Liver
Effect of iodocasein, methionine-deficient	4.02	2.47
Effect of iodocasein, methionine-sufficient	2.41	1.80
Effect of methionine, iodocasein absent	3.39	6.30
Effect of methionine, iodocasein present	4.54	4.66

[1] Numbers in parentheses indicate the number of chicks represented in each GSH mg/100 ml average value entered in the table.

ent studies again decreased growth and feed efficiency, and increased oxygen consumption in vivo. Methionine again counteracted iodocasein in all these respects. Now it is observed in addition that these effects of iodocasein were accompanied by reduced tissue levels of GSH, and those of methionine by increased levels of GSH.

Because 0.05% iodocasein failed to reduce liver levels of GSH with dietary methionine sufficiency, the effects of higher levels of iodocasein were studied; and in view of possible antagonism, higher levels of methionine also. Accordingly an experiment was set up to determine interactions between 4 levels of methionine and 4 levels of iodocasein. These levels, and observed blood and liver content of GSH, are shown in table 2. No noteworthy differences were observed between GSH levels at 5, 8, 11, 14 or 17 days of age; therefore data from all 5 sampling dates are pooled in the averages shown.

From table 2 it appears clear that, at higher levels of dietary administration (0.2 to 0.6%), iodocasein reduced blood and liver contents of GSH at all methionine levels. This conclusion appears questionable only in blood from methionine-deficient chicks. Methionine again led to increased blood and liver contents of GSH, the effect appearing doubtful at the highest level (0.6%) of iodocasein, possibly because of an unexpectedly high basal value.

Thus there can be little doubt that dietary methionine and dietary iodocasein counteracted each other in terms of blood and liver content of GSH in young Leghorn cockerels.

It was desired to evaluate the observations as they might bear on a possible antithyrotoxic role of methionine in metabolism. Since iodocasein cannot at best be regarded as a natural hormone, such application of data obtained with iodocasein

TABLE 2

Blood and liver glutathione in relation to dietary DL-*methionine and iodocasein*

DL-Methionine added to diet	Percentage iodocasein in diet				Averages
	0.0	0.2	0.4	0.6	
%	*mg glutathione/100 ml blood or 100 g liver*				
0.0	*51.5*[1](10)	*52.4*(10)	*46.7* (9)	*50.6*(10)	*50.3*
	98.2 (10)	79.1(10)	87.8(10)	76.3(10)	85.3
0.2	*63.0* (10)	*58.2* (8)	*52.6*(10)	*48.7* (9)	*55.6*
	129.6 (9)	106.7 (9)	84.8(10)	81.0 (9)	100.5
0.4	*64.1* (10)	*56.9*(10)	*51.1*(10)	*52.4* (9)	*56.1*
	143.3 (10)	112.5(10)	105.7(10)	103.1(10)	116.1
0.6	*59.8* (9)	*55.7*(10)	*54.3*(10)	*51.1* (9)	*55.2*
	152.1 (10)	116.1(10)	98.0(10)	83.0 (9)	112.3
Averages	*59.6*	*55.8*	*51.2*	*50.7*	
	130.8	103.6	94.1	85.8	

Significant effects found. ($F_{0.05} = 3.96$; $F_{0.01} = 6.96$)

In blood
for 0.4% iodocasein over 0.0% iodocasein ($F = 8.84$)
for 0.6% iodocasein over 0.0% iodocasein ($F = 23.2$)
for 0.4% methionine over 0.0% added methionine ($F = 5.26$)

In liver
for 0.4% iodocasein over 0.0% iodocasein ($F = 30.1$)
for 0.6% iodocasein over 0.0% iodocasein ($F = 92.5$)
for 0.4% methionine over 0.0% added methionine ($F = 31.2$)
for 0.6% methionine over 0.0% added methionine ($F = 16.2$)

[1] Each number entered in the body of the table is an average of values from the number of chicks indicated in parentheses. The upper italicized number of each pair is the average value for blood, the lower number for liver. Each value is an average of individual values at 5, 8, 11, 14 and 17 days of age; no temporal effect was noted.

might be held invalid. To resolve this question another experiment, similar to that reported in table 2, was conducted with desiccated whole thyroid powder (bovine) as the source of thyroactive substances.

Effects of dietary desiccated thyroid and methionine on blood and liver levels of GSH are shown in table 3. The relationships of blood and liver levels of GSH to other observed criteria at 13 days of age only are shown in table 4. Since no noteworthy differences were observed between GSH levels at 5, 9, 13 or 20 days of age, data from all 4 sampling dates are pooled in the averages shown in table 3.

Again methionine led to increased tissue levels of GSH, slightly more effectively at lower levels of thyroid in the diet. As with iodocasein thyroid powder reduced tissue levels of GSH, with, again, the most questionable instance being in blood at low levels of methionine. At similar levels of dietary administration, iodocasein and thyroid powder have produced in chicks qualitatively similar effects on blood and liver levels of GSH. However, on a gram for gram basis, iodocasein was much the more potent stressor of the two.

Effects of the dietary treatments on the several observed criteria at 13 days of age are shown in table 4. Experience has indicated that these effects usually are not regular until about 2 weeks of age and thereafter. In the present experiment all groups were being depleted of chicks to provide materials for GSH assay, so that after 13 days the numbers of chicks remaining were too small to provide reliable measures for some of the criteria. The data in table 4 indicate, however, that in general methionine increased growth and efficiency of feed conversion to body weight, increased tissue levels of GSH and decreased liver weight relative to body weight. The effects of desiccated thyroid were generally opposite for all criteria used.

Much more striking, however, are the correlations computed from the data of the table. It appears that in the range of thyrotoxic stress encountered in this study the metabolic efficiency of the chicks paralleled concentrations of GSH in the blood

TABLE 3

Blood and liver glutathione in relation to dietary DL-*methionine and desiccated thyroid*

DL-Methionine added to diet	Percentage desiccated thyroid in diet				Averages
	0.0	0.2	0.4	0.8	
%	*mg glutathione/100 ml blood or 100 g liver*				
0.0	*40.0*[1] (14)	*43.6* (14)	*45.3* (15)	*35.5* (14)	*41.1*
	78.7 (17)	77.8 (16)	74.8 (16)	73.7 (16)	76.2
0.2	*47.0* (14)	*49.1* (15)	*48.6* (15)	*41.5* (17)	*46.5*
	116.5 (17)	101.4 (16)	97.6 (17)	95.0 (17)	102.6
0.4	*49.2* (12)	*47.0* (16)	*44.2* (15)	*41.0* (17)	*45.3*
	117.5 (17)	112.4 (17)	103.0 (17)	104.6 (17)	109.4
Averages	*45.4*	*46.6*	*46.0*	*39.3*	
	104.2	97.2	91.8	91.1	

Significant effects found ($F_{0.05} = 3.94$; $F_{0.01} = 6.90$)

In blood
- 0.2% methionine over 0.0% added methionine ($F = 23.5$)
- 0.4% methionine over 0.0% added methionine ($F = 13.8$)

In liver
- 0.4% thyroid over 0.0% thyroid ($F = 6.45$)
- 0.8% thyroid over 0.0% thyroid ($F = 9.34$)
- 0.2% methionine over 0.0% added methionine ($F = 93.6$)
- 0.4% methionine over 0.0% added methionine ($F = 27.3$)

[1] Each number entered in the body of the table is an average of values from the number of chicks indicated in parentheses. The upper italicized number of each pair is the average value for blood, the lower number for liver. Each value is an average of individual values at 5, 9, 13 and 20 days of age; no temporal effect was noted.

TABLE 4

Effects of dietary DL-*methionine and desiccated thyroid on various criteria*

Dietary treatment	Criterion observed at 13 days of age				
	Avg body wt	Avg FUE[1]	Avg LPBW[2]	Avg blood GSH[3]	Avg liver GSH
	g	*%*	*%*	*mg/100 ml*	*mg/100 g*
Basal	77.4(9)[4]	23.7(9)	3.59(4)	47.7(4)	74.7(4)
+0.2% DL-methionine	92.6(9)	44.1(9)	4.11(4)	55.6(4)	165.6(4)
+0.4% DL-methionine	90.6(8)	34.4(8)	3.75(4)	40.0(4)	119.6(4)
+0.2% desiccated thyroid	67.2(8)	27.0(8)	4.01(4)	46.3(4)	85.4(4)
+0.2% desiccated thyroid+0.2% DL-methionine	79.9(8)	35.7(8)	4.28(4)	52.6(4)	135.4(4)
+0.2% desiccated thyroid+0.4% DL-methionine	84.0(9)	41.6(9)	3.19(4)	43.5(4)	99.6(4)
+0.4% desiccated thyroid	67.8(8)	22.5(8)	3.89(4)	41.8(4)	62.1(4)
+0.4% desiccated thyroid+0.2% DL-methionine	77.6(9)	33.5(9)	4.42(4)	45.3(4)	121.4(4)
+0.4% desiccated thyroid+0.4% DL-methionine	83.2(9)	48.2(9)	3.38(4)	44.9(4)	98.9(4)
+0.8% desiccated thyroid	64.2(8)	24.7(8)	3.47(4)	40.4(4)	63.9(4)
+0.8% desiccated thyroid+0.2% DL-methionine	81.4(9)	45.2(9)	3.43(4)	41.4(4)	92.3(4)
+0.8% desiccated thyroid+0.4% DL-methionine	79.6(9)	39.8(9)	3.41(4)	42.5(4)	98.6(4)

Summary of correlations: (with 11 degrees of freedom, $r_{0.05}$ =0.553; $r_{0.01}$ = 0.684)

	Body wt	FUE	LPBW	Liver GSH	Blood GSH
	r	*r*	*r*	*r*	*r*
Blood GSH	0.759	0.630	− 0.712	0.892	—
Liver GSH	0.819	0.699	− 0.640	—	0.892

[1] Feed utilization efficiency in percentage converted to gain.
[2] Liver percentage of body weight.
[3] GSH indicates glutathione.
[4] Each entry is an average of the number of chicks shown in parentheses.

and liver. Further it appears that decreased efficiency was accompanied by increased liver weight relative to body weight.

DISCUSSION

The results reported herein and others referred to leave no reasonable doubt that methionine exerts an antithyrotoxic action in cockerels subjected to toxic dietary amounts of thyroactive materials. The new data of the present report indicate a metabolic mechanism for this antithyrotoxic action, namely via greater synthesis of glutathione. Glutathione at physiological levels previously has been shown (8) to counteract thyroxine in vitro at the mitochondrial level, where thyroxine uncouples oxidative phosphorylation (3–5). Such uncoupling would lead to the very effects we have shown to be correctable in nutritional terms by methionine. Our thesis, then, is that methionine is an antithyrotoxic agent.

The data do not by any means establish that methionine stands alone in this respect. A deficiency of any essential amino acid would be expected to lead to feed wastage and reduced metabolic efficiency. The mechanism operating to cause this could, however, be different in each case. So far as we are aware there is no reported evidence linking any amino acid other than methionine to metabolic effects of excessive thyroid hormone, or, more pertinently, to reversal of thyroid hormonal uncoupling of oxidative phosphorylation. If such action is now specified in the definition of the antithyrotoxic factor, it is doubtful that very many other amino acids than methionine (and presumably cysteine) will be found to have a true antithyrotoxic action in this sense. It should be remembered of course that glycine and glutamic acid enter into the biosynthesis of glutathione.

Ling and Chow (10) have reported that vitamin B_{12} deficiency led to decreased levels of GSH in the blood of rats and of human subjects with pernicious anemia. Folic acid or iron deficiencies did not have this effect.

Register (11) has reported that blood and liver contents of GSH were decreased in rats fed soybean rations deficient in vitamin B_{12}. These effects then would not be unexpected from massive differences in dietary methionine supply. At any rate Register and co-workers (12) observed that in rats not otherwise subjected to stress, methionine deficiency led to large decreases in liver content of nonprotein sulfhydryl (NPSH). They stated that the major NPSH changes were confined to GSH. In their methionine-sufficient animals, application of cold stress led to decreased NPSH; but in methionine-deficient animals, the same stress led to increased NPSH. This reversal of stress effect has not been corroborated in the present studies in chicks, using thyroactive materials as stressors rather than subjection to cold.

The uncoupling action of thyroid hormones is presumed to take place in cell mitochondria generally, in peripheral as well as visceral tissues, throughout the body. Glutathione is known to be widely distributed in animal tissues, and is characterized (13) by an extremely rapid metabolic turnover. The present data show that its concentration is much higher in liver tissue than in blood, and more variable in liver than in blood consequent to the indicated treatments. It is indicated that blood levels are maintained by some homeostatic mechanism within rather narrow limits, below which toxic damage to the organism occurs. Further it appears that liver probably has some capacity to maintain a reserve supply, which in methionine deficiency is partially depleted. Such depletion renders the organism more susceptible to the toxic action of thyroactive stressors by reason of reduced stabilization of circulating levels of protective GSH.

In view of this newly observed action of methionine in opposition to thyroactive stressors, and in view of considerations developed in an earlier paper from this laboratory (2), the existence of a specific, unidentified antithyrotoxic factor as postulated by Ershoff (14), Overby and coworkers (15–19) and others is open to question. In a more recent paper Overby and Fredrickson (20) have provided evidence that deoxycholic acid protected rats from experimental thyrotoxicosis, but that complete protection still required some as yet unidentified principle in liver. The present report does not by any means

eliminate that possibility. But taken together with the complexities of methionine nutrition related in our preceding paper (2), it casts considerable doubt on the notion of an unidentified factor. More definitive evidence will be necessary to establish the fact in either case.

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Dynamic Aspects of Cholesterol Ester Metabolism in Rabbits with Atherosclerosis[1]

LEON SWELL, M. D. LAW AND C. R. TREADWELL
Veterans Administration Center, Martinsburg, West Virginia, and Department of Biochemistry, School of Medicine, The George Washington University, Washington, D. C.

ABSTRACT Rabbits were fed a normal stock diet supplemented with 1 g of cholesterol/day; at 58 days the animals received a meal labeled with cholesterol-4-C^{14} and were then killed at 1, 3 and 5 days. Rabbits with atherosclerosis showed a high percentage of oleic acid and a low percentage of linoleic acid in their aorta, liver and serum cholesterol esters. The specific activity data of the individual aorta and liver cholesterol esters indicate that the 3 major classes of cholesterol esters (saturated, monounsaturated and linoleate) may have different turnover rates. At 1 day, the aorta C^{14}-cholesterol esters had a composition similar to the C^{14}-cholesterol esters present in the serum. After 1 day the composition of the C^{14}-cholesterol esters in the aorta showed a general shift toward the total cholesterol ester fatty acid pattern. The observations of this study support the view that there is an indiscriminate deposition of cholesterol esters into the aorta from the plasma and that the aorta determines the final composition of its cholesterol esters by a selective retention of saturated and monounsaturated esters.

The role of the lipids in the etiology of atherosclerosis and, in particular, the origin of the aortic lipids have not been adequately clarified. Recent studies (1, 2) have provided evidence that the phospholipids of the aorta in man and animals arise primarily from synthesis *in situ;* the triglycerides of rabbit aorta also appear to be derived by synthesis within that tissue (2). Studies (2, 3) on the origin of the free and esterified cholesterol in normal and diseased aorta indicate that these lipid fractions are derived from the serum. Newman and Zilversmit (4) have provided quantitative data on the cholesterol flux into rabbit atheromatous lesions. Increased levels of cholesterol in the lesion were generally associated with higher influxes of cholesterol; also, the rate of entrance of free cholesterol was found to be greater than that of esterified cholesterol. The several reports have dealt primarily with the free and esterified cholesterol fractions as entities. However, it is important to recognize that the esterified cholesterol fraction is composed of a number of individual cholesterol esters and these may have different turnover rates, metabolic origins and, perhaps, functions.

Information on the changes in the cholesterol esters associated with atherosclerosis has been obtained by analyzing the fatty acid composition of the cholesterol esters of the aorta and serum. As yet, there is no general agreement on the changes in cholesterol ester composition in the human aorta associated with atherosclerosis. Studies (5, 6) in animals indicate that there is an increase in the percentage of oleic acid and a decrease in linoleic acid in the serum cholesterol esters of rabbits with atherosclerosis compared with normal animals and that the aortic cholesterol esters of rabbits with atherosclerosis contain a preponderance of monounsaturated fatty acids. These differences in cholesterol ester fatty acid composition between the serum and aorta suggested that there may be a discriminate deposition of certain cholesterol esters in the aorta or that the aorta may selectively retain certain cholesterol esters. The present study was undertaken to provide more definitive information on the dynamic aspects of the metabolism of the individual cholesterol esters in the aorta and other

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tissues of rabbits with atherosclerosis. The data presented suggest that there is a selective retention of certain esters by the aorta following the indiscriminate deposition of cholesterol esters from the serum.

MATERIALS AND METHODS

Animals and diets. New Zealand white, male rabbits weighing 2.5 to 3 kg were used. In the morning, each of 16 animals received 50 g of powdered commercial rabbit pellet chow containing 1 g of cholesterol. The cholesterol was added to the diet by dissolving 10 g of cholesterol in 200 ml of ether and slowly pouring the solution into a mixing bowl with 500 g of powered pellet chow. The diet was thoroughly mixed until the ether had evaporated. The animals consumed this meal in the morning, and in the afternoon were given an additional 75 g of unground pellet chow. A normal control group of 6 animals was fed a similar dietary regimen except that no cholesterol was added to the diet. The diets were fed for 58 days. At that time, the cholesterol-fed rabbits received their regular cholesterol meal to which had been added 10 μc of cholesterol-4-C^{14}. One group of 5 cholesterol-fed animals was killed 24 hours after receiving the labeled cholesterol meal. The 11 remaining cholesterol-fed rabbits were continued on the cholesterol diet and groups of 5 and 6 were killed at 3 and 5 days, respectively. On the 58th day, the control group received their regular diet to which had been added 10 μc of a tracer dose of cholesterol-4-C^{14}. These animals were killed 24 hours after receiving the labeled cholesterol meal. The animals were killed under pentobarbital sodium anesthesia by exsanguination and the liver and the aorta were removed. The aorta was carefully cleared of adherent fat, sliced longitudinally and graded for atherosclerosis from 0 to 4+. Tissues were weighed, ground and extracted with 2:1 chloroform-methanol according to procedures described earlier (7).

Methods. The free and esterified cholesterol fractions in each tissue lipid extract were separated by silicic acid column chromatography (8). Separation and recovery of free and esterified cholesterol were routinely checked with cholesterol-4-C^{14} and known cholesterol-4-C^{14} esters and shown to be quantitative. A portion of the esterified sterol fraction was hydrolyzed. The free sterols in the hydrolyzed esterified fraction and in the original free sterol fraction were precipitated with digitonin and the sterol digitonides washed (9). The purified sterol digitonides were dissolved in 4 ml of methanol. For the determination of C^{14}-activity, 1 ml of the methanol solution was added to a scintillation vial followed by 10 ml of a toluene solution containing 0.4% 2,5-diphenyloxazole and 0.05% 1,4-bis-2(5-phenyloxozolyl)-benzene. The C^{14}-activity was determined in a Nuclear-Chicago Corporation liquid scintillation system; all values were corrected for quench and were counted to within plus or minus 5%.

Free and esterified cholesterol were determined on the lipid extracts by the method of Sperry and Webb (9). The fatty acid composition of the cholesterol ester fraction was determined by gas-liquid chromatography as described previously (10). The cholesterol ester fraction isolated on silicic acid columns was further separated into 4 major fractions (saturated, monounsaturated, linoleate and arachidonate) by a modification of the method of Klein and Janssen (11). To increase the flow rate a mixture of 100 parts of silicic acid to 70 parts of Hyflo Super-cel[2] was used; positive pressure (N_2) was applied to the top of the column to maintain a flow rate of 1 ml/minute. Of particular importance was the conditioning of the silicic acid mixture which was carried out in the following manner: the silicic acid mixture was heated overnight at 120°C and following its removal from the oven it was placed in a shallow pan and placed in a hood with a steam bath for 4 to 5 minutes. The silicic acid mixture was then suspended in benzene and treated according to the directions of Klein and Janssen (11). With this procedure, excellent separation of the major cholesterol ester classes could be achieved in 4 hours.

Recovery and separation of the cholesterol esters were checked with cholesterol-4-C^{14} palmitate, cholesterol-4-C^{14} oleate, cholesterol-4-C^{14} linoleate and cholesterol-

[2] Johns Manville Company, Manville, New Jersey.

4-C^{14} arachidonate prepared as described elsewhere (12). The identity of the tissue cholesterol ester fractions was further verified by gas-liquid chromatography (10). The saturated esters of the various tissues were found to consist primarily of palmitate with small amounts of stearate. The monounsaturated cholesterol ester fraction consisted primarliy of oleate with small amounts of palmitoleate; the linoleate fraction also contained small amounts of trienoic esters. The cholesterol arachidonate fraction contained very small amounts of other polyunsaturated esters. Following the separation of the cholesterol-4-C^{14} esters, their C^{14}-activity was determined and the percentage composition of the C^{14}-cholesterol esters in the blood and tissues calculated.

The specific activity of the individual cholesterol esters in the serum, liver and aorta was calculated from the data obtained on the fatty acid composition of the cholesterol esters, the percentage composition of the C^{14}- cholesterol esters and the levels of the cholesterol esters in those tissues. The assumption was made that the individual serum cholesterol esters were of uniform specific activity. The cholesterol influx into the aorta was calculated as described by Newman and Zilversmit (4). The average specific activities of the plasma free and cholesterol esters were plotted against time and the average specific activity calculated from the area under the curve. This was divided into the total radioactivity of the corresponding free or cholesterol ester fraction in the aorta. This value divided by 3 or 5 gave the influx of free and cholesterol esters per day per aorta.

RESULTS

The free and esterified cholesterol levels in liver, serum and aorta are shown in table 1. The cholesterol levels, both free and esterified, of the experimental animals were markedly elevated compared with those of the control animals. Most of the excess cholesterol present in the tissues was in the esterified form.

The recovery of the fed cholesterol-4-C^{14} in several tissues is shown in table 2. In the serum and liver of the cholesterol-fed rabbits there was a much greater proportion of the recovered C^{14}-cholesterol present in the esterified form than the control animals. The aorta, at the different times, contained a low percentage of the fed C^{14}-cholesterol; it was of the order of 0.1% or less. However, there was an increase in the proportion of the recovered C^{14}-cholesterol in the esterified form in the aorta with time; on the 5th day 70% of the labeled cholesterol which had entered the aorta was in the esterified form. In the animals with atherosclerosis, a much larger percentage of the administered C^{14}-cholesterol was recovered in the serum than in the liver; in the normal control animals, at the end of one day, most of the recovered fed C^{14}-cholesterol was present in the liver. The greatest recovery of C^{14}-cholesterol in liver and serum was not

TABLE 1

Free and esterified cholesterol levels in tissues

Group[1]	Cholesterol			
	Free	Ester	Total	Ester/total
				%
	Liver, mg/g			
Cholesterol-fed	6.5 ± 2.5	29.0 ± 10.0	35.5 ± 12.3	81.6 ± 7.9
Normal-control	1.9 ± 0.5	0.3 ± 0.1	2.2 ± 0.6	13.6 ± 3.4
	Aorta, mg/aorta			
Cholesterol-fed	3.1	4.6	7.7	59.7
Normal-control	1.0	0.1	1.1	10.0
	Serum, mg/100 ml			
Cholesterol-fed	312 ± 110	1054 ± 320	1366 ± 420	77.2 ± 4.5
Normal-control	8.1 ± 3.0	12.3 ± 4.0	20.4 ± 6.5	60.5 ± 3.1

[1] Liver and serum values represent the average ± SD for the 16 cholesterol-fed rabbits and 6 normal-control animals; the aortas of the respective groups were pooled for analysis.

TABLE 2

Recovery of fed cholesterol-4-C^{14} in tissues

Group	Days after cholesterol-4-C^{14} meal	% Fed C^{14}-cholesterol recovered[1]			Ester-C^{14} / total-C^{14}
		Free	Esterified	Total	
					%
			Liver		
Cholesterol-fed	1	1.24 ± 0.30	2.02 ± 0.43	3.26 ± 0.71	62.0 ± 5.4
Cholesterol-fed	3	2.79 ± 0.41	6.44 ± 2.10	9.23 ± 2.50	69.8 ± 9.1
Cholesterol-fed	5	2.27 ± 0.91	4.76 ± 1.84	7.03 ± 2.71	67.7 ± 6.4
Normal-control[2]	1	11.52 ± 2.46	2.35 ± 0.94	13.87 ± 3.33	16.9 ± 3.4
			Aorta		
Cholesterol-fed	1			< 0.1	49.2
Choelsterol-fed	3			< 0.1	63.1
Cholesterol-fed	5			< 0.1	68.8
Normal-control	1			< 0.1	[3]
			Serum		
Cholesterol-fed	1	1.09 ± 0.34	8.62 ± 2.61	9.71 ± 3.00	88.8 ± 6.4
Cholesterol-fed	3	2.67 ± 0.46	11.13 ± 4.12	13.80 ± 4.51	80.7 ± 7.1
Cholesterol-fed	5	1.74 ± 0.51	5.91 ± 1.63	7.15 ± 2.04	82.7 ± 9.0
Normal-control	1	0.66 ± 0.15	1.39 ± 0.31	2.05 ± 0.42	67.8 ± 4.1

[1] Recovery of fed cholesterol-4-C^{14} is given for the total liver, aorta and serum volume; these were computed from the tissue and body weights and hematocrit.
[2] The normal control animals were given a tracer amount (10 μc) of cholesterol-4-C^{14} in their diet.
[3] Only traces of ester-C^{14} activity were observed in normal aorta.

obtained in the cholesterol-fed rabbits until the third day.

The fatty acid composition of the tissue cholseterol esters and the percentage of individual C^{14}-cholesterol esters are shown in table 3. The C^{14}-cholesterol ester composition represents the newly-formed C^{14}-cholesterol esters and their metabolic pattern as a function of time. The fatty acid composition of the cholesterol ester fraction represents the overall chemical level of the individual cholesterol esters in the tissues of the normal and cholesterol-fed rabbits. With respect to the latter, values shown for days 1, 3 and 5 in the table for the cholesterol-fed rabbits are essentially duplicates and indicate the variation among these groups of animals.

One day after administration of the labeled cholesterol meal, the serum and aorta C^{14}-cholesterol esters were very similar in composition. The saturated esters formed the largest fraction of the C^{14}-esters (38 and 41%), followed by the monounsaturated and linoleate esters. The liver C^{14}-ester composition of the cholesterol-fed rabbits was similar to that of the aorta and serum fractions except for a slightly higher percentage of monounsaturated esters. Also, the liver C^{14}-esters of both the normal and cholesterol-fed rabbits were similar in composition one day after the labeled meal. In the normal animals, the liver and serum C^{14}-esters, one day after administration of the labeled cholesterol, were vastly different in composition. The liver C^{14}-cholesterol esters had a higher proportion of monounsaturated esters and a lower percentage of linoleate esters than the serum C^{14}-cholesterol esters. In the cholesterol-fed animals the liver, serum and aorta C^{14}-cholesterol ester compositions showed a general shift with time toward monounsaturated C^{14}-esters with a concomitant drop in the percentage of saturated C^{14}-esters.

The fatty acid compositions of the cholesterol esters of the liver, serum and aorta of the cholesterol-fed rabbits were similar and consisted of a high proportion of monounsaturated fatty acids (50 to 60%) and a low level of polyunsaturated fatty acids (18 to 26%). It is also shown, in confirmation of our previous observations (5), that the serum cholesterol esters of normal rabbits contain considerably more linoleic acid and less oleic acid than the esters deposited in the aorta of animals with atherosclerosis. Also, the differences, while not as marked, suggest that there is less linoleic acid in the aorta cholesterol esters than in the serum cholesterol esters

TABLE 3

Composition of tissue cholesterol esters and cholesterol-4-C^{14} esters

Group[1]	Days after cholesterol-4-C^{14} meal	Composition of cholesterol-4-C^{14} esters				Cholesterol ester fatty acid composition			
		Saturated	Monounsaturated	Linoleate	Arachidonate	Saturated	Monounsaturated	Linoleate	Arachidonate
		% total C^{14} esters				*% total fatty acids*			
				Liver					
Cholesterol-fed	1	31.9	37.3	25.5	5.3	25.6	58.0	14.5	1.9
Cholesterol-fed	3	18.4	52.4	23.6	5.7	23.5	58.5	16.7	1.3
Cholesterol-fed	5	19.8	51.0	24.2	5.0	23.3	59.2	16.5	1.0
Normal-control	1	31.6	44.7	21.4	2.3	35.1	39.9	21.7	3.3
				Aorta					
Cholesterol-fed	1	38.3	32.4	24.7	4.6	29.1	51.6	18.0	1.3
Cholesterol-fed	3	24.8	45.0	26.9	3.3	29.7	51.2	17.8	1.3
Cholesterol-fed	5	27.8	41.9	25.3	5.0	28.1	51.5	19.1	1.3
				Serum					
Cholesterol-fed	1	40.6	31.2	25.6	2.6	25.7	48.2	25.1	1.0
Cholesterol-fed	3	31.3	38.7	27.6	2.6	25.1	51.4	22.2	1.3
Cholesterol-fed	5	26.8	45.9	23.9	3.4	25.2	48.4	25.4	1.0
Normal-control	1	37.6	27.6	31.8	3.0	31.9	24.9	40.7	2.5

[1] Values represent the analysis of pooled samples (5 to 6 animals); normal aorta did not contain sufficient cholesterol esters or C^{14}-activity for analysis.

of the cholesterol-fed rabbits. Comparison of the cholesterol ester fatty acid composition with that of the C^{14}-cholesterol esters, after one day, shows that the aorta, liver and serum C^{14}-cholesterol esters contain a larger proportion of linoleate and a lower percentage of oleate than the total cholesterol esters. After one day, the C^{14}-cholesterol ester composition begins to approach the total cholesterol ester fatty acid pattern in those tissues.

The specific activities of the cholesterol esters and free cholesterol of the tissues are shown in table 4. The liver and serum free cholesterol rapidly approached the same specific activity. This is in agreement with the earlier observations which indicate that the serum and liver free cholesterol pools come into equilibrium very rapidly. On the other hand, the specific activity of the aorta free cholesterol was only one-half the specific activity of the serum and liver free fractions at the latest time (5 days). This indicates that the aorta free cholesterol fraction comes into equilibrium much more slowly with the serum than liver and, perhaps, other tissues. The serum cholesterol esters had the highest specific activities at the earliest time (1 day); the saturated serum cholesterol esters were the highest followed by linoleate and the monounsaturated esters. The specific activity of the saturated serum esters decreased after one day, whereas the monounsaturated and linoleate esters did not reach their peak until after that time. The liver cholesterol esters had a much lower specific activity than the serum esters; the liver ester with the highest specific activity was cholesterol linoleate. The aorta cholesterol esters had varying specific activities which were readily demonstrable particularly at the early times (1 and 3 days). At 3 and 5 days aorta cholesterol linoleate had the highest specific activity followed by the saturated and monounsaturated esters. The specific activities of the aorta cholesterol esters may not have reached their peak at the end of 5 days. Extrapolation of the values beyond the fifth day indicated that the several aorta cholesterol esters would attain the same specific activity as the seurm esters within 7 to 8 days.

Based on the specific activity data obtained on the serum free and ester fractions and the amount of activity associated with the individual esters and free cholesterol in the aorta, the influx of free cholesterol and cholesterol esters was cal-

TABLE 4

Specific activities of tissue free and cholesterol esters

Group	Days after cholesterol-4-C^{14} meal	Free cholesterol	Cholesterol esters[1]		
			Saturated	Monounsaturated	Linoleate
		count/min/mg	*count/min/mg*		*count/min/mg*
		Liver			
Cholesterol-fed	1	377	210	108	296
Cholesterol-fed	3	565	198	226	357
Cholesterol-fed	5	528	125	228	389
Normal-control	1	10,349	10,886	13,560	11,916
		Aorta			
Cholesterol-fed	1	169	82	39	85
Cholesterol-fed	3	176	111	117	202
Cholesterol-fed	5	227	216	179	290
Normal-control	1	225			
		Serum			
Cholesterol-fed	1	419	1,404	576	906
Cholesterol-fed	3	660	1,091	658	1,087
Cholesterol-fed	5	523	554	493	490
Normal-control	1	9,163	16,633	15,361	10,972

[1] Due to the presence of only very small amounts of cholesterol arachidonate in the various tissues and the small amount of counts associated with that cholesterol ester fraction, reliable values could not be obtained on its specific activity.

TABLE 5

Influx and accumulation of free and cholesterol esters in aorta

Days after cholesterol-4-C^{14} meal	Free cholesterol	Cholesterol esters			
		Saturated	Monounsaturated	Linoleate	Total esters
		Influx, μg/day/aorta[1]			
3	432	46.6	180.5	69.8	
5	300	60.0	157.5	60.8	
	366	53.5	169.0	65.3	287.8
		Net accumulation, μg/day/aorta[1]			
	35.0	22.2	39.3	14.0	75.5

[1] Values represent the average influx or net accumulation of cholesterol per aorta per animal.

culated for the 3- and 5-day intervals. These data are shown in table 5. The net accumulation of cholesterol and cholesterol esters per day was calculated from the chemical values obtained for those aorta lipid fractions divided by 60 days (average time fed diet). The difference between the influx and net accumulation per day is indicative of the amount of cholesterol and cholesterol esters being returned to the blood or at least disappearing from the aorta. It is shown that the total influx of free cholesterol into the aorta was greater than the total esterified fraction. However, the net accumulation of the free fraction was less than the ester fraction, suggesting that there was a greater efflux of free cholesterol from the aorta. Inspection of the data obtained on the individual cholesterol esters shows a large influx of the monounsaturated esters into the aorta. The influx was much less for the linoleate and saturated esters. The monounsaturated esters also accumulated to the greatest extent followed by the saturated and linoleate esters. The ratio of net accumulation to influx per day (indicative of the amount of retention of the individual esters by the aorta) suggests that the saturated esters were retained to the greatest extent followed by the monounsaturated and linoleate esters.

DISCUSSION

In agreement with observations reported earlier (5, 6, 13), it is shown in the present study that the aorta cholesterol esters of rabbits with atherosclerosis contain a preponderance of monounsaturated fatty acids and a very low percentage of linoleic acid; the major cholesterol ester fatty acid was found to be oleic acid (50%). Also, the serum cholesterol esters of rabbits with atherosclerosis contain a larger proportion of monounsaturated fatty acids and a low level of linoleic acid when compared with the serum cholesterol esters of normal animals. Similar changes were also noted in the cholesterol ester fatty acid composition of the liver. These observations strongly suggest that there is a derangement of cholesterol ester metabolism in the cholesterol-fed animals and this derangement leads to an increase in tissue cholesterol oleate and a decrease in cholesterol linoleate. These changes appear to be independent of the dietary fat since the rabbits were fed only regular chow and the major fatty acid in the chow is linoleic acid (14). The large accumulation of cholesterol oleate in the serum, liver and aorta of the cholesterol-fed rabbits may be the result of a relative deficiency in the processes whereby cholesterol is catabolized or transported from the blood to the tissues. This relative deficiency may be in the requirement of essential fatty acids for cholesterol transport and metabolism as suggested by previous studies (15, 16).

The specific activity data obtained on the tissue cholesterol esters indicate that the individual esters have different turnover rates. The esters with the slowest turnover are the monounsaturated esters, whereas the linoleate esters in aorta and liver turn over most rapidly. The present data do not entirely eliminate the possibility that the aorta does esterify some incoming C^{14}-cholesterol. However, the

studies of Newman et al. (2), Newman and Zilversmit (4) and Biggs and Kritchevsky (17) indicate that rabbit atherosclerotic aorta derives most of its plaque free and esterified cholesterol from the plasma.

One of the most significant observations of this study relates to the entrance and exit of the individual cholesterol esters in the aorta. The data show that the cholesterol esters entering the aorta have a composition similar to the esters present in the serum. Following the entrance of the cholesterol esters into the aorta there appears to be a selective retention of certain esters, most notably, the saturated esters. However, due to the high influx of cholesterol oleate, the net accumulation of this ester is greater than with the saturated or linoleate esters. Thus, the initial deposition of cholesterol esters in the aorta from the serum may be looked upon as a nonselective passive process, but the final composition of the esters will be determined by the balance between the influx of the individual esters and their selective retention.

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Possible Mechanisms for Dietary Molybdenum Toxicity in the Rat [1]

HERMAN L. JOHNSON [2,3,4] AND RUSSELL F. MILLER [5]
Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia

ABSTRACT Weanling rats were fed 10% casein, semi-purified diets with and without added molybdenum, and nitrogen balance studies were conducted. Digestion and absorption of nitrogen were not affected by dietary molybdenum. Urinary nitrogen excretion was increased when molybdenum was included in the diets. For the first 3 weeks of feeding, urea plus ammonia excretion accounted for most of the increased nitrogen excretion and then an aminoaciduria developed which contributed significantly to the urinary nitrogen excretion. Elevated levels of urinary α-amino nitrogen were observed with an increased level of dietary molybdenum and with increased duration of molybdenum feeding. Urea plus ammonia excretion decreased concomitantly with the increased aminoaciduria. The hypothesis that dietary molybdenum inhibits some facet of protein synthesis is proposed and discussed in conjunction with these observations.

A marked diminution of nutrient utilization by the molybdenum-fed rat has been reported by Johnson and Miller (1) using carefully controlled pair-fed and restricted-fed rats. Monty (2) has demonstrated that the rat could detect which diet contained added molybdenum and, when given a choice, would consume the diet free of added molybdenum. Miller and Price (3) observed that the growth depression in rats fed diets containing 100 ppm added molybdenum decreased as the dietary casein level was increased from 8 to 18%. These observations indicated that the growth depression and decreased utilization of nutrients in the molybdenum-fed rat could be related to nitrogen metabolism. The following experiments were designed to extend these observations and to search for possible mechanisms of molybdenosis in the rat.

METHODS

Male weanling albino rats derived from the Sprague-Dawley strain[6] were housed individually in stainless steel metabolism cages constructed so that the urine and feces were collected separately. The rats were randomly assigned to treatment lots. In each of experiments 1, 2 and 3, four treatments were used: 4 rats, lot A, were fed the basal diet ad libitum; 5 rats, lot B, were fed ad libitum the basal diet containing added molybdenum (600 ppm in experiment one and 400 ppm in experiments 2 and 3); 5 rats, basal diet, lot C, were paired with those in lot B, the latter determining the daily feed allotment; and, 4 rats, lot D, were fed the basal diet restricted in amount so that their average weight gain paralleled the average gain of the rats in lot B. In experiment 4, two treatments of 9 rats each were used; the rats in lot B were fed the basal diet plus 400 ppm molybdenum, ad libitum, and the rats in lot C were fed the basal diet in amounts, daily, equal to the average consumed during the previous 24 hours by the rats in lot B. Two treatments (9 rats/treatment) were used in experiment 5. Rats in lot B were fed as in experiment 4 and each rat in lot D received the basal diet in amounts, daily, equal to 75% of the average consumed by the rats in lot B.

The basal diet was the same as that previously described (1), containing adequate amounts of the essential vitamins

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[3] Studies carried out during the tenure of NIH traineeship, Training Grant 2G673.
[4] Present address: The Samuel Roberts Noble Foundation, Inc., P.O. Box 870, Ardmore, Oklahoma.
[5] Deceased.
[6] Rats were obtained from Dublin Laboratory Animals, Box 846, Dublin, Virginia.

and minerals;[7] and containing casein,[8] vegetable oil and sucrose at 10, 5 and 81.5%, respectively. Fat-soluble vitamins were given *per os* weekly. Tap water was provided ad libitum. Molybdenum was added, when indicated, as an aqueous solution of sodium molybdate.

Urine from each rat was collected in 5 ml of 1 N HCl in 48-hour composites. The feces were collected simultaneously. Both urine and feces were frozen until the end of the collection period and then were analyzed. All collections reported here were made between the 20th and 40th days of feeding.

Dietary, fecal, and total urinary nitrogen were determined by the Kjeldahl procedure. Free ammonia and urea, after urease treatment, were estimated by Nesslerization (4) creatinine and creatine, by the Jaffe reaction (5), and free α-amino nitrogen by the method of Frame et al. (6), after ammonia removal by adjusting pH to 10 to 12 with 10% NaOH and desiccating in vacuo. Values for α-amino nitrogen obtained by this method were comparable to those obtained by the ninhydrin method of Khachadurian et al. (7). An automatic amino acid analyzer was used for the quantitative determination of the individual amino acids of pooled urine samples using the method of Moore and Stein (8, 9). Blood amino nitrogen was determined by the method of Frame et al. (6).

RESULTS

A summary of the average final weights, weight gains and feed and nitrogen consumption is presented in table 1. The rats consuming the basal diet plus 600 ppm molybdenum (exp. 1, lot B) gained almost no weight. To demonstrate the differences in nutrient utilization, some weight gain in the molybdenum-fed rat appeared desirable. This was accomplished in the following experiments by decreasing the level of molybdenum, added to the diet, to 400 ppm. A consistent trend (not statistically significant) toward better weight gain in the pair-fed rats (lot C) in comparison with the molybdenum-fed rats (lot B) was observed. The restricted-fed rats (lot D), although allowed significantly less feed than the molybdenum-fed rats, gained equally well. The molybdenum-fed rats were thus demonstrated to utilize nutrients from the diet less efficiently than paired-fed or restricted-fed controls.

A summary of the nitrogen balance data obtained for 20 days of feeding is presented in table 2. The difference in fecal nitrogen excretion, although statistically significant, is minor and would not contribute significantly to the decreased nutrient utilization. The molybdenum-fed rats retained only 70% as much of the ingested nitrogen as the rats fed the basal diet. This difference in retention was accounted for by an increased urinary nitrogen excretion in the molybdenum-fed rats (62% more than the rats fed the basal diet). The increase in urinary nitrogen excretion was attributed to the increased excretion of urea plus ammonia (41%) and free α-amino nitrogen (540%). The determination of creatine, creatinine, and urinary protein revealed that the excretion of these components was not affected by molybdenum feeding; therefore, these were calculated by difference in these experiments and expressed as the remaining urinary nitrogen. Although the control rats were permitted to consume only 75% as much feed and nitrogen as the ad libitum, molybdenum-fed rats, their retention, expressed as milligrams of nitrogen, was slightly more than that of the molybdenum-fed rats.

Body size affects the amount of urinary nitrogen excretion and, to minimize this variable in the rats of lots A and C, total urinary nitrogen, urea plus ammonia nitrogen, and free α-amino nitrogen excretion was expressed on a body weight basis (fig. 1). Expressed in this way, the total urinary nitrogen excretion by the molybdenum-fed rats was 69 to 143% greater than for rats fed the basal diet. The molybdenum-fed rats consistently excreted more urea plus ammonia, per unit of body weight, than any rats in any of the lots fed the basal diet. The excretion of α-amino nitrogen was elevated 4- to 17-fold, depending upon the time and level

[7] The minerals provided the following in grams per kilogram of diet: $Ca_3(PO_4)_2$, 12.5; NaCl, 6.5; KCl, 5.5; MgO, 0.7; Fe_2O_3, 0.15; MnO_2, 0.10; KI, 0.03; $ZnCO_3$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.013. All salts were C.P. or A.R.

[8] L-Cystine was added to the diet at a level of 0.2%.

TABLE 1

Weight gain and feed consumption as regulated by dietary molybdenum, paired and restricted feeding in rats

Dietary treatment[1]	Days on experiment	Weights		Consumption	
		Final	Gain	Feed	Nitrogen
		g	*g*	*g*	*g*
Experiment 1	7				
Basal diet, ad libitum		166.8 ± 16.2[2]	20.8 ± 3.0	64.4 ± 5.7	914.3 ± 80.4
Basal + 600 ppm Mo, ad libitum		41.2 ± 5.7	0.2 ± 0.4	19.1 ± 2.4	267.0 ± 33.8
Basal diet, pair-fed		59.2 ± 7.3	4.2 ± 1.8	18.6 ± 2.5	264.7 ± 36.0
Basal diet, restricted-fed		49.5 ± 1.9	4.5 ± 0.6	15.0	213.0
Experiment 2-A	10				
Basal diet, ad libitum		111.0 ± 12.7	29.8 ± 6.7	97.2 ± 13.2	1434.8 ± 194.2
Basal + 400 ppm Mo, ad libitum		68.2 ± 6.2	10.0 ± 3.5	55.7 ± 9.6	821.9 ± 142.3
Basal diet, pair-fed		75.6 ± 4.8	16.2 ± 2.2	54.9 ± 9.9	810.5 ± 146.8
Basal diet, restricted-fed		64.2 ± 1.7	14.5 ± 1.0	45.0	664.2
Experiment 2-B	5				
Basal diet, ad libitum		123.8 ± 14.4	12.8 ± 3.9	47.5 ± 12.3	701.5 ± 180.2
Basal + 400 ppm Mo, ad libitum		72.2 ± 9.9	4.0 ± 4.2	29.6 ± 5.0	436.7 ± 73.1
Basal diet, pair-fed		84.8 ± 10.3	9.2 ± 3.4	29.8 ± 4.5	450.0 ± 67.1
Basal diet, restricted-fed		68.8 ± 1.5	4.5 ± 0.6	22.5	332.1
Experiment 2-C	5				
Basal diet, ad libitum		141.2 ± 13.6	17.5 ± 1.9	57.0 ± 5.8	841.6 ± 85.7
Basal + 400 ppm Mo, ad libitum		76.6 ± 10.9	4.4 ± 2.1	28.2 ± 3.7	416.2 ± 51.4
Basal diet, pair-fed		90.8 ± 11.4	6.0 ± 1.9	29.0 ± 3.8	427.6 ± 55.8
Basal diet, restricted-fed		71.8 ± 1.5	3.0 ± 0.0	22.5	332.1
Experiment 3	5				
Basal diet, ad libitum		137.5 ± 10.8	18.8 ± 5.3	57.3 ± 5.4	786.9 ± 74.4
Basal + 400 ppm Mo, ad libitum		70.2 ± 12.0	3.6 ± 1.7	25.8 ± 2.7	354.7 ± 36.9
Basal diet, pair-fed		86.4 ± 8.7	5.2 ± 1.3	26.0 ± 3.1	356.3 ± 43.0
Basal diet, restricted-fed		72.5 ± 4.4	5.0 ± 0.8	21.0	288.2
Experiment 4	5				
Basal + 400 ppm Mo, ad libitum		57.2 ± 14.6	7.1 ± 3.8	27.8 ± 9.4	398.5 ± 134.4
Basal diet, pair-fed		61.2 ± 6.4	9.8 ± 2.2	26.2 ± 3.6	375.4 ± 50.3
Experiment 5-A	5				
Basal + 400 ppm Mo, ad libitum		65.3 ± 8.5	4.4 ± 3.1	29.5 ± 7.2	434.8 ± 105.0
Basal diet, restricted-fed		65.7 ± 2.2	5.2 ± 0.6	22.6	327.8
Experiment 5-B	5				
Basal + 400 ppm Mo, ad libitum		69.1 ± 9.8	3.8 ± 2.4	28.2 ± 5.9	415.7 ± 87.0
Basal diet, restricted-fed		70.3 ± 2.7	4.7 ± 0.9	21.6	317.8
Experiment 5-C	5				
Basal + 400 ppm Mo, ad libitum		74.6 ± 11.0	5.0 ± 2.4	27.9 ± 4.0	411.6 ± 58.6
Basal diet, restricted-fed		76.1 ± 2.6	5.8 ± 1.0	20.7	305.6
Experiment 5-D	5				
Basal + 400 ppm Mo, ad libitum		77.7 ± 12.1	3.1 ± 2.3	28.9 ± 5.2	425.6 ± 76.0
Basal diet, restricted-fed		79.3 ± 2.6	3.2 ± 0.8	21.8	321.0

[1] A, B, C, and D refer to sequential feeding periods of the duration shown, following pre-experimental feeding periods with the same diets for 35 days in experiment 1, 20 days in experiments 2, 4, and 5, and 30 days in experiment 3.

[2] SD.

TABLE 2

Effect of dietary molybdenum on nitrogen balance and urinary nitrogen components in rats (exp. 5)[1]

	Restricted-fed Basal diet	Ad libitum-fed Basal + 400 ppm Mo
	% of consumed	*% of consumed*
Fecal nitrogen	5.0	6.9[2]
Total urinary nitrogen	29.5	47.9[3]
α-Amino nitrogen	1.3	8.5[3]
Urea plus ammonia nitrogen	20.1	28.3[3]
Remaining urinary nitrogen	8.1	11.1[4]
Nitrogen retained (by difference)	65.5	45.2[3]
Consumed (mg nitrogen)	1273	1689
Retained (mg nitrogen)	834	774

[1] Nine rats/treatment; experimental period, 20 days.
[2] Significantly different from the values for the restricted-fed control ($P < 0.01$).
[3] Significantly different ($P < 0.001$).
[4] Significantly different ($P < 0.05$).

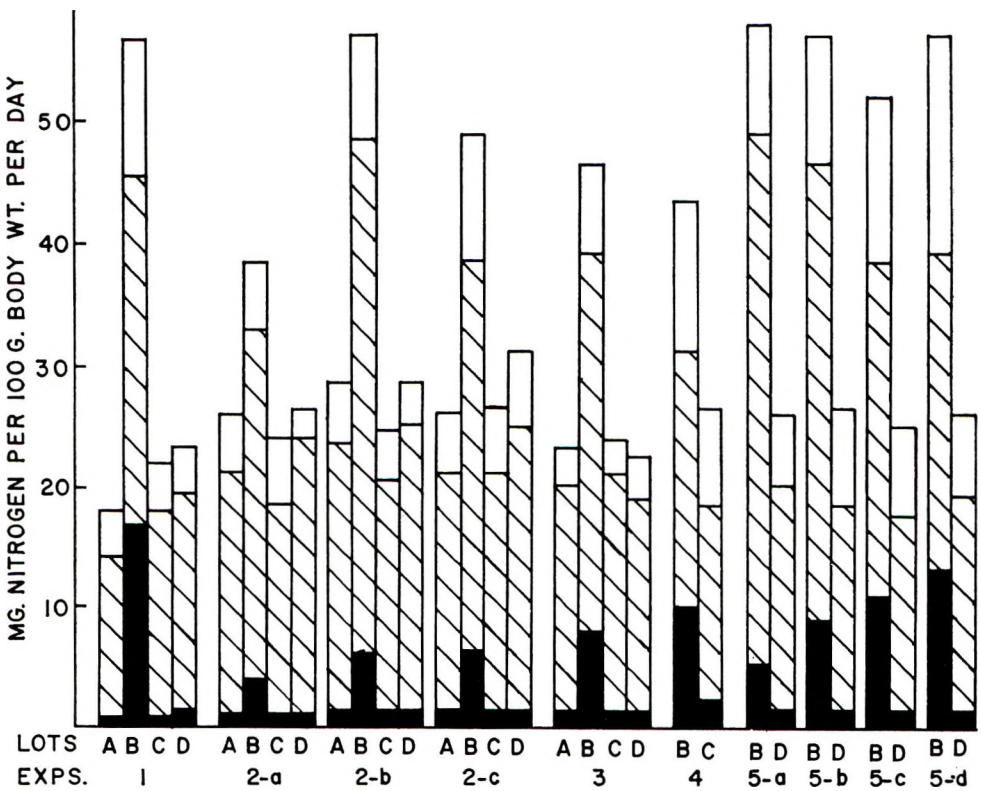

Fig. 1 Urinary nitrogen excretion. Full bar is total urinary nitrogen, black is α-amino nitrogen and cross-hatched is urea plus ammonia nitrogen.

of molybdenum feeding. The increased aminoaciduria with a higher level of dietary molybdenum is demonstrated by comparing lot B, experiment 1 (600 ppm Mo) with lot B, experiment 5-d (400 ppm Mo). Increasing aminoaciduria with an increasing length of feeding period is shown in experiments 2 and 5 where the letters *a* through c and *a* through *d* represent consecutive collection periods. As the aminoaciduria increased, urea plus ammonia excretion decreased. The aminoaciduria could not be detected during the first 14 days of feeding molybdenum and the collections represented here were made between 20 and 40 days. Total urinary nitrogen, urea plus ammonia, and α-amino nitrogen were not significantly affected by the level of feeding of the basal diet devoid of added molybdenum (exp. 1, 2, and 3).

The aminoaciduria accompanying molybdenosis was investigated further. The free amino acid levels of pooled urine samples from rats in lots B and C of experiment 3 are presented (table 3). This analysis was conducted after desiccation at pH 10 to remove excess ammonia and after 6 weeks storage at pH 2.2 and −20°. The values represent approximately an 80% recovery of α-amino nitrogen as determined chemically. Citrulline, a urea cycle intermediate, and the aromatic amino acids, including histidine, were observed only in the urine of the molybdenum-fed rats. The only non-dispensible amino acid excreted in large amounts by the molybdenum-fed rat was threonine. The other hydroxy-amino acid, serine, was also excreted in large amounts.

To determine whether the increased excretion of α-amino nitrogen was a reflection of the blood levels, α-amino nitrogen was determined on blood obtained by heart puncture from rats of experiment 4 after 25 days' feeding and fasting 24 hours. The whole blood α-amino nitrogen level was significantly lower (11.42 ± 1.17 vs. 13.09 ± 0.95 mg/100 ml blood), whereas the plasma level was significantly higher (10.16 ± 0.59 vs. 8.08 ± 0.89 mg/100 ml plasma) in the molybdenum-fed rat compared with the rat consuming the basal diet. Significance was determined by the *t* test ($P < 0.01$).

TABLE 3

Urinary amino acids (exp. 3)[1]

Amino acid	Pair-fed Basal diet	Molybdenum-fed
	μmole/day/rat	*μmole/day/rat*
Histidine	—	8.54
Citrulline	—	7.98
Tyrosine	—	4.28
Phenylalanine	—	3.42
Hydroxyproline	0.09	6.70
Asparagine	0.42	28.21
Serine	0.46	30.76
Threonine	0.38	23.94
Valine	0.04	2.00
Alanine	1.30	39.88
Glycine	2.04	59.54
Isoleucine	0.04	1.14
Glutamic acid	1.20	26.78
Leucine	0.10	2.00
Lysine	0.28	4.56
Proline	2.32	33.34
Ethanolamine	0.32	3.85
Methionine	0.10	0.86
Taurine	4.40	35.90
β-Alanine	0.37	1.85
Aspartic acid	0.38	1.70
L-Methyl histidine	0.23	1.00
Hydroxylysine	0.02	0.09
Ornithine	0.14	0.28
Sarcosine	0.42	0.40
Total	15.05	329.00

[1] Urine collected after 30 days of feeding.

DISCUSSION

Molybdenosis in the rat was accompanied by depressed weight gain and decreased nutrient consumption. These observations are consistent with previous reports (1, 2). The decreased utilization of nutrients by the molybdenum-fed rats was demonstrated by the trend toward better weight gain in pair-fed rats and the decreased consumption, with parallel gain, of the restricted-fed rats consuming basal diet with no added molybdenum. The depression in nutrient utilization may be attributable to either an increased need for energy by the molybdenum-fed rats or a decreased efficiency of utilization of ingested nutrients.

Digestion and absorption of dietary protein was not appreciably impaired in molybdenosis as shown by only minor increases in fecal nitrogen. The increased level of total urinary nitrogen accompanying molybdenosis suggests an alteration in nitrogen metabolism and is consistent with an earlier observation that increased levels

of dietary protein alleviated the molybdenosis syndrome (3). However, increased urinary excretion of nitrogen could also be the result of increased utilization of dietary protein for energy rather than a defect in nitrogen metabolism. The increased excretion of urea plus ammonia by the molybdenum-fed rat suggests that the deamination of amino acids was increased and this could be attributed to increased utilization of amino acids to satisfy caloric requirements. An increased utilization of dietary protein for energy could contribute to the decreased ingestion of diet since food consumption is regulated, extensively, in animals by caloric requirements (10). However, this would not explain the greater rate of body weight gain of the pair-fed controls or the equal gain of the restricted-fed controls unless the molybdenum-fed rat had an increased requirement for energy.

Alternatively, the increased deamination of amino acids could have resulted from an increased catabolism of body protein or a decreased anabolism of dietary protein. Creatine and creatinine excretion by the molybdenum-fed rat was not affected; therefore, increased catabolism appears improbable because these compounds are used to estimate muscle protein catabolism. However, these urinary components are not affected by the turnover of some of the more transient body proteins such as those of the liver and blood and, consequently, more definitive experiments (i.e., using labeled amino acids) are needed to determine the source of the urinary nitrogen. The increased excretion of free α-amino nitrogen by the molybdenum-fed rat represents a loss of metabolizable energy and was a contributing factor to the depressed nutrient utilization.

The aminoaciduria, however (fig. 1) would not account for the depression in nutrient utilization for weight gain because the growth depression was observed immediately after feeding molybdenum, and the aminoaciduria could not be detected until after about 20 days of feeding. Gross kidney damage resulting from molybddenum feeding and contributing to the aminoaciduria appeared unlikely since no proteinuria was observed. However, histopathological studies of the kidneys revealed that some fatty infiltration had occurred. The fat was deposited as fine droplets on or near the basement membranes of the proximal convoluted tubules and a slight degree of regeneration had occurred in the epithelium of the tubules. Since the kidney damage was not extensive, the possibility that it could account for the observed aminoaciduria does not appear probable because the renal threshold for most of the amino acids is high.

It is known that ingested molybdenum is excreted primarily in the urine. The possibility that the animal detoxifies molybdenum by the formation of an amino acid-molybdenum complex prior to excretion was investigated with paper chromatography. Preliminary studies indicated that this was not probable because the molybdenum did not move as a distinct spot and did not correspond to any ninhydrin-positive area. It is possible that a relatively unstable complex was formed and had dissociated either before or during the chromatographic separation so that it was not detected.

An inhibition, by molybdenum, of the resorptive mechanism for amino acids in the kidney is possible. These mechanisms are specific for groups of amino acids (11) and if one or more of them were inhibited, the group or groups of amino acids would be excreted and this selective excretion could result in an "induced" amino acid imbalance. Other heavy metals such as cadmium, lead, uranium, and mercury have caused aminoacidurias (12) and in most cases, serine and threonine were observed in increased concentrations in the urine. This could indicate that the resorptive mechanism for these 2 amino acids was more susceptible to inhibition by heavy metals. Amino acid imbalances are sometimes accompanied by aminoacidurias (13–15) and are more easily induced with low-protein diets. Nine per cent casein diets have been used to induce amino acid imbalances which could be alleviated by threonine supplementation (16, 17). Threonine was the most limiting amino acid in the diet used in these studies since the diet was supplemented with cystine (18). An experiment was designed to determine whether this loss

of threonine caused the growth depression through an imbalance. Both the basal diet and the basal diet plus 400 ppm molybdenum were supplemented with each individually and all combinations of the following at a level of 0.2% of the diet: L-threonine, L-tryptophan, and L-valine. No effect upon weight gain, which could be attributed to the added amino acids, was observed during a 6-week feeding period. Therefore, it was concluded that the excretion of these 3 amino acids did not result in an imbalance of amino acids within the body.

The observations reported here are compatible with the hypothesis that dietary moblybdenum inhibits some facet of protein synthesis. The increased urinary nitrogen excretion would occur if the dietary protein was not being anabolized to body protein. Immediately after feeding molybdenum, the absorbed protein moieties would be utilized for energy; thereby increasing urea plus ammonia excretion and decreasing food consumption. Food consumption would be further diminished since there would be a lesser demand for energy for protein anabolism. The possibility of a block occurring in the urea cycle enzyme synthesis could explain the gradual decline in urea excretion in the molybdenum-fed rats, accompanied by a gradually increasing aminoaciduria. The observed citrullinuria is consistent with this hypothesis. Also, histopathological studies of the livers revealed a uniform lesion in all livers from molybdenum-fed rats consisting of (a) marked fatty infiltration and fatty metamorphosis of the parenchymal cells diffusely distributed throughout the liver lobules, and (b) numerous mitotic figures indicating extensive regeneration. A severe impairment of the liver functions was probable. The protective effect of diets high in protein is also compatible with the inhibited protein synthesis hypothesis. Increasing dietary casein would increase the concentration of reactants for protein synthesis. Furthermore, in vitro studies of the effect of minerals upon the incorporation of amino acids by rat liver preparations (19) have demonstrated an inhibition by molybdenum. These observations are consistent with the proposed hypothesis that molybdenum inhibits some facet of protein synthesis in vivo.

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Nutritional Value of Proteins of Oriental Soybean Foods[1,2]

BLUEBELL R. STANDAL

Department of Nutrition, Hawaii Agricultural Experiment Station, University of Hawaii, Honolulu, Hawaii

ABSTRACT The protein quality of oriental foods of soybean origin has been determined by the net protein utilization (NPU) method at a 10% level of protein in the diet. This method calls for the determination of whole body nitrogen by the Kjeldahl method and its calculation from body moisture on 77 male and female weanling rats fed varying quantity and quality of proteins for 10 days. The correlation coefficient for the body nitrogen values obtained by the 2 procedures was 0.947. This high correlation permitted the computation of whole body nitrogen from body water in subsequent experiments. The proximate compositions of the soybean products were in agreement with those in previous reports. The generous amount of protein in edamame, tofu, and natto is confirmed. The NPU of the control proteins, powdered whole egg and crude casein, was 90.4 and 67.3, respectively, which corresponded to values reported by Miller and Bender using the same method. The NPU values obtained for the soybean products were 72.2, 65.0, 56.0, 44.4, and 35.6 for edamame, tofu, soybean sprouts, natto, and mung bean sprouts, respectively. Edamame and tofu, being both of good quantity and quality in protein, appeared to be dependable vegetable protein sources.

As a source of food, the soybean is looked upon as furnishing protein or oil. In the United States, 90% of the soybean used for food has been for oil (1). In Asia, on the other hand, the bean itself is used in large quantities in various forms of food articles. Hawaii imports 680 to 907 metric tons of soybeans per year from the United States mainland (2) and most of it is used for food. Oriental soybean products are used daily by all nationalities in Hawaii. They constitute important items in the diet. All soybean products used in these islands, e.g., tofu, natto, miso, shoyu, and sprouts, are prepared from the matured beans by the methods used in Japan and China. A certain amount of the beans is grown locally and picked green for use as will be described later. Studies on the quality of proteins of matured soybeans, soybean meal, soybean flour, and soybean milk have been extensive. However, little information is available for some of the oriental preparations of interest here. The biological value of the soybean curd has been reported by Cheng et al. (3), Pian (4), and Chang and Murray (5). The effect on growth by fresh or germinated soybeans was mentioned by Everson and Heckert (6).

In view of the world-wide need for sources of vegetable proteins of good qualities, a study of the quality of the protein of oriental soybean products was undertaken. This study employs the method of determining protein quality as devised by Miller and Bender (7), which was based on the constancy of the ratio of body nitrogen to body water in rats (8).

EXPERIMENTAL

Animals. Albino rats of this Station's Navy[3] colony were used in all experiments. The animals were weaned at 21 days of age and at a weight of about 35 g. The rats were fed the mother's diet[4] until they were 24 or 25 days old and had attained a weight of approximately 50 g. They were divided into experimental and control groups by weight, littermates, and sex. The average weights for the groups did not differ by more than 1 g.

Diets. The non-protein diet contained the following (in per cent): granulated

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[2] Published with the approval of the Director of the Hawaii Agricultural Experiment Station as Technical Paper no. 630.

[3] Originally obtained from the Naval Hospital at Bethesda, Maryland.

[4] This diet contained 29% protein and 6% fat.

sugar, 15; cornstarch, 65; salt mixture, 5 (9); and fat, 15.[5] The protein food under test was substituted for cornstarch in an amount to provide a 10% protein by weight (N × 6.25) in the diet. Contribution to fat by the protein food was adjusted in the amount of the hydrogenated vegetable oil. The contribution was consistently 4.5 to 5% for the soybean products. The vitamin B complex was prepared in 20% ethyl alcohol and 1 ml was fed 3 times a week. Three milliliters of this mixture provided 120 μg each of riboflavin, thiamine, folic acid, and pyridoxine, 600 μg each of pantothenic acid and *p*-aminobenzoic acid, 400 μg of niacin, 12 μg of biotin, 15 mg of inositol, and 30 mg of choline. The fat-soluble vitamins were prepared by mixing cod liver oil[6] and DL-α-tocopherol.[7] Four drops of this mixture were given 3 times a week along with the vitamin B complex mixture. The rats received 600 USP of vitamin A, 60 USP of vitamin D, and 6 mg of vitamin E/week. Only on a rare occasion did a rat leave a portion of the vitamin mixture, as evidenced by the yellow streaks in the vitamin cups.

Protein foods

Whole egg powder. Twenty-three kilograms of spray-processed powdered whole eggs[8] were defatted bulkwise in large cans with petroleum ether several times until the fat content decreased from 46 to 4%. The remaining fat was removed with ethyl ether in a 2.2-liter Soxhlet extraction flask. The fat content decreased from 4 to 1.72% and this preparation was used in the diet. At a level of 10% protein by weight, the contribution of egg fat in the egg diet mixture was 0.25%. This served as the control protein diet.

Tofu. Four brands of tofu, a soybean curd, are available in the grocery stores in Honolulu. The 4 factories visited have similar ways of preparing the tofu. Matured dried soybeans were soaked in water for about 8 hours and divided into 2 portions. One portion was ground coarse and the other ground fine in its own milk. The 2 portions were mixed, steam-cooked, and filtered. A $CaCl_2$ solution was added to the filtrate while warm and this was allowed to stand for 30 minutes. The semi-solid mass was poured into large molds, allowed to stand, and the water pressed out 4 times. The mass was cut into 10-cm cubes. Each cube was put in a cellophane bag, sealed, kept cool and was ready for distribution.

Equal portions of all brands were purchased, drained of water, and mixed well. Water from the mixture was squeezed out using cheesecloth. The cloth was spread over drying trays, and the tofu layer thinned out and dried in a convection air-oven at about 70°C for 18 hours. The dried tofu was ground in a Wiley mill to a homogeneous powder and stored in sealed glass jars at 1°C. This preparation was light tan and contained 56.6% protein and 26.5% fat. A calculated amount was added to the diet to give a 10% protein mixture and adjustment was made for fat.

Natto. This is a fermented soybean preparation. Mature dried beans were soaked overnight and boiled until tender. The water was drained off and allowed to partially air-dry over bamboo trays for 20 minutes. The beans were then put into shallow paper containers and covered with wax paper. The containers were stacked one above the other in large wooden boxes, covered with straw mats, and placed in ovens at approximately 36°C for one day. The finished product had a dark brown color, and the beans retained their shape, but were covered by a stringy, slimy substance. The preparation had a characteristic cheesy odor which attracted flies and apparently stimulated the appetite of rats. Agar smears of the preparation show that it contained an almost pure culture of gram-positive rods in long chains, without spore formation for 2 days at 31°C.

Three brands of natto were available. Equal amounts of the 3 brands were purchased, mixed well, passed through a colander and stored in sealed glass jars in the refrigerator at 1°C. This preparation contained in per cent, protein, 25.7; water, 55.3; and fat 11.4. A calculated portion was added to the diet. This moist

[5] Primex, a hydrogenated vegetable oil, Procter and Gamble, Cincinnati, Ohio.
[6] E. L. Squibb and Sons, New York.
[7] General Biochemicals, Chagrin Falls, Ohio.
[8] Cloverbloom whole egg powder obtained from Armour and Company, Springfield, Missouri.

diet contained 9.92% protein and 11.63% fat. A portion of this diet was dried in the hot-air oven. The dry diet contained 11.5% protein and 15.3% fat. Both diets were used in the experiments.

Edamame. These are locally grown soybeans, *Glycine max* (L.) Merr., of the bansei variety. They are picked green and used as a vegetable. Representative amounts were purchased from different stores, washed, shelled, and steamed for 40 minutes. In the moist treatment, the water which collected in the cooking pan was not added to the beans. These were ground twice and incorporated into the diet. In the dry treatment, the water collected in the cooking pan was added to the beans which were ground twice, dried in the convection hot air oven for 18 hours, ground again through a Wiley mill and incorporated into the diet. Adjustment for fat contained in the preparation was made as described before. The preparation had a pale green color. The protein content of the preparation was 21.2 and 37.5% for the wet and dried material, and the fat content was 7.4 and 14.4%, respectively.

Mung bean sprouts. The sprouts of the mung bean, *Phaseolus aureus* Roxb., were dried, ground as above, and incorporated into the diet. The sprouts so treated were quite brown and had a somewhat bitter taste, although this taste was not evident when incorporated in the diet. The rats did not eat it well; therefore, another batch was dried for a shorter time, ground and added to the diet. It was not possible to grind the sprouts to a fine powder and it was observed that the rats discarded the unground sprouts. This diet was sifted and the unground sprouts dried again, reground, and added to the remainder of the second diet. The rats ate similar amounts of the 3 mung bean diets. The dried mung bean sprouts contained an average of 1.7% fat and 31.1% protein.

Soybean sprouts. These sprouts were larger than the mung bean sprouts and the cotyledons were more fleshy. They were germinated from soybeans, *Glycine max* (L.) Merr. Purchased sprouts were steamed for 5 minutes, dried in a hot-air oven for 18 hours, ground in a Wiley mill to powder and incorporated in the diet. The brown coloration and the toughness of the dried sprouts observed with mung bean did not occur in the soybean sprouts. The powdered sprouts contained 17.4% fat and 42.2% protein.

Casein. The crude casein[9] used contained 0.2% fat and 90.5% protein.

EXPERIMENTAL PROCEDURE

The nitrogen equivalent per gram of food for all diets was determined by the Kjeldahl method. K_2SO_4 and selenized granules were added to the sulfuric acid digestion mixtures. The protein quality of 3 to 4 protein foods was determined at one time, using 8 to 10 rats for each protein. One group of rats was fed a protein-free diet simultaneously. To eliminate the effects of unequal consumption of foods by rats fed different test proteins, control rats for each test protein were raised with the egg diet. These were started and terminated one day later, since these rats received an amount of nitrogen in their diet equivalent to the amount consumed on the previous 24 hours by the rats fed the test protein. The experimental period was 10 days. The amount of food consumed was determined and allowance was made for wastage whenever it occurred. The rats were killed by ethyl ether and weighed carefully. An abdominal incision was made and cut through the length of the rat from the lower jaw to the tail. Each rat was placed in an aluminum foil boat and dried at 95°C for 2 days and in a vacuum oven at 70°C for 6 hours. Body nitrogen was calculated from body moisture according to Miller and Bender (7). Seventy-seven of the dried rats were ground individually, defatted by Soxhlet extraction, and digested in a Kjeldahl flask by carefully controlled heating. The cleared digest was diluted with water, cooled, and made to a 250-ml volume. Six aliquots from each digest were distilled in a micro-Kjeldahl distillation apparatus and titrated with standardized dilute H_2SO_4. Comparison of the values for whole body nitrogen, as obtained by the Kjeldahl method and by calculation from body water, is shown in table 1. After recording the weight

[9] See footnote 7.

TABLE 1

Reliability of whole body nitrogen as calculated from total moisture

Diets	No. of rats	Body N by Kjeldahl	Body N by calculation[1]
		g	*g*
Protein-free	7	1.308 ± 0.111[2]	1.391 ± 0.131
Casein	7	1.922 ± 0.104	1.935 ± 0.084
Tofu	14	1.704 ± 0.149	1.906 ± 0.200
Green soybean	18	1.832 ± 0.123	2.364 ± 0.156
Egg	31	2.047 ± 0.188	2.458 ± 0.239
Correlation coefficient $r^3 = 0.947$			

[1] Log (4.8 − y) = 0.437 − 0.0123n
where n is age in days
y is $\frac{N\ (g)}{H_2O\ (g)} \times 100$
N is whole body nitrogen.

[2] SD.

[3] $r = \frac{\Sigma x_1 x_2}{\sqrt{(\Sigma x_1^2)(\Sigma x_2^2)}}$
where x_1 and x_2 are deviations from the mean of 77 determinations.

change, the amount of nitrogen consumed, and the whole body nitrogen of a rat, the net protein utilization (NPU) was calculated by applying the equation of Miller and Bender (7). Protein efficiency ratio (PER) was calculated for each rat from the weight change divided by the weight of protein consumed by that animal.

RESULTS AND DISCUSSION

Whole body nitrogen. To establish the reliability of the calculation of whole body nitrogen from body water, 77 rats were fed 6 diets containing 6 types of proteins of varying qualities. The rats consumed different amounts of nitrogen in the 6 diets. The values for total nitrogen, as obtained by calculation from body water and as determined by the Kjeldahl procedure, are reported in table 1. The correlation coefficient for the nitrogen values on the 77 rats was calculated according to Snedecor (10). Although the trend was toward lower values by the Kjeldahl method, the correlation coefficient *r*, having the value of 0.947, was highly significant. Body nitrogen values used thereafter in the calculation of NPU were those calculated from body water.

Proximal composition of the soybean products. Of the foods reported in table 2, natto is less popular among the population of the islands. Previous reports on the proximate composition of these foods appeared in 2 publications by Miller (11, 12). Because handling of the raw material by different investigators may vary, the earlier publication of Miller was useful in comparing present data as moisture values were included. With the exception of fat, the food composition data agreed

TABLE 2

Proximate composition of some soybean products[1] of oriental foods

Foods	Moisture	Protein[2]	Fat	Ash	Carbohydrate[3]
	%	%	%	%	%
Edamame (immature green soybeans, bansei var.)	64.57	13.96	7.90	2.29	11.28
Natto (3 brands)	55.30	25.72	11.44	2.37	5.17
Tofu (4 brands)	74.23	14.84	6.58	1.05	3.30
Soybean sprouts (fresh, uncooked)	84.90	7.61	2.40	0.92	4.17
Mung bean sprouts (fresh, uncooked)	93.28	2.16	2.17	0.34	2.05

[1] Samples of products were purchased from 4 to 5 stores and mixed together.
[2] By Kjeldahl method.
[3] 100 − (% protein + % fat + % ash).

well in the present analysis with those reported by Miller (11, 13) for edamame and tofu.

Data in table 2 indicate that edamame, tofu, and natto contained appreciable amounts of protein.

Net protein utilization (NPU). The NPU values obtained for whole egg solid and for casein were 90.9 and 68.0 (table 3). Corresponding data reported by Miller and Bender were 91.0 and 60.0. Good agreement existed between the data obtained in this laboratory and those of Miller and Bender (7) using similar methods. This method was adopted by the present investigator in all studies pertaining to the quality of proteins by biological method.

The highest NPU value obtained in this study was that of steamed edamame (green soybeans). The values were 71.5 for the moist diet and 73.0 for the dry one. Miller and Bender (7) reported a value of 56.0 for soya flour. The immature quality of the green soybeans used in this study may have contributed to the higher NPU value. Everson and Heckert (6) reported that fresh soybeans and germinated soybeans promoted better growth than did unheated mature seeds when used as the source of protein for young-growing rats. The PER calculated for the edamame diets was 2.41 for the moist and 2.27 for the dry diets, respectively. Everson and Heckert (6) obtained PER values of 1.482 for bansei and 1.418 for Fung Delicious soybeans. These beans were tree-ripened and heated at 120°C for 45 minutes before they were incorporated into the diet. Russell (14), on the other hand, reported a PER of 2.1 for soaked, cooked, matured bansei beans. Chang and Murray (5) obtained a PER of 1.73 for Fung Delicious soybeans autoclaved at 110°C for 30 minutes.

Tofu ranked next to edamame with the NPU value of 65.0 and the PER of 1.93. Cheng et al. (3) obtained a biological value of 64 for soybean curd. Chang and Murray (5) obtained a PER of 1.73 for their preparation of soybean curd.

Soybean sprouts appeared to contain protein of relatively good quality, the NPU being 56.0 and the PER 1.36. Of somewhat poorer quality was natto with an NPU of 45.0 and 43.8 for the moist and dry diets, respectively, and a PER of 1.52 and 1.00. These values were similar to the values for ground nut meal and copra meal obtained by Miller and Bender (7). Of poorest quality was the protein of mung bean sprouts with an NPU of 35.6 and a PER of −1.38. Due to smaller consumption of the mung bean diet, rats fed the whole egg control diet were also forced to eat less. The NPU for this group of egg control was calculated separately. The average NPU was 87.3. The amount of nitrogen consumed by this group fed the egg control diet was one-half the amount consumed by the other egg control groups with an NPU of 90.9 (table 3). The rats receiving the minimal intake of a good quality protein showed remarkable economy. On the other hand, the same amount of nitrogen consumed from the mung bean sprout diet did not maintain the rats as evidenced by the negative PER and loss of weight. The amount of protein calories afforded by each experimental diet was calculated from proximate analysis of the diets. Protein calories (P_m) needed to be supplied by each diet for maintenance of weight at ± 1 g over a 10-day period were calculated according to Miller and Payne (15), using the NPU measured at a level of 10% protein by weight (table 3). Comparison of the 2 sets of values showed that mung bean sprouts as a source of protein must afford in protein calories 11.24% of the total calories. From proximate analysis it provided only 9.32%. All other diets supplied more generous amounts of protein calories than were needed for maintenance. Factors other than the protein may be present in the mung bean sprouts which depressed food consumption.

Matured soybeans were known to contain nitrogenous goitrogenic and antitryptic factors. Since these were heat labile, their activities were presumably destroyed in these experiments. From these data it may be concluded that casein and tofu had similar NPU values. Edamame was superior to casein and tofu, whereas soybean sprouts were somewhat inferior. Natto was poor and mung bean sprouts very poor. Natto, which was prepared from matured soybeans gave NPU and

TABLE 3

The quality of proteins of some soybean foods

Diets	Type of diet	No. of rats	No. of replicates	N consumed in 10 days/rat	NPU[1]	PER[2]	Protein[3] calories afforded by diet	Protein[4] calories needed from diet for maintenance (P_m)
				g			*% of total calories*	*% of total calories*
Tofu (soybean curd)	dry	14	2	1.24	65.0 ± 1.2[5]	1.93	10.93	6.15
Natto (fermented soybeans)	moist	6	1	1.56	45.0 ± 8.5	1.52	12.10	8.89
	dry	8	1	1.24	43.8 ± 6.7	1.00	10.80	9.13
Edamame (green soybeans)	moist	9	1	1.55	71.5 ± 11.2	2.41	17.73	5.59
	dry	16	2	1.36	73.0 ± 8.4	2.27	8.60	5.48
Soybean sprouts	dry	17	3	1.04	56.0 ± 3.3	1.36	8.66	7.14
Powdered whole egg control	dry	74	12	1.20	90.9 ± 7.5	3.05	9.31	4.40
Mung bean sprouts	dry	25	3	0.63	35.6 ± 2.6	−1.38	9.32	11.24
Powdered whole egg (control to mung bean sprouts)	dry	15	3	0.66	87.3 ± 14.5	0.93		
Casein	dry	7	1	0.82	68.0 ± 11.0	1.44	9.32	5.88

[1] NPU = net protein utilization = $\frac{\text{retained N in body}}{\text{food N}} \times 100$.

[2] PER = $\frac{\text{Weight change}}{\text{g protein consumed}}$.

[3] By proximate analysis.

[4] $P_m = \frac{4.0 \times 100}{\text{NPU}}$.

[5] SE.

PER values below reported values for the matured beans. It is possible that a substance or substances were formed during the incubation process which contributed to lower values of NPU. Richardson et al. (16) reported retarded growth in poults that received a diet containing mouldy soybean meal. Natto, being a product of bacterial fermentation, may not bear the same defect. No pathological lesions were observed in the rats, such as were seen in the poults. The amount of weight gained was similar to that of rats in the soybean sprout group, but the total body nitrogen was lowered despite generous consumption of nitrogen. Microbiological assay of the essential amino acids shows that natto was low in methionine.[10] The protein score of natto calculated according to FAO (17) was 35. A similar observation was reported for tempeh[11] where lysine and methionine were decreased by fermentation of the soybeans. The nutritive value of the soybeans when fed to the rats decreased with increased fermentation time (18). Experiments with natto diet, supplemented by methionine, are in progress.

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[10] Unpublished data.

[11] Tempeh is an Indonesian preparation. Soybeans were soaked, boiled, and fermented with a species of Rhizopus mold for about two days.