Growth of Rats from Normal Dams Restricted in Diet in Previous Pregnancies

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ABSTRACT Dietary restriction of dams from mating until weaning of progeny, or during lactation alone, resulted in stunting of the offspring. Previous history of this type of dietary restriction failed to influence the growth of the offspring of a subsequent pregnancy during which the dams were not subjected to any dietary restriction.

Recently we reported that in rats, 25 or 50% dietary restriction (from ad libitum consumption) from mating until weaning of progeny or during lactation, or both, stunted the growth of the offspring, even though they were fed ad libitum after weaning (1). This failure to achieve normal growth could be corrected by injection of hypophyseal growth hormone shortly after weaning. The present investigation extends these studies to a determination of whether a history of restriction affects the growth of the offspring of dams in subsequent pregnancies when no restriction is imposed.

EXPERIMENTAL

All animals studied were McCollum-strain rats maintained with an unsupplemented diet of commercial laboratory chow meal.[1]

Two experiments were carried out. In the first of these, baseline performance data for our colony were obtained by measuring the growth of litters from 46 females bred over a period of 8 months. Subsequently, nine of these litters were studied during second matings during which time 50% dietary restriction was imposed throughout the reproductive periods. The same 9 females were then remated with the same males and during this cycle no dietary restriction was imposed. The measured response parameter in this series was the growth of the young, which were all fed ad libitum over a 40-week period. In the second experiment, 4 groups of three previously unmated females each, chosen at random from a population 3 to 4 months of age were studied under our standard mating conditions. These together with 4 males, likewise 3 to 4 months old, comprised 4 breeding groups. Of these groups, two were assigned to the "unrestricted"[2] intake, and the remainder were restricted to levels of one-half the intake of the unrestricted females. The unrestricted levels were established by transferring the 6 females in the unrestricted groups to individual cages at 9 AM on day 1 of breeding and offering each a measured amount, usually 35 g of our stock diet. After 8 hours, the unconsumed food, plus spillage, was measured and the food consumption estimated by difference. Each of these females was then returned to the original breeding cage to reconstitute the original groups. On the second day, the average amount of food consumed, determined for the 6 females maintained with the unrestricted diets, was calculated and quantities corresponding to one-half that amount were weighed into individual feed cups and offered to the 6 females in the restricted groups, after they had been distributed among individual cages in the morning. In most instances, these ani-

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[1] Purina Laboratory Chow, Ralston Purina Company, St. Louis; composition as provided by the manufacturer: (in per cent) crude protein not less than 23.0; crude fat not less than 4.5; crude fiber not more than 6.0; ash not more than 9.0; ingredients: meat and bone meal, dried skim milk, wheat germ meal, fish meal, animal liver meal, dried beef pulp, ground extruded corn, oat middlings, soybean meal, dehydrated alfalfa meal, cane molasses, animal fat preserved with BHA, vitamin B_{12} supplement, calcium pantothenate, choline chloride, folic acid, riboflavin supplement, dried brewer's yeast, thiamine, niacin, vitamin A supplement, 0.5% deflourinated phosphate, 0.5% iodized salt, 0.07% ferric ammonium sulfate, 0.02% $MnSO_4$, trace of ZnO.

[2] No food was made available to these animals between 5 PM and the next morning.

mals consumed the entire ration offered. Similarly on day 2, the intake of unrestricted groups was measured and thereafter daily. These daily figures from the unrestricted rats constituted the baseline for setting the intakes of the restricted groups for the balance of the experiment. This regimen was followed during the period of gestation and lactation. After the litters were cast, each lactating dam was allowed to nurse only 8 pups, regardless of the groups assigned. This procedure was followed to avoid the complications that might arise from infection, unequal distribution of milk, and other variables, which might result from excess litter size.

After weaning, 21 days after birth, the young were transferred to individual cages (38-cm wide by 46-cm deep by 58-cm high) and fed ad libitum. Body weights were recorded weekly for 40 weeks.

The dams were allowed to rest for 8 weeks, during which time the restricted dams, now being fed ad libitum, usually gained in body weight to equal that of the unrestricted dams. All mating groups were then re-established using the same males. However, for the second cycle, dietary restriction was imposed on the previously unrestricted females, whereas those restricted during the first cycle were now unrestricted. After birth and weaning, the young from these second matings were fed stock diet ad libitum. The dams were allowed to rest for 8 weeks and again mated with the same male. Then a third cycle was completed. During the third cycle the animals were maintained on schedules identical with those to which they had been assigned during the first cycle.

The overall experimental design was that of a double cross-over with respect to restriction, so that the 6 sets of litters studied could be divided into 2 groups. Group 1 consisted of successive litters coming from mothers on restricted-to-unrestricted-to-restricted regimens; group 2 litters were from mothers on unrestricted-to-restricted-to-unrestricted regimens.

To study the effect of dietary restriction, or starvation, of rats after birth several procedures were chosen. All rats for this experiment (except those in group D, described later) were born to dams unrestricted from mating until birth of progeny. The controls, (group A) were nursed by dams fed ad libitum during lactation and given stock diet ad libitum after weaning. The animals in group B were born of dams similar to those whose young were in group A, but were starved for 7 days after weaning, until the body weight was reduced by 25% of the weaning weights. The animals in group C were nursed by dams whose dietary intake was reduced by one-half during the lactation period only but were offered our stock diet ad libitum after weaning. The rats in group D were born of dams on whom dietary restriction was imposed from mating until weaning of offspring, and fed ad libitum after weaning.

RESULTS

In table 1 are recorded data on reproduction and growth of young from the 3 groups of the first experiment. The mean

TABLE 1

Effect of dietary restriction of pregnant rats on the growth of their offspring

	Treatment during gestation	No. females delivering	Avg no. of pups/ litter	Avg body wt			
				Birth	3-week	14-week	40-week
				g	*g*	*g*	*g*
A	Unrestricted	46	10.8 ± 0.6 [1]	6.2 ± 0.2	45 ± 2.8 [2]	320 ± 1	410 ± 21
					40 ± 1.1 [3]	210 ± 10	320 ± 18
B	Restricted	9	7.4 ± 1.0	4.8 ± 0.2	31 ± 1.2 [2]	240 ± 11	322 ± 18
					20 ± 1.8 [3]	180 ± 10	225 ± 16
C	Unrestricted	9	11.7 ± 0.7	6.4 ± 0.4	48 ± 3.1 [2]	340 ± 21	460 ± 26
					39 ± 1.1 [3]	220 ± 18	340 ± 11

[1] SE of mean.
[2] Male rats.
[3] Female rats.

litter size, birth weight, and body weights of their offspring (by sex) were smaller in group B than in group A. Subsequently, the performance with respect to these parameters was restored to the treatment level of group A during treatment of group C. These results demonstrate that the dietary restriction from mating until after weaning of the young, and during lactation reduces the litter size and the growth performance without affecting the dams' capacity in a subsequent trial without restriction.

Table 2 summarizes the results of the second experiment. Here again, performance as measured by the number of successful pregnancies, mean birth weight, and weight of young after 14 weeks shows impairment as a result of restriction and restoration to nonrestricted levels in subsequent unrestricted cycles. At least over the number of cycles studied, a previous history of restriction during mating, pregnancy, and lactation appears to have no carry-over effect on the dam during a subsequent unrestricted cycle.

In all 3 cycles, only 2 animals originally assigned to group A and three of the six in group B, became pregnant and delivered.

One of the experimental problems in assessing whether the damage due to any dietary manipulation is "temporary" or "permanent" lies in establishing the length of the interval during which the body weight change of the growing rats must be recorded. We chose a period of 14 weeks in which to allow the retarded rats to attain the weight of the controls. Failure to do so at this time is empirically regarded as evidence of "permanent" damage. This

TABLE 2

Effect of maternal dietary intake on the growth of offspring (cross-over experiment)

Cycle	Treatment	No. pregnancies resulting in live births	Avg body wt		
			Birth wt	14-week	
				Male	Female
			g	*g*	*g*
		Group A			
1	Restricted	3	4.8 ± 0.2 [1]	262	178
2	Unrestricted	5	6.4 ± 0.2	338	225
3	Restricted	3	5.0 ± 0.1	226	167
		Group B			
1	Unrestricted	6	6.2 ± 0.1	336	208
2	Restricted	4	4.8 ± 0.2	281	171
3	Unrestricted	6	6.1 ± 0.2	328	220

[1] Mean ± SE.

TABLE 3

Recovery of body weight of dams subjected to dietary restrictions under varying conditions

Group	Dietary treatment	Rat body wt at various ages					
		Birth	3-week	4-week	6-week	14-week	40-week
		g	*g*	*g*	*g*	*g*	*g*
A	Unrestricted	6.6 ± 0.1 [1] (6) [2]	48 ± 0.5	74 ± 6	150 ± 7	310 ± 20	450 ± 15
B	Dams restricted as pups after weaning	6.6 ± 0.1 (6)	48 ± 0.5	37 ± 3	137 ± 9	326 ± 22	—
C	Dams restricted during lactation	5.2 ± 0.2 (7)	37 ± 1.1	47 ± 2	142 ± 8	328 ± 11	—
D [3]	Dams restricted from mating until weaning of progeny	4.6 ± 0.3 (4)	26 ± 1.0	38 ± 3	97 ± 6	130 ± 8	318 ± 7

[1] SE of mean.
[2] Numbers in parentheses denote number of rats/ery was achieved by this group after 14 weeks of ad
[3] Served as control to indicate that no weight recovgroup.
libitum feeding.

is based on a series of studies on the body weight recovery rates of rats exposed to dietary restriction under different conditions. The results of one experiment on male rats are presented in table 3.

The data in table 3 indicate clearly that at 6 weeks of age there were virtually no differences in body weight between the animals in groups A, B and C whereas those in group D failed to attain the weight of the others, even though they were offered an unlimited food intake as long as 1.5 to 2 years.

DISCUSSION

In man and in animals it is generally assumed that the size of the offspring is dependent upon the size of the parents. There is no report of systematic studies on the effect of maternal nutrition during reproduction on the growth and development of offspring, such as the one presented in this paper. It has been observed that the size of people of a given race usually changes in parallel with changes in the levels of nutrition and medical care, whether these result from migration or local shifts. Such observations, however, do not necessarily permit a firm conclusion that they are effects of nutrition. The data obtained in this communication indicate that the body weight of the pups after birth, even when fed an adequate diet ad libitum, reflects the dietary history of the dam during gestation and lactation. These studies minimize genetic factors since the parents were the same in successive cycles. The same female will produce smaller-size progeny if her diet is restricted during a second pregnancy and the progeny of a third pregnancy can be larger (as large as the first) if the animal is fed ad libitum again. From the data in this study, the food consumption of the dam is demonstrated to be an important factor in determining the body weight of the progeny.

LITERATURE CITED

1. Chow, B. F., and C. J. Lee 1964 Effect of dietary restriction of pregnant rats on body weight gain of the offspring. J. Nutrition, *82*: 10.

Effect of Corn Oil on Metabolic Efficiency of Energy Utilization by Chicks [1]

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ABSTRACT Studies were made of the tissue composition and tissue energy gains of chicks receiving equal intake of metabolizable energy and all other nutrients from diets containing 2.5, 12.5 and 22.5% corn oil substituted isocalorically for glucose. Tissue energy gains per unit of metabolizable energy intake increased as the dietary level of corn oil was increased. It is concluded that replacement of dietary carbohydrate with corn oil increases the metabolic efficiency of energy utilization by chicks. Increases in tissue energy were due solely to increased fat content. Nitrogen retention measured as gain in tissue protein was reduced in one experiment but was unaffected in a second experiment by feeding high levels of corn oil. However, no significant effect of corn oil on growth rate was observed, indicating an inverse correlation between tissue fat deposition and water retention. Based on results reported in the literature for the chick and other species, it appears most probable that the beneficial effect of corn oil on efficiency of energy metabolism is mediated through the heat increment component rather than the basal component of heat production. Further studies will be necessary to determine whether this effect is a general property of well-utilized fats or is due to unidentified substance present in corn oil.

Recent investigations have demonstrated that vegetable oils possess special nutritive properties for the growing chick as evidenced by increased growth rate and improved efficiency of feed utilization. The reports on this subject have been reviewed by Rand et al. (1) and others (2–5).

Increased efficiency of feed utilization may be mainly attributed to the greater concentration of metabolizable energy in diets containing added oils. However, the growth-promoting property of oils appears to be unrelated to dietary energy level. Under conditions equalizing intakes of protein and metabolizable energy, Rand et al. (1) observed increased growth rate and nitrogen retention in chicks when corn oil replaced dietary carbohydrate. Carew et al. (6) observed that growth stimulation by corn oil and soybean oil was independent of changes in metabolizable energy and density of diets. Hopkins and Nesheim (7) concluded that the growth-promoting activity of soybean oil is due to specific components of the oil rather than its energy contribution.

Although the effects of fats on growth rate and gross efficiency have been studied in the chick, very little is known about their effect on energetic efficiency of growth in this species. The purpose of the present studies was to measure comparative efficiency of energy metabolism and tissue composition of chicks fed different dietary levels of a vegetable oil.

MATERIALS AND METHODS

Duplicate lots of 10 Rhode Island Red × Barred Plymouth Rock male chicks were used in each treatment. The chicks were maintained with the basal diet until 2 weeks of age and assigned to experimental groups on the basis of 2-week body weights and gains using a method similar to that described by McKittrick (8). The extremes were discarded and approximately 80% of the original population was used. The experiments terminated when the chicks were 4 weeks of age.

Throughout these studies, chicks were raised in electrically heated, thermostatically controlled battery brooders with raised wire floors in a temperature-controlled laboratory room. Water was given ad libitum.

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TABLE 1

Composition of basal diet

	%	%
Glucose	51.8	
Constant ingredients:	48.2	
Soybean meal (50% protein)		16.0
Crude casein		11.0
Gelatin		2.5
Fish meal, menhaden		4.0
Brewer's yeast, dried		2.5
Whey, dried		2.0
Fish solubles, condensed		2.0
Corn oil		2.5
Chromium oxide mix (30% Cr_2O_3)		1.0
Mineral and vitamin mixtures [1]		4.7
Analytical values [2]		
Crude protein (nitrogen × 6.25), %	28.3	
Ether extract, %	3.0	
Metabolizable energy, kcal/g	3.36	

[1] Mineral and vitamin mixtures supply: (in g/kg) calcium carbonate, 20; dicalcium phosphate, 10; iodized salt, 6.4; K_2HPO_4, 2.2; $MgSO_4$, 1.21; $MnSO_4 \cdot H_2O$, 0.30; $FeSO_4 \cdot 7H_2O$, 0.278; $CuSO_4 \cdot 5H_2O$, 0.0078; NaI, 0.0026; (in mg/kg) niacin, 30; calcium-D-pantothenate, 25; riboflavin, 10; pyridoxine·HCl, 10; thiamine·HCl, 5; folacin, 2; menadione, 1; biotin, 0.4; choline chloride, 1400; *d*-α-tocopheryl acetate, 22; vitamin B_{12}, 0.011; chlortetracycline, 5.5; butylated hydroxytoluene, 100; diphenyl-p-phenylenediamine, 100; (in units/kg) stabilized vitamin A, 10,000 IU; vitamin D_3, 1500 ICU.

[2] Dry matter basis.

The low fat basal diet used in these experiments is presented in table 1. It contained adequate levels of all nutrients known to be required by the chick as well as sources of unidentified factors which have been reported to be necessary for maximal growth rate. All constant ingredients were combined in a single mixture and treated as one dietary component. The experimental diets are shown in table 2. Corn oil was added to the basal diet by isocaloric substitution for glucose on a metabolizable energy basis using the following energy values: glucose, 3.64 kcal/g (9), and corn oil, 8.8 kcal/g (10).

In both experiments the basal diet was fed ad libitum and at restricted levels equal to 50 and 75% of the daily intake of the groups fed ad libitum. In experiment 1, a diet containing 20% added corn oil was also fed at these 3 planes of intake, and in each case energy intake was equalized to that of the respective lots receiving the basal diet. Diets containing 10 and 20% added corn oil were used in experiment 2, and were pair-fed on an energy basis only at the restricted level of 75% of ad libitum basal energy intake.

Chicks receiving restricted energy intakes were fed once daily at approximately the same time each day, and the quantity of feed for each group was placed in a single feeder of sufficient size to provide free access by all chicks. The daily energy intake allocated to chicks on the limited feeding regimens was determined by the previous 24-hour intake of the control lots fed ad libitum, except for the first 24-hour period when energy intake was estimated. Wasted feed was recovered and measured daily by use of a shallow wire mesh covered box under each feeder. Serial analyses of the moisture content of the several diets showed no changes during the 2-week experimental period.

The feeding rates to equalize energy intakes between groups fed the different diets were based on calculated metabolizable energy of the diets using previously determined values for the various components. The actual energy intakes were based on determinations of nitrogen-corrected metabolizable energy for all diets which were made in the course of each

TABLE 2

Composition and energy values of experimental diets [1]

Components	Basal diet	Basal + 10% corn oil	Basal + 20% corn oil
Constant ingredients, %	48.2	55.1	61.9
Glucose,[2] %	51.8	34.9	18.1
Corn oil,[3]	—	10.0	20.0
Metabolizable energy, kcal/g			
Experiment 1	3.36	—	4.25
Experiment 2	3.39	3.87	4.30

[1] Values on a dry matter basis.

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

[3] Mazola, Corn Products Company.

experiment according to the method described by Hill et al. (11) using method 2 for excreta preparation. Excreta were collected when the chicks were 23 to 26 days of age. The determined metabolizable energy values are shown in table 2; they agreed with the calculated values within 1.5%.

Immediately before and after each experimental period, chicks were fasted for 18 hours to permit virtually complete emptying of the digestive tract. In this manner body weights and tissue analyses were unaffected by undigested dietary residues. Two lots of 10 chicks were killed and analyzed at the start of each experiment to supply data on initial carcass composition. Each experimental lot was analyzed at the end of the 2-week feeding period to determine tissue gains of protein and fat. Chicks were killed without blood loss by dislocation of the neck, and frozen for subsequent carcass analysis. Each lot of chicks was prepared for carcass analysis by homogenization in a 4.7-liter Waring Blendor. A weighed amount of water equal to 1.5 times the weight of carcass material was added to facilitate homogenization. A large aliquot of the pooled homogenate for each lot was dried in a Virtis portable freeze-dryer. The aliquot was sufficient to yield at least 60 g of dry matter, and represented about 5 to 10% of the total tissue of a lot of 10 chicks. Loss of water during freeze-drying was measured to determine total carcass moisture and dry matter. Analyses for protein (Kjeldahl nitrogen × 6.25), ether extract and residual moisture were made on the dry material by standard laboratory methods. Quantitative accounting of the total material for each lot was made at each step in the preparation of the samples so that tissue gains could be calculated accurately from the composition data. A more detailed description of the procedure used for carcass analysis has been presented by Carew.[4]

Tissue energy gains were derived by calculation from gains in tissue fat and protein using energy coefficients of 9.35 kcal/g for fat and 4.34 kcal/g for protein. The latter value represents an average gross energy value for tissue protein, 5.66 kcal/g, less the combustible energy of an equimolar amount of uric acid. This energy value for tissue protein was used to make it consistent and comparable with the nitrogen-corrected metabolizable energy values of the respective diets.

Data on weight gains and tissue gains were treated statistically by analysis of variance, and individual degree of freedom comparisons were made (12).

RESULTS

The results of the first experiment are summarized in table 3. The observed mean gains produced by the low fat and high

[4] Carew, L. B., Jr. 1961 Studies of the effects of dietary vegetable oils on growth rate, energy intake, tissue composition and energy metabolism of chicks. Ph.D. Thesis, Cornell University, Ithaca, New York.

TABLE 3

Effect of corn oil on weight gain, tissue composition and energy balance of chicks (exp. 1)

Diet	Level of energy intake	2-Week wt gain	ME [1] intake	Protein intake	Gains		
					Tissue protein	Tissue fat	Tissue energy
		g	*kcal*	*g*	*g*	*g*	*kcal*
Low fat basal	ad libitum	246	1496	125	50.5	18.1	388
Low fat basal	75%	180	1109	93	37.5	9.8	254
Low fat basal	50%	103	741	62	24.0	0.2	106
+20% corn oil	100%	231	1448	124	44.9	21.9	400
+20% corn oil	75%	174	1088	93	34.2	14.4	283
+20% corn oil	50%	99	726	62	21.3	3.1	121
Gains adjusted to equalize energy intake with control lots:							
+20% corn oil	100%	238	1496		46.3	22.9	427
+20% corn oil	75%	177	1109		34.8	14.8	290
+20% corn oil	50%	102	741		21.8	3.6	126

[1] ME denotes metabolizable energy, based on values in table 2.

fat diets at the 3 levels of intake are shown. The respective feeding levels were not identical, due to small discrepancies in expected energy levels and feed wastage; hence the gains of the high fat groups were calculated to a basis of equalized energy intake, using the linear regression of each gain on energy intake for the high fat treatments. These data are also shown in table 3.

Growth rate measured as live weight gain was not significantly different between the 2 diets. The small differences in the observed data were largely eliminated by recalculation to equal energy intake. Protein gains were significantly reduced by feeding the fat supplemented diet ($P < 0.05$) at all levels of intake. At the same time, fat deposition was significantly increased by the high fat diet, and a marked difference was evident at each level of energy intake.

Relative to metabolizable energy consumption, the chicks gained more tissue energy at each level of energy intake when the diet contained 20% added corn oil. A graphic representation of the relation between energy gain and energy intake is shown in figure 1. The relation was linear for each of the respective diets. For the basal diet the calculated regression was $Y = 0.372X - 166$, with a correlation coefficient of 0.994. For the diet supplemented with corn oil the regression was $Y = 0.385X - 151$, with a correlation coefficient of 0.988. The observation that energy gain is linearly related to energy consumption over a wide range of intake

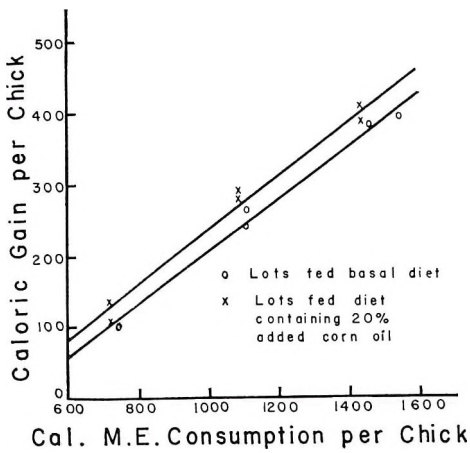

Fig. 1 Relationship between metabolizable energy consumption and tissue energy gain of chicks fed 2 levels of corn oil (exp. 1).

during early growth agrees with previous work in this laboratory (13, 14).

Covariance analysis (12) showed no significant difference between the regression coefficients of the 2 equations, indicating that they could not be distinguished from parallel lines. The analysis did show a significant difference ($P < 0.05$) in the height (mean gains) of the response lines, showing that at any given energy intake within the range studied a greater energy gain was produced by the diet containing 20% corn oil. The magnitude of the effect is shown by the adjusted data for energy gains in table 3.

The results of experiment 2 are presented in table 4. The effect of three levels of corn oil (2.5, 12.5, and 22.5%) on

TABLE 4

Effect of level of dietary corn oil weight gain, tissue composition and energy balance of chicks (exp. 2)

Diet	Level of energy intake	2-Week wt gain	ME [1] intake	Protein intake	Gains		
					Tissue protein	Tissue fat	Tissue energy
		g	*kcal*	*g*	*g*	*g*	*kcal*
Low fat basal	ad libitum	235	1535	112	47.7	24.6	437
Low fat basal	50%	102	746	55	22.2	2.8	122
Low fat basal	75%	175	1117	82	36.2	12.6	274
+10% corn oil	75%	171	1070	84	35.2	14.7	289
+20% corn oil	75%	169	1061	84	34.8	17.4	313
Gains adjusted to equalize energy intake with 75% control lot:							
+10% corn oil	75%	178	1117		36.5	16.0	308
+20% corn oil	75%	177	1117		36.3	19.0	335

[1] ME denotes metabolizable energy.

energy metabolism was measured at only the 75% level of energy intake. The basal diet was fed at 3 levels of energy intake to establish the relationships of gains to energy intake. The effects of small discrepancies in the energy intake of the 3 diets at the 75% level on gains were estimated from these relationships, assuming linearity over the short range involved, and the adjusted gains are also shown in table 4.

As observed in the first experiment, corn oil had no effect on growth rate. Although protein gains tended to decrease as the dietary level of corn oil increased, the small differences were not statistically significant. These differences were eliminated completely when the intake differences were adjusted. Tissue fat gains increased with each addition of corn oil, resulting in greater gains in tissue energy for chicks fed diets with added corn oil.

The relation of metabolizable energy intake to tissue caloric gains for experiment 2 is illustrated in figure 2. The basal response line for chicks fed the control diet has the form $Y = 0.399X - 176$, with a correlation coefficient of 0.994. All points representing lots of chicks fed diets containing added corn oil were located above the control line. This confirmed the results of experiment 1 showing that dietary additions of corn oil increased the net retention of energy in tissues per unit of metabolizable energy intake. These observations further indicated a step-by-step increase in fat and energy gains from feeding 10 and 20% corn oil.

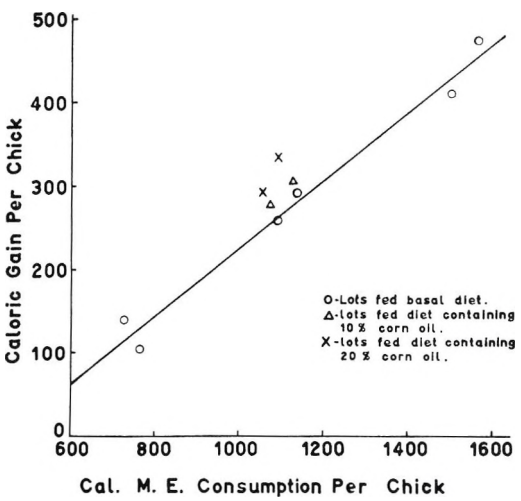

Fig. 2 Relationship between metabolizable energy consumption and tissue energy gain of chicks fed 3 levels of corn oil (exp. 2).

DISCUSSION

The results of the 2 experiments showed that chicks utilize metabolizable energy more efficiently for growth, expressed as tissue energy gain, if part of the carbohydrate portion of the diet is replaced with corn oil. Increases in tissue energy content were solely the result of gains in adipose tissue. No beneficial effect of corn oil on nitrogen retention was observed, whereas in one experiment the gain of tissue protein by chicks fed a high level of corn oil was reduced at all levels of energy intake. These observations suggest that dietary corn oil may influence the metabolism of nitrogenous compounds under certain conditions. Although changes in body composition occurred, no effect of corn oil on weight gains was observed, indicating that fat deposition and water retention were inversely correlated.

Since the differences in energy gain were due entirely to differences in fat deposition, it is appropriate to ask whether it is possible that errors in estimating the metabolizable energy content of the diets could account for the apparent effects of the diets on energy utilization. This was analyzed by taking the increased energy gains produced by the fat-supplemented diets (from the adjusted gains, tables 3 and 4) and converting these to their equivalent in metabolizable energy consumption, using the regression coefficients relating energy gain to intake in each experiment. These ranged from the equivalent of 75 dietary kcal/chick (exp. 1, 20% corn oil at 100% intake) to 153 dietary kcal (exp. 2, 20% corn oil, 75% intake). As a percentage of total metabolizable energy intake for each treatment, the equivalents were 5, 10 and 11% for the 3 levels of intake in experiment 1, and 8 and 14% for the 2 levels of corn oil in experiment 2. In contrast with these large discrepancies, measurements of metabolizable energy content of diets can be expected to be replicable within 1%. It is unlikely, therefore, that errors in metabo-

lizable energy determinations can account for any important part of the observed effects.

The results of the present studies are in contrast with those reported by Rand et al. (1). Using a technique also based on equalized energy intakes, these workers observed increased growth rate and nitrogen retention as the proportion of dietary corn oil increased. It is unfortunate that they presented no data on tissue fat content, since it cannot be determined from their results whether a true increase in efficiency of energy metabolism occurred, or whether the greater synthesis of tissue protein occurred at the expense of fat deposition. The reason for these conflicting results is not known. However, differences in experimental technique such as strain of bird and type of experimental diet may be important.

Investigations concerning the effect of fats on nitrogen metabolism in the rat are equally conflicting. Forbes et al. (15) and Pearson and Panzer (16) reported increased nitrogen retention when high levels of dietary fat were used, whereas Forbes et al. (17, 18, 19) Hoagland et al. (20) and Yoshida et al. (21) reported no differences. Willman et al. (22) and Fox and Swanson (23) observed that the effect of fats on nitrogen metabolism depends on the plane of nutrition. Undoubtedly, many variables enter into this relationship.

The mechanism whereby corn oil promotes greater metabolic efficiency cannot be determined from the present data. This effect represents either a lower energy requirement for basal metabolic processes or a more efficient use of energy beyond basal needs. No experimental evidence has been found to support the former possibility. March and Biely (24) obtained no changes in fasting oxygen consumption when high levels of beef tallow or herring oil were added to chick diets. Although the inclusion of fat in the diet depressed thyroid weight, results on thyroxine secretion were inconclusive. Carew [5] observed no differences in thyroid weight or height of secretory cells when chicks were fed diets containing 1.2 and 13.8% soybean oil.

On the other hand, a large body of evidence has been reported in work with the rat (15, 17) showing that fats decrease the heat increment or specific dynamic effect of dietary mixtures, resulting in more efficient utilization of metabolizable energy. In view of these observations, it seems quite possible that this same effect may account for the increased metabolic efficiency of chicks fed diets containing high levels of oil.

Exactly how fats lower the heat increment when added to dietary mixtures is not known. There are several possible explanations for this effect. 1) An unrecognized vitamin or hormone-like substance which alters the efficiency of metabolic processes may be present in fats and oils. 2) Fats and oils may be metabolized more efficiently than carbohydrates. Part of this effect may represent a saving of energy needed to synthesize fat from dietary carbohydrates. 3) The incorporation of fats per se into the metabolic pool may alter the utilization of absorbed nutrients such that some are more efficiently metabolized. This is supported by the work of Forbes and Swift (25) showing that fats exert an "associative dynamic effect" on diets fed to rats. They observed that the heat increment due to a mixture of protein, carbohydrate and fat was smaller than the sum of the heat increments of the individual components.

In addition to the above possibilities it must also be considered that chicks fed diets high in fat content may have smaller heat losses associated with activity because they either are less active or can perform work more efficiently.

The magnitude of the effect of corn oil in improving energy utilization in the present work is of interest because it bears on the question of the choice of energy units for the evaluation of diets and diet components. As indicated earlier, the metabolizable energy of corn oil for the chick as determined in this laboratory is 8.8 kcal/g. The observed metabolizable energy values of the diet used in the present work are consistent with this value for corn oil. The apparent value of corn oil for deposition of tissue energy was calculated by: 1) converting the energy gain per chick produced by each fat-supplemented diet to its equivalent in basal dietary metab-

[5] See footnote 4.

olizable energy by dividing by the regression coefficient for energy gain per unit metabolizable energy consumed; 2) subtracting the acual metabolizable energy consumption for the fat-supplemented diet from this computed equivalent to estimate the *extra* value of the added fat; 3) dividing this extra value by the amount of supplemental fat consumed per chick, to determine the extra value per gram of corn oil; and 4) adding this extra value to 8.8 kcal/g to estimate the total apparent value of corn oil. The apparent values for corn oil so calculated were 9.4, 11.0 and 10.9 kcal/g for the 3 levels of intake in experiment 1, and 10.9 and 12.1 kcal/g for the 2 levels of corn oil in experiment 2. These values are 107 to 136% of the known metabolizable energy of corn oil, and have a mean of 124%. This discrepancy is sufficiently large to be of considerable importance in evaluating the nutritive worth of a diet, and in calculating the balance of energy to other nutrients in diet formulation.

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Influence of Amount and Type of Fat on Metabolic Efficiency of Energy Utilization by the Chick [1]

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ABSTRACT Studies were conducted to determine the influence of fats of varying fatty acid composition on the efficiency of energy utilization by chicks. When chicks were fed equal intakes of metabolizable energy from low fat diets or diets containing corn oil, beef tallow, soybean oil, or a lightly hydrogenated olive oil, more energy was deposited in the carcasses of chicks fed diets containing the fats than in the carcasses of chicks fed the low fat diet. However, no increase in energy deposition in the carcass was observed if diets containing hydrogenated coconut fat were fed compared with low fat diets. Ten per cent of corn oil in the diet was nearly as effective in improving efficiency of energy utilization as levels up to 34.9% of the diet.

In a preceding paper, Carew and Hill (1) reported that isocaloric substitution of corn oil for dietary carbohydrate increased metabolic efficiency of energy utilization by chicks, measured as total gain in tissue calories per unit of metabolizable energy intake. The previous study, as well as work of Forbes and Swift (2), provides no information as to whether the effects observed on metabolic efficiency of energy utilization are common to all fats, or whether only certain types of fats are effective. Therefore, experiments were conducted to study the effect of several fats of varying fatty acid composition on the metabolic efficiency on energy utilization.

MATERIALS AND METHODS

In the studies reported, duplicate lots of 10 Rhode Island Red × Barred Plymouth Rock male chicks were used per treatment in experiments 1 and 2, whereas triplicate lots of 8 White Plymouth Rock × Vantress male chicks were used in experiment 3. The chicks used in experiment 3 were hatched from eggs laid by hens fed diets low in linoleic acid. In experiment 2 chicks were fed the semipurified basal diet until 2 weeks of age. They were then randomized into experimental groups on the basis of 2-week body weights and gains by a modification of the method of McKittrick (3). In experiments 1 and 3, chicks were fed the purified basal diet until one week of age, and were assigned to the experimental groups so that approximately the same range of body weight was maintained in all groups. All experiments terminated when the chicks were 4 weeks of age.

The basal diets, presented in table 1, were calculated to contain adequate levels of required nutrients, and were supplemented with unidentified factor sources. In experiment 3, the purified basal diet contained 0.5% of hydrogenated coconut oil instead of 0.5% of corn oil. As fats and oils were added to the basal diets, all nutrients were maintained in a constant relationship to the energy concentration by appropriate increases in the constant ingredients premix. Fats and oils were added to the diets by isocaloric substitution for glucose on a metabolizable energy basis, using the following (metabolizable energy) values: (in kcal/g) glucose, 3.64; corn oil, 8.8; degummed soybean oil, 8.9 in experiments 1 and 2, and 9.2 in experiment 3; beef tallow, 6.3; hydrogenated coconut oil 7.85 in experiment 1, 7.7 and 6.8 in experiment 2, and 7.83 in experiment 3; lightly hydrogenated olive oil, 8.76. All of these energy values were obtained from experiments in this laboratory.

Chicks were housed in electrically heated, thermostatically controlled battery

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TABLE 1

Composition of basal diets

	Purified	Semi-purified
	%	%
Glucose	56.5	51.7
Constant ingredients premix	43.5	48.3
Isolated soybean protein [1]	25.0	—
Soybean meal (50% protein)	—	16.0
Crude casein	—	11.0
Fish meal, menhaden	—	4.0
Gelatin	—	2.5
Brewer's yeast, dried	—	2.5
Whey, dried	2.0	2.0
Fish solubles, dried	0.5	1.0
Corn distillers solubles, dried	2.0	—
Corn oil	0.5	0.5
Cellulose [2]	5.0	3.0
Chromium oxide mix (30% Cr_2O_3)	1.0	1.0
Premixes	7.5 [3,4]	4.8 [5]
Analytical values [6]		
Crude protein (nitrogen × 6.25), %	24.2	27.4
Ether extract, %	1.1	1.0
Metabolizable energy, kcal/g	3.23	3.28

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.

[2] Solka Floc, Brown Company, Berlin, New Hampshire.

[3] Supply the following: (in g/kg) DL-methionine, 6.0; glycine, 5.0; $CaHPO_4$, 20.7; $CaCO_3$, 14.8; KH_2PO_4, 10.0; NaCl, 6.0; $MgSO_4$, 3.0; KCl, 1.0; (in mg/kg) $FeSO_4 \cdot 7H_2O$, 333; $MnSO_4 \cdot H_2O$, 333; ZnO, 62; $CuSO_4 \cdot 5H_2O$, 16.7; $NaMoO_4 \cdot 2H_2O$, 8.3; KI, 2.6; $CoCl_2 \cdot 6H_2O$, 1.7; Na_2SeO_3, 0.1; choline chloride, 2100; niacin, 80; calcium D-pantothenate, 40; pyridoxine·HCl, 20; thiamine·HCl, 10; riboflavin, 10; folacin, 3; menadione, 3; biotin, 0.4; vitamin B_{12}, 0.015; ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), 100; chlortetracycline, 10; (in units/kg) stabilized vitamin A, 10,000 IU; vitamin D_3, 1500 ICU; vitamin E, 33 IU.

[4] In experiment 3, vitamin supplementation is the same as described by Hopkins et al. (10).

[5] Supply the following (in g/kg) limestone, 20; dicalcium phosphate, 10; iodized salt, 6.4; K_2HPO_4, 2.2; $MgSO_4$, 1.21; $MnSO_4 \cdot H_2O$, 0.3; (in mg/kg) $FeSO_4 \cdot 7H_2O$, 278; ZnO, 63; $CuSO_4 \cdot 5H_2O$, 7.8; $NaMoO_4 \cdot 2H_2O$, 8.3; NaI, 2.6; $CoCl_2 \cdot 6H_2O$, 1.7; Na_2SeO_3, 0.22; niacin, 30; calcium D-pantothenate, 25; riboflavin, 10; pyridoxine, 10; thiamine·HCl, 5; folacin, 2; menadione, 1; biotin, 0.4; choline chloride, 1400; vitamin B_{12}, 0.033; chlortetracycline, 5.5; ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), 100; (in units/kg) vitamin A, 10,000 IU; vitamin D_3, 1500 ICU; vitamin E, 33 IU.

[6] Dry-matter basis.

brooder units with raised wire floors. Water was given ad libitum. Other experimental procedures, including method of feeding to equalize energy intakes, and the carcass analysis technique, were presented in a preceding report (1). Tissue caloric gains were calculated from tissue gains in fat and protein, using energy values previously obtained (1).

Metabolizable energy values of diets were determined during the last week of each experiment by the method of Hill et al. (4) using method no. 2 for excreta preparation.

Differences in body weight gains and tissue protein gains were treated statistically by analysis of variance. Treatment differences were tested by individual degree of freedom comparisons in experiments 1 and 3 (5) and Duncan's multiple range test in experiment 2 (6). In experiments 1 and 3, differences in tissue energy gains were tested by individual degree of freedom comparisons.

RESULTS

In experiment 1, the effect of 4 levels of corn oil and one level of hydrogenated coconut oil on carcass energy deposition was studied. The diets used in this experiment are shown in table 2. Addition of 34.9% of corn oil to the basal diet replaced all of the glucose in the basal diet. The basal diet and diets containing 20 and 34.9% of added corn oil and 20% of hydrogenated coconut oil were fed ad libitum. In addition, the basal diet and the 4 diets containing added corn oil were equal-fed to give the same metabolizable energy intake for all groups at approximately 75% of the metabolizable energy intake of chicks receiving the basal diet ad libitum.

The results of experiment 1 are summarized in table 3. With ad libitum feeding, chicks fed diets containing added corn oil grew faster than chicks receiving the basal diet. Marked increases in energy intake were observed with the feeding of both levels of corn oil, confirming earlier studies by the authors (7). Increased gains in tissue fat and tissue energy accompanied the ad libitum feeding of diets containing added corn oil. Consumption of the diet containing 20% of corn oil resulted in greater gains in tissue protein (nitrogen retention) than the diet containing 35% of corn oil.

The ad libitum feeding of 20% of hydrogenated coconut oil did not increase growth rate, energy intake, tissue fat gain or total energy gain above that obtained with chicks fed the basal diet. A slight increase in tissue protein gains was observed but to a lesser extent than occurred with

TABLE 2

Composition of experimental diets [1] (exp. 1)

	Purified basal	Diets				
		2.5% corn oil	10% corn oil	20% corn oil	34.9% corn oil	20% coconut oil
	%	%	%	%	%	%
Constant ingredients	43.5	45.1	49.7	55.9	65.1	53.6
Glucose	56.5	52.4	40.3	24.1	—	26.4
Corn oil	—	2.5	10.0	20.0	34.9	—
Hydrogenated coconut oil	—	—	—	—	—	20.0

[1] Values on a dry-matter basis.

TABLE 3

Effect of hydrogenated coconut oil and graded levels of corn oil on weight gains, tissue composition and energy balance of chicks (exp. 1)

Treatment	3-week wt gain	ME [1] intake	Protein intake	Gain in tissue protein	Gain in tissue fat	Gain in tissue energy
	g	*kcal*	*g*	*g*	*g*	*kcal*
Ad libitum series						
1 Low fat basal	292	1746	131	60.0	23.1	477
2 +20% corn oil	340	2033	151	68.9	31.9	597
3 +34.9% corn oil	323	1875	145	62.2	35.5	602
4 +20% hydrogenated coconut oil	290	1677	139	63.2	20.1	462
Restricted series						
5 Low fat basal	219	1300	98	43.8	12.0	300
6 +2.5% corn oil	226	1312	99	45.9	11.9	310
7 +10% con oil	231	1319	99	46.7	14.8	341
8 +20% corn oil	233	1304	99	46.8	16.4	357
9 +34.9% corn oil	218	1278	102	41.8	17.5	345

Statistical comparisons	Mean squares		
	3-week wt gain	Gain in tissue protein	Gain in tissue energy
1, 2, 3, 4 vs. 5, 6, 7, 8, 9	33012 *	1548 *	—
1 vs. 2, 3, 4	940 *	33 *	—
2, 3 vs. 4	2290 *	7 *	—
2 vs. 3	299	5 *	—
5 vs. 6, 7, 8, 9	85	6	2254 *
6 vs. 7, 8, 9	15	1	2094 *
7 vs. 8, 9	0.3	8	120
8 vs. 9	104	26	141

[1] ME indicates metabolizable energy.
* designates significance at 5% level of probability.

the diet containing 20% added corn oil. The widely different energy consumptions under ad libitum feeding conditions make difficult assessment of the effect of the fats on energy efficiency.

Although the value of 7.85 kcal of metabolizable energy/g for hydrogenated coconut oil was used in formulating the diet in experiment 1, this sample had an actual metabolizable energy value of 6.14 kcal/g, which slightly changed the energy-to-protein balance of the diet. However, the lack of responses in growth rate, energy intake, tissue fat gains and tissue energy gains from the feeding of hydrogenated coconut oil probably were more a result of the properties of this type of fat per se than changes in the energy-to-protein balance.

Results obtained with restricted but equal intakes of energy in experiment 1 are presented in the lower part of table 3. The paired feeding was very effective in this experiment and only small differences in energy intake were observed.

Although weight and tissue protein gains of groups receiving added corn oil were generally greater than basal, these differences were not significant. In contrast, tissue fat content was increased in chicks receiving the diets containing more than 2.5% of corn oil. These changes in carcass composition were reflected in gradual increases in tissue energy gains of chicks fed the diets containing up to 20% of corn oil. Based on statistical analysis of gains in tissue energy, significantly greater energy gains occurred in chicks receiving the diets containing 10% of corn oil compared with the gains of chicks receiving the low fat diet. However, this level was as effective as the higher dietary levels of corn oil.

An additional experiment was conducted to compare the effect of feeding diets containing corn oil, soybean oil, beef tallow and hydrogenated coconut fat on metabolic efficiency of energy utilization. The 6 diets used in experiment 2 to compare the 4 types of fat are shown in table 4. In this experiment all chicks were equal-fed at a level approximately 75% of their estimated ad libitum intake, based on daily energy intake values taken from similar studies. Two diets containing hydrogenated coconut oil were used in this study. Each sample was assigned a different metabolizable energy value, since previous experience had shown that it was difficult to establish energy values for different samples of hydrogenated coconut oil. Two values, 6.8 kcal/g and 7.7 kcal/g, were chosen as the most expected metabolizable energy values. The actual values determined in the study were 7.23 kcal/g and 6.96 kcal/g, respectively.

The results of experiment 2 are shown in table 5. Greater variability in energy in-

TABLE 4

Composition of experimental diets [1] *(exp. 2)*

	Diets					
	Semi-purified basal	20% corn oil	20% soybean oil	20% beef tallow	20% coconut oil (6.8 kcal/g)	20% coconut oil (7.7 kcal/g)
	%	%	%	%	%	%
Constant ingredients	48.3	60.8	61.1	54.3	55.7	57.9
Glucose	51.7	19.2	18.9	25.7	24.3	22.1
Corn oil	—	20.0	—	—	—	—
Soybean oil, degummed	—	—	20.0	—	—	—
Beef tallow	—	—	—	20.0	—	—
Hydrogenated coconut oil	—	—	—	—	20.0	20.0

[1] All values on a dry-matter basis.

TABLE 5

Effect of various fats on weight gains, tissue gains and energy balance of chicks (exp. 2)

	2-week wt gain [1]	ME [2] intake	Protein intake	Gain in tissue protein	Gain in tissue fat	Gain in tissue energy
	g	*kcal*	*g*	*g*	*g*	*kcal*
Group 1						
1 Low-fat basal	206^{ab}	1272	106	44.0^{a}	15.9	339
2 20% Beef tallow	212^{a}	1283	105	42.3^{ab}	22.8	397
3 20% Hydrogenated coconut oil (7.23 kcal/g)	214^{ab}	1298	103	41.8^{ab}	18.9	358
Group 2						
4 20% Corn oil	197^{b}	1237	104	38.5^{b}	20.9	362
5 20% Soybean oil	202^{ab}	1249	105	40.9^{ab}	19.6	362
6 20% Hydrogenated coconut oil (6.96 kcal/g)	200^{ab}	1222	106	42.6^{ab}	14.9	324

[1] Treatment averages not followed by the same letter are significantly different ($P < 0.05$).
[2] ME indicates metabolizable energy.

takes than expected was encountered in this experiment owing to deviations in the actual metabolizable energy values of the fats from those used in the original calculations. However, the 6 treatments could be divided into 2 groups of 3 treatments each that had similar energy intakes. In group 1, chicks fed the basal diet served as the negative control for chicks fed the diets containing beef tallow and the sample of hydrogenated coconut oil with an actual metabolizable energy value of 7.23 kcal/g. In group 2, corn oil served as the positive control for comparison with soybean oil and the sample of hydrogenated coconut oil with an actual metaboliazble energy value of 6.96 kcal/g.

Among treatment groups only slight differences in growth rate were observed.

Tissue protein gains of chicks in all groups fed diets containing added fat were lower compared with the basal group. The depressed protein gain was statistically significant only for chicks receiving the diet containing corn oil. In group 1, chicks receiving the diet containing beef tallow had greater gains in tissue fat above basal than did chicks receiving the diet containing hydrogenated coconut fat. In group 2, chicks receiving hydrogenated coconut fat had less tissue fat gains than chicks receiving diets containing corn oil or soybean oil. These differences in tissue composition indicated that hydrogenated coconut oil did not increase energy gains as effectively as did the other fats studied, and this is confirmed by the results shown for tissue energy gains.

On the basis of total gains in tissue energy presented in table 5, beef tallow, corn oil and soybean oil effectively increased efficiency of energy utilization, whereas hydrogenated coconut oil produced only a very small increase in metabolic efficiency above the basal treatment.

The results of experiment 2 suggested that hydrogenated coconut oil did not improve efficiency of energy utilization to the same degree as soybean oil, corn oil and tallow. Since hydrogenated coconut oil was nearly devoid of linoleic acid, a possible explanation for the differences observed among these fats could be that an adequate intake of linoleic acid was required for maximal efficiency of energy utilization. A slightly hydrogenated olive oil was prepared which contained only 1.2% of linoleic acid, and the effects of this oil, soybean oil and hydrogenated coconut oil were compared in experiment 3.

The diets used in this experiment are shown in table 6 and the results obtained are shown in table 7. The chicks that received high dietary levels of oil in this experiment were restricted to 75% of the average caloric intake of chicks fed the basal diet ad libitum. The energy intake of chicks fed the experimental diets varied to some extent, owing to differences in determined metabolizable energy values of the fats compared with the expected values used when the diets were formulated. However, the interpretation of the results is clear despite these differences in energy intake. Chicks receiving diets containing soybean oil or the lightly hydrogenated olive oil had significantly greater gains in tissue protein, fat, and energy compared with those of chicks receiving the diets containing hydrogenated coconut oil or the basal diet. Apparently the composition of fats does influence their effect on

TABLE 6

Composition of experimental diets (exp. 3)

	Purified basal	15% Hydrogenated coconut oil	15% Olive oil	15% Soybean oil
	%	%	%	%
Constant ingredients	43.96	51.55	53.24	54.09
Glucose	56.04	33.45	31.76	30.91
Hydrogenated coconut oil	—	15.00	—	—
Lightly hydrogenated olive oil [1]	—	—	15.00	—
Soybean oil, degummed	—	—	—	15.00

[1] Prepared by Swift and Company, Chicago. Had the following fatty acid composition: 16:0, 11.9%; 16:1, 0.9%; 18:0, 7.4%; 18:1, 78.6%; 18:2, 1.2%.

TABLE 7

Effect of hydrogenated coconut oil, lightly hydrogenated olive oil and soybean oil on energy deposition by chicks (exp. 3)

	Treatment	22-day wt gain	ME [1] intake	Protein intake	Gain in tissue component [2]		
					Protein	Fat	Energy
		g	*kcal*	*g*	*g*	*g*	*kcal*
1	Low fat basal	308	1757	14.2	64.4	17.0	438
2	15% Hydrogenated coconut oil	316	1689	14.2	64.8	16.1	432
3	15% Hydrogenated olive oil	334	1686	14.2	70.2	21.9	510
4	15% Soybean oil	338	1735	14.2	71.7	19.4	492

[1] ME indicates metabolizable energy.
[2] By analysis of variance and individual degrees of freedom comparison, the gains in tissue fat, protein and energy of treatment 1 and 2 are significantly different from values for treatments 3 and 4 ($P > 0.05$).

efficiency of caloric deposition in the carcass, but this effect does not appear to be related to the linoleic acid content of the fat.

DISCUSSION

These studies indicate that the effect of dietary fats on metabolic efficiency of energy utilization varies with the amount and composition of the fat used. Beef tallow, corn oil, soybean oil and lightly hydrogenated olive oil increased efficiency of energy utilization by the chick when added to diets containing approximately 1% fat. Hydrogenated coconut oil was the least effective in this respect of the fats studied, and only slight improvements in efficiency of energy utilization were observed from the use of high levels of this fat.

In experiment 1, the degree of improvement in efficiency of energy utilization was not directly proportional to the amount of fat present in the diet. Low amounts of dietary fat (10%) were as effective as levels up to 34.9% in producing the maximal increase in efficiency of energy utilization. These observations are similar to results reported by Forbes et al. (8) when rats were fed graded levels of lard.

The effects of the various fats used on tissue protein gains were not consistent. In experiment 2, corn oil significantly depressed tissue protein gains, whereas in experiment 3 soybean oil and lightly hydrogenated olive oil significantly increased protein gains. Rand et al. (9) reported that additions of corn oil to isocaloric, isonitrogenous diets for chicks increased nitrogen retention, whereas Carew and Hill (1) observed depressions in tissue protein gains as the level of dietary corn oil increased. The reason for these varying effects of fats on nitrogen metabolism is not easily explained.

The observation that hydrogenated coconut oil was relatively ineffective in improving efficiency of energy utilization suggested that certain fatty acids may be important in determining this effect. However, from the results presented, a specific linoleic acid requirement does not appear to be involved since both beef tallow and lightly hydrogenated olive oil containing 3.5 and 1.2% linoleic acid, respectively, were effective in increasing efficiency of energy utilization. Nevertheless, since hydrogenated coconut fat is composed of nearly all relatively short-chain (C_{12}, C_{14}) saturated fatty acids, the metabolism of these fatty acids may be less efficient compared with that of the fatty acids in the more unsaturated fats. The lack of effect on efficiency of energy utilization observed from feeding diets containing hydrogenated coconut fat may reflect the fatty acid composition of this specific fat, or may indicate the effect to be expected from any fat nearly devoid of unsaturated fatty acids.

The deposition of energy in the carcass of chicks as studied in these experiments represents the end result of the efficiency of pathways used in the anabolism and catabolism of dietary components. The increased efficiency of energy utilization that

occurs when fats are included in the diet may be a reflection of the efficiency with which energy is stored as preformed dietary fat. Storage of energy by chicks fed diets low in fat content may be less efficient because of the necessity for fat synthesis.

Although the effect of fats on efficiency of energy utilization appears to be related to their fatty acid composition, other components of fats may be important in this respect.

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Effect of Linoleic Acid on Nutritional Muscular Dystrophy in the Chick [1]

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ABSTRACT Experiments were conducted which showed that in the absence of dietary linoleic acid, muscular dystrophy does not occur in chicks even when the diet is low in both vitamin E and sulfur amino acids. The addition of graded levels of linoleic acid to the diet resulted in proportionate increases in the incidence and severity of muscular dystrophy. The vitamin E requirement for prevention of muscular dystrophy was increased with increasing levels of linoleic acid up to 0.5% linoleic acid. No increase was observed in the vitamin E requirement for prevention of muscular dystrophy when the level of linoleic acid was increased from 0.5 to 2.5% of the diet. Vitamin E at a level which completely prevented muscular dystrophy had no effect on the level of linoleic acid in muscle lipids, which increased in both dystrophic and non-dystrophic chicks in proportion to the amount of linoleic acid in the diet.

Investigations by Calvert et al. (1), Scott (2, 3) and Scott and Calvert (4) have shown nutritional interrelationships between vitamin E, selenium, cystine and antioxidants in the prevention of muscular dystrophy in the chick. These observations strongly indicate that vitamin E, selenium and antioxidants may be concerned with the metabolism of sulfur within the tissues. However, all attempts thus far to elucidate this metabolic function have been unsuccessful.

Since vitamin E is known to act both in vivo and in vitro as an antioxidant, a natural hypothesis to explain the function of vitamin E is that it is simply required to prevent peroxidation of the unsaturated fatty acids of the lipoproteins and phospholipids in the cell walls of the tissues. This antioxidant effect of vitamin E appears to be its main function in the prevention of encephalomalacia (5–10).[3,4] Although selenium is of primary importance in the prevention of exudative diathesis (11), the antioxidant properties of vitamin E may have some bearing on the prevention of this disease (12–14). However, the effect of oxidation of fatty acids upon production of muscular dystrophy in the chick has not been investigated extensively, primarily because the work of Dam et al. (15) and Machlin and Shalkop (16) did not demonstrate an effect of dietary fat on this disease.

Experiments were conducted, therefore, to determine whether dietary linoleic acid influences the course of nutritional muscular dystrophy in chicks fed an alcohol-extracted basal diet free of linoleic acid and containing levels of selenium and an antioxidant[5] adequate for the prevention of exudative diathesis and encephalomalacia, respectively. Some of the chicks used in these experiments were hatched from hens receiving low levels of dietary linoleic acid to reduce the amount of this fatty acid in the tissues of the chicks.

EXPERIMENTAL

All experiments were conducted with White Plymouth Rock or White Plymouth Rock × Vantress chicks hatched from Cornell Poultry Farm flocks maintained with special diets.

Chicks were housed at 1 to 2 days of age in thermostatically controlled, electri-

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[3] Century, B., and M. K. Horwitt 1958 Some factors in production of encephalomalacia in the vitamin E-deficient chick. Federation Proc., *17:* 473 (abstract).

[4] Sugai, M., M. Inoue, H. Tsuchiyama and F. A. Kummerow 1960 The interrelationship of vitamin E, linoleic and long chain keto acids. Federation Proc., *19:* 421 (abstract).

[5] 1,2-dihydro-6 ethoxy-2,2,4-trimethylquinoline.

cally heated battery brooders with wire-mesh floors in air conditioned rooms.

Prior to mixing the diets, all implements were washed thoroughly with ethanol and the mixing operations were programmed so that diets deficient in linoleic acid were mixed first, followed by diets containing increasing amounts of linoleic acid. The cans in which feed was stored were lined with polyethylene liners and separate plastic feed scoops used for each can. All diets were refrigerated during the experimental period. The feed and water were supplied ad libitum.

To obtain chicks with low linoleic acid stores, the first 3 experiments were conducted with chicks hatched from hens receiving the linoleic acid-low breeding diet shown in table 1. Chicks used in the last experiment were hatched from eggs from a flock of hens receiving a diet [6] low in vitamin E.

The composition of the basal chick diets used in these experiments is shown in table 2. The casein in diet 1 and isolated soybean protein in diet 2 were extracted with technical grade methanol for 48 hours prior to mixing into the experimental diets. This procedure reduced the fat content of the protein source to 0.4% as determined after acid hydrolysis. Changes and variables used in the various experiments will be indicated in the description of each experiment. All linoleic acid additions to the diets were made with linoleic acid [7] of 95% purity as determined by gas-liquid chromatography. The linoleic acid preparations were found to contain small amounts of oleic and linolenic acids as the major contaminants.

TABLE 1

Composition of hen diet for production of linoleic acid-depleted chicks

	%
Sucrose	55.00
Hydrogenated coconut fat	1.00
Soybean meal, 50% protein	34.80
Dicalcium phosphate	3.00
Ground limestone	4.00
Mineral mixture [1]	1.15
Vitamin mixture [2]	1.00
DL-Methionine	0.05

[1] Supplied the following per kg of diet: (in grams) NaCl, 5.230; $KHCO_3$, 3.304; $MgSO_4$, 2.203; $FeSO_4 \cdot 7H_2O$, 0.330; $MnSO_4 \cdot H_2O$, 0.330; $CuSO_4 \cdot 5H_2O$, 0.017; ZnO, 0.072; and (in milligrams) $CoCl_2 \cdot 6H_2O$, 2.0; $Na_2MoO_4 \cdot 2H_2O$, 3.0.

[2] Supplied the following per kg of diet: (in milligrams) biotin, 0.22; menadione sodium bisulfite, 1.65; pyridoxine·HCl, 4.4; folic acid, 4.4; thiamine·HCl, 9.9; riboflavin, 9.9; Ca pantothenate, 22.0; niacin, 49.6; choline chloride, 578; ethoxyquin, 125; and vitamin D_3, 991 ICU; vitamin E, 30 IU; vitamin A, 9910 IU; and vitamin B_{12}, 11 μg.

TABLE 2

Composition of basal chick diets

	Diet 1	Diet 2
	%	%
Casein	15.00	—
Isolated soy protein	—	20.00
Gelatin	10.00	—
Glucose [1]	65.57	69.84
Cellulose [2]	3.00	3.00
Hydrogenated coconut oil [3]	0.50	0.50
Vitamin mixture [4]	0.48	0.48
Mineral mixture [5]	5.45	5.45
Glycine	—	0.76
Amino acid mixture [6]	—	0.97

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Solka Floc, Brown Company, Berlin, New Hampshire.
[3] Hydrol, Durkee's Famous Foods, Inc., Chicago.
[4] The vitamin mixture supplied the following per kg of diet: (in milligrams) thiamine·HCl, 10.0; riboflavin, 10.0; pyridoxine·HCl, 4.5; niacin, 50; inositol, 250; folic acid, 4.0; Ca pantothenate, 20.0; biotin, 0.20; menadione, 0.50; choline Cl (70%), 2200; ethoxyquin, 125; and vitamin B_{12}, 20 μg; vitamin A, 5600 IU; vitamin D_3, 4410 ICU.
[5] The mineral mixture supplied the following per kg of diet: (in grams) $CaHPO_4$, 21.51; $CaCO_3$, 14.92; KH_2PO_4, 8.68; $KHCO_3$, 2.1; NaCl, 6; and (in milligrams) $MnCl_2 \cdot 4H_2O$, 463; $FePO_4 \cdot 4H_2O$, 265; MgO, 680; KI, 2.6; $Cu(C_2H_5O_2)_2$, 11.9; ZnO, 60; $CoCl_2 \cdot 6H_2O$, 1.7; $NaMoO_2 \cdot 2H_2O$, 8.3; and Na_2SeO_3, 2.2.
[6] The amino acid mixture supplied the following: (in grams/100 g of diet) L-leucine, 0.20; DL-phenylalanine, 0.50; and DL-tryptophan, 0.06.

Equal numbers of male and female chicks were used in each experiment unless otherwise specified. Group weights were recorded for the chicks at the beginning of the experiment and at weekly intervals thereafter. Muscular dystrophy was measured by careful, periodic observation of the chicks for signs and symptoms, beginning at about two weeks and by positive diagnosis by autopsy at the end of the experimental period. The duration of each experimental period will be reported under results of the experiment.

Samples of breast muscle to be analyzed for fatty acid composition were removed immediately after the chicks were decapitated. The samples were frozen immediately in liquid nitrogen and stored at −29° until the analyses were conducted. Muscle lipid was extracted with chloroform and methanol using a modification of the method of Bligh and Dyer (17), and

[6] Nesheim, M. C. 1959 Studies on the effects of selenium and other factors on vitamin E deficiency in the chicks. Ph. D. Thesis, Cornell University.
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methyl esters of the fatty acids were prepared according to the procedure of Stoffel et al. (18). The methyl esters were subjected to gas-liquid partition chromatography in an F & M 609 gas chromatograph with a flame ionization detector. The temperatures of the injection block, column and detector were 300°, 170° and 260°, respectively. A 2-meter × 4-mm i.d. aluminum column packed with 16% ethylene glycol succinate coated on 80–100 mesh Gas-Chrom P was used for the separation of the methyl esters of the muscle fatty acids. Helium was used as the carrier gas to a flow rate of 150 ml/minute at a pressure of 4.22 kg/cm².

RESULTS

A. *Positive effect of linoleic acid in the etiology of muscular dystrophy.* The first experiment was designed to determine whether linoleic acid is involved in the production of muscular dystrophy and if the amount of linoleic carried over from the egg to the chick influences this involvement. To accomplish this, chicks received diet 1 (table 2) with and without 0.5% linoleic acid. This amount of linoleic acid approximates that found in the 4.0% stripped lard which had been used routinely in most of our previous experiments on muscular dystrophy. A comparison was also made of the effects of linoleic acid in linoleic acid-depleted chicks hatched from hens receiving the basal diet shown in table 1, and non-depleted chicks from hens receiving the same diet with 5.0% added safflower oil.

The results of the experiment are presented in table 3. Linoleic acid was found to be involved under the conditions of this experiment in the production of nutritional muscular dystrophy. Depletion of linoleic stores of the chicks at hatching did not affect the results.

To determine whether this muscular dystrophy-producing effect is specific for linoleic acid, a further experiment was conducted to determine whether oleic acid (which represents (49% of lard) also has a detrimental effect, either alone or together with linoleic acid. Since 0.5% linoleic acid used in the previous experiment produced only a 50% incidence of muscular dystrophy, further studies also were made to determine the level of linoleic acid which produced a maximal incidence of muscular dystrophy. Linoleic acid was added alone at levels ranging from zero to 1.0% of the diet. Combinations of 4 levels of linoleic acid with oleic acid were used to determine whether an interrelationship exists between linoleic acid and oleic acid in the production of muscular dystrophy. All of these treatments were compared with the effect of 4.0% stripped lard. The basal diet used in this experiment was changed to diet 2 (table 2) to determine whether the effect of linoleic acid on muscular dystrophy occurred with

TABLE 3

Linoleic acid and nutritional muscular dystrophy in the chick [1]

Treatment		Weight, 5 weeks	Muscular dystrophy, 5 weeks	Score [2]
		g		
Basal [3]	chicks from linoleic acid-depleted hens [5]	465	0/24 [4]	—
+0.50% linoleic acid		503	11/24	0.7
Basal [3]	chicks from non-depleted hens [6]	468	0/23	—
+0.50% linoleic acid		528	12/24	0.6

[1] Ten male and 10 female chicks/lot at start.

[2] Chicks were scored using a scale based upon zero for no dystrophy to 3 for severe degeneration of the breast muscles. Group score = $\frac{\text{total score for group}}{\text{number of survivors}}$.

[3] Diet 2, table 2.

[4] Numerator indicates number of chicks showing muscular dystrophy; denominator shows total number of chicks examined.

[5] Chicks for these 2 treatments obtained from a flock of hens receiving the basal diet shown in table 1.

[6] Chicks from hens receiving the same diet (table 1) except that 5.0% safflower oil was added to hen diet to supply linoleic acid.

a different protein source. The results of this experiment are presented in table 4.

These results show an increasing incidence and severity of muscular dystrophy as increasing amounts of linoleic acid were added to the diet. Oleic acid, at a level comparable to that present in 4.0% lard, was not effective in producing muscle lesions, and the combinations of linoleic acid and oleic acid had no greater effect on muscular dystrophy than occurred with linoleic acid alone. Both 0.5% linoleic acid alone and 1.95% oleic acid plus 0.50% linoleic acid produced the same degree of incidence and severity of muscular dystrophy and the same as that observed with 4.0% lard, indicating that linoleic acid is the only fatty acid in lard which influences the production of muscular dystrophy. Other unsaturated fatty acids such as linolenic and arachidonic acids, however, should be examined for their possible influence on muscular dystrophy.

Century et al. (19) and Century and Horwitt (20) reported that dietary linoleic acid influenced the amount of linoleic acid in brain tissue of the chicks in their experiments, and at the same time was directly related to the production of encephalomalacia.

Studies were conducted, therefore, to determine the extent to which linoleic acid influences the linoleic acid content of chick muscles, and to determine the linoleic acid content of dystrophic muscles as compared with those in which dystrophy has been prevented by supplementing the high linoleic acid diets with vitamin E.

The muscles of chicks from the previous experiment, which had received zero, 0.5 and 1.0% linoleic acid, and of chicks from another experiment which had received 1.0% linoleic acid or 1.0% linoleic acid plus 5.0 mg of D-α-tocopheryl acetate, were analyzed for linoleic acid content. The results of this study are presented in table 5.

The increasing levels of dietary linoleic acid caused an increase in the linoleic acid content of muscle lipid. This was accompanied by an increase in the incidence and severity of muscular dystrophy. Addition of vitamin E to the diet containing 1.0% dietary linoleic acid prevented muscular dystrophy but did not affect the level of linoleic acid incorporated into muscle lipid. These results suggest that the incidence and severity of muscular dystrophy may be related to the amount of linoleic acid present in the tissue, but they do not account for the role of vitamin E in the prevention of the disease.

B. *Effect of dietary level of linoleic acid on the vitamin E requirement for prevention of muscular dystrophy in the chick.* Century et al. (19) have postulated that the vitamin E requirement for prevention of encephalomalacia is directly dependent upon the dietary level of linoleic acid. To determine whether a similar relationship exists, an experiment was conducted on the effect of linoleic acid on the vitamin E requirement for the prevention of muscular dystrophy in the chick. In this ex-

TABLE 4

Comparative effects of linoleic and oleic acids on muscular dystrophy in the chick [1]

Treatment	Muscular dystrophy, 5 weeks	Score [2]
Basal [3]	0/19 [4]	
+0.25% linoleic acid	9/20	0.4
+0.50% linoleic acid	11/19	0.8
+1.00% linoleic acid	18/19	1.4
+1.95% oleic acid	0/20	—
+1.95% oleic acid+0.25% linoleic acid	5/20	0.2
+1.95% oleic acid+0.50% linoleic acid	15/20	0.8
+1.95% oleic acid+1.00% linoleic acid	17/20	1.4
+4.0% stripped lard	13/20	0.8

[1] Ten male and 10 female chicks/lot at start.
[2] See footnote 2, table 3.
[3] Diet 1, table 2.
[4] See footnote 4, table 3. Numerator indicates number of chicks showing muscular dystrophy; denominator shows total number of chicks examined.

periment the linoleic acid content of the diet was increased by adding stripped corn oil or additional stripped lard to a basal diet containing 4.0% stripped lard. Selenium at 1.0 mg/kg of diet (as sodium selenite) was added to reduce the vitamin E requirement below 10 mg of D-α-tocopheryl acetate/kg of diet (1). The results of this experiment are presented in table 6. Although the effect of linoleic acid upon the vitamin E requirement was highly significant between the levels of zero and 0.5% linoleic acid, statistical analysis of the data by a chi-square test for interaction (21) revealed no effect of linoleic acid on the vitamin E requirement for the prevention of muscular dystrophy when the dietary level of this fatty acid was increased from 0.5 to 2.5%. Therefore, the effect of linoleic acid on the vitamin E requirement for the prevention of nutritional muscular dystrophy is evident only in diets containing less than 0.5% linoleic acid. And under these conditions, wherein the basal diet contained sufficient synthetic antioxidant to prevent rancidity of the linoleic acid and to prevent encephalomalacia in the chicks, the vitamin E requirement for prevention was not directly proportional to the linoleic acid level of the diet.

DISCUSSION

The studies reported here have shown an involvement of linoleic acid in the production of muscular dystrophy, even though the basal diet contained adequate amounts of ethoxyquin and selenium for the prevention of encephalomalacia and exudative diathesis. A comparison

TABLE 5

Effect of dietary linoleic acid on the linoleic content of muscle lipid [1]

Treatment	Experiment 1			Experiment 2		
	Muscular dystrophy	Score	Linoleic acid	Muscular dystrophy	Score	Linoleic acid
			%			%
Basal [2]	0/8 [3]	0 [4]	1.0 [5]			
+0.5% linoleic acid	5/8	1.1	7.3			
+1.0% linoleic acid	8/8	1.6	15.4	11/12 [6]	1.7	14.1
+1.0% linoleic acid+5.0 mg D-α-tocopheryl acetate/kg				0/12	0	15.2

[1] Equal numbers of male and female chicks used for analysis.
[2] Diet 1, table 2.
[3] See footnote 4, table 3.
[4] See footnote 2, table 3.
[5] Linoleic acid determined by gas-liquid chromatography and presented as percentage of total muscle fatty acids.
[6] Numerator indicates number of chicks showing muscular dystrophy; denominator shows total number of chicks examined.

TABLE 6

Effect of increasing levels of linoleic acid (in corn oil or lard) upon vitamin E requirement for prevention of muscular dystrophy

Treatment	(1) (0.5% LA) [1]	As (1) + 5.0% lard (1.0% LA) [1]	As (1) + 2.5% corn oil (1.5% LA) [1]	As (1) + 5.0% corn oil (2.5% LA) [1]
	Muscular dystrophy 5 weeks	Muscular dystrophy 5 weeks	Muscular dystrophy 5 weeks	Muscular dystrophy 5 weeks
Basal [2]	9/19 [3]	9/20	8/20	7/19
+2.5 mg D-α-tocopheryl acetate/kg	1/20	7/20	3/19	4/20
+5.0 mg D-α-tocopheryl acetate/kg	0/20	2/20	1/20	3/20
+7.5 mg D-α-tocopheryl acetate/kg	0/20	0/20	0/20	0/20
+10.0 mg D-α-tocopheryl acetate/kg	0/20	0/20	1/20	0/20

[1] Calculated total amount of linoleic acid.
[2] Diet 2, table 2, contained 1.0 mg Se/kg of diet and 0.05% added DL-methionine. All diets were isocaloric.
[3] Numerator indicates number of chicks showing muscular dystrophy; denominator shows total number of chicks examined.

of these results with those obtained by Dam et al. (15) and by Machlin and Shalkop (16) indicates that the extensive alcohol extraction of the protein in the diet and the fact that vitamins A and D_3 were added in dry form to the diet instead of being administered in oil as in Dam's work, may be responsible for no dystrophy occurring in the absence of added linoleic acid, whereas it had been shown to occur without added fat in the experiments of Dam et al. (15) and Machlin and Shalkop (16). These results establish that linoleic acid is involved in the etiology of muscular dystrophy, but the function of linoleic acid in the development of the dystrophic lesions was not revealed by these experiments.

During the preparation of the present report, Hutcheson et al. (22) suggested that linoleic acid may enhance muscular dystrophy through an antagonism of selenium. These workers observed that chicks receiving a basal diet having no added fat and with no added selenium or antioxidant (DPPD), developed muscular dystrophy, but when low amounts of both selenium and DPPD were added, dystrophy did not occur. These investigators reported that the addition of 4.0% lard or of linoleic acid equivalent to that present in 4.0% lard overcame the effect of added selenium and DPPD and caused a high incidence of muscular dystrophy. Oleic acid, linolenic acid and a saturated fat (hydrogenated coconut oil) were not antagonistic to the effectiveness of selenium and DPPD in preventing dystrophy.

The results of the present experiments support the observations of Hutcheson et al. (22) showing that no muscular dystrophy occurs in chicks receiving a vitamin E-deficient, sulfur amino acid-low basal diet containing low levels of selenium and ethoxyquin. The present experiments, however, indicate that linoleic acid plays a more important role in the development of muscular dystrophy than as a single antagonist of low levels of selenium and ethoxyquin. If linoleic acid functions as an antagonist of a low level of dietary selenium, then increasing amounts of selenium should overcome its deleterious effect. Previous results from this laboratory[8] have shown, however, that increasing the level of selenium to 10 mg/kg of diet had no effect on muscular dystrophy in chicks receiving diets containing 4.0% lard or 0.5% linoleic acid.

Other studies (1,4) have shown the protective mechanism for prevention of muscular dystrophy in the chick to be intimately involved with interactions of vitamin E, selenium and the sulfur amino acids. The development of muscular dystrophy, like encephalomalacia, may be a result of in vivo oxidation of the linoleic acid in important phospholipids or lipoproteins with the formation of peroxides which initiate the degeneration of the muscle tissue. However, 2 important questions remain to be answered before this explanation can be accepted. 1) Why does muscular dystrophy occur in the presence of a dietary level of antioxidant which completely prevents encephalomalacia; and 2) why are methionine and cystine not effective against encephalomalacia when they are completely effective in preventing muscular dystrophy? With these questions unanswered, the etiology of nutritional muscular dystrophy and the metabolic roles of vitamin E, selenium, linoleic acid and antioxidants in the prevention of this disease remain to be determined.

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Serum and Aortic Lipids in Rabbits Fed Cholesterol and Linoleic Acid Stereoisomers [1]

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ABSTRACT Rabbits fed 1 g of cholesterol and 6 g of elaidinized linoleic acid (a mixture of linoelaidic stereoisomers) daily for 84 days showed no higher concentration of free cholesterol, cholesterol ester, triglyceride, phosphatidyl ethanolamine, phosphatidyl serine, lecithin, sphingomyelin or other lipid fractions in their serum than did control rabbits fed 1 g cholesterol and 6 g linoleic acid for a similar period. In the rabbits fed the elaidinized linoleic acid the concentration of *trans* isomer after 84 days was 13.2 ± 11.3% in the serum cholesterol ester fatty acids and 11.3± 8.2% in the triglyceride fatty acids, whereas it was usually less than 2.5% in the phospholipid fatty acids. The rabbits fed elaidinized linoleic acid showed little, or no more, atherosclerosis of the aortic arch, no more atherosclerosis in the thoracic and abdominal aorta, and only a slightly higher cholesterol content in their aortas than did those rabbits fed natural linoleic acid and cholesterol. The relationship of these results to those of similar experiments in which supplements of elaidinized oleic acid or olive oil were fed with cholesterol to rabbits is discussed.

Natural linoleic acid has the structure of 9-*cis*, 12-*cis*-octadecadienoic acid. A large variety of positional isomers are theoretically possible. If the double bond positions are fixed to the 9 and 12 positions, 4 geometric or stereoisomers are possible; 9-*cis*, 12-*cis*; 9-*cis*, 12-*trans*; 9-*trans*, 12-*cis*, and 9-*trans*, 12-*trans*. These various isomers may be formed during the hydrogenation of highly unsaturated oils and fats. Only the 9-*cis*, 12-*cis* stereoisomer has essential fatty acid activity (1). The other stereoisomers have been reported to be inactive (2, 3). It has been suggested that the *trans* isomers might interfere or compete with the utilization of the 9-*cis*, 12-*cis* isomer; however, Mattson et al. (4) observed that although the 9-*cis*, 12-*trans* and 9-*trans*, 12-*trans* isomers of linoleic acid have no essential fatty acid activity, they do not interfere or compete with the activity of the natural 9-*cis*, 12-*cis* isomer having essential fatty acid activity. The natural 9-*cis*, 12-*cis* isomer which is present at high concentrations in some vegetable oils has been implicated (5) as an important dietary factor regulating the serum cholesterol level. Although a considerable number of studies (6) have been made on the effects of the natural 9-*cis*, 12-*cis* isomer of linoleic acid and the vegetable oils that contain predominantly this fatty acid, comparatively little is known about the metabolism of the *trans* isomers of this fatty acid (7, 8) or the effects of feeding the *trans* isomers of this fatty acid on serum lipid concentrations (9), or on the severity of atherosclerosis either in animal or man. McOsker et al. (9) reported that partially hydrogenated vegetable fat containing up to 8.0% *cis*, *trans* linoleic acid had no elevating effect on the serum cholesterol concentration when fed to human subjects at a level of 20.6% (41% of calories) in the diet for 4 weeks. Blank and Privett (8) reported that rats fed methyl 9-*cis*, 12-*trans*-linoleate did not gain as much weight and exhibited gross signs of essential fatty acid deficiency compared with control rats fed methyl 9-*cis*, 12-*cis*-linoleate. *Trans* isomers were detected in the tissue lipids of the rats fed the 9-*cis*, 12-*trans* isomer; however, concentrations deposited were not reported. Dhopeshwarkar and Mead (10) have reported the deposition of methyl elaidate in fat-deficient guinea pigs fed methyl elaidate.

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[2] Research Associate, National Heart Foundation of Canada.

In previous studies from this laboratory, the effect on serum lipids and atherosclerosis in rabbits fed cholesterol and oleic acid was compared with the effect of feeding cholesterol and elaidic acid (11), and the effect of feeding natural olive oil, a vegetable oil which contains mainly oleic acid, was compared with elaidinized olive oil (12).

In the present paper, utilizing rabbits fed cholesterol, the effects of feeding a dietary supplement of the natural 9-*cis*, 12-*cis* stereoisomer of linoleic acid are compared with the effects of feeding its elaidinized counterpart containing the mixture of *cis-trans* isomers formed by elaidinization. Observations were made on aortic atherogenesis, on the transport of lipids in the serum and the deposition of lipids in the tissues.

METHODS

Rabbits and diets. Male, white, New Zealand rabbits weighing about 2 kg, and about 3 months old, at the start of the feeding period were divided into 4 groups and each group was fed one of four different test diets. The first group was fed a daily food ration containing 1 g cholesterol and 6 g natural linoleic acid mixed with 53 g basal food pellets. The second group was fed 1 g cholesterol and 6 g elaidinized linoleic acid mixed with 53 g basal food pellets. The third group was fed a daily food ration containing 1 g cholesterol and 79 g basal food pellets. The fourth group was fed 80 g of the basal food pellets per day without any added cholesterol or fatty acid. The test diets were prepared in bulk and stored at 10°. The basal food pellets, a commercial preparation, contained the following ingredients: (in %) barley, 20; wheat middlings, 25; wheat flour, 4; malt sprouts, 20; dehydrated alfalfa meal, 20; soybean meal, 0.5; molasses, 6; calcium carbonate, 2; dicalcium phosphate, 1; sodium chloride, 1, and trace minerals and vitamins 0.5. The basal food pellets contained 3% fat of vegetable origin originating from these ingredients. This fat in the basal food pellets showed no *trans* isomer by infrared analysis; however, it diluted the concentration of linoleic acid incorporated into the diet of the first group and it also diluted the concentration of elaidinized linoleic acid in the diet of the second group. The fatty acid composition of the total daily food ration fed to the 4 groups of rabbits is shown in table 1 where the values indicate the total derived from the fatty acid supplement and that derived from the natural fat in the basal food pellets.

TABLE 1

Total fatty acids consumed per rabbit per day from both food pellets and 6 g fatty acid supplement

Fatty acid or isomer	Fatty acid consumed per rabbit per day			
	Cholesterol + linoleic acid group	Cholesterol + linoelaidic acid group	Cholesterol + no fatty acid group	No cholesterol + no fatty acid group
	mg	*mg*	*mg*	*mg*
Linoleic acid isomers:				
Cis-9-*cis*-12	5216	1526	716	716
Cis-9-*trans*-12	—	1170	—	—
Trans-9-*cis*-12	—	1080	—	—
Trans-9-*trans*-12	—	1440	—	—
Oleic, *cis*-9	1340	740	380	380
Elaidic, *trans*-9	—	600	—	—
Linolenic *cis* isomer	87	75	69	69
Linolenic *trans* isomers	—	12	—	—
Stearic	207	207	27	27
Palmitic	443	443	203	203
Palmitoleic	60	18	—	—
Palmitolaidic	—	42	—	—
Total/day	7353	7353	1395	1395

The natural linoleic acid used as the supplement for the diet of the first group and used for preparing the elaidinized linoleic acid was a commercial preparation that actually contained 75% linoleic, 18% oleic, 7% saturated fatty acids (mainly palmitic and stearic), 0.5% linolenic, and traces of other fatty acids. No *trans* isomer could be detected in this material and it was assumed that the double bonds in the 75% linoleic acid present had the natural *cis*, *cis* stereoisomeric configuration. Ultraviolet spectrophotometric analysis showed no evidence of conjugation and it was assumed that these bonds were in the natural 9,12 position. The linoleic acid was elaidinized by a method similar to that described previously (13, 14). The elaidinized linoleic acid was washed with water until free from nitric acid, nitrous acid and nitrite ions. For feeding purposes it was not chromatographed, recrystallized or purified any further and the material fed to the rabbits contained nitro addition products.

Figure 1 shows the infrared spectrophotometric curves of the linoleic acid supplement and the elaidinized linoleic acid supplement as used for feeding purposes but in its methylated form. In figure 1, arrows 2 and 3 indicate the absorption bands due to the nitro addition groups formed during the course of the elaidinization reaction. The nitro addition

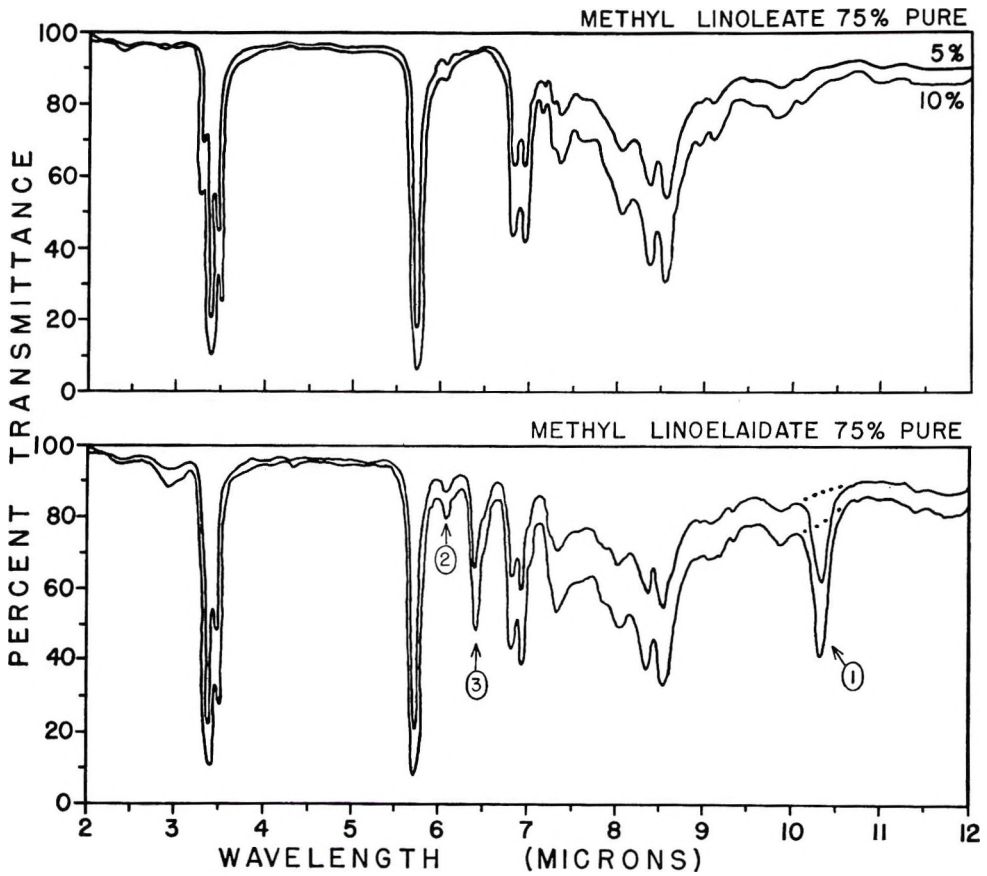

Fig. 1 Infrared spectrophotometric recordings (touched-up) of the "linoleic acid" and "linoelaidic acid" fatty acid supplements used for feeding purposes. Each sample was methylated (to remove the strong carboxyl absorption band of free fatty acids which occurs in the 10.5- to 11.5-μ region) and read at 5 and 10% concentration in carbon tetrachloride in 0.1 mm sodium chloride cells. Arrow 1 indicates the band due to *trans* double bonds and arrows 2 and 3 indicate nitro addition groups formed during elaidinization.

compounds could have been removed chromatographically or by recrystallization; however, this was not done since this further purification would have caused the elaidinized linoleic acid to differ further from the natural linoleic acid. Prior to chemical analysis for the exact composition of the fatty acids used as food supplements, they were methylated using boron trifluoride (15).

Samples of the methylated, elaidinized linoleic acid supplements used for feeding purposes were chromatographed on silicic-silver nitrate [3] columns to separate the *cis, cis* isomer from the *trans, trans* isomer and from the *cis, trans* and *trans, cis* isomers. After infrared analysis of these fractions for *trans* concentration, gas chromatography (16, 17) and ultraviolet spectrophotometric analysis, it was possible to estimate the fatty acid composition consumed per rabbit per day as shown in table 1 where the amounts of fatty acid indicated are from both the fatty acid supplements and from the food pellets. The percentage of linoleic acid isomers formed by elaidinization was approximately 18% 9-*cis*-12-*cis;* 26% 9-*cis* 12-*trans;* 24% 9-*trans*, 12-*cis;* and 32% 9-*trans*, 12-*trans*. These data indicate that the elaidinization was incomplete compared with elaidinization to the point of equilibrium as reported by Litchfield et al. (14) who were able to bring the isomer concentrations to 6% 9-*cis*, 12-*cis;* 19% 9-*cis*, 12-*trans;* 19% 9-*trans*, 12-*cis*, and 56% 9-*trans*, 12-*trans*.

After the rabbits had been fed the special diets for 84 days, they were bled and sera obtained for cholesterol analysis (18) and for lipid analysis by silicic acid chromatography. The rabbits were then killed, the aortas removed and cleaned free from adventitia and inspected for severity of atherosclerosis as previously described (12). The aortas were then frozen, later freeze-dried, weighed, and then extracted for 24 hours in a Soxhlet apparatus with boiling ethanol and for 24 hours with diethyl ether at room temperature.

Methods of lipid analysis. The serum and aortic lipid extracts were chromatographed by silicic acid chromatography by a method described previously (19) which was a modification of a previous method (20). Two changes in technique were made. First, the so-called cephalin fraction consisting of phosphatidyl ethanolamine and phosphatidyl serine was subfractionated on a silicic acid-silicate water column into phosphatidyl ethanolamine and phosphatidyl serine as described by Rouser et al. (21, 22). Second, using acetone as an eluent (23) a so-called "oxidized lipid fraction" was collected. Since the ratio of lipid to phosphorus in this fraction was nearly 25 to 1, this fraction

[3] Methods used will be described in detail elsewhere.

TABLE 2

Composition of eluents and lipid fractions eluted

Composition of eluent	Volume	Fraction eluted
	ml	
Petroleum ether	250	hydrocarbons
2% Diethyl ether in petroleum ether	600	cholesterol esters
5% Diethyl ether in petroleum ether	600	triglycerides
20% Diethyl ether in petroleum ether	600	free cholesterol
50% Diethyl ether in petroleum ether	100	mono-and diglycerides
20% Methanol in chloroform	200	cephalin (phosphatidylethanolamine+ phosphatidyl serine)
40% Methanol in chloroform	50	phosphatidyl inositide
40% Methanol in chloroform	300	lecithin (phosphatidyl choline)
60% Methanol in chloroform	200	sphingomyelin
100% Acetone	200	oxidized phospholipid
100% Methanol	200	lysolecithin, lysocephalin
Subfraction of the cephalin fraction (Rouser et al. (21)		
1% Ammonia in methanol: chloroform (1:4)	70	preparation of silicic acid-silicate water column
20% Methanol in chloroform (1:4)	500	phosphatidyl ethanolamine
100% Methanol	200	phosphatidyl serine

is referred to as oxidized phospholipid; however, its exact identity is not known. Table 2 shows the composition of the eluents used and lipid fractions eluted for the primary lipid fractionation and for the subfractionation of the cephalin fraction into phosphatidyl ethanolamine and phosphatidyl serine. The *trans* isomer concentrations in the cholesterol ester, triglyceride and phospholipid fatty acids were determined by infrared spectrophotometric analysis at 10.36 μ as previously described (19, 24–26). No attempt was made to resolve the various fatty acid *trans* stereoisomers as was done for the analysis of the elaidinized linoleic acid fed the rabbits, because not enough fatty acid was available from each lipid fraction to make possible a further subfractionation into fatty acid stereoisomers. The *trans* isomer value obtained for each lipid fraction was the total isolated *trans* concentration expressed as a percentage relative to a methyl elaidate standard assumed to be 100%. The methyl elaidate standard was checked against secondary standards obtained from the American Oil Chemists' Society, Spectroscopy Committee.[4]

RESULTS

All values shown in the tables are expressed as means plus or minus the standard deviation. Apparent differences of means between groups were analyzed for significance by Fisher's *t* test (27). In this study as in previous studies (11, 12), when comparing the serum lipids and severity of atherosclerosis of experimental groups of rabbits, we have concluded that with the high variability of response to factors tested, to be reliable, a minimum of 20 rabbits is required per group and that mean values are significantly higher or lower only when the *t*-test shows a *P* value less than 0.05. In the tables and text, the control group of rabbits fed the diet containing the natural 9-*cis*, 12-*cis* linoleic acid is referred to as the linoleic group, whereas the group of rabbits fed the elaidinized mixture of stereoisomers is referred to as the linoelaidic group.

Effect on serum lipids. Table 3 shows the cholesterol concentration in the serum, as determined by the Schoenheimer-Sperry method (18), after the rabbits had been fed the diets for 84 days. The concentrations of total, free and esterified cholesterol in the serum of the linoelaidic group were not significantly different ($P > 0.2$) from the values for the linoleic group. The values in these 2 groups were somewhat higher than values for the group of rabbits fed the diet contaning cholesterol without any fatty acid supplement. Table 4 shows the nature of the lipids in the serum after the rabbits had received the diets for 84 days as determined by silicic acid chromatography. Comparison of data on sera from 4 rabbits from the linoleic group with data from 12 rabbits from the linoelaidic group indicates a great similarity both qualitatively and quantitatively in the nature of the lipids in the serum. Infrared spectrophotometric analysis showed that, in the fatty acids esterified to cholesterol

[4] Secondary standards through courtesy of Dr. R. T. O'Connor, Chairman, Box 19687, New Orleans 19, Louisiana. J. Am. Oil Chemists' Soc., 36: 627, 1959.

TABLE 3

Terminal, 84-day serum cholesterol concentrations as determined by the Schoenheimer-Sperry method (18)

Dietary group	Cholesterol + linoleic acid	Cholesterol + linoelaidic acid	Cholesterol + no fatty acid	No cholesterol + no fatty acid
No. of rabbits	20	23	23	36
Cholesterol	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Free	281 ± 173 [1]	345 ± 186 [2]	226 ± 171	15 ± 9
Esterified	1132 ± 743	1183 ± 597 [3]	749 ± 473	42 ± 15
Total	1413 ± 905	1528 ± 776 [4]	975 ± 637	57 ± 20

[1] Values indicated are means ± SD.
[2] Linoelaidic group vs. linoleic group, $P > 0.2$.
[3] Linoelaidic group vs. linoleic group, $P > 0.8$.
[4] Linoelaidic group vs. linoleic group, $P > 0.6$.

TABLE 4

Terminal, 84-day serum lipid concentrations as determined by silicic acid chromatography

Dietary group	Cholesterol + linoleic acid		Cholesterol + linoelaidic acid		Cholesterol + no fatty acid		No cholesterol + no fatty acid	
No. of rabbits	4	4	12	12	21	21	8	8
	mg/100 ml	*% total lipid*	*mg/100 ml*	*% total lipid*	*mg/100 ml*	*% total lipid*	*mg/100 ml*	*% total lipid*
Total lipid	2651	100.0	3195 ± 1524 [1]	100.0	3303 ± 1671	100.0	373.1 ± 31.5	100.0
Cholesterol ester	1516	57.3	1862 ± 932	57.7 ± 9.2	1142 ± 696	34.7 ± 14.8	125.5 ± 31.8	33.6
Free cholesterol	349	13.2	429 ± 237	13.4 ± 3.1	281 ± 186	8.8 ± 3.1	61.4 ± 10.8	16.5
Triglyceride	195	7.4	239 ± 189	7.7 ± 4.6	353 ± 328	11.7 ± 8.4	55.6 ± 25.0	14.9
Mono-diglyceride	6	0.2	7.0 ± 2.8	0.3 ± 0.4	72 ± 113	1.9 ± 1.8	5.8 ± 3.0	1.6
Phosphatidyl inositide	21	0.8	26 ± 20	0.8 ± 0.6	9 ± 7	0.3 ± 0.2	3.6 ± 2.3	1.0
Phosphatidyl ethanolamine	—	—	87 ± 49	2.7 ± 1.3	212 ± 331	5.9 ± 5.0	32.2 ± 11.2	8.6
Phosphatidyl serine	—	—	69 ± 87	2.0 ± 1.4	169 ± 133	5.1 ± 2.8	25.9 ± 4.6	6.9
Phosphatidyl (ethanolamine + serine)	127	4.8	156 ± 116	4.8 ± 2.0	381 ± 389	10.9 ± 5.8	58.1 ± 10.9	15.6
Lecithin	103	3.9	126 ± 85	3.6 ± 1.7	203 ± 120	7.9 ± 5.5	19.6 ± 7.9	5.3
Lysolecithin	150	5.7	184 ± 102	5.2 ± 1.4	125 ± 71	4.0 ± 1.8	18.8 ± 5.8	5.0
Sphingomyelin	127	4.8	156 ± 134	4.2 ± 3.3	130 ± 120	4.0 ± 1.8	12.9 ± 8.3	3.5
Oxidized phospholipid	50	1.9	61 ± 37	1.7 ± 0.8	54 ± 34	1.8 ± 1.1	6.1 ± 4.5	1.6
Total phospholipid	578	21.9	647 ± 334	20.4 ± 6.6	907 ± 556	28.7 ± 9.4	119.1 ± 22.0	31.9
Hydrocarbon	7	0.2	9 ± 5.6	0.3 ± 0.2	486 ± 504	14.3 ± 13.8	6.6 ± 2.2	1.8

[1] Mean ± SD.

from the serum of the rabbits fed the linoelaidic diet, the proportion of *trans* isomer was 13.2 ± 11.3%, whereas the proportion of *trans* isomer in the fatty acids of the serum triglycerides of these rabbits was 11.3 ± 8.2%. No *trans* isomer was detected in any of the cholesterol ester or triglyceride fractions from the rabbits fed the other diets. In the rabbits fed the linoelaidic diet the proportion of *trans* isomer in the methyl esters prepared from the phospholipid fatty acids was as follows: phosphatidyl ethanolamine, 2.5%; phosphatidyl serine, 2.2%; lecithin, 2.4%; lysolecithin, 2.1%. No *trans* isomer could be detected in any of the serum phospholipid fatty acids from the rabbits fed the other diets. *Trans* isomer values indicated are relative to a pure methyl eliadate standard assumed to be 100%

Effect on aortic atherosclerosis. Table 5 shows the extent of atherosclerosis in the aortas as determined by estimating the percentage of intimal area involved with visible lesions. This method does not directly assess the thickness nor the nature of the lesions. The normal group fed no cholesterol and no fatty acid showed no atherosclerotic involvement of the aorta. There was great variability of aortic involvement in the rabbits fed the other 3 diets as indicated by the high standard deviations. The value of 33 ± 36 for the entire aorta of the rabbits fed cholesterol and linoelaidic acid was not significantly higher ($P > 0.2$) than the value of 21 ± 26 for the rabbits fed cholesterol and linoleic acid. There was no significant difference ($P > 0.3$) in the extent of aortic atherogenesis between these groups and the group of rabbits fed cholesterol without any fatty acid supplement in their diet. Again, when the individual aortic segments were compared, there was no significant difference ($P > 0.05$) in the extent of atherosclerosis produced by the various diets containing cholesterol. Table 5 also shows the cholesterol content for whole aorta of the various groups by determination of cholesterol concentration in an aliquot of the aortic extract by the Sperry method (18). The value of 5.73 ± 4.97 mg total cholesterol/whole aorta for the rabbits fed the linoelaidic acid diet was not significantly higher than the value of 3.83 ± 3.68 for the rabbits fed the linoleic acid diet. Therefore, in experiments of 84 days' duration, on the basis of both visual inspection of the aortas and analysis of their cholesterol content, it is concluded that the diet of cholesterol and linoelaidic acid is not significantly more atherogenic to rabbits than a diet of cholesterol and linoleic acid and that both of these diets have about the same degree of atherogenicity as a diet containing cholesterol but without these fatty acid supplements.

TABLE 5

Severity of aortic atherosclerosis as determined by estimating the percentage of intimal area involved with lesions and as determined by cholesterol analysis of the aortas

	Linoleic acid + cholesterol	Linoelaidic acid + cholesterol	Cholesterol + no fatty acid	No cholesterol + no fatty acid
No. of rabbits	20	23	23	36
	%	%	%	%
	Intimal area involved with lesions			
Whole aorta	21 ± 26 [1]	33 ± 36	28 ± 30	0
Arch	37 ± 36	49 ± 40	35 ± 39	0
Thoracic	21 ± 26	33 ± 37	28 ± 31	0
Abdominal	10 ± 20	25 ± 33	20 ± 28	0
	Cholesterol per whole aorta			
	mg	*mg*	*mg*	*mg*
Free [2]	1.91 ± 1.62	3.11 ± 2.96	1.71 ± 1.77	0.31 ± 0.05
Esterified [2]	1.92 ± 2.11	2.61 ± 2.53	2.36 ± 2.74	0.13 ± 0.11
Ester [3]	3.32	4.51	4.08	0.22
Total [2]	3.83 ± 3.68	5.73 ± 4.97	4.07 ± 4.43	0.44 ± 0.12

[1] Mean ± SD.
[2] Values were determined by Schoenheimer-Sperry method.
[3] Cholesterol ester values were calculated by multiplying esterified values by the factor 1.76.

TABLE 6

Lipids isolated by silicic acid chromatography from pooled aortic lipids

Dietary group	Linoleic acid + cholesterol		Linoelaidic acid + cholesterol		Cholesterol + no fatty acid	
	Lipid isolated/ aorta		Lipid isolated/ aorta		Lipid isolated/ aorta	
No. of rabbits	20		23		23	
	mg	*% total lipid*	*mg*	*% total lipid*	*mg*	*% total lipid*
Lipid fraction						
Total lipid	18.0	100	28.5	100	16.2	100
Cholesterol ester	3.9	21.7	9.0	31.5	2.8	17.5
Esterified cholesterol	2.3	12.6	5.2	18.2	1.6	10.1
Free cholesterol	2.9	16.1	4.8	16.8	3.3	20.5
Triglyceride	5.6	30.9	6.2	21.8	2.9	17.9
Mono- diglyceride	0.6	3.3	0.9	3.3	1.7	10.5
Inositol phosphatide	0.16	0.9	0.3	1.0	0.2	1.1
Phosphatidyl ethanolamine	2.5	14.1	3.1	11.0	2.1	13.2
Phosphatidyl serine	1.1	5.9	1.2	4.2	1.1	6.8
Total cephalin	3.6	20.0	4.3	15.2	3.2	20.0
Lecithin	0.4	2.1	1.1	4.0	0.8	4.9
Lysolecithin	0.35	1.9	0.9	3.0	0.3	2.0
Total lecithin	0.7	4.0	2.0	7.0	1.1	6.9
Sphingomyelin	0.17	1.0	0.4	1.4	0.3	1.8
Oxidized phospholipid	0.13	0.7	0.2	0.8	0.1	0.7
Total phospholipid	4.8	26.6	7.3	25.4	4.9	30.5
Hydrocarbon	0.25	1.4	0.3	1.0	0.5	3.0
% *Trans* isomer in fatty acids of:						
Cholesterol ester	0.0	—	14.8	—	0.0	—
Triglyceride	0.0	—	14.9	—	0.0	—

The nature of the lipids in the aorta. Table 6 shows the various lipids isolated by silicic acid chromatography from the pooled aortas of each group. The values are expressed as milligrams of lipid per aorta. The total lipid isolated per aorta from the rabbits fed the linoleic acid diet was 18.0 mg/aorta compared with a value of 28.5 mg/aorta for the rabbits fed the linoelaidic acid diet. The lipid fractions isolated from both groups were, however, very similar with the exception that in the group fed the linoelaidic acid diet, infrared spectrophotometric analysis showed the presence of *trans* fatty acids. No *trans* isomer was detected in any of the aortic lipid fractions from the rabbits fed the other diets. In the rabbits fed the linoelaidic acid diet, the proportion of *trans* fatty acids observed in the aortic cholesterol ester was 14.8%, whereas in the aortic triglyceride fatty acids the proportion of *trans* fatty acids was 14.9%. These single values for aortic lipids are in the same range as the 13.2 ± 11.3% *trans* isomer proportion in the cholesterol ester fatty acids in the serum of these same rabbits and in the same range as the 11.3 ± 8.2% *trans* isomer proportion for the triglyceride fatty acids in their serum.

Histologic findings. The 3 groups of rabbits fed (a) cholesterol and linoleic acid, (b) cholesterol and linoelaidic acid, and (c) cholesterol alone, without added fatty acid, all showed a marked degree of lipophage infiltration in the spleens and livers as well as a marked amount of stainable fat in these organs and a slight amount of liver fibrosis. In contrast, the control group of rabbtis fed neither cholesterol nor fatty acid in the diet showed none of these changes. There was, however, no significant difference between any of the 3 groups fed diets containing cholesterol with respect to the degree of lipophage infiltration in the spleens and livers, in the amount of stainable fat or in the amount of liver fibrosis.

DISCUSSION

In 2 previous experiments that compared the effects of feeding natural and elaidinized oleic acid (11) and natural olive oil and elaidinized olive oil (12) in rabbits also fed cholesterol, the feeding of these elaidinized dietary supplements was accompanied by the development of higher serum lipid levels and a little more atherosclerosis of the aortic arch than in the rabbits fed the natural *cis* lipid supplements. In the present experiment no such result was obtained with natural and elaidinized linoleic acids, although the trend of the data was not opposed to the previous results.

The reason or reasons that may underlie the difference in results between this experiment with linoleic acid and the previous experiments wherein oleic acid and olive oil were tested can only be speculated about since the experimental designs do not permit further interpretation. The dose or ratio of oleic to elaidic acids and their ratio to other fatty acids in the diet that will bring about a result like that referred to above where there was an increase in atherogenicity has not been established. It may be that the effects previously reported are limited to elaidic acid. In the present experiment, there were various stereoisomers of elaidinized linoleic acid in the fat supplement given in the food. Three-tenths was natural 9-*cis*, 12-*cis* isomer, two-tenths was 9-*cis*, 12-*trans*, two-tenths was 9-*trans*, 12-*cis* and only three-tenths was 9-*trans*, 12-*trans* isomer.

Although it would have been desirable to test the specific effects of the pure *trans* stereoisomers fed in the basal diets for 84-day periods, the unavailability and the difficulty of preparing the pure isomers in the quantities required for such a feeding study made such an investigation impractical. Whether the feeding of an exclusively 9-*trans*, 12-*trans* supplement would have altered the result is not known, nor is it known whether there are dietary ratios of the various linoleic stereoisomers that might produce an effect like that obtained with elaidic acid. However, the results suggest that the mixed *trans* stereoisomers of linoleic acid do not enhance atherosclerosis in rabbits fed cholesterol any more than does the natural 9-*cis*, 12-*cis* isomer

Any speculative interpretation of the present result should note that, as transported in the serum, the percentage of total *trans* fatty acid esterified with cholesterol or with glycerol in this experiment was about the same as in the previous 2 experiments, although the exact nature of the isomers is not known and it should not be assumed to be identical in the various experiments. Therefore, the mere transport of *trans* isomers of esterified fatty acids in the serum is not the only factor concerned in our previous observations, but that some additional factor, perhaps concerned with the amount or nature of the particular *trans* isomer, must be considered. In addition, both control and experimental groups of rabbits in this experiment developed about as much hypercholesterolemia and as much aortic atherosclerosis as did the rabbits that were fed natural olive oil and cholesterol in one of the previous experiments, indicating that certain types of *trans* isomers of fatty acids can be transported in the serum, in the presence of hypercholesterolemia and developing atherosclerosis without necessarily producing quantitative differences in the level or severity of either the hypercholesterolemia or the aortic atherosclerosis.

A point in the results which has given us great concern has been the low *trans* isomer concentrations detected in the serum phospholipid fatty acids compared with the *trans* isomer concentrations noted in the cholesterol ester and triglyceride fatty acids. These results are difficult to explain at the present time; however, we have observed similarly low values in the phospholipid fatty acids of rabbits fed cholesterol and elaidic acid and also in rabbits fed cholesterol and elaidinized olive oil. Because of the interference of the phosphate group with the infrared measurement of the *trans* double bond at 10.36 μ, the fatty acids were split or removed enzymatically using a crude pancreatin preparation to avoid the possible artifactual formation of *trans* bonds during hydrolysis or interesterification with alkaline agents or catalysts. Although this

pancreatin preparation had marked phospholipase A activity and hydrolzed trielaidin to elaidic acid, we have no estimate on how efficient it would be in splitting a synthetic phospholipid containing *trans* fatty acids. We do know, however, that this same pancreatin preparation was able to split the *trans* fatty acids from the serum phospholipid fractions isolated from the serum of rabbits injected with Triton WR-1339 [5] and fed elaidic acid; these showed high concentrations of *trans* fatty acids in their serum phospholipid fractions. We therefore have some confidence in our technique as a suitable method for the preparation of methyl esters from phospholipids.

Two other minor points may be mentioned. There is no reason to believe that a deficiency of essential fatty acids arose in the present experiment since there was excess unaltered *cis*-9-*cis* 12 linoleic acid in the dietary lipid supplements and in the basic food pellets which, of course, were not changed. Lastly, nitro addition products were present in the elaidinized lipid of both this and the previous 2 experiments. Although the possible effects of such products in experiments like these are unknown, they must either be variable, negligible or absent with respect to the development of hypercholesterolemia and aortic atherosclerosis in the 3 experiments; however, this will have to be established definitely by using pure stereoisomers from which the nitro addition products have been removed.

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Thyroxine and Antithyrotoxic Effects in the Chick [1]

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ABSTRACT Concentrates prepared from soy protein, lactalbumin or liver residue retained their antithyrotoxic activities but no longer had any chick growth-stimulating properties, and the two "factors" are, therefore, not identical. Most substances which exhibited good antithyrotoxic activity in the rat also had some protective effect in the chick. The administration of thyroid hormone to chicks or ducks had no effect on the basal metabolic rate or on the α-glycerophosphate dehydrogenase activity in any tissue studied.

A possible relationship between the antithyrotoxic activity of a test supplement and an unidentified "soy factor" for chick growth was suggested by the parallel activities exhibited by certain proteins in these 2 tests (1, 2). Antithyrotoxic substances block the calorigenic as well as the growth depression effects of exogenously administered thyroid hormone (3–5). The reason for the different growth effects produced by various proteins in chicks has not been clarified. The results obtained in this study showed that the 2 activities could not be attributed to the same substance, since concentrates could be prepared which retained their original antithyrotoxic activities but no longer stimulated chick growth.

Another objective of this study was a comparison of the antithyrotoxic effectiveness of various test supplements in the chick with the results previously obtained in the rat (4, 5). In addition to the usual criteria of growth and survival, measurements of the metabolic rate and liver α-glycerophosphate dehydrogenase (GPD) activity were planned as further indicators of antithyrotoxic activity (5). However, the administration of thyroid hormone to the chick or the duck did not give an increased metabolic rate or tissue GPD activity; and the only criteria readily available for detecting thyrotoxicity in these birds were decreased growth and survival. Such growth and survival studies were not completely satisfactory in the chick, but it was possible to show that most of the substances possessing good antithyrotoxic activity in the rat also had some protective effect in the chick.

EXPERIMENTAL

The diet used for the growth studies was identical with the soy factor assay diet described previously (2) except that the chlortetracycline and diphenyl-*p*-phenylenediamine were omitted. This diet contained: (in grams) casein, 20; gelatin, 10; methionine, 0.6; fish solubles, 20; soy oil, 3.5; salt mixture, 8.3; inositol, 0.1; choline, 0.2; a vitamin mixture, and enough starch to make 100; it gave a maximal growth rate in chicks when further supplemented with 20% soy protein at the expense of an equal weight of starch. Any growth stimulation produced by the addition of a supplement to this diet in place of the soy protein was, therefore, attributed to the "soy factor."

For a study of antithyrotoxic activity, this diet was modified as follows. 1) The soy protein and fish solubles were reduced to 10% of each to minimize the amount of antithyrotoxic activity present in the basal diet; these amounts still supported maximal growth in the absence of added thyroid hormone. 2) Ten per cent corn oil[2] replaced the 3.5% soy oil since the higher fat content of the diet proved beneficial in the thyrotoxic rat (4) and the corn oil was relatively free of antithyrotoxic activity. 3) The vitamin content of the diet was increased to conform with that previously used in antithyrotoxic studies in rats (3); the higher concentrations of

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[2] Mazola, Corn Products Company, Argo, Illinois.

niacin, choline and inositol already present in the chick diet were retained in the antithyrotoxic diet, and the 2-methyl-1, 4-naphthoquinone concentration was increased to 2 mg/kg. These precautions were taken to prevent any vitamin deficiencies in the hyperthyroid chicks. The starch content of the diet was adjusted to total 100%.

Groups of 8 one-day-old male White Leghorn chicks were maintained in heated brooders with raised wire-mesh bottoms, and fed the diets ad libitum. Body weights were recorded weekly. Metabolic rates were determined as previously described (3) and the measurement of α-glycerophosphate dehydrogenase activity in chick and duck tissues was made by the procedure described for rat liver (5). The optimal pH for determining GPD activity in chick intestine was the same 7.6 found for rat liver. Whole tissue homogenates gave as much GPD activity as did the centrifuged mitochondrial precipitate obtained from both normal or thyroxine-treated chicks; the supernatant fractions had no GPD activity at pH 7.6.

The I^{131} iodide uptakes by the thyroid glands were determined 24 hours after the subcutaneous injection of 1 μc of carrier-free radioactive iodide. The thyroid glands were dissected, weighed and counted in a gamma crystal scintillation well counter, and the I^{131} uptake values were calculated as a percentage of the administered dose.

RESULTS

Lack of identity of growth factor and antithyrotoxic activity. Table 1 shows the growth stimulation that was obtained when various supplements were added to the soy factor assay diet. The 4-week body weights were increased from the basal level of approximately 230 g to 280 to 290 g by the addition of soy protein, lactalbumin, liver residue or cottonseed meal. All concentrates prepared from these materials by alkaline hydrolysis and subsequent precipitation with acid (5) retained the antithyrotoxic activities of the original materials when assayed in rats, but only the cottonseed meal concentrate retained some of the chick growth-stimulaing properties of the original meal. Other substances tested for both antithyrotoxic activity in the rat and growth-stimulating effects in the chick (table 1) did not exhibit a good correlation between the 2 activities. For example, hemoglobin was a very effective antithyrotoxic agent which had little effect on chick growth, whereas egg yolk had only a modest antithyrotoxic activity but gave a good growth stimulation.

TABLE 1

Growth response of chicks to various supplements which were previously assayed for antithyrotoxic acivity in rats

Substance tested	% of diet	4-week chick wt [1]	AT activity in rats [2]
		g	
Basal diet [3]	—	227	—
Soy protein	20	281	20
Soy protein conc	2	223	22
Liver residue [4]	10	290	32
Liver residue conc	2	221	34
Cottonseed meal [4]	10	288	48
Cottonseed meal conc	3	270	> 42
Lactalbumin	20	281	25
Lactalbumin conc	1.7	207	28
Hemoglobin	5	254	50
Fibrin	20	270	21
Fermentation residue	10	263	23
Egg yolk	10	297	17
Dehydrocholic acid	0.2	252	19
Desoxycholic acid	0.2	237	—
Liver residue fats	5	245	—
Distillers solubles	10	273	16

[1] The values listed are averages of 3 or more independent experiments.

[2] AT activity denotes antithyrotoxic activity of the test supplements in hemoglobin units by the rat GPD assay procedure; a 10% hemoglobin diet was arbitrarily rated 100 hemoglobin units (5).

[3] Basal diet: 20 casein + 10 gelatin + 0.6 methionine + 20 fish solubles + 3.5 soy oil + 8.3 salts + vitamins + starch to 100%; supplements replaced an equal weight of starch.

[4] The liver residue was tested as received; the cottonseed meal was extracted with trichlorethylene to remove the lipids.

Antithyrotoxic studies in the chick. The 4-week body weight of chicks fed the basal diet used in the antithyrotoxic studies was 300 g, and 22 of the original 24 birds survived (average of 3 independent experiments with 8 chicks/group). When 0.2% iodinated casein[3] was added to this diet, the corresponding 4-week body weight averaged 230 g and only 15/24 survived. The addition of the following substances [4] to the thyrotoxic diet at the expense of starch restored body weights to at least 250 g and increased survival to 19 to 21/

[3] Protamone (1% thyroxine equivalent), Cerophyl Laboratories, Kansas City, Missouri.

[4] The sources of supply were given previously (5).

24: 5% liver residue fat, 10% liver residue, 10% fish meal, 10% distillers solubles, 10% fermentation residue, 20% lactalbumin, 20% fibrin or 0.2% desoxycholic acid. Cholesterol (1%) or 20% more casein had no protective effect on survival, but the casein improved growth somewhat.

The fish solubles assay diet previously described (2) contained: (in grams) soy protein, 35; methionine, 0.75; glycine, 0.4; soy oil, 3.5; salts, 8.3; inositol, 0.1; choline, 0.2; a vitamin mixture and sufficient starch to make 100. When supplemented with 10% fish solubles at the expense of starch, it gave a 4-week body weight of 305 g with 23/24 survival. When 0.2% iodinated casein was also added, the weight was 285 g with 21/24 survival. Soy protein was clearly antithyrotoxic by comparison with the casein-gelatin diet. In general, those substances which exhibited good antithyrotoxic activity in the rat also had some effect in the chick.

Thyroid administration to birds. Unlike the results obtained with rats (5), the administration of thyroid hormone to chicks or ducks had no major effect on the metabolic rate or on the GPD activities in any of the tissues that were studied (tables 2 and 3). Negative results were obtained: 1) by the feeding of 0.35% iodinated casein or 0.6 to 2 mg L-thyroxine (T_4)/100 g diet to one-day-old chicks or ducks for 2 or 4 weeks; 2) by the subcutaneous injection of 250 μg/day of triiodothyronine (T_3) or T_4 into one-day-old ducks for 2 weeks; or 3) by the subcutaneous injection of 25 μg T_4/day into 4-week-old chicks for an additional 4 weeks. Hence, the lack of any effect of the thyroid hormone on the GPD activity or metabolic rate in the chick or duck could not be attributed to the analogue administered (iodinated casein, T_4, T_3), the mode of administration (orally or subcutaneously), large or small doses, the duration of administration (11 to 28 days) or the age of the bird (1 to 56 days). Similarly the lack of any calorigenic effect from the thyroid hormone could not be attributed to an antithyrotoxic factor in the diet, since the same negative results were obtained when the chicks were fed a purified diet free of antithyrotoxic activity or a commercial poultry chow.

TABLE 2

Effect of thyroid hormone administration on the metabolic rate of the intact bird and the α-glycerophosphate dehydrogenase (GPD) activity in chick tissues

Exp	Treatment	α-Glycerophosphate dehydrogenase (GPD)					4-week body wt	Survival at 23 days	MR [1]
		Small intestine [2]		Liver	Kidney	Spleen			
		Mid 1/6	Cecal 1/6						
		μl O_2 10 min/150 mg fresh tissue					*g*		
1 [3]	Normal controls	39 ± 2.9	19 ± 1.7	7 ± 1.8	3 ± 1.8	12 ± 2.2	305	30/32	8.7 ± 0.3
	Iodinated casein, 0.35%	31 ± 2.8	25 ± 3.0	6 ± 1.6	4 ± 1.1	9 ± 2.5	230	20/32	8.7 ± 0.3
2 [4]	Iodinated casein or T_4	—	23 ± 3.4	5 ± 1.1	2 ± 0.6	—	—	—	7.0 ± 0.4
3 [5]	T_4 injected	34 ± 3.1	16 ± 1.5	5 ± 0.7	9 ± 1.5	—	—	—	—

[1] MR = metabolic rate in liters O_2 consumed/square meter body surface/hour, measured at 25 days.
[2] One-sixth of the total length was removed from the middle and terminal (cecal) portions of the small intestine and analyzed separately.
[3] Exp. 1: one-day-old chicks (34 g) fed experimental diet (20 casein + 10 gelatin + 0.6 methionine + 10 soy protein + 10 or 20 fish solubles) ± 0.35% iodinated casein for approximately 4 weeks before analysis. Values are mean ± SE for groups of 5 to 7 birds.
[4] Exp. 2: one-day-old chicks (36 g) fed experimental diet (20 casein + 10 gelatin + 0.6 methionine + 20 fish solubles + 10 soy protein) with 0.35% iodinated casein or 20 mg/kg of L-thyroxine (T_4) for 2 weeks before analysis. Mean ± SE for 8 birds. Both T_4 and iodinated casein gave the same results.
[5] Exp. 3: 3.5-week-old chicks fed chow and injected subcutaneously with 25 μg L-thyroxine (T_4) daily for 4 weeks and then 100 μg T_4 daily for an additional 5 days. Body weight at killing, 550 g. Mean ± SE for 7 birds.

TABLE 3

Effect of administering thyroid hormone (T_3, T_4, iodinated casein), propylthiouracil (PTU) or dinitrophenol (DNP) on the α-glycerophosphate dehydrogenase activity (GPD) and metabolic rate (MR) in the duck [1]

	Untreated controls	T_3 or T_4 250 μg/day sc	0.35% iodinated casein or 0.6 mg T_4/100 g of diet	PTU 0.02% in diet	DNP 0.15% in diet
GPD, μl O_2/10 min/150 mg					
Intestine, mid 1/6	41 ± 2.6(23)	51 ± 4.7(18)	62 ± 5.5(18)	40 ± 5.2(13)	46 ± 12.1(5)
Intestine, cecal 1/6	37 ± 3.7(19)	38 ± 2.6(18)	62 ± 4.4(19)	34 ± 5.2(13)	27 ± 2.8(5)
Brain	82 ± 5.3(14)	70 ± 6.2(11)	78 ± 9.5(4)	70 ± 7.3(5)	87 ± 12.1(5)
Liver	4 ± 1.0(15)	6 ± 1.2(11)	3 ± 1.0(4)	2 ± 1.6(5)	5 ± 0.9(5)
Kidney	4 ± 1.1(15)	4 ± 0.6(11)	4 ± 1.8(4)	2 ± 1.7(5)	1 ± 0.8(5)
Heart	10 ± 1.1(23)	8 ± 1.3(18)	9 ± 1.0(19)	12 ± 2.9(12)	7 ± 0.4(5)
Lung	5 ± 0.7(13)	7 ± 0.9(11)	6 ± 0.9(4)	5 ± 0.9(5)	6 ± 0.7(5)
Skeletal muscle	20 ± 2.6(2)	21 ± 2.2(18)	17 ± 1.7(19)	17 ± 2.8(13)	22 ± 2.6(5)
Metabolic rate, liters $O_2/m^2/hr$ [2]	11.3 ± 0.3(20)	11.0 ± 0.3(25)	11.9 ± 0.3(26)	8.6 ± 0.5(13)	13.7 ± 0.5(5)
Thyroid wt, mg/100 g body wt	8.0 ± 0.5(21)	4.4 ± 0.3(14)	3.5 ± 0.2(23)	57.5 [3]	9.5 ± 0.8(9)
I^{131} uptake by thyroid, % of dose	10.8 ± 1.6(9)	1.4 ± 0.3(6)	0.05 ± 0.01(4)	1.4 ± 0.5(4)	10.5 ± 1.0(5)
Heart wt, mg/100 g body wt	631 ± 14 (16)	751 ± 17 (7)	820 ± 15 (23)	597 ± 30 (15)	890 ± 24 (9)

[1] All values are mean ± SE for the number of ducks given in parentheses. One-day-old ducks (46 g) maintained with commercial laboratory chow (Purina Chow, Ralston Purina Company, St. Louis) and treated as indicated for 11 to 15 days.

[2] Approximate body weight at time of MR measurement was 300 g for first 4 groups and 170 g for the DNP group.

[3] Average of 32 ± 4.2 (7) and 83 ± 9.9 (8) in 2 separate experiments.

Although the administration of thyroid hormone had no effect on metabolic rate or tissue GPD activity, it produced a deleterious effect on chick growth and survival, and it decreased the thyroid weights and the uptake of I^{131} by the thyroid glands in the duck (table 3). The duck heart weight was also increased by thyroid administration but the effect was relatively small by comparison with the effect of thyroid hormone in rats.

The metabolic rate of the untreated chick was the same or slightly higher than the rat, but the metabolic rate in the untreated duck was appreciably higher. The feeding of propylthiouracil significantly reduced the metabolic rate of ducks, but had no apparent effect on tissue GPD activity. Feeding thiouracil to rats[5] gave easily measured decreases in the GPD activity of liver, kidney, heart and skeletal muscle. In the duck, the GPD activity in liver and kidney was so small that any possible reduction was difficult to measure. However, any decrease in heart or skeletal muscle as a result of feeding propylthiouracil could have been detected, but did not occur. Feeding dinitrophenol did not increase the GPD activity in any tissue of the rat or duck, but depressed growth and increased the heart weight in the duck.

DISCUSSION

There is no reason to doubt that the thyroid gland is functional in the chicken, duck and birds in general. Follicles appear in the chick embryo thyroid about the 11th day of incubation, and thereafter incorporate I^{131} into the colloid as mono- and diodotyrosine and T_4, together with small amounts of T_3 and 3, 5-diiodothyronine (6, 7). The gland is easily stimulated by thyrotropin with a concomitant loss of thyroidal iodine and T_4 (7). Pigeons and chickens exhibit seasonal variations in thyroid weight and iodine content, and the heavier thyroidal weights in cold temperatures are associated with histological evidence of greater secretory activity, a higher basal metabolic rate (BMR) (8), and a fairly sizeable increase in thyroxine secretion rate (9, 10). Goiters are produced in chickens by feeding an iodine - deficient diet or thiouracil - type drugs. The reversal of the thiouracil enlargement by the administration of T_4 is a clear indication that the gland normally produces a thyroid hormone under the stimulus of thyrotropin from the pituitary, and the daily output of T_4 has been measured in chickens, ducks and turkeys by this technique (11, 12). An active secretion of thyroid hormone is also implied by the atrophy of the gland which can be produced by the administration of excess thyroxine (13). On the basis of the antigoiterogenic response, the chick pituitary responds to T_4, T_3, triiodothyroacetic acid and tetraiodothyroacetic acid, and the chicken plumage responds to T_4, T_3 and triiodothyroacetic acid (14). By comparison with mammals, chicken and duck plasmas have a low value for protein bound iodine, which is due to the absence of the thyroxine-binding protein; unlike the mammal, T_4 is just as active as T_3 in the fowl (15).

It has generally been assumed that the thyroid hormone has the same effect on the BMR in birds that it has in mammals, but this assumption is not supported by adequate evidence. The higher BMR which is produced in chickens by exposure to cold temperature is associated with a higher output of thyroxine, but the thyroidectomized goose can adjust to cold with an increased BMR as readily as the normal (16), and there is no assurance that this increased BMR in chickens can be attributed to the thyroid hormone. Lee and Lee (16) observed a 15 to 33% decrease in the BMR of thyroidectomized geese, and Winchester (7) obtained decreases of 6 to 14% in the BMR of thyroidectomized hens; in both laboratories the administration of thyroid hormone restored these values to approximately the normal levels, but the BMR could not be increased substantially above this normal value by the administration of excess thyroid hormone. Similarly, the administration of excess thyroid hormone to intact chickens gave only minor increases of 8 to 17% in the BMR (18–21). Feeding 0.1 to 0.2% thiouracil in the diet to chickens decreased the BMR 16 to 24% (18–20); feeding 0.2 to 0.5% thiouracil for 13 to 18 weeks or putting 0.1% thiouracil in the drinking water gave decreases of 35

[5] Unpublished.

to 40% (22, 23). Such decreases were restored to normal by the simultaneous administration of T_3 or T_4 (23). The lack of any real increase in the BMR after thyroid administration to birds is in marked contrast with the two- to threefold increase readily obtained with rats. The thyroid hormone could be involved in establishing the normal BMR in birds, but does not produce any appreciable elevation in BMR when present in excess.

The function of the thyroid hormone in birds is by no means clear. The most prominent features of thyroidectomy in an adult bird (17, 24–31) are: 1) changes in plumage, 2) cessation in development or regression of the gonads and secondary sex characteristics, and 3) the deposition of increased amounts of fat. The destruction of the thyroid gland in young chicks by I^{131} also severely inhibited growth and produced a limited survival period. Since such birds developed relatively normally with widely varying amounts of administered T_4 (32) a minimal amount of the thyroid hormone can be considered essential for the young chick, but there appears to be no finely adjusted requirement and no serious consequences of a moderate excess. Feeding a high concentration of thiouracil (0.3 to 0.5%) produced thyroidectomy-like changes in the plumage (33) and smaller concentrations (0.1 to 0.2%) were sufficient to increase fat deposition and inhibit comb growth (19, 20, 22, 34). The administration of excess thyroid hormone to intact chickens had little or no effect on any organ, other than the thyroid gland itself (13); it decreased subcutaneous and carcass fat (35), and was effective in reducing the lipemia and fatty liver produced by cholesterol feeding (36, 37). The most characteristic effect of thyroid administration to birds is an increased rate of growth of the plumage together with an alteration in the morphology and pigmentation of the regenerating feathers (38, 39).

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An Antithyrotoxic Assay Based upon the Response of Rat Liver α-Glycerophosphate Dehydrogenase [1]

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ABSTRACT A simplified procedure was developed for the determination of mitochondrial α-glycerophosphate dehydrogenase (GPD) activity. The marked increase in rat liver and kidney GPD activity which resulted from thyroid administration was inhibited by the addition of antithyrotoxic substances to the diet; this provided the basis for the development of a new assay procedure for the antithyrotoxic factor. This assay curve was not influenced by coprophagy, food restriction or exposure of the rats to a moderately cold temperature, and the basal enzyme level was not affected by a low protein diet, by starvation or by starvation and refeeding. Liver GPD activity was also correlated with the steady-state metabolic rate so that the former could be used as supplementary evidence in the metabolic rate, growth and survival type of experiment. The fractionation of liver residue, cottonseed meal, lactalbumin and soy protein by alkaline hydrolysis followed by precipitation with acid recovered essentially all of the original antithyrotoxic activity in the precipitate.

Previous studies from this laboratory (1) demonstrated that the antithyrotoxic effect of liver residue was due to a nullification of the calorigenic effect of the thyroid hormone administered in the diet. A new assay procedure for antithyrotoxic activity was based upon the reduction in metabolic rate which occurred when liver residue (or other source of antithyrotoxic activity) was included in the thyroid-containing diet (2). Although this metabolic rate assay procedure was well adapted to the screening of substances for antithyrotoxic activity, it required too much time and material to provide a convenient assay for fractionation studies. The procedure described herein requires only one-half as much time and one-third as much material to complete the assay. And it also provides additional evidence that the antithyrotoxic factor nullifies in some way the usual effects of thyroid administration.

This assay was based upon the six- to tenfold increase in the activity of rat liver mitochondrial α-glycerophosphate dehydrogenase (GPD) which results from the administration of thyroid hormone (3). Maximal enzyme activity was reached in about 9 days, and was relatively constant until at least the 14th day. When liver residue or some other source of antithyrotoxic activity was included in the diet along with the thyroid hormone, the magnitude of this increase in liver GPD was proportional to the relative amounts of the 2 factors present. These differences were reproducible under standardized conditions and could, therefore, be quantitated for assay purposes. The method of determining the GPD activity in rat liver mitochondria was simplified and made practical for this purpose. Correlations have also been made between the steady-state metabolic rate (MR) and the liver GPD activity so that a determination of the latter could be used as supplementary information in the metabolic rate assay, or in simple growth and survival experiments.

EXPERIMENTAL AND RESULTS [2]

The same basal diet was used in these studies as previously described for the

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[2] In addition to the sources of supply previously listed (1, 2), the crude casein (high protein), hemoglobin (bovine), zein and cellulose (non-nutritive fiber) were obtained from General Biochemicals, Chagrin Falls, Ohio; crystalline hemoglobin, linoleic acid, dry bovine plasma, phenazine methosulfate, and L-thyroxine from Nutritional Biochemicals Corporation, Cleveland; liver residue from Wilson and Company, Chicago; distillers solubles (Soludri) from Schenley Distillers Inc., New York; fish solubles from Starkist Foods, Terminal Island, California; rats from Holtzman Company, Madison, and CFW mice from Carworth Farms, Inc., New City, New York. The fresh hemoglobin and crude cottonseed oil were prepared in the laboratory, and saffiower oil was obtained at a local food market.

MR assay (1, 2) except for the substitution of: 1) 30% crude casein for the 30% vitamin-test casein; 2) 10% corn oil for the 10% cottonseed oil; and 3) a vitamin A concentrate for the pure vitamin A. The corn oil was used because it appeared to be relatively free of antithyrotoxic activity. The other substitutions were made for economy. Both basal diets gave the same MR assay curve. The liver residue used in the present studies was not extracted with alcohol and chloroform as in the previous studies, but was tested in the diet as received. Hemoglobin was used as the reference standard for antithyrotoxic activity, with the thought that it might be more reproducible than liver residue. Substitutions in the diet were made for sucrose.

Weanling male rats of the Holtzman strain, weighing approximately 50 g, were fed the various diets for the times indicated. Where indicated, metabolic rates were determined as described previously (1). Iodinated casein (1% thyroxine equivalent) was used as the source of thyroid hormone activity.

Determination of liver α-glycerophosphate dehydrogenase. Livers were obtained from decapitated rats, and the GPD activity was determined manometrically by the following procedure. The body of a Warburg flask contained: 1.0 ml of 0.15 M KCl, 0.1 ml of 0.15 M $MgSO_4$, 0.3 ml of 0.01 M KCN, 0.5 ml of 0.6 M potassium phosphate buffer (pH 7.7), and 0.5 ml of a 1:20 liver homogenate in 0.15 M KCl (25 mg fresh liver). The side arm contained 0.2 ml of 0.6 M DL-α-glycerophosphate disodium salt,[3] and 0.2 ml (2 mg) of phenazine methosulfate (both prepared fresh daily). The center well contained 0.2 ml of 20% KOH plus a filter paper roll. After equilibrating for 10 minutes at 30°, the contents of the side arm were tipped in and the incubation was continued for another 5 minutes to "activate" the enzyme (4). Manometer readings were then made at 5-minute intervals for the next 20 minutes, and the oxygen consumption was recorded as microliters of O_2 per 10 minutes per flask containing 25 mg fresh liver. With this small amount of liver, the endogenous oxygen consumption in the absence of the α-glycerophosphate substrate averaged only 2 μliters/10 minutes, and this was routinely subtracted from the values obtained in the presence of the substrate. With larger amounts of tissue the endogenous respiration was determined simultaneously and subtracted. The net oxygen consumption due to the substrate, was linear throughout the reading period when the activity of the liver was below 20 (μl O_2/10 min/25 mg). With higher activities, there was some gradual fall-off in rate as the reaction progressed.

This procedure was based upon the method of determining GPD activity in mitochondria published by Lee et al. (3). In checking possible variations in the original procedure, the following observations were made.

1) Homogenization of the liver in 0.15 M KCl at 0° clumped the mitochondria (5) and the latter were, therefore, more easily centrifuged than were the mitochondria prepared in 0.25 M sucrose; the GPD activity in the precipitate collected at 6800 × *g* for 20 minutes from the KCl homogenate was also 30 to 40% greater than that obtained from a sucrose homogenate.

2) The activity in a whole liver homogenate could be determined directly without isolation of the mitochondria and without interference from the soluble GPD. Interference from the latter enzyme was a potential complication because only the mitochondrial enzyme is affected by the administration of thyroid hormone (3). However, such interference was not expected to be significant because: a) the pH optimum of the soluble enzyme is 10.4 for the oxidation of GPD to dihydroxyacetone

[3] All GPD values reported in this paper were obtained by the use of 120 μmoles/flask of DL-α-(di) sodium glycerophosphate·$6H_2O$ (obtained from Mann Research Laboratories, Inc., New York). Higher substrate concentrations gave higher GPD values; 300 μmoles gave about 95% of the maximal values obtained with 400 to 500 μmoles. When the same livers were analyzed with both 120 and 300 μmoles of substrate, the higher substrate concentration gave GPD values which were consistently 30% above the values obtained in the routine determination with 120 μmoles; this 30% increase was obtained at all levels of enzyme activity from 6 to 60 (μl O_2/10 min/25 mg liver). Hence the values and relationships reported would have been 30% higher but otherwise unchanged if the higher substrate concentration had been used. The higher substrate concentration did not prevent the small fall-off in enzyme activity during the determination, and there were no obvious advantages to the use of the higher concentration. A saturation level of 400 μmoles would have provided 86 mg of substrate to be acted upon by the enzyme present in 25 mg fresh liver.

phosphate, and at pH 7.4 to 7.6 the mitochondrial enzyme is 200 times more active than the soluble enzyme (6); b) the soluble enzyme requires DPN for its activity; no DPN was added to the assay system and this cofactor could be expected to be deficient at the dilutions of the liver homogenate used. By direct comparison, the same GPD activity was observed in the whole homogenate and in the washed mitochondrial precipitates for both normal and hyperthyroid livers (typical results: 80 mg normal rat liver gave a GPD activity of 15 for whole homogenate and 15 for the washed KCl-mitochondrial precipitate obtained at 6800 $\times$ *g* for 20 minutes; 15 mg of hyperthyroid liver gave 26 and 27, respectively; no activity was found in the centrifuged supernate of normal or hyperthyroid liver homogenates at pH 7.4 to 7.6).

3) All of the manipulations could be carried out at room temperature. The activity of the whole homogenate prepared at room temperature was at least as high as the corresponding preparation at 0°. (e.g., 80 mg normal liver had a GPD activity of 15 when prepared at room temperature and 12 when prepared at 0°; similarly the activity of the KCl mitochondrial precipitate was unimpaired when it was prepared and washed at room temperature and compared with a similar preparation made at 0°.)

4) The presence or absence of added Mg^{++} had little or no effect on the GPD activity in the whole homogenate, but omitting cyanide from the reaction vessel decreased the measured activity. (Typical results: when the complete system containing 25 mg of hyperthyroid liver had an activity of 25, omitting Mg^{++}, CN^{-} or both gave values of 25, 19 and 21, respectively.) All concentrations of phenazine methosulfate from 1 to 4 mg/flask gave the same results, but no activity was observed in the absence of the dye; other dyes were not tested since phenazine methosulfate worked well by preparing it fresh daily and exercising ordinary precautions against undue exposure to light. Also it has been reported to be a better electron acceptor for this enzyme than methylene blue, cytochrome c, et cetera (7).

5) The same activity was obtained when the amount of pH 7.7 phosphate buffer added per flask varied from 50 to 300 μmoles (e.g., all values for 25-mg aliquots of a hyperthyroid liver fell between 24 and 26). The pH of the solution after mixing all components was 7.6, and it remained at this value throughout the run when 100 to 300 μmoles of phosphate buffer were used. In confirmation of the report by Ringler and Singer (7), the pH optimum for GPD was found to be 7.6 in this procedure.

6) Any amount of liver could be used which would give suitable readings in the Warburg procedure. When increasing amounts of the same normal or hyperthyroid liver homogenate were added to Warburg flasks, the resulting measured activities were the same when calculated to a constant amount of liver (e.g., 60, 30 and 20 mg of a hyperthyroid rat liver gave GPD values of 72, 35 and 23). For routine assay purposes, 25 mg of all types of livers were used. This provided relatively small measurements for the normal livers, but precision in the normal range was neither required nor of any particular benefit in this assay procedure; when precise basal values were desired, 150 mg of fresh liver/flask were used.

Correlation of liver GPD with the steady-state metabolic rate. A correlation between the liver GPD activity and the steady-state metabolic rate existing in the intact rat was made by determining the enzyme activity in a large number of rats that were being used in the routine metabolic rate assay (2). In that procedure weanling male rats were fed the standard 30% casein diet containing 0.1% iodinated casein ± the antithyrotoxic test supplement (primarily hemoglobin and cottonseed meal concentrates in these studies) for 19 to 30 days. After determining the MR in duplicate, rats were killed and the liver GPD activity was determined. The data were then arranged into groups with similar metabolic rates (irrespective of the diets fed) and the results are shown in figure 1. The liver GPD increased rapidly as the steady-state metabolic rate increased from 6 to 10, and then continued to increase more slowly with higher metabolic rate.

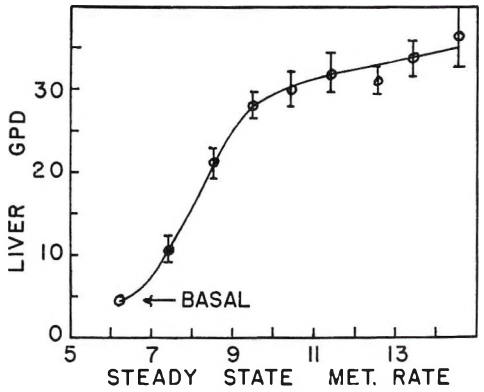

Fig. 1 Correlation between the steady-state metabolic rate (MR) of the intact rat (in liters O_2/square meter body surface/hour) and the corresponding liver α-glycerophosphate dehydrogenase activity (liver GPD, in μl O_2/10 minutes/25 mg fresh liver). The standard error of the mean is indicated by the vertical line. The basal (non-thyroid) values were: MR = 6.2 ± 0.17; GPD = 4.6 ± 0.6. Values from 22 to 33 individual rats were averaged to obtain each value below MR = 11; 15 determinations were averaged for each value between 11 and 13; 7 values for each point above 13.

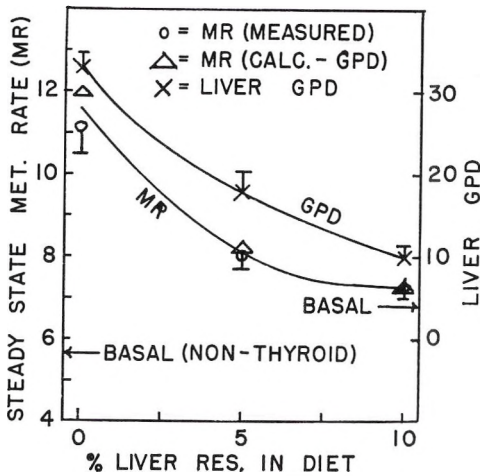

Fig. 2 Correlation between the steady-state metabolic rate (MR in liters O_2/square meter body surface/hour) assay curve and the corresponding liver α-glycerophosphate dehydrogenase activity (in μl O_2/10 minutes/25 mg fresh liver). Each point represents the average of 13 weanling male rats fed 30% casein diets containing 0.1% iodinated casein plus zero, 5 or 10% liver residue for 19 to 30 days. The standard error of the mean is indicated in only one direction, by the vertical bar. Although plotted on the same graph for convenience, the relationship between MR and GPD is not linear; the calculated MR was obtained from figure 1 and represents the MR which corresponded to the experimental GPD values.

Since a correlation existed between the steady-state MR and the liver GPD activity, it was possible to use the latter as a further index of antithyrotoxic activity in addition to the usual criteria of growth, survival and metabolic rate. A direct test of this possibility was made by feeding weanling male rats the standard 0.1% iodinated casein ± 5 or 10% liver residue diets, measuring the metabolic rate between the 19th and 30th days, and then determining the liver GPD. The metabolic rate assay curve in this experiment and the corresponding values obtained for liver GPD in the same rats are shown in figure 2. By reading these enzyme values against the curve in figure 1, the corresponding "calculated" metabolic rate was obtained. The good agreement between the "calculated" and experimentally determined metabolic rate shows that either measurement could be used to assess antithyroidal activity.

Antithyrotoxic assay based on liver GPD response. An antithyrotoxic assay procedure was developed which utilized only the determination of liver GPD activity. With 0.025 or 0.05% iodinated casein in the diet, the liver GPD reached a maximal value in 9 days, and for routine assay purposes was measured between the 11th and 14th day. Since one purpose in developing the enzyme assay procedure was to conserve the amount of antithyrotoxic material expended in the assay, the amount of iodinated casein used in these diets was reduced to 0.025%.

Figure 3 shows 2 typical assay curves obtained in the routine assay procedure in which weanling male rats were fed a 30% casein diet containing 0.025% iodinated casein and increasing amounts of hemoglobin for 11 to 14 days before determining the liver GPD. Different groups of rats which were treated identically showed such variations in response, and each curve in figure 3 is actually the average of 7 or 8 independent runs that were subsequently grouped together on the basis of a "high" or "low" response. The reason for such variation is unknown. When the usual assay procedure with 0.025% iodinated casein yielded the "low" curve, a

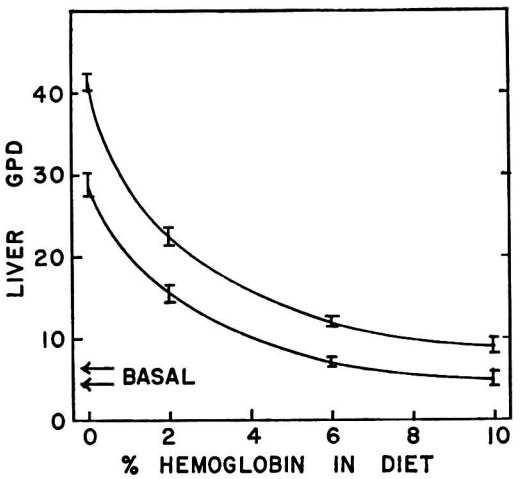

Fig. 3 Typical assay curves for antithyrotoxic activity based upon the determination of liver α-glycerophoshpate dehydrogenase activity (GPD in μl O_2/10 minutes/25 mg fresh liver). Weanling male rats were fed a 30% casein diet containing 0.025% iodinated casein (1% thyroxine equivalent) plus the indicated amounts of hemoglobin for 11 to 14 days before determining the liver GPD manometrically. Each point is the average of 40 rats for the low curve and 60 to 70 rats for the high curve, and the standard error of the mean is given by the vertical bar. The upper curve also illustrates the results obtained with 0.05% dietary iodinated casein at the same time that the lower curve was obtained with 0.025% iodinated casein.

simultaneous run with 0.05% iodinated casein gave the same "high" curve shown in figure 3; hence small errors in this part of the procedure could not account for the differences encountered. The basal level for those rats which gave a low curve averaged 4.34 ± 0.37 (38 rats), whereas the corresponding value for the rats giving a high curve was 6.35 ± 0.30 (60 rats). This represents a significantly larger output of endogenous thyroxine or an increased sensitivity of the tissues to thyroxine in those rats giving the high assay curve. However variations in the endogenous thyroxine output would not alter the assay curve since the amount administered was at least 10 times greater. The amount of thyroxine administered in the routine assay approximated the minimal amount of L-T_4 in the diet (0.3 mg/100 g) required to give a maximal liver GPD response of approximately 40.

From a practical standpoint, these fluctuations in the assay curve were controlled by including a zero, 2 and 6% hemoglobin standard in each run; unknowns were read from this standard curve obtained simultaneously. The same assay curve was obtained when zero to 10% hemoglobin was substituted for either sucrose or casein, and the same basal values were obtained when the diet contained 30 or 40% casein. Ten per cent hemoglobin completely nullified the effect of 0.025% iodinated casein on the enzyme in the low assay curve; in the high assay curve there was a residual effect reminiscent of the "residual MR" previously described (1). The conditions established for this assay appeared to be optimal for utilizing the entire potential range of the assay curve, and at the same time providing the maximum sensitivity for testing antithyrotoxic activity.

The major advantage of this enzyme assay as compared with the metabolic rate procedure, was the saving of time and assay material. The major disadvantage was that other supplementary evidence could not be obtained simultaneously, and small groups of rats did not provide any high degree of precision. All the rats survived and the growth retardation produced by this small amount of iodinated casein for this short period of time was too small to be of any value. Metabolic rate measurements were also of no value as supplementary evidence because: 1) the liver GPD activity increased more rapidly than the metabolic rate and there was no good parallelism between the two when measured at 11 to 14 days, and 2) the increased metabolic rate at 11 to 14 days with 0.025% iodinated casein was relatively small, not yet stabilized at the steady-state value, and therefore, difficult to measure with any confidence.

Kidney GPD. Rat kidney GPD activity was influenced by the relative balance between dietary thyroidal and antithyrotoxic factors in the way previously described for liver GPD, but the magnitude of the change in the kidney was less. Figure 4 shows the values obtained simultaneously for liver and kidney GPD activity in the same rats when the diet contained 0.025% iodinated casein plus zero to 6% hemo-

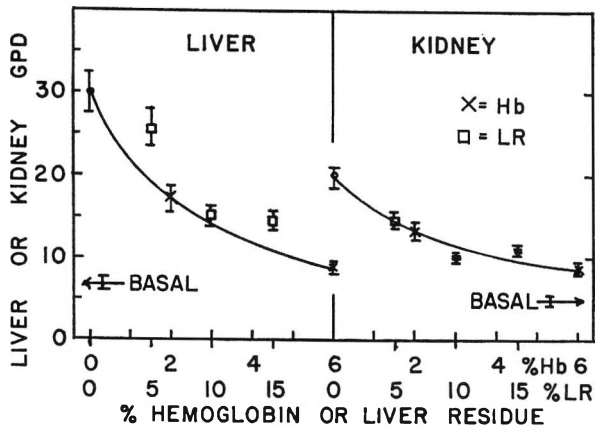

Fig. 4 Comparative assay curves with liver and kidney α-glycerophosphate dehydrogenase activities (both in μl O_2/10 minutes/25 mg fresh tissue). Weanling male rats were fed the basal 30% casein diet ± 2 or 6% hemoglobin (Hb) or 5, 10, or 15% liver residue (LR) for 12 to 14 days before determining the GPD activity in the livers and kidneys of the same rats. Each point is the average of 13 to 16 rats, and the standard error of the mean is indicated by the vertical line.

globin or 5 to 15% liver residue. The hemoglobin was about 3 times as active as the liver residue in the assay with kidney GPD and in numerous additional assays by the liver GPD procedure.

Figure 5 shows the relationship between kidney and liver GPD activity when determined simultaneously in the same rats in a large number of experiments. The degree of hyperthyroidism was reflected by larger changes in liver GPD, but there was a consistent relationship between this enzymatic activity in liver and kidney in the hyperthyroid state. In the normal and hypothyroid states the weanling rats used routinely in these studies followed a slightly different curve from 2 groups of adult rats that were being studied for other purposes.

Factors possibly affecting the assay. Coprophagy: Coprophagy was a potential complication in this assay procedure for 2 reasons: 1) approximately 50% of the dietary thyroxine was excreted in the feces under these conditions, and a variable recycling of this material through the intestinal tract by means of coprophagy might have given erratic results; and 2) if coprophagy was a factor, then certain test supplements might have been antithyrotoxic simply by preventing coprophagy. To study the role of coprophagy the usual standard assay curve with zero to 10% hemoglobin diets was run in the usual way with 2 groups of young male rats: 1) with tail cups (8) to prevent coprophagy, and 2) in the usual way without tail cups. L-Thyroxine was used in these experiments instead of the iodinated casein to correlate the results with other studies utilizing I^{131}-labeled thyroxine. The results in table 1 show that the liver GPD values were very close to those obtained in the standard assay curve (also shown in table 1 for comparison), and that there were no major differences between the results obtained with or without tail cups.

Restricted food intake: Since the thyroid hormone was fed in the diet in this assay procedure, gross fluctuations in the daily food intake might alter the response. This was studied by comparing the assay curve obtained by ad libitum feeding with the corresponding values obtained in rats that were given individually only 4 g of the same diets/day. The results in table 2 show that this restriction of total food intake had no major effect on the liver GPD values, even though the rats did not grow significantly with this limited amount of food. The 0.025% iodinated casein in the diet produced a maximal increase in liver GPD in both the restricted and ad libitum fed groups. The rats receiving only 4 g of food/day were getting about 40% of the thyroxine con-

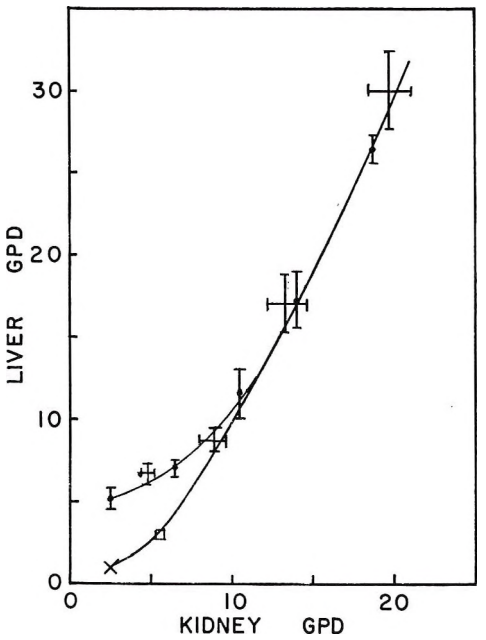

Fig. 5 The relationship between rat liver and kidney GPD (in μl O_2/10 minutes/25 mg fresh tissue) in weanling male rats fed the basal diet $\pm$ 0.025% iodinated casein $\pm$ 2 to 6% hemoglobin for 12 to 14 days. Each point is the average of 7 to 19 rats. The standard error of the mean is indicated by the bars; vertical lines only were from a scatter diagram which disregarded diets and were grouped in accordance with kidney GPD values; points given by both vertical and horizontal lines are the results shown in figure 4. The upper curve was obtained with weanling rats; the 2 points on the lower curve were obtained with mature intact (□) and thyroidectomized (×) rats (32 to 36/group), and the standard errors for these points were too small to illustrate (less than 10% of the mean).

sumed by the rats fed ad libitum but by the end of the experiment they were also less than half as large; hence, the thyroxine intake calculated on a body weight basis was roughly comparable in both groups. The thyrotoxic effect of any diet would appear to depend upon the net balance between thyroactive and antithyrotoxic factors present; the total amount of food consumed was relatively unimportant because the balance per se was not affected. The data also show that the same additional amounts of this enzyme were "synthesized" in response to the thyroxine stimulation, even though the simultaneous formation of other proteins necessary for growth was restricted by the dietary limitation.

Room temperature: Fluctuations in room temperature might affect the assay curve since the thyroid gland is involved in the regulation of body temperature upon exposure to cold. This point was checked by determining the assay curve obtained with rats maintained at 15.5° (60°F), and comparing such results with those obtained simultaneously in rats kept at the usual room temperature of 23.3° (74°F). Both liver and kidney GPD values were obtained at 2 different substrate concentrations (as supplementary data). The results in table 3 show that this variation in room temperature had no effect on the assay curve. While the metabolic rates of the intact rats at 10 to 11 days were measured too soon to be at the steady-state levels, there was no indication of any effect of the room temperature variation

TABLE 1

A comparison of the liver glycerophosphate dehydrogenase (GPD) assay curve obtained in the usual way and when coprophagy was prevented by the use of tail cups [1]

	Liver GPD				
	Basal	T_4	T_4 + 2% Hb	T_4 + 6% Hb	T_4 + 10% Hb
	μl O_2/10 min/25 mg fresh weight				
Usual assay procedure	6 $\pm$ 0.3(3) [2,3]	24 $\pm$ 1.3(4)	20 $\pm$ 2.8(5)	6 $\pm$ 1.3(5)	5 $\pm$ 2.7(3)
With tail cups	4 $\pm$ 0.7(3)	25 $\pm$ 2.9(7)	12 $\pm$ 2.6(7)	5 $\pm$ 1.1(5)	5 $\pm$ 1.3(6)
Average assay curve [4]	4 $\pm$ 0.4	29 $\pm$ 1.4	15 $\pm$ 1.1	7 $\pm$ 0.6	—

[1] Starting weight = 81 g; average weight gain = 5.5 g/day/rat for 11 days for basal $\pm$ 6 or 10% Hb, 4.3 g for T_4 group and 4.9 for T_4 + 2% Hb. Tail cups reduced these values by 15%. Average food consumption = 9 g/day/rat for all groups without tail cups and 20% more with tail cups. All diets except the basal contained 0.5 mg of L-thyroxine (T_4)/100 g diet.

[2] All values are the mean $\pm$ SE.

[3] Numbers in parentheses indicate number of animals per group.

[4] Average assay curve obtained in 7 experiments with 0.025% iodinated casein and a total of 40 rats/group.

TABLE 2

Effect of a restricted food intake on the liver glycerophosphate dehydrogenase (GPD) assay curve [1]

	Liver GPD				
	Basal	0.025% Iodinated casein	Iodinated casein + 2% Hb	Iodinated casein + 6% Hb	Iodinated casein + 10% Hb
			μl O_2/10 min/25 mg fresh liver		
Usual ad libitum intake	6 ± 0.6 [2]	41 ± 2.8	16 ± 3.5	9 ± 1.2	9 ± 0.8
Food restricted to 4 g/day	5 ± 0.5	44 ± 3.2	20 ± 1.9	13 ± 1.4	10 ± 1.3
Average assay curve	6 ± 0.3	41 ± 1.1	22 ± 1.1	12 ± 0.7	—

[1] Starting body weight = 55 g; average weight gain (ad libitum) = 5.7 g/day/rat for all basal ± hemoglobin groups, 4.8 g/day for the iodinated casein group; average weight gain when food intake was restrcited to 4 g/day was: 0.4 g/day/rat for basal ± 6 or 10% hemoglobin; −0.4 g/day/rat for iodinated casein ± 2% hemoglobin. The usual food consumption ad libitum was 8 to 10 g day.

[2] Values are mean ± SE for 8 rats/group, except that the average assay curve was obtained from groups of 60 to 70 rats.

on the metabolic rates achieved with the different diets.

A decrease in room temperature from 23.3 to 15.5° was enough to decrease the growth rate of the rats, and increase slightly the liver and kidney GPD in the basal groups. This relatively small effect on tissue GPD is actually equivalent to a 30% increase in endogenous thyroxine output [4] and this is approximately the magnitude of the effect on the thyroid that would be predicated from the data published by Dempsey and Astwood (9). In the groups fed iodinated casein, the amount of thyroxine provided exogenously would suppress the thyroid gland and render it nonfunctional at both temperatures.

Diet variations:[5] Feeding an 8% casein diet to weanling male rats for 11 days gave the same liver GPD (7.9 ± 0.70) as did the usual 30% casein basal diet (7.8 ± 0.33); hence protein deficiency had no effect on this liver enzyme. Starvation for 2 days immediately preceding the analysis, or starvation for 2 days followed by 2 days of refeeding the basal diet gave values of 6.1 ± 0.48 and 5.0 ± 0.68, respectively. These relatively small differences from the basal diet rule out any major effect of short-term fasting and any participation of this enzyme in the rebound or adaptation phenomenon. Adding 10% hemoglobin to the basal 30% casein diet (at the expense of sucrose) gave a liver GPD of 6.3 ± 0.36, whereas feeding a commercial chow[6] gave 3.5 ± 0.36. Other studies have shown that this chow contains antithyrotoxic activity.

Mouse liver GPD. The GPD activity in the livers of weanling mice fed the basal diet for 14 days was 29 ± 1.8 (mean ± SE for 23 mice, in μl O_2/10 min/25 mg) or about 5 times the value found in normal rat liver. When weanling mice were fed the basal diet containing 0.025% iodinated casein for 2 weeks, this was increased to 49 ± 1.7 (23 mice); the addition of 5% hemoglobin to the iodinated casein diet reduced this value to 38.5 ± 1.7 (30 mice). Hence mice responded to these various diets like the rats, but the magnitude of the response to thyroid feeding and the degree of protection afforded by hemoglobin was less in mice than in rats.

Antithyrotoxic assay results.[7] Table 4 shows the average results obtained when a variety of substances were repeatedly assayed (usually 3 to 5 times) for their antithyrotoxic activities by this enzyme procedure. The values have been given in "hemoglobin units," i.e., the relative activity of the test substance in comparison with the hemoglobin standard when the

[4] Unpublished data.

[5] These values are averages of 8 or 9 rats/group, as determined on 80 mg and recalculated to 25 mg fresh liver.

[6] Big Red Rat Food, Grange League Cooperative, Ithaca, New York.

[7] Prior to the development of the GPD enzyme assay, the following acids from California Biochemicals, Inc. were found to be inactive by the MR procedure at the percentage dietary concentration given in parenthesis: 9, 10-dihydroxystearic (0.2), 12-hydroxyoleic (0.2), α-hydroxystearic (0.17), α-hydroxy-*n*-valeric (0.03), α-hydroxy-*n*-hexacosanoic (0.03), 8,9,16-trihydroxy palmitic (0.17), 12-hydroxystearic (0.2). Selachyl alcohol (0.03) and pig brain cerebrosides (0.2) were also inactive.

TABLE 3

Effect of two room temperatures on the liver and kidney glycerophosphate dehydrogenase (GPD) assay curve [1]

Diet	Room temperature	Body wt gain	Metabolic rate at 10–11 days	Liver GPD [2]		Kidney GPD [2]	
				120 μmoles substrate	300 μmoles substrate	120 μmoles substrate	300 μmoles substrate
	°C	g/day					
Basal	23.3	7.2	7.8 ± 0.29 [3]	7.6 ± 1.1 [4]	8.4 ± 1.3 [4]	7.8 ± 0.5 [4]	—
0.025% iodinated casein	23.3	6.3	10.7 ± 0.59	42 ± 5.5	57 ± 8.0	23 ± 1.9	33 ± 2.4
Iodinated casein + 2% Hb	23.3	6.7	9.1 ± 0.92	28 ± 5.7	35 ± 6.3	16 ± 1.8	21 ± 2.3
Iodinated casein + 6% Hb	23.3	7.8	8.8 ± 0.53	8 ± 1.4	9 ± 1.6	8 ± 0.5	10 ± 0.6
Basal	15.5	5.1	7.9 ± 0.48	9.5 ± 1.1 [4]	10 ± 0.7 [4]	8.8 ± 0.2 [4]	—
0.025% iodinated casein	15.5	5.2	10.6 ± 0.82	41 ± 1.8	52 ± 3.8	26 ± 0.9	34 ± 1.6
Iodinated casein + 2% Hb	15.5	6.2	8.3 ± 0.38	18 ± 2.7	23 ± 3.2	16 ± 1.9	22 ± 2.3
Iodinated casein + 6% Hb	15.5	6.2	8.7 ± 0.32	8 ± 0.8	10 ± 0.9	11 ± 1.7	13 ± 1.9

[1] Starting weight = 63 g; 6 rats/group; metabolic rate in liters O_2/square meter body surface/hour.
[2] Liver and kidney glycerophosphate dehydrogenase (GPD) in μl O_2/10 minutes/25 mg fresh tissue as determined with 120 or 300 μmoles substrate.
[3] Mean ± SE.
[4] Determined on 75 mg tissue and calculated to 25 mg.

TABLE 4

Antithyrotoxic activity of various test supplements as determined by the glycerophosphate dehydrogenase (GPD) assay procedure

Substance tested	% of diet	Hb [1] units
Fibrin [2]	20	21
Gelatin [2]	20	8
Zein [2]	20	8
Fish meal	10	19
Dry fish solubles	10	26
Distillers solubles	10	16
Fermentation residue	10	23
Cellulose	10	6
Egg yolk	10	17
Egg yolk (nonlipids)	4	5
Egg yolk (lipids)	6	10
Crystalline hemoglobin	1	23
Fresh beef hemoglobin	1	13
Dry bovine plasma	10	11
Dehydrocholic acid	0.2	19
Na glycocholate	0.2	20
Crude cottonseed oil	5	10
Safflower oil	5	12
Soy oil	5	3
Lactalbumin [2]	20	25
Lactalbumin conc	1.7	28
Soy protein [2]	20	20
Soy protein conc	2.0	22
Egg albumin [2]	20	12
Egg albumin conc	0.8	4
Egg albumin conc	(2.0)	16
Liver residue (unextracted)	10	32
Liver residue conc	(1)	17
Cottonseed meal	8	38
Cottonseed meal conc	(3)	> 42
Hemoglobin	10	100
Hemoglobin conc	1.2	67

[1] The results have been recorded as hemoglobin (Hb) units, i.e., the relative activity of the test supplement in comparison with a 10% hemoglobin diet (= 100 Hb units).
[2] Dietary casein was reduced from the usual 30 to 20%; total protein = 40%.

activity of a 10% hemoglobin diet was arbitrarily assigned the value of 100 hemoglobin units. Liver residue was about one-third as active as hemoglobin in this test system. The correspondence between the results previously obtained by the metabolic rate assay (2) and the results obtained by this enzyme procedure was reasonably good; no substance exhibited good activity by one procedure and poor activity by the other. The fish solubles used previously were relatively inactive, but the new supply possessed good antithyrotoxic activity. Of the additional substances tested, zein was a relatively inactive protein (like gelatin, casein and egg albumin). Two-thirds of the activity in egg yolk was present in the lipid-soluble fraction (etha-

nol, then trichloroethylene extract combined) and moderate activity was also present in crude cottonseed oil and safflower oil. Whereas the activity of such lipids has generally been attributed to the linoleic acid present, the addition of 0.5% or 2% linoleic acid (95 to 98% pure) to the diet had little or no antithyrotoxic effect. Other substances which exhibited little or no activity (zero to 8 Hb units) in this assay included 10% cellulose, 0.6% chlorophyll, 0.25% hematoporphyrin, and 30 mg/kg of procaine penicillin. Fresh hemoglobin and crystalline hemoglobin were at least as active as the dried preparation used for the standard, and the activity of the hemoglobin cannot be attributed to a plasma contaminant.

Fractionation procedures [8]

Cottonseed meal (CSM) was stirred with 3 volumes of trichloroethylene (TCE), filtered, and washed with TCE to remove most of the oil; 800 g of the dry defatted CSM were refluxed for 7 hours with 8 liters of 1 N HCl (technical) in the presence of a few boiling chips and Dow-Corning antifoam A. After cooling to room temperature, the insoluble precipitate was filtered off and washed generously with water. No activity could be recovered from the acid filtrate by adsorption with charcoal (as such or after adjusting to pH 5) and eluting the charcoal with 0.5 N NH_4OH.

Three batches of the moist acid precipitate were combined (700 g dry weight from 2400 g CSM) and refluxed for 22 hours with 8 liters of 1 N NaOH in the presence of chips and antifoam. The solution was filtered hot with the aid of Celite,[9] and washed well with hot water. Approximately 150 to 180 g of alkali-insoluble residue remained with the Celite, and it had little or no activity. If the alkaline solution was cooled before filtration, about one-sixth of the solids that could be recovered subsequently by acid precipitation (and a higher percentage of the activity) was found in the alkali insoluble residue; this activity could be leached away from the truly alkali-insoluble residue by dissolving in hot water. The activity appeared to be in the form of a sodium salt that had a limited solubility in cold NaOH (or other salts).

The alkaline solution was cooled to room temperature, acidified with HCl to a pH of 2 to 3, filtered and washed well with water. The air-dried precipitate was powdered, soaked with 3 volumes of TCE overnight, filtered and washed with TCE to remove any remaining fatty acids. The TCE removed about 6% of the solids from this fraction and the lipid extract was essentially inactive. Approximately 200 g of the acid-insoluble, lipid-free precipitate was obtained from each kilogram of TCE-extracted CSM used originally. Since there was no loss of activity in the processing, this fraction represented a fivefold purification (original CSM = 0.5 metabolic rate unit/g; this fraction equals 2.5 metabolic rate units/g).

This fractionation precedure for CSM was based upon the method originally developed for liver residue by Overby.[10] He avoided the preliminary acid hydrolysis and refluxed the defatted liver residue in 2 N NaOH for 18 hours. The acid-insoluble precipitate was then redissolved in NH_4OH, precipitated with $CaCl_2$ as the Ca salt, and the Ca removed by leaching with HCl. The Ca salt precipitation could also be applied to the CSM fractionation, but it provided little additional purification of the CSM concentrate.

In the fractionation of lactalbumin and other proteins the procedure was stripped to a simple alkaline hydrolysis and acid precipitation, as follows: 800 g of the protein were refluxed for 22 hours with 8 liters of 1 N NaOH. After cooling to room temperature, the solution was acidified with HCl to a pH of 2 to 3. The acid-insoluble precipitate was filtered off, washed well with water, air-dried, and tested for antithyrotoxic activity by the enzyme assay. The amount of precipitate obtained from each protein is indicated in table 4, along with the assay results, since each concentrate was assayed at a level corresponding to the amount obtained from the original test supplement, (e.g., 1.7 g of concentrate were obtained from 20 g of lactalbumin).

[8] The cottonseed meal fractionation was developed by use of the MR assay procedure; the GPD enzyme assay of the starting and final products are also given in table 4.

[9] Diatomaceous silica filter aid, Johns Manville Products Corporation, Manville, New Jersey.

[10] Personal communication.

The values given in parentheses were tested at a different level (e.g., 0.725 g of concentrate were obtained from 10 g liver residue). The antithyrotoxic activity of the original starting material was well retained in all of the concentrates prepared by this procedure.

DISCUSSION

Alkaline hydrolysis followed by acid precipitation gave a water-insoluble fraction which possessed essentially all of the antithyrotoxic activity of the original starting material. Either the relatively pure proteins (lactalbumin, soy protein) contained, in addition to the amino acids, an appreciable amount of other material which resisted the alkaline hydrolysis, or the relatively large amount of insoluble precipitate obtained from the protein hydrolysate was an artifact formed during the hydrolysis. Such a water-insoluble fraction was not obtained when a mixture of amino acids was refluxed in 1 N NaOH for 22 hours. This fraction has traditionally been referred to as the ill-defined "humus" obtained during the hydrolysis of proteins, and has generally been considered to be composed of degradation products. If such were the only explanation, it is a coincidence that the same amount of activity was produced in forming this fraction as was present in the original protein.

It is also possible that a portion of the original protein molecule is unusually resistant to alkaline hydrolysis, and that it is this portion of the molecule which confers antithyrotoxic activity upon the original protein and is subsequently isolated as the acid-insoluble precipitate after alkaline hydrolysis. Those proteins which possessed good antithyrotoxic activity (e.g., lactalbumin, soy protein) gave the largest yield (8 to 10%) of this acid-insoluble fraction after alkaline hydrolysis. Those proteins which were relatively inactive (casein, egg albumin) gave only half as much or less of this fraction; however the concentrate which was obtained from egg albumin possessed good activity when compared with the other protein concentrates on the same weight basis. The greatest difficulty with this concept is the relatively low nitrogen content (1 to 2%) of these fractions; any undegraded peptide chain should theoretically have a higher nitrogen content.

Although the same kind of antithyrotoxic concentrate was obtained from all the different starting materials, the activity in each was not due to the same chemical substance. About three-fifths of the liver residue concentrate solids were soluble in 1 N alcoholic KOH, and this also solubilized 80 to 90% of the activity. By contrast, alcoholic KOH did not solubilize any significant proportion of the cottonseed meal concentrate solids or activity. Autoclaving the cottonseed meal concentrate in 2 N NaOH for 22 hours did not destroy the activity, and three-quarters of the original weight of this fraction was recovered by acid precipitation. The activity in the liver residue concentrate was less stable to autoclaving. Such results suggest that this particular property of binding or otherwise nullifying the biological effect of the thyroid hormone is not limited to a single chemical substance.

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Studies of Thiamine Metabolism in the Rat [1]

I. METABOLIC PRODUCTS FOUND IN URINE

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ABSTRACT The catabolic products of the metabolism of pyrimidine carbon C^{14}-labeled thiamine in rat and rabbit urine were examined by the application of column and paper chromatographic techniques. The presence of a minimum of 22 different metabolites of thiamine was detected. It is considered that most of these are "true" metabolites since at the pH of rat urine and at room temperature, thiamine was quite stable.

There is abundant literature concerning the question of thiamine balance in both experimental animals and man, but little is known of the metabolic fate of the thiamine molecule. A variety of forms of thiamine (other than thiamine phosphate and "active aldehydes") have been shown with reasonable certainty to be present in the tissues and excretory products of mammals. These include free thiamine, thiochrome, thiamine disulfide (1), 5-(2-hydroxyethyl)-4-methylthiazole (1–3), and of necessity, some form of the pyrimidine moiety of thiamine. Early reports suggesting that thiamine pyrophosphate is present in mammalian urine (4, 5)[2] have not been confirmed by more recent investigators (1, 6, 7). A claim for the presence of thiamine 5-acetic acid in human urine (8) has recently been withdrawn (6). The isolation from a perfusate of rat liver of thiamine disulfate, *o*-acetyl thiamine, and a second acetyl derivative in which the acetyl group was not bound to oxygen (9, 10) has recently been reported. At least a dozen unidentified degradation products of C^{14} thiazole-labeled thiamine have been reported by Iacono et al.[2] and Iacono and Johnson (1) to occur in rat urine.

Although several groups of workers (11-13) have provided indirect evidence for the occurrence of some form of the pyrimidine moiety of thiamine in urine, only Kawasaki and Okada (14) have succeeded in isolating such a compound. These workers reported the isolation of 2-methyl-4-amino-5-hydroxymethyl pyrimidine from human urine after giving large doses of thiamine to test subjects. Whether the isolated compound was really a metabolic product of thiamine metabolism appears to be open to some doubt. The authors themselves point out that the extremes of temperature and pH used during their isolation procedure may well have led to the generation of their compound by hydrolytic cleavage of thiamine present in the urine. In addition, Suhara and Iritani (15) have recently reported they were unable to detect the presence of this compound in the urine of rats.

The present study was undertaken particularly to clarify the metabolism of the pyrimidine moiety of thiamine. This was accomplished by the use of thiamine labeled with C^{14} in the pyrimidine part of the molecule.

EXPERIMENTAL PROCEDURE

Except where noted, adult female Sprague-Dawley rats were used. They were housed in pairs in stainless steel metabolism cages constructed to permit the separate collection of urine and feces. Twenty-four-hour urine specimens were collected under toluene in glass bottles

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[2] Iacono, J. M., G. Wolf and B. C. Johnson 1953 Metabolism of radioactive thiamine in the rat. Federation Proc., *12*: 223 (abstract).

containing 1 ml of 2 N acetic acid and a few crystals of thymol. The samples of urine were then pooled, centrifuged to remove any precipitate, and stored at −20° until ready for use.

A thiamine-deficient basal diet was used throughout the experiment. It had the following percentage composition: vitamin-free casein, 18; cottonseed oil, 5; sucrose, 69; succinylsulfathiazole,[3] 1; nonnutritive fiber,[4] 3; and salt mixture,[5] 4. The following vitamins were added at the levels indicated: (mg/100 g of basal diet) vitamins A and D, at 400 and 40, I.U. respectively; riboflavin, 0.80; niacin, 4.00; Ca pantothenate, 4.00; biotin, 0.04; folic acid, 0.20; pyridoxine, 0.50; *p*-aminobenzoic acid, 10.00; choline chloride, 100.00; α-tocopheryl acetate 4.0; and menadione, 0.5.

A single New Zealand-strain rabbit was used in one short experiment. This animal was housed in a standard metabolic cage and fed a stock pelleted diet. Urine was collected for 12-hour periods, under toluene, in a glass bottle containing 5 ml of 2 N acetic acid and a few crystals of thymol.

In all experiments the animals were allowed food and water ad libitum.

The synthesis of thiamine labeled with C^{14} in the pyrimidine ring was accomplished using the pyrimidine synthesis described by Grewe (16). The method used for coupling the pyrimidine and thiazole moieties was that of Gravin (17). Since malononitrile-1-C^{14} was used in this synthesis, 2 species of labeled thiamine molecules were produced. One species was labeled in the 4-position of the pyrimidine ring and the other was labeled in the methylene bridge. The specific activity of the thiamine-C^{14} synthesized was 0.296 μc/μmole. The final product showed an ultraviolet spectrum similar to that of authentic thiamine chloride hydrochloride at 2 pH values. The molecular extinction coefficients of both compounds were the same and a mixed melting point determination showed no depression. Both the authentic and labeled compounds behaved identically on ion exchange and paper chromatography. The labeled compound yielded a fluorescent product on alkaline oxidation that migrated on paper chromatography with the same R_F value as authentic thiochrome. Finally, the synthesized compound supported the growth of *Lactobacillus viridescens* and *Phycomyces blakesleeanus*, cured acute thiamine deficiency in the rat, and supported the normal growth of rats maintained with a thiamine deficient diet for 10 months.

Acid-washed charcoal was used to obtain the urinary metabolites of thiamine-C^{14} in a form free of inorganic salts. The charcoal[6] was prepared by refluxing 100 g of charcoal for 3 hours in one liter of 2 N hydrochloric acid. The charcoal was allowed to settle, the hydrochloric acid decanted, and the same procedure was repeated. After recovery of the charcoal by Büchner filtration the charcoal was washed exhaustively with distilled water until the pH of the washings was 5.0 or greater. It was then washed with 4 liters of ethanolic ammonia (ethanol/ammonium hydroxide/water, 50/5/45, by volume) and with distilled water until neutral before air-drying. In the desalting procedure the pH of the urine was adjusted to 6.0 to 6.5, 1 g of acid-washed charcoal added for every 100 ml of urine, and the suspension stirred for 1 hour at room temperature. The charcoal was removed by centrifugation (5000 × *g* for 10 minutes) and suspended in 100 ml of pyridine/ethanol/water (10/45/45 by volume) for each gram of charcoal originally added to the urine. This mixture was shaken for 3 hours at 37°, centrifuged, and the supernatant containing the radioactive metabolites was decanted. Some 85 to 90% of the radioactivity in the urine could be obtained free of inorganic salts by this procedure.

For chromatography the charcoal eluates were reduced in volume in a rotary evaporator under vacuum at 40° to approximately 5 ml. The flask was washed with 5 ml of distilled water which was added to the original concentrate and its pH was adjusted to 5.5. After centrifugation at 5000 × *g* for 10 minutes the super-

[3] Nutritional Biochemicals Corporation, Cleveland.
[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[5] Hubbel, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14*: 273.
[6] Norite-A, Matheson Coleman and Bell, East Rutherford, New Jersey.

natant was placed on an Amberlite CG-50 column [7] (1.0 × 40.0 cm; 200–400 mesh; H^+ form) which had been previously washed with distilled water until the pH of the eluant was 4.5 or greater. After all of the sample had drained into the resin two 2-ml portions of distilled water were permitted to drain into the resin and the radioactive metabolites were eluted with water to 0.01 N hydrochloric acid gradient. Five hundred milliliters of eluant were used and 10- to 12-ml fractions collected.

To prepare the radioactive peaks from the Amberlite CG-50 columns for paper chromatography, the combined column fractions were reduced in volume under vacuum at 40° to approximately 3 ml and then lyophilized. The lyophilizate was dissolved in 0.1 ml of the chromatographic solvent system to be used and applied to the paper in 3-cm bands at a point 4 cm from the bottom of the filter paper sheet.

It was found necessary to subject the initial radioactive peak from the Amberlite CG-50 columns to further ion exchange chromatography on a neutral column of Dowex-1-resin [8] (1.0 × 20.0 cm; 100–200 mesh; chloride form) prior to paper chromatography. This was accomplished by reducing the combined fractions of the peak to approximately 5 ml under vacuum at 40°, adjusting to pH 6.5, and placing on the Dowex-1 column. After the sample had passed into the column the resin was washed twice with 2-ml portions of water and the column eluted first with 100 ml of distilled water followed by 0.001 N hydrochloric acid.

Ascending paper chromatography was performed on acid-washed sheets of Whatman filter paper (nos. 1 and 3 MM). The sheets were acid-washed by descending chromatography with 500 ml of 0.1 N hydrochloric acid followed by 500 ml of 50% (by volume) ethanol.

The metabolites of thiamine-C^{14} were detected by radioautography, by fluorescence or quenching under ultraviolet light, and by a combination of these methods.

Radioautographs were prepared using Eastman "No-screen" X-ray film. The film was exposed to the paper chromatogram for periods varying from 2 to 30 days depending on the amount of radioactivity and developed in the conventional manner.

Radioactivity measurements were made using a Nuclear-Chicago D-47 gas-flow detector equipped with a model M5 sample changer, or a Packard Tri-Carb liquid scintillation counter.

The fractions collected from the ion exchange columns were checked for radioactivity by plating 0.5-ml aliquots of the column fractions on aluminum planchets, drying, and counting in the gas-flow counter. In the majority of the fractions this volume gave a plate which could be considered to be infinitely thin. Because of the large number of fractions assayed no attempt was made to correct for sample absorption in those fractions not drying to an infinitely thin layer.

Scintillation counting was used to determine the recovery of the administered radioactivity from the urine. This method was also used to determine the radioactivity in a particular sample, when accuracy greater than that provided by uncorrected gas-flow counting was required. The scintillation fluid used for counting the radioactivity in these aqueous samples was that of Bray (18). Quenching as a result of the color or high salt content, or both, of a particular sample was corrected for by adding to a triplicate sample 0.2 to 0.5 ml of the scintillation fluid containing a known amount of thiamine-C^{14}.

RESULTS

A typical pattern of the radioactive metabolites of thiamine-C^{14} eluted from an Amberlite CG-50 column is shown in figure 1. The pattern of eluted radioactivity is representative of a pooled desalted urine sample from 10 rats, each of which received an intraperitoneal injection of 100 μg of thiamine-C^{14} daily for 30 days prior to collection. Five major peaks of radioactivity are evident. Peak no. 1 is only slightly cationic in character since it was readily eluted. Peaks no. 2 through 5 contain compounds of increasing basicity. Peak no. 5 contains thiamine-C^{14} as well as some 10 other metabolites of thiamine-C^{14}. The activities in the various

[7] Mallinckrodt Chemical Works, St. Louis, Missouri.
[8] AG-1-X8, Bio-Rad Laboratories, Richmond, California.

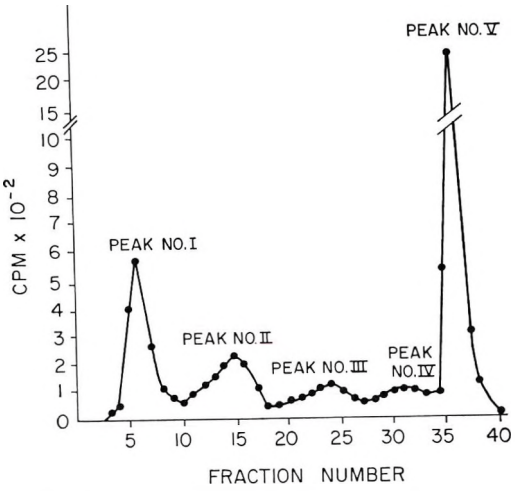

Fig. 1 Column chromatography of desalted rat urine. Exchanger, Amberlite CG-50 (200–400 mesh) in the H^+ form, 1 × 40 cm. Flow rate 60 ml/hour, fraction size 12 ml. The activity in counts per minute as determined by gas-flow counting is that of 0.50-ml aliquots of the 12-ml fractions.

peaks were found to vary both qualitatively and quantitatively depending upon the amount of thiamine-C^{14} administered, the state of thiamine nutrition of the animal, and the length of time the animal had been receiving radiothiamine. Peaks 1 and 2 contain a greater percentage of the total radioactivity in rats injected with thiamine-C^{14} for a longer period of time than in rats injected for a shorter period. A time delay of about 2 days in the appearance of these 2 peaks has also been observed in animals which had just started receiving thiamine-C^{14}.

It was necessary to subject peak no. 1 to further ion exchange chromatography on Dowex-1-chloride prior to paper chromatography. The pattern of radioactivity from such a column is shown in figure 2. This pattern represents the radioactivity of a pooled desalted 24-hour urine from 10 rats, each of which received intraperitoneal injections of 100 μg of thiamine-C^{14} /day for 39 days prior to collection. This procedure separated the bulk of the radioactivity in peak 1 from contaminating solid material so as to permit satisfactory paper chromatography. For this purpose the main radioactive peak (fractions 15–17) was reduced to dryness in the manner previously described.

Ascending paper chromatography of the lyophilized samples of the 5 radioactive peaks shown in figure 1 was carried out for 18 hours on acid-washed Whatman 3 MM paper. The paper was then dried and a radioautogram prepared by placing it in contact with Eastman "No-screen" X-ray film for an average of 10 days. The pattern of radioactive metabolites obtained and their average R_F values is schematically represented in figure 3. In all, 22 metabolites of thiamine-C^{14} were detected in urine of rats by radioautography. It is probable that this is a minimal figure since, with one exception, no attempts were made to rechromatograph individual radioactive bands in another solvent to determine whether they contained more than one radioactive component. It is also possible that a film exposure time greater than that used might have revealed the presence of additional minor metabolites. Metabolite no. 16, figure 3, is thiamine. The percentage of the radioactivity in the urine represented by thiamine-C^{14} was determined by cutting the area corresponding to thiamine from the chromatogram, eluting the radioactivity with distilled water, and counting in the Packard Tri-Carb scintillation counter. The

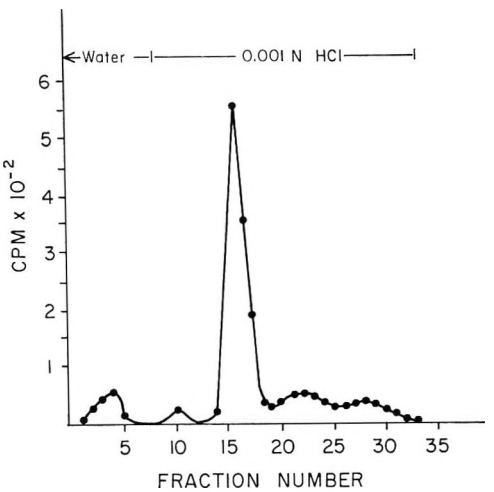

Fig. 2 Ion exchange chromatography of peak no. 1 from the Amberlite CG-50 column (see fig. 1). Exchanger, Dowex-1-chloride (100–200 mesh), 1 × 20 cm. Flow rate 60 ml/hour, fraction size 12 ml. The activity in counts per minute as determined by gas flow counting is that of 0.50-ml aliquots of the 12 ml-fractions.

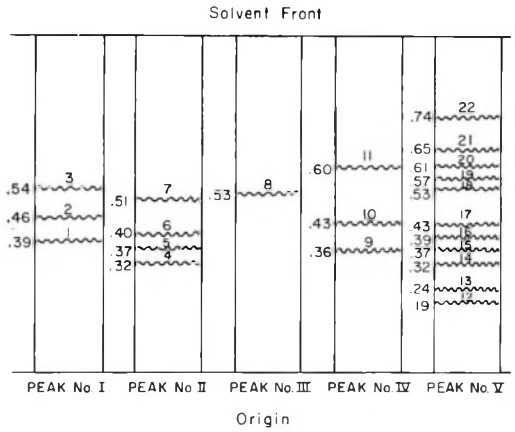

Fig. 3 Schematic representation of the radioautograms of the radioactive peaks from an Amberlite CG-50 column of the desalted urine of rats receiving thiamine-C^{14} (see fig. 1). Solvent *n*-propanol/water/1 M acetate buffer pH 5.0 (70/20/10), by volume (22). Ascending technique for 18 hours.

thiamine-C^{14} excreted in a 7-day period was found to represent 48 to 51% of the total activity of the urine collected from 10 rats injected daily with 100 µg of thiamine-C^{14} for 40 days or more. However, this proportion of the total radioactivity as thiamine-C^{14} is characteristic only at an intake level of 100 µg/day since the percentage of the total urinary radioactivity which is thiamine-C^{14} will vary in proportion to the amount of thiamine administered.

There was a significant variation of the R_F values from one radioautogram to the next. The range of R_F values is shown in table 1. Metabolite no. 16 in table 1 is thiamine-C^{14}. The R_F value of authentic thiamine-C^{14} when spotted from distilled water solution is 0.38. Metabolites no. 9 and 22 were detected only once on a radioautogram which had been exposed to X ray film for approximately 3 weeks. The remaining metabolites were detected a minimum of 3 times on separate radioautograms of separate urine collections. The variation of R_F values may be ascribed to 2 factors, both of which are dependent upon the amount of urine placed on the chromatogram. The first is the amount of brown oily urinary pigment contained in each radioactive peak from the Amberlite CG-50 column. This tends to increase the R_F values of the metabolites apparently in direct proportion to its concentration. The second factor is the amount of solid material in the sample to be chromatographed. These solids of indeterminate nature tend to decrease R_F values and make the radioactive bands more diffuse. This effect was particularly evident when the Amberlite CG-50 column peaks obtained from the pooled 24-hour urine of 20 or more rats were chromatographed on paper.

TABLE 1

Range of R_F values obtained for the metabolites of radiothiamine shown in figure 3

Metabolite no.	Range of R_F values	Metabolite no.	Range of R_F values
1	0.35–0.42	12	0.18–0.21
2	0.43–0.50	13	0.21–0.27
3	0.50–0.57	14	0.31–0.34
4	0.30–0.36	15	0.34–0.41
5	0.33–0.39	16	0.35–0.45
6	0.36–0.43	17	0.39–0.48
7	0.50–0.54	18	0.53–0.56
8	0.53–0.54	19	0.56–0.61
9	0.36	20	0.59–0.61
10	0.42–0.43	21	0.64–0.65
11	0.59–0.61	22	0.74

An alternate method of developing the Amberlite CG-50 column consisted of elution with water for 200 ml followed by elution with pyridine/acetic acid/water (7.5/1/91.5, by volume). The pattern of radioactivity obtained by use of this method is shown in figure 4. The advantage of this elution procedure is that thiamine-C^{14} can be separated from all but one of its radiometabolites. A schematic representation of radioautographs of the 4 radioactive peaks obtained by this elution procedure is shown in figure 5. For paper chromatography it was necessary to subject peak no. 1, figure 4, to the same Dowex-1 treatment described for peak no. 1, figure 1.

Because of the similarity of the initial elution procedure, the radiometabolites of peaks no. 1 and no. 2, figure 5, are of the same number and approximate R_F values as the same peaks in figure 3. Radioactive bands 8, 9, 10, and 11 from figure 3 are now a part of peak no. 3 in figure 5. Considering the similarity in R_F values, radioactive bands 9 and 15 in figure 3 are probably combined to form radioactive band no. 11 in figure 5. In addition, radioactive bands 10 and 17 as well as 11 and 20 from figure 3 are probably combined

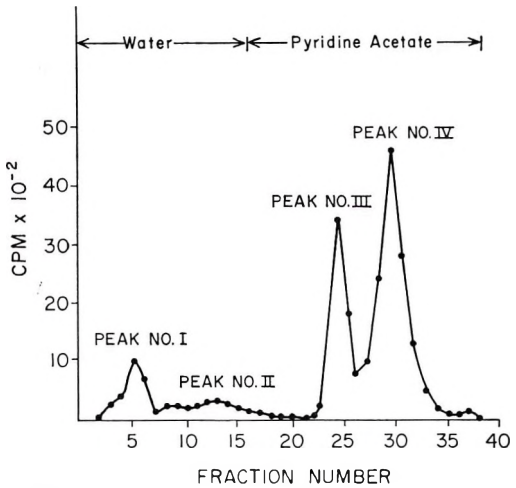

Fig. 4 The chromatography of desalted urine using a pyridine/acetate eluent. Exchanger, Amberlite CG-50 (200–400 mesh) in the H^+ form, 1 × 40 cm. Flow rate 60 ml/hour, fraction size 12 ml. The pattern of radioactivity shown is representative of that found in a pooled desalted 24-hour urine sample from 10 rats each of which received 200 μg of thiamine-C^{14} by way of 2 intraperitoneal injections of 100 μg 12 hours apart. The activity in counts per minute as determined by gas-flow counting is that of 0.50-aliquots of the 12-ml fractions.

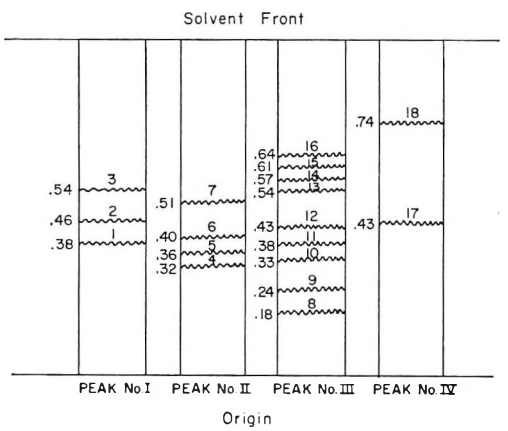

Fig. 5 A schematic representation of the radioautograms of the radioactive peaks from an Amberlite CG-50 column of desalted urine eluted with pyridine/acetate (see fig. 4). Solvent for paper chromatography *n*-propanol/water/1 M acetate buffer pH 5.0 (70/20/10, by volume (22). Ascending technique for 18 hours.

to form bands 12 and 15, respectively, in figure 5, metabolite no. 17 in figure 5 is thiamine-C^{14}.

A New Zealand rabbit weighing 2.93 kg was injected intraperitonealy with 1.265 mg (1.11 μc) of thiamine-C^{14} on the first day and with 1.000 mg (0.88 μc) on 3 succeeding days. An aliquot consisting of one-third of the volume of each of the 3-day urine collections was examined chromatographically for breakdown products of thiamine-C^{14}.

The qualitative pattern of radioactivity eluted from CG-50 columns was similar to that shown in figure 1 for the rat. Radioautographs of the CG-50 peak 1 from rabbit urine showed radioactive bands coinciding with bands 1 and 2, figure 3. Peak 5 radioautographs from rat and rabbit urine are compared in figure 6. Three radioactive bands detectable in rat urine were not observed in rabbit urine. Two obvious quantitative differences were evident. (1) The radioactive band with an R_F of 0.17 in rabbit urine contained a greater percentage of the total activity in the peak than did the band in rat urine with an R_F of 0.19. The latter band was actually absent from some chromatograms. (2) The rat urine band with an R_F of 0.61 was present in much larger quantity than the corresponding band in rabbit urine. It should be emphasized, however, that a strict comparison is not entirely valid since the labeled thiamine was administered to the rabbit for only 4 days and his tissue stores were not as

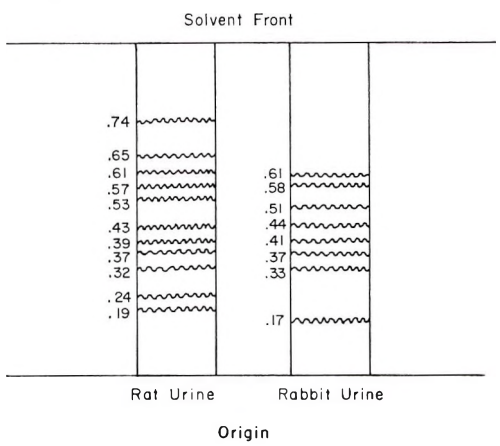

Fig. 6 A schematic comparison of the radioautograms of peak no. 5 from an Amberlite CG-50 column of desalted rabbit urine with the corresponding peak from rat urine. The solvent in both cases was *n*-propanol/water/1 M acetate buffer pH 5.0 (70/20/10, by volume) (22). Ascending technique for 18 hours.

completely labeled as those of the rats studied. Because of the small amount of radioactivity present, peaks 2, 3, and 4 were not subjected to paper chromatography.

It became evident early in this study that for the radioautographic data to have any validity, a determination of the stability of thiamine in urine would have to be undertaken. To this end, a group of 14 Sprague-Dawley rats were fed a thiamine-free diet for one week. Then, 7 of the rats were given daily injections of 30 μg of thiamine-C^{14}. Separate urine collections were made from the injected and noninjected animals. An amount of thiamine-C^{14} equal to or in some cases greater than that injected was added to the pooled urine of the noninjected animals. To correlate easily the R_F values of any compounds resulting from the possible breakdown of the thiamine-C^{14} which was added to the urine with those resulting from the injection of thiamine-C^{14}, the Amberlite CG-50 peaks of radioactivity of both urines were chromatographed on the same piece of filter paper.

In the first experiment the urine containing the added thiamine-C^{14} was adjusted to pH 8.6 and incubated at 37° for 3 hours. Subsequent ion exchange chromatography of the desalted urine indicated the presence of a small amount of radioactivity corresponding to peak no. 1, figures 1 and 4, with the remainder of the activity corresponding to peak no. 5 in figure 1. Radioautographs of these peaks from the urine samples of both groups of animals revealed 2 breakdown products of the thiamine-C^{14} as a result of incubation with the urine. The R_F values of these breakdown products were similar to those of band nos. 1 and 18 in figure 3. The radioactivity data from the Amberlite CG-50 column indicated that the breakdown product with an R_F value similar to metabolite no. 1, figure 3, represented about 2.5% of the total activity incubated with the urine. No attempt was made at quantitating the amount of the breakdown product corresponding to band no. 18, figure 3, but from the darkening of the film it appeared to be quantitatively as great as the metabolite with a similar R_F value from the urine of the injected rats. Two additional radioautographs under the same conditions of incubation gave results almost identical to those described. In a further study of thiamine stability, the pooled urine of the noninjected animals was adjusted to pH 9.0, 350 μg of thiamine-C^{14} added and the urine incubated at room temperature for 24 hours. Subsequent column chromatography gave the same pattern of activity as in the first incubation study except that the radioactivity of the early peak made up about 10% of the total. Comparative radioautography with the urine of the injected rats again indicated the presence of bands which were similar in R_F to bands nos. 1 and 18, figure 3, plus an additional radioactive band with an R_F value similar to that of band no. 3, figure 3. Finally, thiamine-C^{14} was added to the urine collected without acidification. At the end of the 24-hour collection period the pH of the pooled urine was measured and found to be 7.6. Subsequent column chromatography followed by radioautography showed the presence of only a trace of a breakdown product with an R_F similar to that of radioactive band no. 18, figure 3. This experiment was repeated under the same conditions except that the pooled 24-hour urine was then incubated for 4 hours at 37° and was permitted to stand at room temperature for an additional 20 hours. The subsequent radioautograph again revealed the presence of only a trace amount of a material with an R_F value similar to band no. 18, figure 3.

DISCUSSION

A minimum of 22 different products of C^{14}-pyrimidine-labeled thiamine were detected in the urine of rats. Although the bulk of the evidence indicates otherwise, the possibility exists that 3 of the products were formed as a result of breakdown of the radioactive thiamine in the urine.

It is difficult to believe that each of these 20 or more metabolites is a separate chemical species. What is more probable is that a portion of these metabolites represent products of the action of the various detoxification enzymes of a lesser number of metabolites of the thiamine molecule. For example, the glucuronide, phosphate, or sulfate ester of the same metabolite

might be formed. Another source could be the formation of a series of mixed disulfides of thiamine. In future work, the most promising way of assigning a general structure to these metabolites would be by using 2 forms of thiamine-C^{14}, one of which is labeled in the pyrimidine ring, and another labeled in the thiazole ring. Using this approach it should be possible, using the purification and separation methods described, to distinguish between metabolites that are some form of pyrimidine moiety, some form of the thiazole moiety, and some form of both the pyrimidine and thiazole moieties.

The thiamine stability studies indicated that thiamine is quite stable for extended periods of time at room temperature at the normal pH of urine. These results are in agreement with those obtained by Melnick and Field (19). These workers reported that for any breakdown of thiamine to occur in urine incubated at 37.5° for 6 hours, it was necessary to adjust the pH to 9.0 or higher. Our results demonstrate that there is a negligible breakdown of thiamine in urine kept at pH values less than 8.6. At or above pH 8.6, however, some breakdown occurs leading to the appearance of 2 or 3 different compounds depending upon the pH.

The mechanism by which thiamine is broken down remains to be investigated. The relative stability of thiamine-C^{14} in urine indicates that the bulk of the radio compounds are true metabolic products. It appears that there are 2 possible sources of these metabolites: 1) compounds formed as a result of thiamine acting as a coenzyme, as for example, the compound 2 - (1 - hydroxyethyl) - thiamine pyrophosphate which is formed as a consequence of the participation of thiamine pyrophosphate in the pyruvate decarboxylase reaction (20), and 2) products of the actions of degradative enzymes such as the thiaminases on thiamine. In any event, further work on the structure of the metabolites of thiamine should give valuable information concerning the reactions involved in providing the rather large number of metabolites of the thiamine molecule.

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Studies of Thiamine Metabolism in the Rat

II. ISOLATION AND IDENTIFICATION OF 2-METHYL-4-AMINO-5-PYRIMIDINECARBOXYLIC ACID AS A METABOLITE OF THIAMINE IN RAT URINE [1,2]

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ABSTRACT A compound isolated from the urine of rats receiving thiamine-C^{14} has been identified as 2-methyl-4-amino-5-pyrimidinecarboxylic acid. Column and paper chromatographic evidence indicates this same compound is also present in the urine of germfree rats and in rabbit and human urine.

In a previous publication (1) the detection of 22 different metabolites of thiamine-C^{14} in the urine of rats receiving daily intraperitoneal injections of labeled vitamin was described. Subsequently, attempts have been made to identify the major pyrimidine metabolites of thiamine in mammalian urine.

It would be expected that some form of the pyrimidine moiety of thiamine would be present in the urine of the rat and rabbit because it is well established that the thiazole moiety of thiamine is excreted in the urine of these species (2–4). One form of the pyrimidine moiety which might be expected to occur in urine is 2 - methyl - 4 - amino - 5 - hydroxymethylpyrimidine (HMP). This compound would be generated as a result of hydrolytic cleavage of the covalent bond between the methylene bridge and the thiazole ring and is known to be a product of the action of a specific bacterial thiaminase (5). Although Kawasaki and Okada (6) have reported the isolation of HMP from human urine, repeated attempts in our laboratories to detect this compound in the urine of rats receiving pyrimidine-C^{14}-labeled thiamine were unsuccessful. The use of HMP-C^{14} as an intermediate in our synthesis of thiamine made it convenient to look for HMP in human urine by using it as a marker. In 2 separate experiments, HMP-C^{14} was added to human urine and re-isolated by the column and paper chromatographic methods used in the isolation of metabolites of thiamine-C^{14} from rat urine. Since no decrease in the specific activity of the added HMP-C^{14} was observed in either case, we must conclude that this compound does not normally occur in rat or human urine. The failure to detect HMP in mammalian urine led us to investigate its possible conversion into another product. The results of this study, which are recorded here, provide evidence that 2 - methyl - 4 - amino - 5-pyrimidinecarboxylic acid is a major product of the mammalian catabolism of thiamine.

EXPERIMENTAL PROCEDURE

The methods for determining radioactivity as well as the procedures for isolation and detection of the metabolites of thiamine-C^{14} have been described previously (1).

In a study designed to determine the amount of HMP-C^{14} converted to $C^{14}O_2$, rats were placed in a standard respiration chamber and the expired carbon dioxide collected by drawing the expired air through 175 ml of 10% sodium hydroxide. The trapped carbon dioxide was precipitated as barium carbonate by addition of 20% barium chloride to a suitable aliquot of the sodium hydroxide. The barium carbonate precipitate was collected by cen-

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[2] A portion of the data was taken from a thesis submitted by R. A. Neal to the faculty of the Graduate School of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

trifugation and washed twice by resuspension in 1% ammonium chloride and in turn in water followed in each case by centrifugation. The barium carbonate was then dried in an oven at 110° for 12 hours. The activity of the dried barium carbonate was determined by the method of Ott et al. (7).

The animals and animal diets, as well as the procedures for collection of the urine have been described previously (1). In studies with germfree rats[3] the animals were fed a sterile thiamine-deficient diet similar to that described previously except that cornstarch replaced the sucrose and the succinylsulfathiazole. Six germfree rats were individually housed in wire-screen cages which were set over a funnel to allow urine collection. The duration of this experiment was 7 days. It was not practical to collect the urine daily during this study so the pooled 7-day urine sample was collected in toto on termination of the experiment.

The chemical synthesis of 2-methyl-4-amino-5-pyrimidinecarboxylic acid was accomplished by the hydrolysis of 2-methyl-4-amino-5-cyanopyrimidine with sodium hydroxide. The experimental details are as follows: 250 mg of 2-methyl-4-amino-5-cyanopyrimidine [4] which had been recrystallized from absolute methanol, was hydrolyzed with 5 ml of 10% sodium hydroxide on a boiling water bath. Termination of the hydrolysis was indicated by the cessation of the evolution of ammonia (about 10 minutes). The reaction mixture was acidified with 5 N hydrochloric acid and the pH of the solution adjusted to 4.5 at which time a copious precipitate formed. The resultant compound was twice recrystallized from a mixture of methanol/ethanol/water (40/40/20, by volume). Elementary analysis [5] of the synthesized compound was as follows: calculated for $C_6H_7N_3O_2$: C, 47.03; H, 4.61; N, 27.45; found: C, 46.98; H, 4.48; N, 27.35.

RESULTS

Since attempts at detecting HMP in the urine of rats or man were unsuccessful, the possibility that it was extensively degraded was investigated by measuring the expiration of $C^{14}O_2$ following the intraperitoneal injection of HMP-C^{14} into rats. In the initial experiment 60 μg (0.113 μc) of HMP-C^{14} were injected into a rat which had been fed a stock diet ad libitum. The expired CO_2 was collected in 3-hour increments for 12 hours following the injection. The radioactivity in the precipated barium carbonate amounted to 1.26% of that administered. In a second experiment, in which 60 μg of HMP-C^{14} were injected into a rat that had been fasted for 24 hours, 1.27% of the administered radioactivity appeared as $C^{14}O_2$ in the 12 hours following the injection.

Because these experiments indicated that injected HMP-C^{14} was not extensively degraded, the compound was again injected intraperitoneally into rats and the urine examined for metabolites by the methods used in the studies with thiamine-C^{14} (1). The pattern of radioactivity resulting from ion exchange chromatography of the desalted urine of rats receiving HMP-C^{14} is shown in figure 1. Because of the large amount of contaminating material, it was necessary to subject the pooled fractions of peak no. 1, figure 1, to further ion exchange chromatography on Dowex-1-C1 prior to paper chromatography. The pattern of radioactivity resulting from rechromatography of this peak is shown in figure 2. The pattern of activity (fig. 2) resembles that obtained by rechromatography of the corresponding peak for the Amberlite CG-50 column of the desalted urine of rats receiving thiamine-C^{14}.

Fractions 16 and 17, figure 2, as well as fractions 23 to 25 (peak no. 2) and 34 to 37 (peak no. 3), figure 1, were reduced to dryness and chromatographed (ascending) on acid washed Whatman no. 3 MM paper, for 18 hours in *n*-propanol/water/1 M acetate buffer pH 5, (70/20/10, by volume) (8). The subsequent radioautograph of the radioactive peak of figure 2 showed the presence of 3 radioactive bands with R_F values of 0.39, 0.46, and 0.54. These 3 bands corresponded in both R_F value and characteristic shape with 3 radioactive bands isolated in identical

[3] The studies with germfree rats were made possible through the generosity of Dr. Floyd Daft and Mr. E. R. McDaniel of the National Institutes of Arthritis and Metabolic Disease, National Institutes of Health.

[4] Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

[5] Clark Microanalytical Laboratory, Urbana, Illinois.

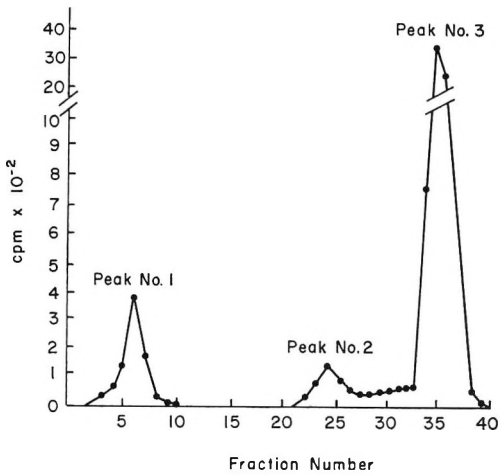

Fig. 1 The chromatography of the urine from rats receiving 4,7-C^{14}-2-methyl-4-amino-5-hydroxymethylpyrimidine (C^{14}-HMP). Exchanger, Amberlite CG-50 (200–400 mesh), 1.0 × 40.0 cm, in the H^+ form. Flow rate 60 ml/hour, fraction size 12 ml. The activity in counts per minute as determined by gas-flow counting is that of 0.5-ml aliquots of the 12-ml fractions.

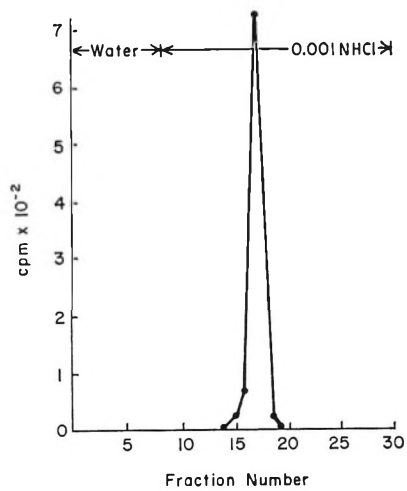

Fig. 2 Ion exchange chromatography fractions 5–7, figure 1, on Dowex-1-chloride (100–200 mesh), 1 × 20 cm. Flow rate 60 ml/hour, fraction size 12 ml. The activity in counts per minute as determined by gas-flow counting is that of 0.5 ml aliquots of the 12-ml fractions.

fashion from the urine of rats receiving thiamine-C^{14}.

The radioautograph corresponding to peak no. 2, figure 1, indicated the presence of a radioactive band with an R_F value of 0.59. The R_F value for the radioactive band resulting from radioautography of the corresponding peak of radioactivity from an Amberlite CG-50 column of the urine of rats receiving thiamine-C^{14} is 0.53.

The radioautograph of peak no. 3, figure 1, revealed the presence of 2 radioactive bands. One of these, with an R_F value of 0.56 was identified as unchanged HMP-C^{14} on the basis of its ultraviolet spectrum. The second band had an R_F of 0.39 which is also the R_F value of thiamine in the same solvent system. This band, however, did not fluoresce when sprayed with ethanol/10% sodium hydroxide/2.5% potassium ferricyanide (2/1/0.05, by volume), a mixture which converts thiamine and its esters into their corresponding thiochrome derivatives. Unfortunately, this radioactive band was in an area on the paper chromatogram which contained a large amount of a brown pigment which may have quenched traces of thiochrome fluorescence.

Peak no. 1, figure 1, accounted for 12 to 18% of the total activity eluted from 3 separate columns and the radioactive band in this peak with an R_F of 0.46 contained 73 to 82% of the total radioactivity in the peak. The bands of radioactivity with R_F values of 0.39 and 0.54 contained about equal amounts of the remaining activity. The 3 radioactive compounds in the corresponding peak from the urine of rats receiving thiamine-C^{14} exhibited about the same quantitative ratio.

Peak no. 2, figure 1, contained 5 to 6% of the activity eluted from the column with peak no. 3 accounting for the remainder. Approximately 99% of the radioactivity in peak no. 3 was due to HMP-C^{14}.

When peak no. 1, figure 1, was isolated from the urine of rats receiving 100 μg/day of thiamine-C^{14}, it was found to contain 6 to 12% of the total activity eluted from the column. Thus each rat excreted 5 to 9 μg of the injected radioactive thiamine in the form of 3 acidic metabolites which behaved the same on column and paper chromatography as compounds resulting from the metabolism of injected HMP-C^{14}.

The acidic nature and quantitative importance of the radioactive band with an R_F value of 0.46 isolated from the urine

of rats receiving thiamine-C^{14} prompted further investigation of its structure. A pyrimidine structure for this compound was tentatively assigned on the basis of the similar chromatographic behavior of the metabolite resulting from injection of HMP-C^{14} into rats. The glucuronide, sulfate, or phosphate derivatives of HMP were first considered as likely possibilities because of the acidic behavior of the compound upon column chromatography.

Since free HMP should be generated upon hydrolysis of these compounds an amount of the appropriate radioactive band with an activity equivalent to 60 μg of thiamine was subjected to hydrolysis with 2 ml of 0.1 N hydrochloric acid at 121° for one hour in an autoclave. The solution was then neutralized with 0.2 N sodium hydroxide and examined for free HMP by column chromatography. An HMP peak was not detected. All of the radioactivity remained in its original position (peak 1, fig. 1).

The fact that the unknown compound was more acidic than HMP led next to the consideration that it might be 2-methyl-4-amino-5-pyrimidinecarboxylic acid (PCA). This compound would result from oxidation of the hydroxymethyl group in the 5-position of HMP. To test this hypothesis a sample of synthesized PCA was subjected to column chromatography and found to be eluted from columns of Dowex-1-C1 and Amberlite CG-50 with about the same volumes of eluate as was observed for the unknown compound. When the synthesized PCA was chromatographed on Whatman no. 3 MM paper in *n*-propanol/water/1 M acetate buffer pH 5.0, along with the lyophilized fractions from a Dowex-1-C1 column containing the unknown compound, its R_F value was 0.08 lower than that of the unknown. Elution of the unknown compound and PCA from the paper and rechromatography under the same conditions resulted in a decrease in the R_F of the unknown compound to that of the synthesized PCA. An R_F value for PCA somewhat lower than that of the unknown compound was also observed when the Dowex-1-C1 fractions containing the unknown compound were chromatographed, along with PCA, in both *i*-propanol/ammonium hydroxide/water (255/3.9/45, by volume) and *i*-propanol/0.1 N hydrochloric acid (255/45, by volume). Subsequent elution and rechromatography of the unknown compound decreased the R_F value to that or nearly that of PCA. The range of R_F values obtained with the unknown compound and with PCA in 4 solvent systems is shown in table 1. The decrease in the R_F value of the unknown compound upon rechromatography on paper was thought to be due to a decrease in the amount of brown urinary pigment present.

The ultraviolet spectra of the unknown compound and authentic PCA were next compared. An amount of the unknown compound equivalent to 120 μg of thiamine-C^{14} was partially purified on a Dowex-1-C1 column and chromatographed on acid-washed Whatman 3 MM paper in *n*-propanol/water/0.1 M acetate buffer pH 5.0. The area of radioactivity corresponding to the unknown compound was detected by radioautography, cut out, eluted with distilled water, and applied as a 0.5-cm spot to acid-washed Whatman no. 1 filter paper. The paper chromatogram was developed

TABLE 1

Range of R_F values for the unknown compound and for 2-methyl-4-amino-5-pyrimidinecarboxylic acid (PCA) in various systems

Solvent system	R_F Values	
	Unknown compound	PCA
n-Propanol/water/1 M acetate buffer pH 5	0.43–0.50	0.39–0.41
Isopropanol/ammonium hydroxide/water	0.25–0.34	0.25–0.26
Isopropanol/0.1 N hydrochloric acid	0.24–0.33	0.26
Isobutyric acid/ammonium hydroxide/water	0.45–0.49	0.46

in the first direction with *i*-propanol/ammonium hydroxide/water for 18 hours ascending and in the second direction in *i*-propanol/0.1 N hydrochloric acid for the same period of time. The resulting chromatogram showed an ultraviolet quenching spot with an R_F of 0.29 in the first direction and 0.26 in the second. This spot was cut from the paper, eluted with 0.1 ml distilled water and its ultraviolet spectrum compared with that of synthetic PCA at pH 2.0 and 8.0. The spectra of the 2 compounds are virtually identical (fig. 3).

To determine further whether the unknown compound from urine was PCA, co-chromatography of the 2 compounds was carried out in 3 solvent systems. These were *i*-propanol/ammonium hydroxide/water (255/3.9/45, by volume), *i*-propanol/0.1 N hydrochloric acid (255/45, by volume), and *i*-butyric acid/ammonium hydroxide/water (199/3/98, by volume), In all 3 systems the radioactive areas, as determined by radioautography, and the ultraviolet quenching areas coincided. Further evidence as to the identity of the unknown compound was obtained by crystallization of a mixture of the unknown compound and synthesized PCA to constant specific activity. In this experiment 10 mg of the synthesized PCA were added to an amount of the unknown compound equivalent to 100 μg of thiamine-C^{14} and the mixture repeatedly crystallized from methanol/ethanol/water (40/40/20, by volume). The results are shown in table 2. The specific activity stabilized after the first crystallization and remained constant thereafter.

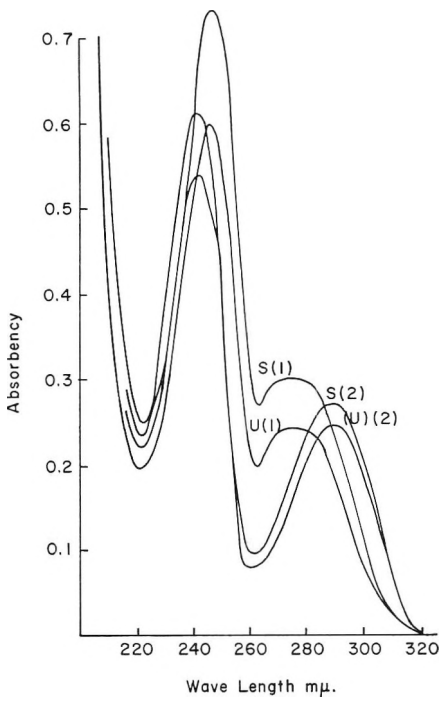

Fig. 3 The comparison of the ultraviolet spectra of the unknown compound (U) and the synthesized PCA (S) at pH 2.0 (1), and pH 8.0 (2), in a Cary continuous recording spectrophotometer.

TABLE 2

Specific activity [1] *of the precipitates from a mixture of 2-methyl-4-amino-5-pyrimidinecarboxylic acid (PCA) and the unknown urinary compound*

Crystallization no.		Optical density at 247 mμ	
	count/min/ml		*count/min/μmole*
1	41	0.460	955
2	68	0.870	837
3	61	0.795	823
4	58	0.730	850

[1] Solutions of PCA in 0.01 N hydrochloric acid were found to obey Beer's Law in the optical density range of 0.075 to 1.15. Therefore a sample of the crystals was taken, dissolved in 1 ml of 0.01 N hydrochloric acid and the optical density of an aliquot of this in 2 ml of 0.01 N hydrochloric acid was determined in a Beckman DU spectrophotometer. The activity of a 1-ml aliquot of this solution was determined in the Packard Tri-Carb scintillation counter.

During these studies it became apparent that the possibility of a microbial origin of this compound in the gut would have to be excluded before it could be considered a product of mammalian metabolism and that the most unequivocal method of clarifying this point would be to identify the compound in the urine of germfree rats. Analysis of the pooled 7-day urine collection from 6 germ-free rats that had been injected with thiamine-C^{14} indicated that 7% of the radioactivity in the urine appeared in the first radioactive peak eluted from an Amberlite CG-50 column. Paper chromatography of this peak of radioactivity indicated clearly the presence of the unknown compound in the urine of germ-free rats.

Human urine was then examined for presence of this compound. In this study

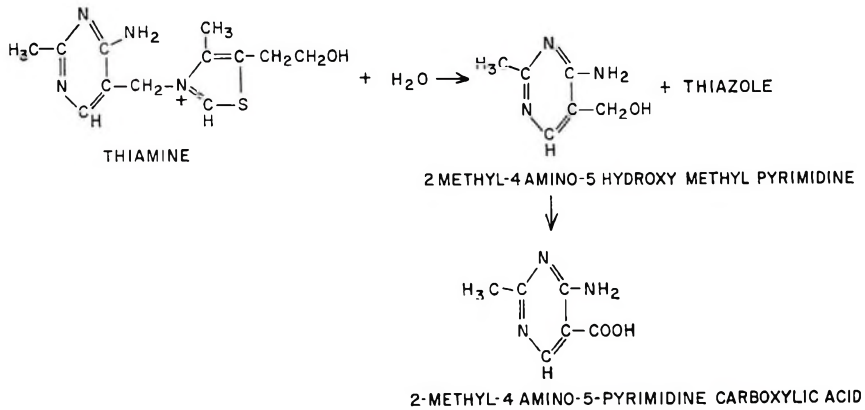

Figure 4

a healthy human volunteer (male, Caucasian, age 65 years) ingested 1 mg (0.88 μc) of thiamine-C^{14} daily for 5 days. A portion of the 24-hour urine samples was analyzed for the presence of the unknown compound using the same techniques described for rat urine. Although the specific activity of the urine was low, the column and paper chromatographic evidence indicated strongly that the compound in question was also present in human urine. As reported in a previous publication (1), a compound corresponding to PCA is also present in rabbit urine.

DISCUSSION

The evidence presented indicates that an acidic compound excreted in the urine of rats receiving thiamine-C^{14} has the structure 2-methyl-4-amino-5-pyrimidinecarboxylic acid. Column and paper chromatographic evidence demonstrates that this compound is also present in rabbit and human urine as well as in the urine of germfree rats. Quantitatively it represents from 5 to 10% of the total activity excreted in the urine of rats receiving 100 μc of thiamine-C^{14} daily.

Although no data have been obtained on the mechanism of the derivation of this compound from thiamine, a logical sequence would be hydrolytic cleavage (enzymatic or nonenzymatic) of the covalent bond between the methylene carbon and the thiazole ring nitrogen to yield 5-(2-hydroxyethyl)-4-methylthiazole and HMP followed by enzymatic oxidation of the HMP to PCA. Such a provisional scheme is shown in figure 4. The existence of HMP as an intermediate is suggested by the observation that this compound, when injected, is partially converted to PCA. Evidence for the formation of a carboxyl group from a 5-hydroxymethyl group of a pyrimidine has been reported by Fink et al. (9). These workers reported chromatographic evidence for the formation of uracil-5-carboxylic acid from thymine by rat liver slices with 5-hydroxymethyl uracil being a possible intermediate. The same workers have also reported similar observations in *Neurospora crassa* with the addition that 5-formyl uracil was also identified as a metabolic product.[6] Although it is possible that a similar oxidative sequence might apply to the metabolism of the pyrimidine moiety of thiamine, we have not examined this possibility.

ADDENDUM

Two reports bearing on this subject have recently come to our attention. Shintani (10) has reported the detection of PCA in the urine of the rabbit after injection with HMP. His absorption spectra closely resemble those recorded here. Matthies and Peters (11) have recently studied the cytostatic effects of HMP derivatives in mice. PCA was found to be considerably less toxic than HMP, suggesting that it may serve as a detoxification product of the latter compound.

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Urolithiasis in the Rat [1]

IV. INFLUENCE OF AMINO ACID SUPPLEMENTS ON THE OCCURRENCE OF CITRATE CALCULI

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ABSTRACT Urolithiasis was investigated in the NMRI-D strain of rat fed a purified type of diet containing 15% casein and 4% salts. The predominant component of the stones produced was calcium citrate. Although previous studies indicated that increased dietary protein eliminated calculi, the present study shows that DL-methionine or L-lysine monohydrochloride supplementation are also effective in reducing the occurrence of calculi and together completely prevent the syndrome. The methionine effect is thought to be via the conversion of methionine-sulfur to inorganic sulfate which provides an acidic group in the urine. The lysine monohydrochloride effect also is believed to be due to the acidic group provided, in this case chloride. These suggestions concerning the effects of DL-methionine and L-lysine were supported by experiments in which dietary inorganic sulfate and chloride reduced the proportion of rats which developed calculi.

Previous studies in this laboratory have shown that the NMRI-D albino rat is particularly susceptible to the formation of bladder and kidney stones composed predominately of calcium citrate (1). The etiology of stone formation in these studies appears to be distinct from the citrate stones reported by Schneider and Steenbock (2) in rats fed diets low in phosphate, since supplementation with phosphate did not eliminate the lithiasis (3). Calculi are formed in the NMRI-D rat fed a diet containing 15% casein and 4% Hubbell, Mendel and Wakeman salt mixture (4), but are prevented by increasing the protein level to 30% with either casein or soybean protein althongh gelatin is ineffective (3). The present report presents results of a series of experiments in which the calculi-producing diet was supplemented with individual amino acids.

EXPERIMENTAL

Male, weanling rats of the NMRI-D strain were used in all experiments. This strain originated from the Osborne-Mendel rat which was inbred for more than 15 years with selection for susceptibility to dental caries. The animals were 21 to 22 days of age at the start of experiments and weighed between 25 and 45 g. Rats were distributed at random into groups, maintained in individual stainless steel cages, housed in an air conditioned room, and provided food and distilled water ad libitum. The experimental feeding period was always 5 weeks, although if an animal died before this, an autopsy was performed in an attempt to ascertain the probable cause of death and whether stones were present in the kidney or bladder.

The basal purified diet, designated 15P4 to indicate the casein and mineral levels, had the following percentage composition: vitamin-test casein, 15.0; sucrose, 76.8; salt mixture (4), 4.0; vitamin mixture,[3] 2.2; corn oil, 1.0; and linoleic acid, 1.0. Previous studies with the NMRI-D rat had shown that an additional 15% casein would completely prevent stone formation;

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[1] The opinions or assertions contained in this article are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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[3] The vitamin mixture provided the following quantities: (per 100 g of diet) vitamin A, 2000 IU; vitamin D, 220 IU; (in mg) α-tocopherol, 11; ascorbic acid, 100; inositol, 11; choline chloride, 66; menadione, 5; *p*-aminobenzoic acid, 11; niacin, 10; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; (in μg) biotin, 44; folic acid, 200; vitamin B_{12}, 3.

therefore, it was decided to supplement the 15P4 diet with individual amino acids, each at a level which would be found in 15 g of casein. If the protective effect of casein was due to its amino acid content, it was thought that the amino acids supplied by gelatin would not be effective since this latter protein did not protect against calculi. On this assumption the first amino acids to be tried were those present in much higher concentrations in casein than in gelatin. The following values were used for the amino acid composition of casein: (in per cent) tyrosine, 6.4; cystine, 0.4; methionine, 3.4; valine, 7.0; threonine, 3.9; phenylalanine, 5.2; glutamic acid, 21.8; isoleucine, 6.5; leucine, 9.9; tryptophan, 1.3; and lysine, 7.9. All supplements were as the free amino acids except for lysine which was provided as the monohydrochloride.

At the termination of the 5-week experimental period the rats were anaesthetized and a blood sample obtained by heart puncture. The kidneys, ureters, bladder and urethra were examined in each animal for evidence of stone formation. Some of the tissues were fixed in buffered formalin for histological examination and the remaining kidneys were saved for the determination of calcium accummulation.

Calcium determinations were performed using a modification of the method of Williams and Moser (5). For kidney calcium, the tissues were digested in concentrated nitric acid, the excess nitric acid driven off, and the dried sample taken up in water before calcium analysis. Phosphorus was determined by a modification of the procedure of Fiske and Subbarow (6). Plasma urea was assayed by an automatic procedure of Skeggs (7).

RESULTS

The effects of feeding supplemental amino acids on the occurrence of calculi in the NMRI-D rat are shown in table 1. In a series of experiments, among the 3 control groups provided the basal stone-producing diet, about 50% of the animals were observed to have calculi. Tyrosine, cystine, valine, glutamic acid, threonine, isoleucine, or leucine added to the basal diet in quantities equivalent to that found in 15 g of casein were ineffective in reducing the incidence of urolithiasis. Supplementation with phenylalanine or tryptophan appeared to increase the incidence of stones, although whether the increase was a significant one is not known. Further studies with these latter amino acids have not been pursued; however, it would be of some interest if they could be shown to have an influence on the etiology of stone formation. When DL-methionine was added to the basal diet at 0.51% there was a marked decrease in the proportion of rats developing calculi although stones were not eliminated. In a subsequent trial, the quantity of methionine was doubled; nevertheless, this failed to eliminate stone formation, and roughly the same proportion of rats developed calculi as with the lower level of methionine. The only other amino acid which demonstrated a protective effect against calculi was L-lysine-monohydrochloride, but again there was only a reduction in the number of rats with stones and not a complete elimination of calculi.

Since methionine and lysine were the only 2 amino acids which appeared to have a preventive influence on stone formation, the additive effect of the amino acids was investigated. The data presented in table 2 generally confirmed the previous observation concerning the influence of methionine and lysine supplementation individually and show that with both amino acids, urolithiasis was eliminated. These latter observations have now been repeated in 2 additional experiments and have confirmed the effectiveness of a combination of methionine and lysine. Of 40 rats given both amino acids in these latter experiments, small flakes of unidentified composition were observed in the pelvis of the kidneys of 2 animals. These flakes do not resemble the discrete stones found normally and it is not certain whether difficulties would develop in long-term experiments. The discrete uroliths formed in the amino acid studies were analyzed and found to be predominately citrate.

The levels of plasma calcium, phosphorus and urea and kidney calcium were investigated in rats of the methionine-lysine experiment (table 3). The data have been subdivided into groups of animals which developed stones and those

TABLE 1

Effect of amino acid supplementation on calculi formation

Diet	Supplement	No. of rats		Weight	
		With stones	Without stones	Start	5-weeks
	%			g	g
15P4	none		8	35.2±2.1 [1]	120.7± 5.7
		8		32.7±0.9	111.3± 8.2
15P4 + L-Tyrosine	0.96		7	32.7±5.2	111.5± 9.8
		9		32.5±5.4	101.7± 7.2
15P4 + L-Cystine	0.06		8	33.1±2.2	129.3±10.8
		8		33.7±2.3	123.7± 6.0
15P4 + L-Valine	1.05		9	32.4±0.9	104.7± 8.5
		7		35.0±1.9	99.0± 5.6
15P4 + L-Phenylalanine	0.78		5	37.4±1.5	145.4± 6.0
		10		35.1±4.3	127.5± 6.4
15P4 + L-Glutamic acid	3.27		5	36.0±4.3	120.4±12.6
		7		31.0±0.8	95.8± 8.8
15P4	none		7	35.7±2.1	106.0± 7.6
		8		30.3±4.8	82.3± 7.9
15P4 + DL-Methionine	0.51		16	32.0±1.0	118.0± 4.9
		4		30.2±1.3	99.2± 8.5
15P4 + L-Threonine	0.60		9	36.7±1.5	109.3± 6.3
		9		36.0±1.2	102.4± 5.2
15P4 + L-Tryptophan	0.20		6	32.1±1.5	90.3±12.5
		14		31.7±1.4	93.0± 6.4
15P4 + L-Isoleucine	0.98		7	33.5±1.7	106.1± 8.0
		8		34.5±1.8	111.0± 6.3
15P4	none		2	38.0	126.0
		2		32.0	119.0
15P4 + DL-Methionine	1.02		12	34.7±1.5	122.9± 4.3
		3		35.0	98.6
15P4 + L-Leucine	1.05		6	30.8±2.1	121.6± 7.5
		9		29.4±0.9	92.5±10.0
15P4 + L-Lysine·HCl	1.48		12	38.1±1.7	149.6± 5.1
		3		34.6	128.3

[1] Mean value ± SE.

TABLE 2

Effect of methionine and lysine supplementation on calculi formation

Diet	Supplement	No. of rats with stones/total	Weight	
			Start	5-weeks
	%		g	g
15P4	none	14/20	34.7±1.0 [1]	130.2±4.8
15P4 + DL-Methionine	0.51	5/20	34.8±1.0	153.3±6.1
15P4 + L-Lysine·HCl	1.48	2/20	34.0±0.9	137.0±1.2
15P4 + DL-Methionine + L-Lysine·HCl	0.51 1.48	0/20	34.7±1.0	156.7±2.7

[1] Mean value ± SE.

which did not. With lysine supplementation, so few rats developed calculi that no standard error of the mean was calculated. There was little difference among groups (either with or without stones) receiving the varied diets, with respect to plasma calcium, which was about 12 mg/100 ml, or phosphorus which was a little less than 6 mg/100 ml. Plasma urea concentrations were somewhat higher in rats with urolithiasis than those without stones. A level of 17 to 18 mg urea/100 ml of plasma was noted in rats without stones. The higher level of plasma urea in stone-bearing rats was undoubtedly a reflection of the fact that many animals with calculi demonstrated distended bladders and frequently had abnormal kidneys.

TABLE 3

Mean plasma calcium, phosphorus, urea and kidney calcium of rats with and without urolithiasis

Diet		Calcium	Phosphorus	Urea	Kidney calcium
		mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 g dry wt*
15P4	with stones	12.3 ± 0.3 [1]	5.8 ± 0.3	24.5 ± 2.0	106 ± 10
	without stones	12.7 ± 0.3	5.8 ± 0.3	16.8 ± 2.6	118 ± 8
15P4 + Methionine	with stones	11.9 ± 0.6	6.4 ± 0.6	35.0 ± 8.7	134
	without stones	11.6 ± 0.3	5.1 ± 0.2	18.2 ± 1.7	92 ± 8
15P4 + Lysine	with stones	12.2	5.9	24.0	153
	without stones	12.4 ± 0.3	6.2 ± 0.3	18.3 ± 0.5	115 ± 7
15P4 + Methionine + lysine	without stones	12.2 ± 0.2	5.3 ± 0.1	17.3 ± 0.8	101 ± 6

[1] Mean value ± SE.

The concentration of calcium in the kidneys of rats without calculi was about the same regardless of the dietary conditions and the means varied from 92 to 118 mg/100 g of dry weight. Kidney calcium in rats having calculi was variable, being normal in rats receiving the 15P4 diet and above normal in rats fed the methionine- or lysine-supplemented rations. When the rats were killed all visible calculi were removed from the kidneys. However, calcium deposits in the kidney tubules could not be removed and thus the high values may reflect this.

A preliminary attempt was made to clarify the action of methionine in reducing the proportion of rats with calculi. It is known that the sulfur of methionine, cystine and cysteine can give rise to inorganic sulfate which is the highest oxidation state of sulfur in animal tissues. It is also known that methionine tends to acidify the urine via this mechanism (8). An experiment was conducted in which the basal 15P4 diet was supplemented with 0.45% ammonium sulfate which is equivalent to the amount of sulfate which could be formed if all of the supplemental methionine-S of the previous experiment was converted to sulfate. The data in table 4 indicate that this level of sulfate will markedly reduce the proportion of rats developing calculi with only one out of 20 animals showing stones after 5 weeks.

Methionine can also act as a methyl donor and is vital in the synthesis of choline (9). An attempt was made, therefore, to determine whether supplemental choline would reduce the occurrence of urolithiasis. A group of rats was fed a diet containing the same molar proportion of choline as the methionine supplement of the first experiments. The data in table 4 indicate that choline was ineffective in preventing or reducing the number of rats with calculi.

Another feeding experiment was performed simultaneously with the last two and this concerned magnesium. Hammar-

TABLE 4

Occurrence of stones with dietary supplements of ammonium sulfate, choline, or magnesium salts

Diet	Supplement	No. of rats with stones/total	Weight	
			Start	End
	%		*g*	*g*
15P4	none	11/20	31.1 ± 1.2 [1]	153.6 ± 5.8
15P4 + $(NH_4)_2SO_4$	0.45	1/20	29.8 ± 1.2	146.3 ± 3.8
15P4 + Choline chloride	0.48	14/20	30.3 ± 1.0	136.8 ± 6.1
15P4 + $MgSO_4 \cdot 7H_2O$ [2]	0.83	6/19	32.7 ± 0.7	138.3 ± 1.9
$MgCO_3$	0.28			

[1] Mean value ± SE.
[2] Supplements raised the magnesium level of the ration to 200 ppm. One-half of the supplemental magnesium from magnesium sulfate and one-half from magnesium carbonate.

sten (10) and others (11) have implicated magnesium deficiency or inadequacy in the etiology of some urolithiasis. The basal 15P4 diet used in many of our experiments contains 35 mg Mg/100 g, an amount which usually is considered adequate for normal growth of the rat, but which some investigators consider inadequate for the prevention of calcification of some of the soft tissues. A group of rats was provided the 15P4 diet supplemented with sufficient magnesium salts to bring the total magnesium concentration to 200 mg/100 g. One-half of the magnesium was provided as magnesium sulfate and one-half as magnesium carbonate to prevent a gross disturbance of the acid-base relationship. Although there was a smaller proportion of rats with calculi fed the magnesium ration than fed the control 15P4 diet, six out of 19 rats still developed stones (table 4). Since the supplement brought the magnesium level to 5 times the normal level, but did not completely prevent stone formation, it does not appear possible that magnesium insufficiency plays any role in the etiology of citrate urolithiasis in the NMRI-D rat.

Further studies were conducted to clarify the reason for lysine being effective in the prevention of stone formation. Since the lysine supplementations used in the previous studies were in the form of the monohydrochloride, it was considered that its effectiveness may have been due to the acid group. To test this and to determine the influence of different levels of lysine, an experiment was conducted in which groups of NMRI-D rats were fed 0.59, 1.18 and 2.36% lysine in the form of the monohydrochloride. The actual quantities of the lysine monohydrochloride fed were: 0.74, 1.48, and 2.96%, respectively. Other groups of rats were given diets containing the equivalent quantities of HCl, that is, 0.15, 0.30, and 0.60% HCl. The HCl was provided by treating the salt mixture with the appropriate quantity of concentrated HCl before incorporating it into the diet. The results of this study (table 5) indicate that fourteen of the 20 rats fed the basal 15P4 diet developed stones and that there were progressively fewer animals with calculi as the level of lysine was increased, with complete prevention at the highest level of 2.36% lysine. Similarly, HCl appeared to be very effective in protecting against urolithiasis with the highest concentration of 0.60% resulting in no rats with stones.

Neither the methionine nor the lysine effect can be attributed to increased water intake. A number of animals from each group were placed in metabolism cages and their water intake measured, but these studies failed to show much difference in water consumption due to supplementation. The pH of freshly voided urine was

TABLE 5

Occurrence of calculi with dietary supplements of lysine-monohydrochloride or hydrochloric acid

Diet	Supplement	No. of rats		Weight	
		With stones	Without stones	Start	End
	%			g	g
15P4	none	14		34.8 ± 1.3 [1]	134 ± 6
			7	37.0 ± 0.8	152 ± 7
15P4 + Lysine·HCl	0.74	8		35.0 ± 1.8	138 ± 6
			11	35.7 ± 2.2	144 ± 5
15P4 + Lysine·HCl	1.48	2		31.0	139
			16	36.0 ± 0.4	153 ± 4
15P4 + Lysine·HCl	2.96	0			
			20	34.9 ± 1.0	138 ± 9
15P4 + HCl	0.15	4		37.8 ± 1.6	127 ± 4
			16	35.3 ± 1.4	123 ± 3
15P4 + HCl	0.30	1		35.0	121
			16	36.0 ± 1.1	117 ± 4
15P4 + HCl	0.60	0			
			20	35.3 ± 1.1	110 ± 4

[1] Mean value ± SE.

measured at the time of autopsy. The mean pH of urine from animals fed the basal 15P4 diet was 6.4 for both those animals developing and those not forming stones. Similarly, for rats not developing stones with methionine, lysine or methionine and lysine additions, the urinary pH was 6.3, 6.3 and 6.2, respectively. It is recognized that the pH of freshly voided urine may not accurately reflect the pH of the urine at the site and time of stone initiation; therefore, great credence should not be placed in these values.

GENERAL DISCUSSION

Hunt (8) has shown that by varying the dietary content of sulfur either by increasing the total protein content of the diet or by supplemental methionine, the urinary output of acidic groups can be made to increase by the output of sulfate. In some subjects of his study almost all of the urinary output of acid could be ascribed to the oxidation of sulfur. Following the observations of Hunt (8), Miller et al. (12) studied the influence of methionine supplementation on the growth of stones induced in rats by the introduction of a small metallic foreign body in the bladder as described by Vermeulen (13). It was found that magnesium ammonium phosphate stones in male rats of the Harlan strain could be prevented by the methionine therapy. On the other hand, the calcium phosphate calculi in females of the Holtzman strain, were unaffected by methionine supplementation of the ration. Miller et al. (12) indicate that the methionine supplements did not lower urinary pH as much as reported by others (14), but apparently was sufficient to dissolve the magnesium ammonium phosphate stones and inadequate to dissolve the calcium phosphate calculi.

In the present studies, methionine supplementation at 0.51% of the diet (about one-half of that used by Miller (12)) reduced the proportion of NMRI-D rats with calculi but failed to completely prevent stones. Doubling the concentration of methionine was no more effective than the lower level. Considering the data of others, it appears likely that the methionine effect is related to its conversion to sulfate in the animal body and the excretion of this anion in the urine. It is suggested that the higher level of methionine was no more effective than the lower concentration because of a saturation of the systems involved in the conversion of methionine-S to sulfate. This will have to be confirmed by experimental procedures. Cystine can also provide sulfur for conversion to inorganic sulfate; however, in our studies cystine at a level of 0.06% of the diet did not show any protective effect. This may have been too low a concentration, and thus these studies will now have to be extended to cover a wide range of concentrations.

A dietary source of inorganic ammonium sulfate was very effective in lessening the occurrence of the calculus disease. The level of sulfate fed, although equivalent to the amount of sulfur in 0.51% methionine, does not mean that this amount was absorbed and excreted. In fact, it is quite likely that a considerable portion of the sulfate did not enter the circulation since the sulfate ion is notoriously poorly absorbed from the gastrointestinal tract. Carefully controlled balance studies will be necessary to completely evaluate the role of sulfate in lithiasis. Acidification of the urine has been reported to decrease the excretion of citric acid (15). It is thus anticipated that metabolic studies will indicate that methionine or sulfate supplementation of the diet will lead to decreased citrate excretion. The role of protein supplementation in reducing the excretion of citric acid has already been reported in studies using the NMRI-D rat (16). With high dietary protein, however, the prevention of calculi may be enhanced by factors other than sulfate formation, such as, increasing the fluid intake and the excretion of excess amino acids which have been reported to increase the solubility of calcium salts (17).

It appears likely that the effect of lysine supplementation in reducing the occurrence of stones is related to the fact that the commercial form of the amino acid is the monohydrochloride and this would be expected to provide additional chloride ions in the urine with a concomitant reduction in the number of citrate ions. The lysine monohydrochloride appears to be an effective means of preventing stone

formation even though lysine has been reported to stimulate the absorption of calcium from the gastrointestinal tract under some conditions (18).

Our current working hypothesis concerning the formation of citrate stones in the NMRI-D rat is that it is primarily a result of the high carbonate content of the HMW salt mixture (4) resulting in an alkaline-type diet. To maintain ionic equilibrium in the urine, there is a production of citrate ions which reaches a high enough concentration to exceed the solubility product of calcium citrate. Increasing the protein level of the diet or supplementing the diet with methionine provides additional sulfate groups which reduces the requirement for citrate excretion. The higher protein may also promote aminoaciduria with a tendency to solubilize calcium. The lysine monohydrochloride effect may be looked upon also as a mechanism for providing an acidic group to the urine with a concomitant reduction in the production of citrate. Studies are in progress to provide further experimental evidence to support or disprove the above. It is hoped that with additional evidence we may be able to approach an understanding of the difference in susceptibility to calculus formation demonstrated by the NMRI-D rat in comparison with the Sprague-Dawley and Long Evans strains.

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Observations on Feeding Tung Oil to Chickens [1,2]

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ABSTRACT The inclusion of commercially available crude tung oil in the rations of chicks at levels as low as 0.5% caused slow growth. Higher levels (2.0% to 5%) of tung oil in the diet caused high mortality. Upon autopsy the affected chicks showed enlarged gallbladders, fluid in the abdominal, thoracic and pericardial cavities, variable liver size, enlarged hearts and had an odor of tung oil. Methyl esters of the fatty acids from tung oil were not toxic. The unsaponifiable fraction from tung oil isolated after alkaline hydrolysis also was not toxic.

The extreme toxicity to young chickens of commercially available tung oil was observed in an experiment conducted to study the role of conjugated fatty acids in essential fatty acid nutrition of chickens. The toxicity of factors in tung oil meal is well documented (1–3). However, the effects of feeding tung oil (4) and purified conjugated fatty acids (5–7) to rats are not clearly defined in the literature. It was the objective of these studies to determine 1) the characteristics of crude tung oil toxicity in young chickens, 2) the level of tung oil in the diet necessary to produce the toxicity, and 3) whether the toxicity of the crude oil was in the fatty acids or in some other fraction.

EXPERIMENTAL

Four experiments with tung oil are reported. White Plymouth Rock cockerels were used in all experiments. Two pens of 10 chicks each received the experimental diets in trials 1 and 2 of the first experiment and in each of the last 3 experiments. The basal casein-gelatin glucose diet [3] contained only traces of lipids. The chicks were housed in wire-floor brooders and received ad libitum the experimental diets and water.

The crude tung oil [4] was almost a pure triglyceride when examined by thin-layer chromatography. The fatty acid composition as determined by gas-liquid chromatography was as follows: (expressed as a percentage of the total fatty acids) palmitic, 1.7; palmitoleic, 0.2; stearic, 1.7; oleic 7.5; linoleic, 8.0 and elaeostearic, 80.1. The saponifiable and unsaponifiable fractions were obtained by a standard method (8) and the methyl esters of the tung oil fatty acids were prepared as described by Knight et al. (9) for preparing methyl esters of oleic acid. These products were examined by thin-layer chromatography. The methyl esters contained a trace of triglyceride but were free of monoglycerides and diglycerides. The unsaponifiable material contained over 90% of one compound as distinguished by gas-liquid chromatography and thin-layer chromatography. The thin-layer chromatography indicated that the remaining approximately 10% was a mixture of six or more compounds.

When desirable, the data collected were subjected to statistical analysis, and treatment significance was determined by the multiple range test of Duncan (10) as modified by Kramer (11).

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[2] This investigation was supported in part by a Public Health Service research grant no. 6338 and a research career program award no. 18,411 from the Arthritis and Metabolic Disease Institute.

[3] The composition of the basal diet used in these experiments was as follows: (g/100 g diet) glucose monohydrate, 65.30; casein, 18.44; gelatin, 9.22; DL-methionine, 0.34; cellulose, 1.00; choline·Cl concentrate (70.7%), 0.29; NaCl, 0.75; KCl, 0.60; $MgCO_3$, 0.19; $CaHPO_4$, 2.86; $CaCO_3$, 0.54; 1,2,dihydro-6-ethoxy-2,2,4-trimethylquinoline, 0.03; vitamin mixture, 0.22; and mineral mixture, 0.22. The vitamin mixture supplied the following: (mg/100 g diet) vitamin A, (384,000 USP units/g), 204; vitamin E (296 IU/g), 22.3; vitamin D_3 (950,000 USP units/g), 0.06; thiamine·HCl, 1.98; riboflavin, 1.98; Ca pantothenate, 3.30; pyridoxine·HCl, 0.99; niacin, 3.96; folic acid, 0.66; biotin, 0.07; vitamin B_{12} (1 mg/g), 3.30; *p*-aminobenzoic acid, 16.5; inositol, 165.0; and menadione sodium bisulfite complex, 0.53. The mineral mixture supplied the following: (mg/100 g diet) $FeSO_4 \cdot 7H_2O$, 11.0; $CuSO_4 \cdot 5H_2O$, 1.1; $CoCl_2 \cdot 6H_2O$, 1.1; KI, 1.1; $Na_2MoO_4 \cdot 2H_2O$, 0.11; $ZnSO_4 \cdot 7H_2O$, 16.5; $MnSO_4 \cdot H_2O$, 17.0.

[4] Tung Oil, Pacific Vegetable Oil Corporation, Richmond, California.

RESULTS

The results of the first experiment in which 5% of tung oil alone and in combination with 5% corn oil were included in rations fed to chickens are shown in table 1. These results illustrate the extreme toxicity of the tung oil to chicks and show that corn oil offers no protective effect, as evident from growth rate and mortality data.

The second experiment was conducted to determine the effect of level of tung oil on growth rate and mortality. These results presented in table 2 indicate that the lowest level of tung oil fed (0.5%) was capable of depressing growth rate significantly. It was necessary to feed a somewhat higher level of tung oil to cause high mortality during the short experimental period used. Two per cent tung oil in the diet caused 35% mortality. No definite pattern evolved from the liver weight data.

Lower levels of tung oil were tested in the third experiment and the methyl esters of tung oil fatty acids were also tested. These results presented in table 3 indicate that although 0.2% tung oil may be borderline in inhibiting growth rate, 2.0% will inhibit growth rate and 0.02% definitely will not. Tung oil caused high mortality in this experiment only when fed at the 2% level. This experiment was of a fairly short duration because of a shortage of methyl esters of tung oil fatty acids. Significant differences in liver weight as a percentage of body weight occurred but no definite pattern evolved.

The fourth experiment was conducted to determine the effect of feeding both the saponifiable and unsaponifiable fractions of tung oil. The saponifiable fraction was not toxic and it stimulated a growth response equivalent to that obtained from feeding corn oil (table 4). The unsaponifiable material did not influence growth rate or mortality.

The gross pathology exhibited by individual animals fed a certain level of tung oil was not consistent. However, certain unusual conditions continued to appear when animals were examined after receiving tung oil in the ration; these in-

TABLE 1

Toxicity of crude tung oil

Supplements to basal diet	Avg wt			Mortality		
	2-weeks	3-weeks	4-weeks	2-weeks	3-weeks	4-weeks
	g	*g*	*g*	%	%	%
Trial 1						
None	—	272	381	—	0	10
5% Tung oil	—	200	312	—	95	95
5% Corn oil	—	379	449	—	5	10
5% Tung oil + 5% corn oil	—	109	—	—	95	100
Trial 2						
None	197	—	423	0	—	0
5% Tung oil	116	—	178	85	—	90
5% Corn oil	215	—	516	0	—	0
5% Tung oil + 5% corn oil	109	—	250	65	—	95

TABLE 2

Growth rate, liver size and mortality as influenced by level of tung oil in the diet

Supplements to basal diet	Avg wt, 21 days	Liver wt	Mortality
	g	*% body wt*	%
None	321 [1]	4.18	5
0.5% Tung oil	251	4.90	5
1.0% Tung oil	195 |	4.99	10
2.0% Tung oil	158 ||	4.03	35
4.0% Tung oil	97 |	5.27	70

[1] Means bordered by the same adjacent vertical line are not significantly different ($P < 0.01$).

TABLE 3

Effect of feeding methyl esters of fatty acids from tung oil on growth, liver size and mortality

Supplements to basal diet	Avg wt, 12 days	Liver wt	Mortality
	g	*% body wt*	*%*
None	143	5.29	0
0.02% Tung oil	168	5.23	0
0.2% Tung oil	137	6.17	0
2.0% Tung oil	81	4.43	20
2.0% Methyl esters of fatty acids from tung oil	158	5.92	5

Ranking treatment means [1]

Weight, g	81.8	137	143	158	168
Liver wt, % body wt	4.43	5.23	5.29	5.92	6.17

[1] Means underscored by the same line are not significantly different ($P < 0.01$).

TABLE 4

Effect of feeding saponifiable and unsaponifiable protein of tung oil on growth and mortality of chicks

Supplements to basal diet	Avg wt, 25 days	Mortality
	g	*%*
None	323	0
2.0% Corn oil	387	10
2.0% Tung oil	105	95
Unsaponifiable material from tung oil $\cong$ 2%	338	10
Saponifiable material from tung oil $\cong$ 2%	428	5

Ranking of treatment means [1]

Weight, g	105	323	338	387	428

[1] Means underscored by the same line are not significantly different ($P < 0.01$).

cluded; 1) enlarged gallbladder filled with fluid the color and consistency of normal bile; 2) an abnormally large amount of fluid in the abdominal thoracic and pericardial cavities; 3) variable-size livers with whole lobes occasionally not developed; 4) enlarged heart; and 5) tissues having an odor similar to that of tung oil. Combinations of these symptoms occurred in chicks fed as little as 0.5% tung oil in the diet. When the chicks were fed the methyl esters of tung oil or fatty acids of tung oil (exps. 3 and 4), the only noticeable abnormality was the odor of tung oil in tissues. When the chicks were fed the unsaponifiable material equivalent to 2% of tung oil (exp. 4), six of the 18 chicks examined at the end of the experiment had abnormally large amounts of fluid in the abdominal cavity and in the pericardial sac. They did not show any other abnormalities.

Microscopic examination of hearts and livers from chicks fed 2% of tung oil for 21 days in experiment 3 revealed the following abnormalities:[5] (a) Chick no. 7959: the liver showed diffuse degenerative changes characterized by the hepatic cells pulling away from the supporting tissue. There were a few areas of proliferation by epithelium in the bile duct. The heart muscle showed some degeneration and heterophyl infiltration. (b) Chick no. 7947: the liver showed mild diffuse changes which could not be differentiated

[5] Dr. S. C. Schmittle, Poultry Disease Research Center, U. of Georgia, carried out the microscopic examinations and some of the gross examinations reported.

from early postmortem decomposition. The hepatic cells appeared coalesced and were pulling away from the matrix.

DISCUSSION

These data indicate that commercially available tung oil contains appreciable quantities of a toxin, probably identical to the toxin described as soluble in fat solvents by Lee and Watson (1) and later characterized chemically and physically by Holmes and O'Conner (3). This toxin contained a number of ester linkages, which would be hydrolyzed by the saponification procedure used in preparing the saponifiable and unsaponifiable fractions of tung oil. Therefore, it is reasonable that neither of these fractions were toxic, unless the conjugated fatty acids as such are toxic.

Holman (5) and Holman and Greenberg (6) have reported that conjugated fatty acids are toxic to rats causing retarded growth and early death. However, Aaes-Jørgensen (7) fed rats for 17 weeks 1% of conjugated linoleate or eleostearate and reported no harmful effects. The studies reported in the present paper substantiate his results since the methyl esters of tung oil were not toxic to the chicks.

The gross symptoms of crude tung oil toxicity are similar to those observed in chicks fed fat containing chick edema factor (12). However, the former can be distinguished on the basis of the tung oil odor of the carcass.

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Phosphorus Metabolism in Lambs Fed Varying Phosphorus Intakes [1,2]

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ABSTRACT A growth and metabolism trial was conducted using 24 lambs. Three P levels were fed, namely 0.12, 0.15 and 0.29% P in a complete ration. Phosphorus deficiency, based upon reduced plasma inorganic P levels, was evident after one week in those lambs fed the 2 lower levels of P. Body weight gain and feed intake were adversely affected within 2 weeks. After P deficiency was established (7 weeks), P^{32} was injected subcutaneously and the metabolism of P and P^{32} was studied during a 2-week metabolism study. Metabolic fecal P and P balance increased with increasing increments of dietary P. Fecal elimination of the injected P^{32} was greater for the lambs fed adequate amounts of P. The percentage of endogenous P present in the rumen fluid increased with increasing levels of dietary P. Total endogenous P in the rumen fluid increased markedly with increasing P intake. It is concluded that endogenous P secretion into the rumen is decreased by a deficiency of P probably as a result of decreased plasma inorganic P levels. Equations expressing the P requirement of lambs in terms of total and available P are described.

The phosphorus (P) requirement and metabolism of ruminants have been studied by several workers (1–8). Many criteria have been used to evaluate P adequacy including body weight gain, feed efficiency, plasma inorganic P levels, bone ash and P content, P balance and various forms of pica. Most studies indicate that a P intake of 53 mg/kg body weight or 0.18% of an air-dried ration (90% dry matter) is near the minimal P requirement for growing cattle and lambs.

The metabolic function which becomes the first limiting function under a borderline P deficiency in ruminants is a subject of debate. South African workers (9) indicate that the concentration of water-soluble P in the rumen remains relatively constant even though the animals are P-deficient. Since the saliva contained inorganic P in a concentration which was 4 to 15 times that of the blood, it appears that this endogenous secretion is responsible for maintaining rumen P concentration when the dietary intake is inadequate. On the other hand, Iowa workers [3] concluded that the P requirement of the rumen microorganisms is greater than that for the host animal. This conclusion is based upon the observation that gains are increased by higher intakes of P than are required for the maintenance of a normal level of inorganic P in the blood. Thus, there appears to be some disagreement between the South African and the Iowa workers.

Resolution of this difference was one purpose of the study to be reported. An attempt was made to determine whether endogenous P secretion into the rumen was altered by varying P intakes. It is known, for instance, that endogenous secretion of nitrogen into the rumen is increased with decreasing intake of dietary nitrogen (10). Data were also obtained from which a quantitative estimate could be made of the total and available P requirement of the growing lamb. Elimination of radioactive P^{32} was also studied as affected by varying P intakes. A preliminary report of this work has appeared previously.[4]

METHODS

Twenty-four Texas wether lambs were sorted into 8 outcome groups on the basis

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[3] Burroughs, W., A. Raun, E. Cheng and C. C. Culbertson 1956 Different kinds and amounts of phosphorus in fattening cattle rations. A. H. Leaflet 209, Iowa Agricultural Experiment Station, Ames.

[4] Preston, R. L., and W. H. Pfander. Phosphorus metabolism in lambs fed varying phosphorus intakes 1962. Federation Proc., 22: 492 (abstract).

of phenotype and weight. One lamb from each outcome group was randomly placed in each of 3 treatment groups. The average initial weight of these 3 groups was 27.3, 26.7 and 26.6 kg. The lambs were individually fed ad libitum a completely mixed ration containing either 0.12, 0.15 or 0.29% P, respectively, as shown in table 1. These levels of P were thought to be deficient, borderline and adequate, respectively. Vitamin A (4.5 mg) was fed once each week during the entire experiment. Three samples of each ration taken at spaced intervals throughout the trial were analyzed, the averages of which are shown in table 2. The gross energy of the rations was 3.90 kcal/g.

The lambs were weighed each week at which time feed intake was totaled. The lambs were placed as a group in a lot with shelter and water except while they were fed in an adjacent barn. Plasma inorganic P was determined (11) weekly on blood samples withdrawn immediately prior to

TABLE 1

Ration composition

Ingredient	Treatment ration		
	0.12% P	0.15% P	0.29% P
	kg	*kg*	*kg*
Molasses beet pulp	60.00	60.00	60.00
Corn grits (degerminated)	20.00	20.00	20.00
Corn starch	10.00	9.95	9.76
Animal fat (stabilized) [1]	3.00	3.00	3.00
Blood meal	3.00	3.00	3.00
Dehydrated alfalfa meal	1.10	1.10	1.10
Urea (feed grade) [2]	1.00	1.00	1.00
$NaHCO_3$	0.70	0.70	0.70
Limestone	0.70	0.58	0
Dicalcium phosphate [3]	0	0.17	0.94
Salt (iodized)	0.50	0.50	0.50
	mg	*mg*	*mg*
$CoSO_4 \cdot 7H_2O$	0.88	0.88	0.88

[1] Butylated hydroxytoluene (95%), 0.01%; butylated hydroxyanisol, 0.01%; and citric acid, 0.01%.
[2] Supplied by E. I. Du Pont de Nemours & Company, Wilmington, Delaware.
[3] Supplied by International Minerals and Chemical Corporation, Skokie, Illinois.

TABLE 2

Chemical composition of rations

Component	Treatment ration		
	0.12% P	0.15% P	0.29% P
Moisture,%	9.21	9.18	9.24
Nitrogen, %	1.97	2.04	2.01
Ether extract, %	3.66	3.64	3.55
Crude fiber, %	10.5	10.4	10.5
Ash, %	4.97	4.92	5.11
Ca, %	0.56	0.55	0.49
P, %	0.12	0.15	0.29
Na, %	0.77	0.75	0.75
K, %	0.80	0.80	0.80
Mg, %	0.11	0.11	0.12
S, %	0.15	0.16	0.17
Cu, ppm	7.2	7.0	6.9
Mn, ppm	25	25	25
Co, ppm	0.38	0.38	0.41
Zn,[1] ppm	8.9	8.6	8.9
Mo, ppm	0.082	0.088	0.104

[1] Although this was the zinc content of the ration, the feed was fed in galvanized feeders which no doubt increased the zinc intake of the lambs.

the morning feeding. On the 49th day of the experiment, after the lambs were weighed, they were shorn.

After 49 days, it appeared that the lambs being fed the lower P levels were definitely being affected by a P deficiency as indicated by reduced gains and lowered plasma inorganic P. At this time, half of the lambs on each P treatment were implanted subcutaneously with 2 mg diethylstilbestrol (DES), and 2 controls and 2 implanted lambs from each P level were placed in metabolism stalls where urine and feces were collected. At this time, the amount of feed fed to the lambs was reduced to 85% of their voluntary consumption during the preceding week. Following a 4-day preliminary period, P^{32} (2.5 mc/lamb) was injected as a buffered (pH 7.2) solution subcutaneously over the right shoulder.[5] Excreta collections were made for 2 weeks at which time the lambs were killed for bone and rumen content analyses.

The remaining lambs were treated in a similar manner after 66 days on experiment. Their feed intake during the metabolism trial was reduced to 80% of their voluntary consumption during the previous week. The amount of P^{32} injected was 2.8 mc/lamb. Excreta collections were again made for a 2-week period.

Feces, urine and rumen fluid samples were analyzed for P (11) after wet ashing with concentrated HNO_3 followed by 70% $HClO_4$. Bone P was similarly determined after dry ashing. Gross energy determinations were made on the feed and feces.[6] Corrections were made for nitrogen and sulfur oxidations.

Radioactivity of the samples collected was determined using a proportional counter and scaler.[7] Fecal samples were ground, mixed and counted as a pellet similar to that outlined by MacKenzie and Dean (12). Blood, plasma and urine samples (0.4 or 0.5 ml) were counted on aluminum planchets (2.54-cm diameter). Trichloroacetic acid (10%) filtrates of plasma were counted using a liquid scintillation counter.[8] In all cases, standards of the injection solutions were counted under similar geometrical and chemical conditions and these in turn were standardized against a primary P^{32} source for absolute counting rate. All samples, with a few exceptions, were counted to less than a 3% counting error. All absolute amounts of P^{32} are those corrected to the date of injection. Since the amount of P^{32} injected was similar in both injections, much of the data have been averaged for purposes of presentation.

The left femur bone from the lambs used in the first collection study was removed at time of killing. The length was measured and the bone divided into 3 equal lengths, described as proximal, shank and distal portions, respectively. These were dried, extracted with ether and dry-ashed similar to procedures outlined by Benzie et al. (13). The ash was then dissolved in 1 N HCl and made up to volume. The P content was determined in a manner similar to the plasma inorganic P, and P^{32} determined in a manner similar to that used for whole blood, plasma and urine samples.

White blood cell and differential counts were made on blood samples taken from the lambs subsequent to P^{32} injection. The number of leucocytes in each of the 4 corners of 2 counting chambers were counted; 200 cells were counted for the differential counts.

Histological sections were prepared [9] of the liver, kidneys and rumen mucosa from the lambs used in the first collection study. Hematoxylin-eosin stain was used.

Statistical evaluation of the data was by analysis of variance and correlation analysis as decribed by Snedecor (14).

RESULTS AND DISCUSSION

Body weight changes during the first 49 days of the experiment are shown in figure 1. Average daily gains over this period were 5, 28 and 168 g, respectively, for the 3 increasing P levels. The adequate level of P supported greater gains ($P < 0.01$) than the 2 lower levels of P, which were not significantly different (SE of mean = 21). The average amount of wool clipped from each lamb at 49 days was 1.18, 1.18 and 1.45 kg, respectively. Total

[5] Oak Ridge National Laboratory, cat. no. P-32-P1. Injection solution contained a total of 7.4 mg P.
[6] Emerson adibatic bomb calorimeter.
[7] Picker X-ray Corporation.
[8] Scintillators: primary 2,5-diphenyloxazole; secondary 1,4-bis-2-(5 phenyloxayolyl)-benzene. Packard Company liquid scintillation counter.
[9] Prepared and interpreted by Dr. L. D. Kintner, Prof. Veterinary Pathology, University of Missouri.

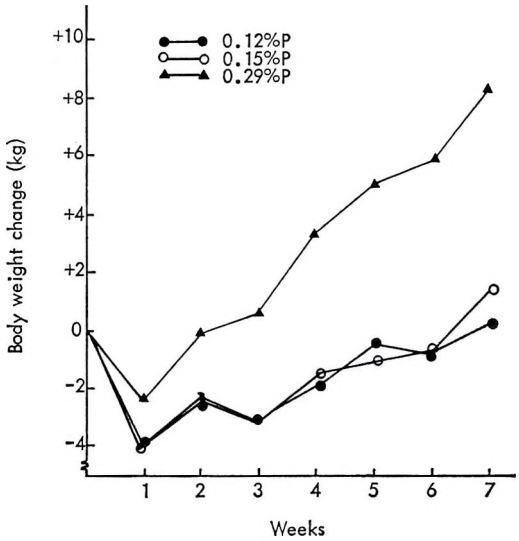

Fig. 1 Body weight change in lambs fed phosphorus at varying levels.

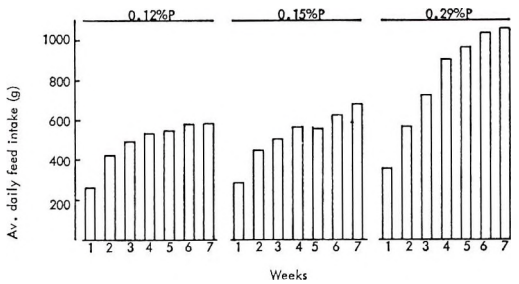

Fig. 2 Weekly variations in daily feed consumption in lambs fed phosphorus at varying levels.

feed consumption was less ($P < 0.01$) on the lower levels of P (fig. 2). Average daily feed consumption over the 7-week period was 490, 524 and 800 g, respectively, for the 0.12, 0.15 and 0.29% P levels (SE of mean = 44 g).

When average daily gain for each treatment is plotted against average P intake (mg/kg body weight), as shown in figure 3, a significant correlation resulted ($P < 0.01$) permitting the P requirement of lambs to be expressed by the equation

$$P = 0.0194W(1 + 0.0171G),$$

where P is the daily phosphorus requirement (g), W is the body weight (kg), and G is the daily gain (g). The indicated P required, therefore, by a 35-kg lamb gaining 180 g/day would be 2.78 g/day. This compares with an estimated requirement of 2.7 g/day (15).

Plasma inorganic P decreased with time on the 2 lower levels of dietary P ($P < 0.01$; SE of mean = 0.30), as shown in figure 4. The initial difference between treatments may be the result of the blood

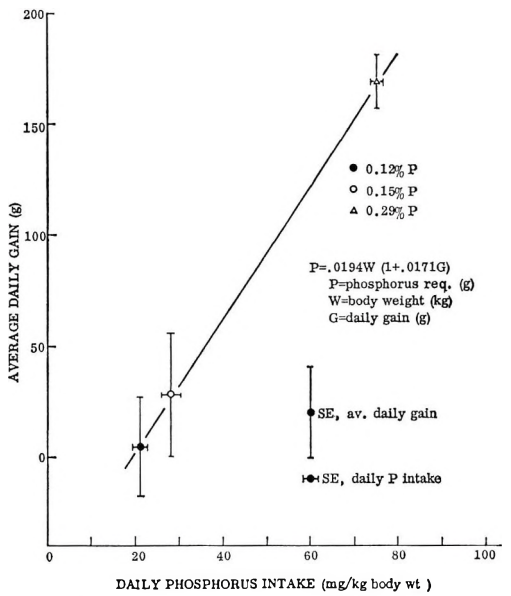

Fig. 3 Phosphorus requirements of lambs based on body weight gain.

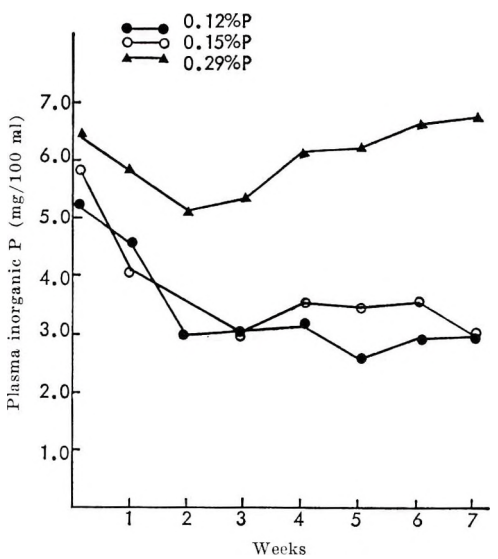

Fig. 4 Plasma inorganic phosphorus change in lambs fed phosphorus at varying levels.

samples being withdrawn on the second day of the experiment. Although equal initial values cannot be assumed, other work has shown a marked decrease in plasma inorganic P during the first week after lambs were fed a ration deficient in P. This has also been reported by Henderson and Weakley (16). Plasma levels for the lambs fed the 0.29% P level are within the "normal" range listed for whole blood (17) of 5.7 to 7.1 mg/100 ml with the exception of those observed on the second and third weeks, which were 5.13 and 5.35, respectively. Since the P intake of the lambs fed the 0.29% P ration during the first, second and third weeks was 40, 66 and 78 mg/kg, respectively, it appears that a P intake near 70 mg/kg would be required to maintain a "normal" plasma inorganic P level in the growing lamb. Thus, dietary levels of P which will give "normal" levels of plasma inorganic P will permit a daily weight gain near 150 g. Since gains somewhat greater than this were observed in this study, it may be that maximal gains will not be permitted if the dietary intake of P is sufficient to only maintain "normal" plasma inorganic P levels.

Figure 5 shows the P^{32} activity (μc/g feces) during the 2 weeks after injection. Peak activities were reached 3.2, 2.5 and 2.3 days after P^{32} injection for the 3 increasing levels of P, respectively. This response, coupled with the higher activities on the first day after injection for the higher P levels, may indicate a faster feed passage due either to the P level in the ration or more probably to a higher feed intake. Since peak activity in the plasma is reached within the first half-hour after subcutaneous injection (18), a given level of activity in the plasma can be assumed to be reflected in the feces approximately 3.2, 2.5 and 2.3 days after P^{32} injection, respectively, for the 3 increasing dietary P levels. There appears to be a second order component to the 3 fecal excretion curves but collections were not made for a sufficient length of time to confirm this response.

Specific activities of the plasma inorganic P decreased with increasing dietary P intake as shown in figure 6. This inorganic fraction appeared to account for a

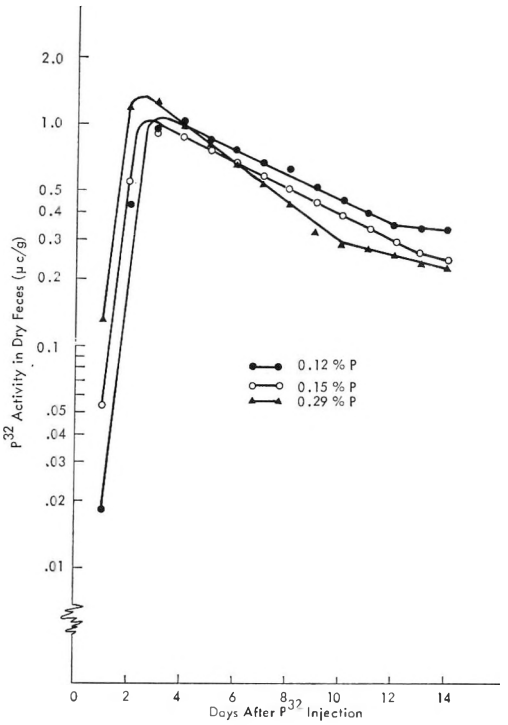

Fig. 5 Radioactivity in feces following P^{32} injection into lambs fed phosphorus at varying levels.

rather constant percentage of the activity in the plasma (61%) regardless of date of bleeding or dietary P level. The activity in the plasma accounted for a decreasing percentage of the activity in whole blood with time after injection (51, 46 and 40% of the activity in whole blood 3, 7 and 14 days, respectively, after P^{32} injection). There were no differences in this respect due to dietary P level.

The procedure used to calculate metabolic fecal P was similar to that used by Lofgreen (18). Specific activities of the plasma inorganic P used in these calculations were determined as follows. Since the peak activity of the fecal P occurred 3.2, 2.5 and 2.3 days after P^{32} injection for the 3 P treatments, respectively, and since plasma activities are maximal shortly after injection, it was assumed that this represents the time lag for a given level of activity in the plasma to be reflected in the feces. Referring to figure 7, the average metabolic P of the feces collected during x would have the same specific

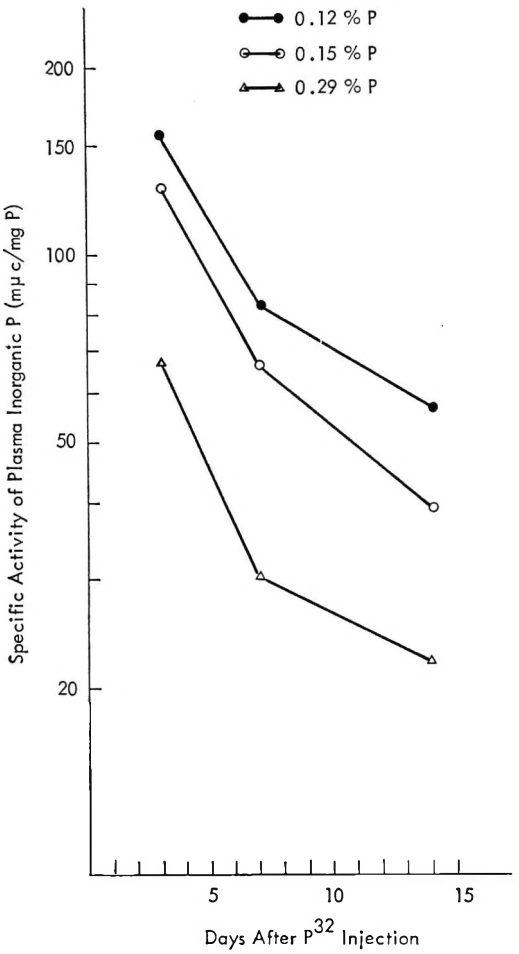

Fig. 6 Specific activity of plasma inorganic phosphorus.

activity as plasma inorganic P determined at $a + \frac{1}{2}x - a$ (or $x/2$) days after administration of P^{32}, and for feces collected during y, the specific activity of plasma inorganic P at $a + x + \frac{1}{2}y - a$ (or $x + y/2$) days after P^{32} administration would be used. Thus, the plasma specific activities used during the first week of collection (after the peak activity occurred in the feces) for the 3 P treatments were those observed 1.9, 2.2 and 2.4 days after P^{32} injection; those used for the second week of collection were those observed 7.3, 8.0 and 8.2 days after P^{32} injection. The actual plasma specific activities on these days were determined from a graphic plot of the plasma specific activities similar to the one shown in figure 6.

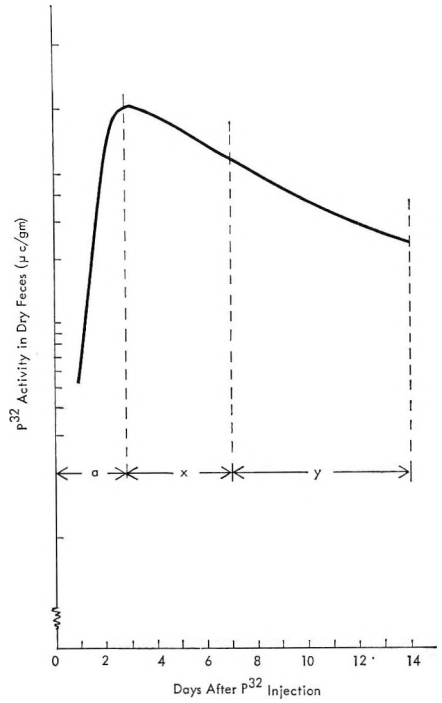

Fig. 7 Typical plot of fecal specific activity and method used to calculate metabolic fecal phosphorus (see text for description).

Table 3 shows the results obtained during the balance trial. The apparent P digestibility was low and variable between treatments. Metabolic fecal P increased progressively with increasing P intake ($P < 0.01$; SE of mean = 0.093), and was equal to 70, 73 and 80% of the observed fecal P. The true digestibility of the ration P was very much higher, therefore, than the apparent digestibility. Metabolic fecal P was decreased in those lambs implanted with DES (0.581 g/day) compared with controls (0.742 g/day), a difference which approached significance ($P = 0.06$). This effect was especially true in those lambs fed the ration containing 0.29% P, which resulted in a significant interaction ($P < 0.05$) between dietary P levels and DES implantation upon the amount of metabolic fecal P.

True digestibility of the ration P increased with increasing dietary P level, owing to the high true digestibility (calculated by difference) of the added dicalcium phosphate (91.5%). Urinary P and P^{32} excretion was almost negligible. Phos-

TABLE 3

Phosphorus digestibility, balance and P^{32} elimination in lambs fed varying phosphorus intakes

	Treatment ration		
	0.12% P	0.15% P	0.29% P
Feed intake, g/day	516	566	828
P intake, g/day	0.620	0.837	2.419
Fecal P, g/day	0.397	0.517	1.653
Apparent P digestibility, %	36.0	38.2	31.7
Metabolic fecal P, g/day:			
1st Week	0.267	0.347	1.356
2nd Week	0.294	0.408	1.297
True P digestibility, %	81.2	83.4	86.5
True P digestibility of dicalcium phosphate, %	—	92.9	90.1
Urinary P, mg/day	12.1	11.0	16.5
P balance, g/day	0.210	0.309	0.750
Fecal elimination of P^{32}, % :			
1st Week	10.90	11.52	22.78
2nd Week	6.24	6.42	8.07
Urinary elimination of P^{32}, % :			
1st Week	0.39	0.34	0.45
2nd Week	0.23	0.23	0.24

phorus balance increased progressively with increasing P intake ($P < 0.01$; SE of mean = 0.067). The DES implantation increased P balance (0.465 g/day) over the controls (0.380 g/day) which approached statistical significance ($P = 0.15$). This effect was much more pronounced in those lambs fed the 0.29% P ration, which resulted in an interaction which approached significance ($P = 0.12$).

Plotting the daily P intake (mg/kg body weight) against daily P balance, as shown in figure 8, gave a relationship which is significantly correlated ($P < 0.01$). Thus, the dietary P required for varying P balance can be described according to the equation

$$P = 0.0039W(1 + 23.1B),$$

where P is the daily phosphorus requirement (g), W is the body weight (kg), and B is the daily P balance (g).

Combining this equation with that shown for daily gain gives some interesting mathematical possibilities. With a P intake which does not permit any body weight gain (19.4 mg/kg body weight), a daily P balance of 0.17 g would be expected.

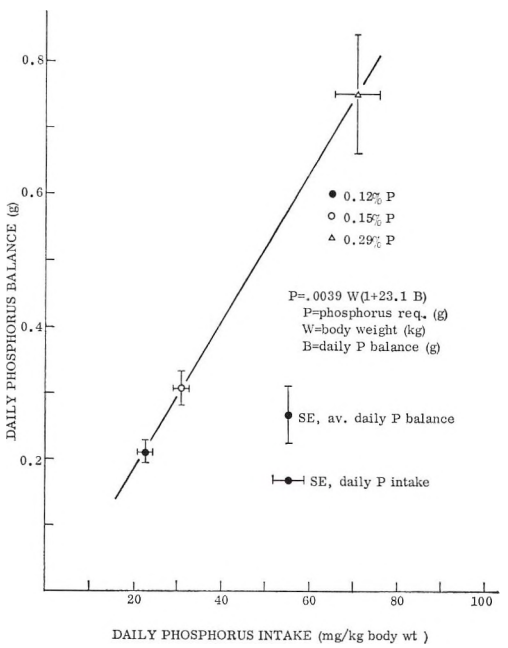

Fig. 8 Phosphorus requirement of lambs based on phosphorus balance.

This would presumably be completely used in bone growth. A P intake which would permit a daily gain of 25 g (27.7 mg/kg body weight) should result in a daily P balance of 0.265 g. If it is assumed that soft tissue and bone contain 0.2 and 4.0% P, respectively, it can be calculated that 77% of the gain would be soft tissue and 23% would be bone. Similarly, with daily gains of 100 and 200 g, it can be calculated that 91 and 93% of the gain, respectively, would be soft tissue; 9 and 7% would be bone.

Since the availability of the P fed in this experiment was determined, it is possible to express the available P requirement of the growing lamb by the equation

$$P = 0.0157W(1 + 0.0187G),$$

where P is the daily phosphorus requirement in grams of available P, W is the body weight (kg), and G is the daily gain (g). For the example lamb shown above, the available P requirement would be 2.40 g/day. Using the above equation and the availabilities of P in feed and mineral P sources, the total P required in any lamb ration can be determined.

The total amount of P^{32} eliminated via the feces was 17, 18 and 31%, respectively, for the 3 P levels ($P < 0.01$, SE of mean = 1.52). Thus, internal radiation exposure from injected P^{32} would be less with an adequate compared with a deficient P intake. Implantation with DES reduced the elimination of P^{32} compared with controls (14.7 and 18.2%, respectively). This difference approached significance ($P = 0.08$). Considerably less P^{32} was excreted during the second week (11.2%) than during the first week (22.6%) after P^{32} injection, as would be expected ($P < 0.01$).

The average gross energy digestibility coefficients for the 0.12, 0.15 and 0.29% P treatments were 86.7, 85.9, and 83.6, respectively ($P < 0.05$). This trend is no doubt accounted for by the greater feed consumption by the lambs fed the adequate level of P. Certainly, gross energy digestibility was not impaired by P deficiency. Using the equations set forth by Garrett et al. (19), it is possible to compute the average net energy requirement of the lambs on each level of P. Dividing this figure by the actual digestible energy intake, it was found that 46.7, 48.0 and 52.8% of the digestible energy was available as net energy. Thus, the efficiency of energy utilization in the P-deficient lambs may have been impaired. This has been shown by Riddell et al. (20) and Kleiber et al. (21).

Calculation of endogenous P concentration in rumen fluid was made by comparing the specific activity of the plasma inorganic P with the specific activity of the rumen fluid P, determined on samples obtained at the time of killing at the end of the first collection period. These calculations revealed that endogenous rumen P did not increase with decreasing P intake. The percentage of P in rumen fluid which was of body origin tended to increase with increasing P intake (table 4). The total endogenous P present in the rumen fluid increased progressively with increasing P intake. Thus, the transfer of endogenous P into the rumen decreases with an inadequate intake of P. This result does not agree with that reported by the South African workers (9). It does lend support to the idea presented by the Iowa workers [10] indicating that the P re-

[10] See footnote 3.

TABLE 4

Total phosphorus and endogenous phosphorus in rumen fluid of lambs fed varying phosphorus intakes

	Treatment ration		
	0.12% P	0.15% P	0.29% P
P concentration, mg/g	0.692	0.931	1.17
Specific activity, mμc/mg P	72.0	51.9	21.7
Endogenous P, %	60.8	66.8	64.8
Total rumen fluid P, g [1]	1.37	2.04	3.20
Total endogenous P, g	0.83	1.36	2.08

[1] Assuming rumen contents equal 15% of body weight and that one-half of the contents was rumen fluid.

quirement for the rumen microorganisms may be greater than that for the host animal. This idea is also supported by the amount of P required for maintenance of plasma inorganic P versus that required for maximal gains, presented earlier. The amount of endogenous P found in the rumen fluid of the lambs fed the 0.29% P ration (2.08 g) compares with a total estimated amount of 4.75 g secreted daily into the rumen of lambs (5).

The bone data indicate changes typical of those observed in P deficiency. The length of the femur increased slightly with increasing dietary P (16.4, 16.8 and 16.8 cm, respectively); also the amount of ash in the dry fat-free bone (45.5, 46.4 and 48.1%, respectively). This increase in ash was accounted for by an increase in the ash of the shank portion of the bone (60.7, 63.3 and 63.8%, respectively) and the distal portion of the bone (38.3, 40.1 and 42.2%, respectively). The P content of the bone ash (17.8%) was not altered by the dietary intake of P. The P content in the ash of the shank portion was slightly higher (18.0%) than in the distal (17.6%) or proximal (17.5%) portions. Specific activity tended to remain constant in the distal portion (52, 56 and 55 mμc/g P), and to increase in the shank (14, 15 and 22 mμc/g P) and proximal (34, 43 and 48 mμc/g P) segments, respectively, with increasing dietary P. Total P^{32} in the femur bone was 230, 246 and 291 mμc, respectively. Since only 4 lambs on each treatment were observed for bone changes, none of the above differences approached statistical significance ($P < 0.05$).

In general, there was a decrease in the number of leucocytes following P^{32} injection. Since all lambs were injected with P^{32}, it is impossible to say that this was the effect of internal radiation exposure. The average white blood cell count was 9300/mm³ for all 3 P treatments prior to P^{32} injection. This number decreased to an average of 5670/mm³ 7 days after P^{32} injection, after which some increase took place. A lower average count was observed for the deficient P levels after P^{32} injection (5870, 6700 and 7660/mm³ for the lambs fed 0.12, 0.15 and 0.29% P, respectively). Because of considerable variation, however, only the overall decrease after P^{32} injection was significant. In general, a decreasing percentage of lymphocytes accompanied the decreased numbers of leucocytes but this was extremely variable.

The histological sections indicated infiltration of many of the portal triads in the liver with leucocytes (especially mononuclear cells) in the lambs fed the 0.12 and 0.15% P rations, although some leucocytic infiltration was observed in the lambs fed the 0.29% P ration. Three out of 4 kidneys from the lambs fed the low P rations contained cellular and mineral casts in the collecting tubules. The kidneys from the lambs fed the 0.29% P did not contain any observed casts. The ration fed in this experiment resulted in rumens which were covered with several layers of vesicular cells. This occurred on all levels of dietary P.

During the course of this experiment no marked evidence of "pica" was observed.

ACKNOWLEDGMENT

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Vitamin B_6 Deficiency and the Initial Acquisition of Behavior [1]

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ABSTRACT Littermate rats paired by weaning weights were assigned at random to either a vitamin B_6-deficient group or to one of two control groups. The vitamin B_6-deficient group was fed our B_6-deficient diet ad libitum. One control group was fed ad libitum this same basal diet with added pyridoxine. The second control group was fed the same diet as the first control group; however, their intake was restricted to an amount which maintained each control rat at the same weight as its deficient partner. In one study, deficient and control rats were compared in the acquisition of a lever press response to avoid an electric shock. In a second study deficient and control rats were compared in the time taken to learn to press a lever to obtain water after being deprived of water. Differences in vitamin B_6 deficiency status were independently established by differences in growth, by xanthurenic acid excretion after a tryptophan load, and by gross observation of typical deficiency symptoms. In both studies the performance of the deficient animals was inferior ($P < 0.05$) to that of the control animals. No behavioral differences were observed between the 2 control groups.

Neurological changes associated with pyridoxine deficiency in swine (1, 2) and reported behavioral changes associated with vitamin B complex deficiency (3–5) suggest that vitamin B_6 plays an important role in the acquisition and maintainance of behavior. Meier and Moss (6) reported abnormal behavior in rats from pyridixone-deprived mothers. Vitamin B_6 deficiency has been associated by some, but not others, with increased maze running time (7)[2] with a breakdown in discrimination in a classical conditioning situation (8, 9) and with changes in thermoregulatory behavior (10). One of the most significant ways in which any agent may affect behavior is to change the time its takes to learn a new response. If a nutritional deficiency results in "slower" learning, this may have many implications, not only for our understanding of the biochemical substrate of behavior, but also clinically and socially. In this paper we report some studies on pyridoxine deficiency and the initial acquistion of behavior.

METHOD

A. Preparation of animals. McCollum-strain rats from our stock colony were weaned at 21 days. Littermate pairs were matched by weight and sex. One member of each pair was assigned at random to the deficient group, the other member was assigned to one of two control groups. The deficient group was fed ad libitum our vitamin-free casein [3] base pyridoxine-deficient diet (table 1) first described by Tulpole and Williams (11). The control diet was made by adding 30 mg/kg pyridoxine·HCl to this vitamin B_6-deficient

TABLE 1

Composition of experimental diet

	%
Vitamin-free casein [1]	20.41
Corn oil	4.08
Sucrose	70.28
Salts [2]	4.08
Vitamin mixtures [3]	1.15

[1] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] For composition refer to Hegsted et al. 1941 J. Biol. Chem., *138:* 459.
[3] Each 100 g of diet contained 2.55 mg α-tocopherol; 25.48 mg percomorph oil (vitamin A 1500 USP units, vitamin D 200 USP units); 0.25 mg vitamin K; 221.73 mg niacin; 9.0 mg inositol; 9.0 μg biotin; 2.25 mg *p*-aminobenzoic acid; 1.8 μg folic acid; 9.0 mg choline chloride; and 0.45 μg vitamin B_{12}.

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[1] This research was partially supported by Grant HD 00475-05 from the National Institute of Child Health and Human Development.
[2] Yeh, S. D. J. 1960 Studies on vitamin B_6 deficiency in relationship to vitamin B_{12} absorption. Unpublished dissertation, Johns Hopkins University.
[3] Vitamin-free Casein, obtained from General Biochemicals, Inc., Chagrin Falls, Ohio. The manufacturer states this contains 0.218 μg pyridoxine/g.

diet. In one control group each animal was fed the control diet ad libitum. In the second control group each animal was fed an amount of control diet each day sufficient to maintain it at the same weight as its deficient partner. This procedure is similar to pair-feeding except that an attempt is made to match body weights rather than food consumption. As no differences were observed between the 2 control groups in any of the behavioral measures, these 2 groups were combined for analysis.

B. Establishment of deficiency status. Animals were weighed regularly, and observed for indications of acrodynia, ataxia, convulsions, or other possible signs of vitamin B_6 deficiency.

At age 20 weeks the animals were fasted for 24 hours and then given a tryptophan load of 5 mg/100 g body weight. Twenty-four hour urine collections were made. Total xanthurenic acid in the urine was then determined by the method of Rosen et al. (12) as a measure of relative vitamin B_6 deficiency.

C. Behavioral apparatus. Commercial experimental chambers, each containing a lever, a grid floor for delivering electric shock, a small observation window, and accessory equipment were used.[4] Intensity and timing of electric shock were automatically controlled. A feeder, consisting of a motor driven spoon containing 3 drops of water, could be made available to the animal for a 3-second interval in one chamber. A second chamber used in the study had a feeder which dispensed measured water reinforcements into a small reservoir. This feeder was replaced by a motor driven spoon on the third day of use because of malfunctioning. A buzzer sounded throughout the operation cycle of all feeders. Experimental contingencies were automatically controlled and recorded by external relay equipment, timers, counters, and a cumulative recorder, except where the experimenter controlled the presentation of reinforcements as indicated in a later section. The lever-pressing behavior of the animal was automatically recorded on counters and on a cumulative chart recorder.

D. Behavioral procedures. 1. Acquisition of lever pressing to avoid or escape from electric shock. Five pairs of vitamin B_6-deficient and control animals were compared in the initial acquisition of a lever pressing response to avoid or escape from electric shock. On the first day of the 11th week (12th for one pair) each animal was placed in the experimental chamber for one-half hour to habituate to the new situation. No apparatus other than a ventilating blower was operative at this time. On the second day, each animal was placed in the chamber, the lights went on, and the animal received a train of 1.6 ma electric shocks, each shock lasting 0.4 seconds, and a new shock starting every 3 seconds. This train of shocks continued until the animal chanced to depress the lever in its attempts to escape the shock. The lever press terminated the train of shocks for 12 seconds. Every lever press delayed the onset of shock 12 seconds from the response, but if 12 seconds elapsed without a response the shocks started anew and continued until a lever press occurred. The procedure was thus technically Sidman avoidance, with a 3-second shock-shock interval and a 12-second response-shock interval (13). The session continued until the animal received 100 shocks. The number of responses emitted before the animal received the 100th shock was recorded, a greater number of responses being associated with "superior" performance and more rapid learning. The session terminated with the 100th shock.

On the following day the animals were exposed to the avoidance contingencies for 10 minutes to insure that the avoidance behavior was well-established. On the next day a new procedure was added. Five minutes of avoidance was alternated with 5 minutes of escape, until the animal had received 25 minutes of each condition. The escape procedure differed from the avoidance procedure in that once the shock started, it continued until the animal responded, and responses before its onset were ineffective in avoiding it. Thus the animal could not *avoid* the shock, it could only *escape* the shock once it commenced. Two deficient animals had to be removed from the chamber before completing the session to prevent damage from too much

[4] Foringer and Company, Rockville, Maryland. Chamber 1102 R.

continuous shock, and a recording malfunction resulted in no data for a third; hence the procedure was repeated on the next day. On this day the cumulated time the shock was on during the 5-minute escape periods was recorded for each animal.

2. Acquisition of lever pressing with water reinforcement. In this study the vitamin B_6-deficient animal and the control animal in each of 7 pairs were deprived of water, and then compared with respect to the time it took each animal to learn to press a lever to obtain water.

The study was not conducted with the intention of studying initial learning; the animals were trained to press the lever only as the first step in acquiring a more complex repertoire. However, data were recorded from the beginning of training and these data were examined retrospectively when our interest in initial rates of acquisition was aroused by the observations concerning the initial learning of avoidance and escape behavior.

Seven pairs of vitamin B_6-deficient and control animals were restricted in water intake starting in the tenth week, to maintain and control motivation for water. The 2 members of each pair were always equally deprived of water to try and insure equal motivation. After training started there were sometimes differences *between* pairs in degree of water restriction, as pairs whose behavior suggested low motivation were more seriously restricted than others to increase motivation. Details of the water restriction regimen are presented in table 2. Pairs 1 through 3 are those that were originally studied in the chamber containing the motor driven spoon. The remaining pairs were run in the second chamber.

The day's ration of water was given to each animal immediately after the training session, or at a time corresponding to this on the days before the animals started training. Each animal consumed its water immediately upon receiving it.

Starting in the eleventh week, 3 days of "magazine training" were given. Each animal was placed in the experimental chamber for one hour daily and the feeder and buzzer were operated at irregular intervals by a film timer, so that the animals would learn to approach the feeder and drink immediately upon hearing the buzzer.

Starting on the fourth day of the eleventh week each animal was trained to press the lever. The experimenter controlled the presentation of water reinforcements with a hand switch. Training was carried out by presenting differential reinforcement for successive approximations to the desired response. At the start of training for each animal, any response which was related remotely to the desired

TABLE 2

Water restriction: daily ration of water given each animal in home cage

Training day(s) [1]	Pairs [2]	Daily ration	Type of training next day [3]
		g/g body wt	
Before week 10	all	ad libitum	
1–2	all	none	none
3–6	all	0.05	
7	all	none	
8	1–3	0.05	
8	4–7	none	magazine training
9	all	0.05	
10	all	0.05	
11	1–3	0.05	
11	4–7	none	lever pressing
12	all	none	
13–16	4–7	0.025	

[1] For this table the first day of week 10 is labeled day 1, and subsequent days are numbered accordingly.

[2] To equate motivation, each member of a pair was offered the same amount of water. All pairs, however, did not always receive the same amount of water.

[3] For an explanation of the training procedures see text.

lever press, as moving into the half of the chamber containing the lever, was reinforced. When the animal had learned this response, the criterion for the form of response to be reinforced was changed, the new criterion specifying a form of response which more closely resembled the lever press. By a series of such successive approximations the final response was acquired by all animals. Each daily session for an animal was continued until the animal was satiated, as evidenced by failure to approach the feeder and drink when the buzzer sounded. Each response (lever press) made by the animal resulted in the automatic presentation of a water reinforcement. The date on which each animal started responding regularly was recorded. These dates were the data used to measure speed of initial acquisition.

RESULTS

A. Establishment of deficiency status. Relative vitamin B_6 deficiency status was established by following weight gains, by differences in xanthurenic acid excretion, and by observing clinical symptoms. In figure 1 the range of weights for all animals fed the vitamin B_6-deficient diet and that for all animals fed the control diet

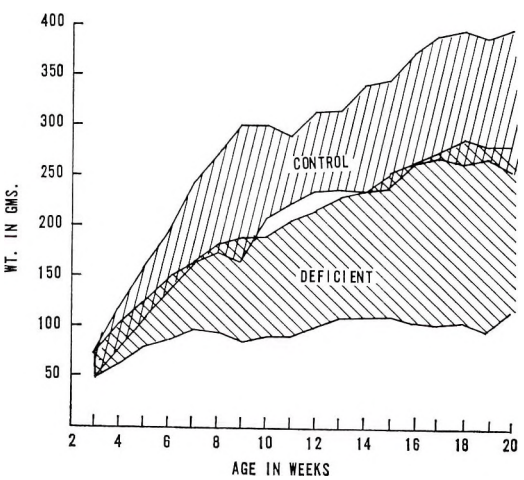

Fig. 1 Weight ranges of rats fed the vitamin B_6-deficient and control diets ad libitum. In the tenth week water rerstriction was started for some animals, and in the eleventh week the behavioral testing was started. Measures of xanthurenic acid excretion were obtained in the 20th week.

ad libitum are shown. The 2 groups are clearly differentiated. The overlap between the 2 groups is accounted for by the low weights of several control rats on a restricted water regimen.

Xanthurenic acid excretion was measured in all animals except 2 deficient rats that died before this test was completed (apparently due to vitamin B_6 deficiency), and in the one pair of animals that was older than the others. Thus xanthurenic acid excretion in 9 deficient and 11 control animals was measured. The mean amounts of xanthurenic acid excreted and the standard errors are 5.2 ± 1.1 mg for the deficient animals and 1.1 ± 0.4 mg for the control animals.

Acrodynia and ataxia were observed in all deficient animals, and were not observed in any control animals. At the time the behavioral measurements were made the acrodynia was limited in some deficient animals to a slight redness and coarseness of the hair on the head, back, and haunches, and the ataxia was not severe. No convulsions were observed.

B. Differences in acquisition of lever pressing to avoid or escape from electric shock. The number of responses emitted before receiving 100 shocks by each vitamin B_6-deficient animal and by each control animal on the initial day of avoidance training was recorded (table 3). In each pair the control animal made more responses (indicated by a plus sign). On the first day of escape training for which data is available, the cumulated shock time was recorded (table 3). In every pair the performance of the control animal was superior to that of the vitamin B_6-deficient animal for both avoidance and escape, and the differences are substantial. A one-tailed sign test (14) for matched pairs indicates that the control animals performed significantly better than the deficient animals in both measures ($P < 0.05$).

C. Differences in rate of acquisition of lever pressing with water reinforcement. The number of days before each vitamin B_6-deficient animal and each control animal learned to press the lever regularly is shown in table 4. In 5 pairs the control animal learned more rapidly than deficient animals; in 2 pairs there was no difference. In no pair did the vitamin B_6-defi-

TABLE 3

Initial acquisition of avoidance and escape responses

Pair	Animal	Avoidance [1]	Sign [2]	Escape [1]	Sign [2]
		no. of responses		*seconds*	
1	Deficient	39	+	63	+
	Control	265		38	
2	Deficient	42	+	77	+
	Control	337		12	
3	Deficient	52	+	> 150 [3]	+
	Control	291		25	
4	Deficient	81	+	126	+
	Control	450		30	
5 [4]	Deficient	541	+	84	+
	Control	1198		67	

[1] The avoidance score indicates the number of responses made before receiving 100 shocks on the first day of avoidance. The escape score indicates the total time that the shock remained on the first day of the escape procedure for which data were available.
[2] A plus sign indicates that the control animal of the pair was superior in performance. A one-tailed sign test for matched pairs indicates significant differences between both avoidance and escape scores ($P < 0.05$).
[3] Session terminated as animal ceased responding entirely.
[4] This pair of animals older than others.

TABLE 4

Number of training sessions required by deficient and control animals in each pair to learn to press lever regularly for water reinforcement

Pair	Animal	No. of sessions	Sign [1]
1	Deficient	1	0
	Control	1	
2	Deficient	2	+
	Control	1	
3	Deficient	3	+
	Control	2	
4	Deficient	6	0
	Control	6	
5	Deficient	7	+
	Control	4	
6	Deficient	6	+
	Control	1	
7	Deficient	7	+
	Control	3	

[1] A plus sign indicates that the control animal of that pair learned more quickly, a zero that there was no difference. A one-tailed sign test for matched pairs is significant at $P < 0.05$.

cient animal learn more rapidly than the control. Thus, the vitamin B_6-deficient animals learned significantly more slowly than the control animals ($P < 0.05$, one-tailed sign test for matched pairs).

DISCUSSION

In studying laboratory animals, "good" nutrition has usually been defined in terms of growth and reproductive capacity. However, the ability to acquire and maintain adequate behavioral repertoires is also essential to the well-being of the animal. An animal that cannot learn, that cannot maintain its behavior under stress, or that does not have sufficient endurance to meet the demands placed upon it, cannot survive. Thus, it is important to investigate the behavioral significance of components of nutrition in determining the adequacy of a diet, as well as the effect upon growth and reproduction.

Because of the multiple roles of the coenzyme pyridoxal 5-phosphate in metabolism, we felt it would be worthwhile to determine whether vitamin B_6 deficiency is reflected in behavior. Because of the sensitivity of many behavioral measures to pharmacologic actions, the production of deficiency by dietary manipulation rather than by antimetabolities appeared wiser. The fact that scores from the pair-fed controls and ad libitum-fed controls did not differ, and that the differences between the scores of the deficient animals and the control animals were large, indicates that general inanition was not a significant factor. The independent assessment of relative vitamin B_6 deficiency suggest that this deficiency is the important factor; however, some unknown difference between deficient and control animals which occurred coincidently with the pyridoxine deficiency cannot be excluded.

It is possible that the behavioral scores do not reflect a difference in "ability" per se. Motor impairment, a change in activity level associated with deficiency, or some

change in "emotionality" may account for the differences in observed performances. From our observation of the animals, and from currently incomplete data we do not believe that the differences are the result of any of these "peripheral" variables; however, this is yet to be established.

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

SHERATON-BLACKSTONE HOTEL, CHICAGO, ILLINOIS
APRIL 13–17, 1964

COUNCIL MEETINGS

The Council of the American Institute of Nutrition met Saturday evening, April 11, and Sunday morning and evening, April 12. The actions of the Council were presented at the Institute business meetings and are included in the report of those meetings.

SCIENTIFIC SESSIONS

A total of 270 abstracts was submitted to the Institute; 36 of them were transferred to other societies; 65 were accepted from other societies. These abstracts were arranged into 25 regular and 3 intersociety (atherosclerosis) sessions. The following 5 half-day symposia were presented:

1. Relation of Nutrition to Food Science
2. Balance and Imbalance in Nutrition
3. Animal Nutrition Research, a Key to Some World Food and Nutrition Problems
4. Interrelationships among Vitamin E, Coenzyme Q and Selenium
5. Nutrition in Clinical Medicine

BUSINESS MEETINGS

Business meetings were held on Monday, April 13 and Thursday, April 16, 1964. Dr. Grace A. Goldsmith presided at both meetings.

I. *Minutes of 1963 meeting*

The minutes as published in the *Journal of Nutrition*, *80*: 447, 1963, were approved.

II. *Elections*

The 652 ballots were counted by Drs. Robert L. Squibb and Benjamin T. Burton. The following were elected:

President: R. W. Engel
President-elect: J. B. Allison
Councilor (three-year term): N. S. Scrimshaw
Nominating Committee:
R. E. Hodges, chairman
G. F. Combs
Gladys Everson
H. E. Sauberlich
B. S. Schweigert

These individuals assume office on July 1, 1964.

III. *Membership Status*

As of April 1, 1964, there were 938 members of the Institute: 849 active, 75 retired and 14 honorary. There was an increase of 64 members over last year. The Clinical Division had a total of 138 members.

The following members died during the past year:

Alfred W. Bosworth, June 8, 1963
Farris Hubbert, Jr., December 28, 1963
John F. Lyman (Charter Member), November 19, 1963
Sydney S. Negus, May 17, 1963
Paul L. Pavcek, March 30, 1964
Sybil Smith, July 13, 1963
Stewart G. Tuttle, October 23, 1963

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

> RESOLVED: That the American Institute of Nutrition, assembled at Chicago, Illinois, in its annual meeting, April 13, 1964, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of one of its charter members, John Franklin Lyman.
>
> Dr. Lyman was an effective teacher who introduced many students to the field of nutrition. He was well-known for his research on the fat-soluble vitamins and the nutrition of the newborn animal. In the course of this work, he showed that the ion-protein balances in milk had much to do with the ability of an animal to effectively utilize calcium, phosphorus, and protein. He and his students showed that these factors affected fat absorption. Much of this work laid the foundations for the industrial development of infant foods based on cow's milk formulae.

IV. *New Members*

The Membership Committee considered the requests of 106 nominees. The following 69 nominees recommended by the Committee were approved for membership at the business meeting:

NEW MEMBERS — 1964 *

Juan Manuel Baertl
Herman Baker
Andre Bensadoun
Stanley S. Bergen, Jr.
Donald Berkowitz
Marvin L. Bierenbaum
J. Wm. Boehne
Robert James Bolt
Richard C. Bozian
G. E. Bunce
W. O. Caster
H. C. Choitz
William A. Cochrane
R. C. Cragle
Marvin Cornblath
C. F. Cramer
George L. Curran
Krishnamurti Dakshinamurti
W. N. Dannenburg
E. J. Day
Seymour Dayton
Antoon P. DeGroot
Hector F. DeLuca
Edwin H. Ellison
R. S. Emery
Frank Falkner
Richard A. Freedland
George B. Jerzy Glass
George G. Graham
W. R. Graham, Jr.
Seymour J. Gray
M. G. Hardinge
Betty E. Hawthorne
Mohamed M. Abdel Kader
Samuel G. Kahn
Hans Kaunitz
Thomas DeArman Kinney
Abbas E. Kitabchi
Eva H. Kwong
Rachel H. Larson
J. W. Lassiter
J. G. Lecce
W. P. Lehrer
Leo Lutwak
Ellen H. Morse
Eduardo Angel Porta
Ananda S. Prasad
Nicholas Raica, Jr.
Robert H. Roberson
Quinton R. Rogers
Zakaria I. Sabry
J. Seronde, Jr.
A. Leonard Sheffner
Harry Swachman
Irvin D. Smith
Fiorenzo Stirpe
Daniel Boxall Stone
James F. Sullivan
Louis W. Sullivan
Hertha H. Taussky
Reinhardt Thiessen, Jr.
Ian J. Tinsley
Pran Nath Vohra
Shreepad R. Wagle
Lloyd E. Washburn
Harold L. Wilcke
William L. Williams
Gerald N. Wogan
Lois M. Zucker

* For institutional affiliations and addresses of new members, see the Federation Directory to be published in the Fall of 1964.

HONORARY MEMBERS

The following scientists were elected to Honorary membership:

Edward John Bigwood
Emeritus Professor and Honorary Rector
Brussels University, Belgium

Eric John Underwood
Dean, Faculty of Agriculture and
Director, Institute of Agriculture
University of Western Australia
Nedlands, Western Australia

V. *Treasurer's Report*

Dr. Douglas V. Frost, presented the Treasurer's report which was approved. The Auditing Committee, Drs. Edwin L. Hove and Paul E. Johnson, reported that they had examined the books and records of the Treasurer and found them in order.

TREASURER'S REPORT

April 11, 1963 — April 3, 1964

Balance in checking account, April 18, 1963	$ 4,849.79	
Balance in savings account	6,087.50	
U. S. Series K Bond	500.00	$11,437.29
Income		
American Institute of Nutrition dues, 838 members	4,189.00	
Federation dues, 848 members	5,088.00	
Journal of Nutrition, 775 subscriptions	7,550.00	
ASCN dues, 138 members	276.00	
AJCN, 215 subscriptions	1,827.50	
Wistar Institute for Editorial Office, 3 quarters at 2425	7,275.00	
Wistar Institute for Additional Net Subscriptions, 103 at 11.25	1,158.75	
Dues, 1962	18.00	
Federation rebate from 1963 annual meeting	4,417.00	
U. S. K Bond Interest	13.80	
Citizens National Bank and 1st Western Savings, interest	270.91	
Overpayment from members	23.50	
Nutrition Foundation and Borden Company for Award Winner Reception	439.45	
FASEB (return of working fund loan for travel grants to 6th International Congress)	250.00	
Page charge collections from *Journal of Nutrition*	12,500.00	
Other (Foreign exchange and mailing charges)	11.39	$45,308.30
Total receipts and balance brought forward		$56,745.59
Expenses		
Federation Office:		
Assessment on 892 members	5,352.00	
Other Federation services	797.14	

Wistar Institute:		
Journal of Nutrition subscriptions for 1963–64	5,360.00	
Journal of Nutrition subscriptions for 1962–63	270.00	
Printing charges	478.45	
ASCN dues and subscriptions	2,103.50	
Journal of Nutrition, Editorial Office	13,690.25	
Secretary's office expense	1,055.13	
Treasurer's office expense	155.20	
Travel (Council meetings, as needed; Delegates, Nutrition Society of Canada)	1,022.83	
IUNS (Dues — 1963–64; International Nutrition Committee, postage)	81.00	
Printing charges	254.33	
Overpayment of dues	23.00	
Miscellaneous	19.95	
Bank charges	26.29	
Total expenditures		$30,689.07
Balance on Hand, April 3, 1964		
Checking account	9,321.99	
Savings accounts:		
Citizens National Bank of Waukegan, 4%	6,194.02	
First Western Savings and Loan, 4.85%	10,040.51	
U. S. Series K Bond	500.00	
		$26,056.52

VI. *Dues*

The Council recommended and the membership agreed that the dues for the coming year should remain at their current level.

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

Editing and Publication Operations

	Calendar year		
	1961	1962	1963
Volumes published	73, 74, 75	76, 77, 78	79, 80, 81
Pages published (including papers, biographies, announcements and proceedings)	1399	1447	1436
(Scientific papers only)	(1357)	(1403)	(1383)
Papers published (including 3 biographies)	203	209	204
Papers submitted	287	321	312
Papers rejected	59	85	113
Rejection rate (based on number of papers submitted)	20%	26%	36%
Supplements published	—	1	—
Operating schedule			
Av. time from date of receipt of manuscript to mailing to Wistar:			
Av. no days with reviewer	20.6	20	21.2
Av. no days out for revision	18.3	17.9	24.8
Av. no. days in office, in mail or in unavoidable delay	21.8	32.6	34.9
Average total days	60.7	70.5	80.9
Av. time from date of receipt of manuscript to mailing by Wistar:			
Av. no months with Editorial Office	2.0	2.4	2.6
Av. no. months with Wistar Press	3.2	3.4	3.2
Av. *total* months from date of receipt to mailing of Journal	5.2	5.8	5.8

Summary of Finances in the Operation of the Editor's Office, *Journal of Nutrition*

July 1, 1963 — June 30, 1964

Balance brought forward	$(-2,290.25)
Receipts, AIN	14,290.25
Total receipts and balance available	12,000.00
Expenditures, partially estimated, per schedule	12,118.65
Estimated balance July 1, 1964 (deficit)	$(-118.65)

The Council approved the budget proposed by Dr. Barnes for the Editor's Office for the year starting July 1, 1964.

VIII. *Interim Business Management*

The Council approved an arrangement whereby the Federation Business Office will handle the billing of Institute members for dues and journal subscriptions; also collect from the authors the charges for pages in the *Journal of Nutrition*, maintain a current accounting of Institute fiscal activities, provide an audit of Institute financial transactions and prepare the Federal income tax form. The charge for this service which will cover both AIN and ASCN members will be $2.00 per year for each active member. This service will start June 1, 1964.

IX. *President's Report*

An effort was made to eliminate multiple Federation dues for members of two or more affiliated societies. Representatives of other societies argued that since such dual membership permitted the individual to present papers before each society to which he belonged, the dual dues assessment should continue.

The Federation Directory will no longer be a part of the Federation Proceedings. It will be issued separately to all members late in October.

The Editorial Board of Federation Proceedings which formerly consisted of the Secretaries of the Societies will now be composed of appointed representatives from each society. This Board will be responsible for reviewing papers other than those from symposia.

X. *Executive Secretariat*

Dr. W. A. Krehl, for the Committee (Drs. F. S. Daft, G. M. Briggs, W. A. Krehl, E. L. Severinghaus and P. L. White), presented a proposal to establish a secretariat at Beaumont House (the Federation Office). The membership was not willing to approve the suggested publication phase of the secretariat. The complete proposal follows. Comments and suggestions about it are solicited.

A motion was passed urging the Council to proceed with the establishment of an executive secretariat but omitting the publication phase of the proposal.

THE AMERICAN INSTITUTE OF NUTRITION SECRETARIAT

It is proposed that the American Institute of Nutrition establish the office of permanent executive secretary in Beaumont House at the Federation Headquarters in Bethesda, Maryland. The office should be initiated as soon as possible in 1964.

Objectives: The executive secretary should aid in (1) advancing the science of nutrition and its applications to human well-being more effectively than the American Institute of Nutrition has been able to do as now organized; (2) improving the quality of nutrition research and teaching by the promotion of better graduate and postdoctoral training programs; (3) facilitating communication between nutritionists and individuals in closely related fields; and (4) relieving the excessive burden now placed on the president, secretary, and treasurer.

Qualifications required for Executive Secretary: The person must have demonstrated continued interest in and support of the science of nutrition. Preferably, the AIN secretary should be a member or qualified for membership in the American Institute of Nutrition. He must have demonstrated a high degree of maturity and judgment in administration and organization. The individual must work cooperatively and productively with others, particularly at the executive level.

Tenure, Salary, and Assistance: The position should be established on a five-year tenure basis. The Committee for the Executive Secretariat should report to the Council annually with an exhaustive Council review and evaluation of the position at the end of three years. At that time, a decision would be made either to continue the term of office or to terminate it at the

end of the five-year period. Responsibility of the Executive Secretary is to the membership of the AIN via the Council. The Executive Secretary will serve as an ex-officio non-voting member of the Council.

Salary: A salary range from $18,000 to $22,000 is recommended. Fringe benefits applicable to Federation employees would be applied.

Secretarial assistance: The Executive Secretary would require initially, one secretary with salary commensurate with similar positions in the Federation offices. (A salary range of $4,000 to $5,000 would be required.)

Housing of the AIN Secretariat: Beaumont House would provide office space for the Executive Secretary and his assistant at the rental rates applicable to Federated Societies. It is anticipated that the Federation facilities and record keeping, billing and distribution of communications to AIN Society members would be available on a "cost" basis.

Activities of the Secretariat: The Secretariat will be responsible for handling all of the Society's routine financial business and correspondence. The Executive Secretary will be responsible for arranging the annual meetings. He should envision a broadened base of communication with the AIN membership to solicit its reactions regarding the programs of the AIN, enhance the quality and quantity of papers submitted by members to the annual meeting, and keep the members apprised of the activities of the Society. The basic objective of the Secretariat should be to stimulate interest in AIN membership and make every effort to bring cohesiveness of objective into the admittedly divergent areas of nutrition represented by our membership. Activities and relations with the International Union of Nutrition Sciences and other international nutritional business matters will be handled by the Executive Secretary. It is anticipated that the efficient management of the routine duties of the Secretariat as indicated above, should not require more than one-fourth of the time of the Executive Secretary. The assignment of additional duties for the Executive Secretary should be directed only through the Council.

Responsibilities of the AIN Secretariat in the area of professional education and nutrition research and development: In proposing the establishment of the AIN Secretariat and the appreciable cost of operating such an office, the AIN membership recognizes its responsibility and broadened social awareness of the role and scope of nutrition in problems of health. Perhaps the outstanding overall responsibility, opportunity and challenge, then, of the AIN Secretariat is to plan, develop and execute a program of nutrition education aimed primarily at the professional level and to explore further the possibilities of engaging in a program of public service designed to advance the science of nutrition.

Specifically, the following are possible areas of interest for development by the secretary:

I. Assessment and organization of resources in nutrition.

A. Establish lines of contact and communications with organizations directly or peripherally interested in nutrition. It is anticipated that the AIN Secretariat should develop a clearing house for those individuals, organizations and trade associations who have demonstrated an interest in, or who are actively participating in some phase of the science of nutrition.

B. Develop a register of individuals working in the field of nutrition, their qualifications, capabilities and contributions. Such a register could serve the appropriate governmental, academic and industrial organizations who have an interest in developing programs requiring individuals with skills in the science of nutrition. It could also be used in the development of themes and programs in nutrition information and education as may be needed and approved by the Council.

II. The development of professional education and research in nutrition as it relates to health.

A. Cooperation in the development of "Careers in Nutrition"

brochure. There is need to bolster the interest of good students in the science of nutrition in our graduate school programs. Interest is lagging on the part of U. S. students in our nutrition schools and departments at nearly all levels.

B. Aid in the development of programs of medical and postgraduate medical education in nutrition. Clinical nutrition should be encouraged as a sub-specialty of medicine. Every effort should be made to improve nutrition education in the medical program at all levels so that physicians recognize more clearly the place that nutrition has in the care of the patient both in health and disease.

The Executive Secretary cannot work alone. In all of these suggested areas, he should enlist the aid of outside nutrition interests and attempt to unify with them the program for nutrition development. For example, both the AMA Council on Foods and Nutrition and the National Vitamin Foundation have programs in nutrition education for the practicing physician. Cooperation with these resources should be given, bringing to bear the various resources of the Society. In the same way, the Executive Secretary should cooperate with the nutrition education programs sponsored by the dietetics and home economics groups to enhance their programs. The AIN Secretariat should not enter into competition with the programs that are sponsored by other closely allied nutrition organizations. Ours should be a cooperative and collaborative function.

III. The Executive Secretary shall have the responsibility for the development of plans for the editing and publishing of a nutrition journal for the dissemination of professional nutrition information to the AIN membership and all others with an interest in the science of nutrition.

A. This journal reporting the current concepts in nutrition would deal not only with the interpretation and significance of the scientific areas of nutrition but would function as a guide in formulating patterns of nutrition education so as to improve and preserve the scientific stature of nutrition, particularly at the level of the nutrition teachers and educators.

In association with this journal, a newsletter for members would carry significant AIN news, Council and Committee assignments and reports and other matters of information considered vital to the interests of the AIN membership.

B. The Executive Secretary will be editor of the new journal and have an editorial board of associate or preferably contributing editors, who would assume the responsibility in a systematic way, to make contributions to the journal. In addition, "outside contributors" would be selected and screened. The membership of the editorial board would be selected jointly by the editor in collaboration with the Council and both the editor and editorial board would be responsible to the AIN Council.

C. It is anticipated initially that this would be a quarterly journal with a circulation base of approximately 10,000 although conceivably this could be expanded to as much as 15,000 or 20,000. Expansion of the journal would be based on quality of material available and demand.

Financing the AIN Secretariat: It is proposed that a grant be sought from a foundation to cover the initial budget of the secretariat. To insure continuity for a reasonable period, the grant request should be for five years.

Anticipated Budget:

Executive Secretary's salary	$21,000	($18,000–$22,000)
Assistant Secretary	4,500	(4,000–5,000)
Fringe benefits, 12%	3,000	
Rent and office expense	6,500	
Total	$35,000	

During the period that the grant is in effect, efforts must be made to improve the financial position of the AIN commensurate with its increased financial responsibilities. A reserve or contingency fund should be accumulated from the following sources:

A. A modest increase in dues.
B. Revenue from page charges from the *Journal of Nutrition* should be accumulated over and above the necessary financial obligations to the editorial management of the journal not covered by Wistar.
C. The share of the registration fees from the annual meeting should be placed in this contingency reserve.
D. The Council should explore institutional memberships which should be instituted as soon as the appropriate changes can be made in the AIN Constitution.
E. All funds accumulated in the contingency fund in excess of $2,000 each year be used to support the office of the AIN Secretariat and reduce thereby the grant needs by this amount. We are asking support to underwrite the start and early development of the AIN Secretariat with reasonable expectation that our contingency reserve would accumulate a substantial amount of money each year and hence reduce the grant support need accordingly.

This Committee is facing the membership and Council with one of the most important decisions in the history of the AIN. However, this proposal emphasizes that to further the knowledge of nutrition is a charge of our Constitution. We therefore ask that the AIN assume the responsibility of leadership which recognizes that food and nutrition science properly employed may be the single most important environmental factor in improving the health of man. We ask also that our Society assume its rightful leadership in the development of sound nutrition education programs for those professional people interested in nutrition and bring to it the unifying force and demonstrated abilities so abundantly evident in our membership. If we accept these broadened responsibilities, we must also assume the associated increased cost and burden. The Secretariat Committee strongly urges that this not be a delaying decision, but one reached with mature judgment and promptly.

XI. *Reports of Committees and Representatives*

A. *Committee on Nutrition Training and Fellowships:* Dr. L. C. Norris, chairman, and Dr. J. McGinnis, co-chairman.

Dr. Norris presented a suggested outline for a curriculum in nutrition which covered both undergraduate and graduate training. This report was accepted by the Council with the recommendation that it be turned over to a committee to be appointed by the incoming president for use in preparing a brochure on careers in nutrition and training required. The Committee's report follows.

Programs of undergraduate and graduate studies have been prepared by the American Institute of Nutrition for the purpose of indicating the type of training needed by students in preparation for careers of teaching, research and technical service in the field of nutrition. The programs have been developed for the purpose of guidance only and are not intended to represent rigid requirements. The undergraduate program will of necessity vary somewhat depending upon the requirements of the institution in which the student is registered.

In colleges of Arts and Sciences, undergraduates may have difficulty in taking work in nutrition. In this case dependence must be placed upon courses in physiology and biochemistry to provide some background in nutrition. In colleges of agriculture and home economics it may not always be possible to include physical chemistry in the undergraduate curriculum. However, physical chemistry, except in special circumstances should not be omitted, since it is necessary for work in advanced biochemistry. Additional work in biological and animal science may be taken to advantage when this does not conflict with the student's special requirements for graduation.

Laboratory work has not been specified in all courses in the biological sciences in the undergraduate program. It is desirable in these instances, provided the lecture part of the course gives a broad coverage.

The graduate program for the Ph.D. degree in nutrition is not as detailed as the undergraduate program, largely because graduate work usually represents levels of learning and accomplishment rather than acquiring a certain number of specific units of credit. Thus, advanced courses are taken for the purpose of attaining these ends and vary somewhat among institutions. The work needed to support the major will also vary depending upon the student's special interests in nutrition. The minimum time required to complete work for the Ph.D. degree is three years, but four years may be needed, if the student's preparation is insufficient.

The graduate program for the M.S. degree in nutrition is similar to that for the Ph.D. degree but less work is required. At some institutions two types of graduate programs may be taken for this degree. In general, however, the program calling for a written thesis, based upon research work with its accompanying training in experimental procedures is to be preferred, particularly for students planning upon careers of teaching and/or research in nutrition. One year, at least, is required to complete work for the M.S. degree.

Suggested Undergraduate Program for Students Preparing for Graduate Work in Nutrition

	Units
Mathematics:	
Analytical geometry and calculus	6–9
Physical sciences:	
General and qualitative chemistry*	8–10
Quantitative analysis *	3–4
Physical chemistry	6
Organic chemistry *	6–10
Physics *	6–8
Biological sciences:	
General biology or zoology *	6–8
Physiology	6
Microbiology *	4–5
Biochemistry *	6
Nutrition and food science	6
Humanities and social sciences:	
English	6
Foreign language	6–8
Restricted electives (not less than 3 subjects)	15–18
Anthropology, economics, geography, history, philosophy, political science, physchology and sociology	
Average for nutrition preparation	95–100
Additional college requirements and electives	25
TOTAL for graduation	120–125

* To include laboratory.

Suggested Graduate Program for the Ph.D. Degree in Nutrition

	Units
Major field:	
Advanced nutrition	10–12
Experimental nutrition laboratory	3–4
Nutrition seminar	2 per yr
Biostatistics	4–6
Foreign language (reading knowledge of two)	
Thesis: research project in animal, human, or clinical nutrition	
Minor fields:	
Advanced biochemistry	8–10
Biochemical techniques laboratory	4–6
Advanced physiology	6
Experimental physiology laboratory	3–4

Substitute minor fields:

Advanced microbiology, advanced chemistry, food science and technology, or pathology and related subjects, lectures and appropriate laboratory courses, plus at least 6 units each of lectures in advanced biochemistry and advanced physiology to support the nutrition major. Total, approximately 25 units.

Optional special interest subjects:

Public health and anthropology may be taken to supplement the above minor fields, or in particular instances developed into minor fields provided at least 6 units of lectures in advanced biochemistry and advanced physiology are taken in support of the nutrition major.

Suggested Graduate Program for the M.S. Degree in Nutrition

The graduate program in nutrition covers the amount of training expected of students preparing for the Ph.D. degree. Students preparing for the M.S. degree would ordinarily take about 18 to 20 units of the course work expected of Ph.D. degree students, plus a thesis research project in animal, human or clinical nutrition, or in some instances approximately 30 units of this course work together with a report on a special problem, requiring little or no research.

Dr. McGinnis pointed out that the Resident Associateships sponsored by the National Science Foundation and NAS–NRC do not list nutrition as a field of study for postdoctoral Fellows. No nutrition laboratory is listed among the governmental institutions where these Fellows may work. The Federal Civil Service does not include nutrition as a specialty and the Educational Testing Service does not list nutrition as an advanced area test for scholarship training programs.

To correct these deficiencies, Dr. McGinnis introduced the following resolutions which were approved at the business meeting:

WHEREAS the science of nutrition with its different areas of specialization is recognized in many colleges and universities through the offering of organized programs of study and training leading to the Master's and Ph.D. degrees, and in some cases to Bachelor's degrees, and in some universities by the existence of nutrition departments and by industrial firms through employment of scientists classified as nutritionists, adequate recognition of nutritionists and the science of nutrition as a distinct scientific discipline by agencies and departments of the Federal Government is lacking;

WHEREAS opportunities for postdoctoral research experience are not available in the fields listed by NAS–NRC under the Postdoctoral Research Associateship Program sponsored by different Government laboratories and research centers;

WHEREAS students interested in pursuing graduate study in nutrition must take advanced area tests as a part of their application for various types of Fellowships awarded by NSF, which tests are not felt to be entirely appropriate for testing their mastery of subject matter in physical and biological sciences offered at the undergraduate level; and,
WHEREAS the failure to recognize nutritionists and the science of nutrition as a discipline detracts from our efforts to attract adequate numbers of the most capable students into graduate study in nutrition and, in addition, hampers our efforts to find the most favorable and appropriate employment of nutritionists and to provide opportunities for postdoctoral research experiences comparable to those in other fields, the following resolutions were adopted by the American Institute of Nutrition:

RESOLVED: That the American Institute of Nutrition, assembled at Chicago, Illinois, in its annual meeting, April 16, 1964, urge the National Academy of Sciences — National Research Council, to recognize nutrition as a discipline in the basic and applied natural sciences and further, urge that nutrition be listed among the many different fields (approximately 50) that are available in the Postdoctoral Resident Research Associateship Program administered by NAS–NRC, and, further, that the AIN recommend that the different laboratories and research centers of the Federal Government conducting nutrition research support such Research Associateships in Nutrition and designate laboratories or research centers at which Research Associateships in Nutrition are tenable.
RESOLVED: That the American Institute of Nutrition, assembled at Chicago, Illinois, in its annual meeting, April 16, 1964, recommend to the Fellowships Section, National Science Foundation, Washington, D. C., that plant, animal and human nutrition be included among the many separate and distinct fields of interest or study eligible for NSF Fellowship support and that the AIN further recommend to the Educational Testing Service, Princeton, New Jersey, the development of a Graduate Record Examination Advanced Test in Nutrition, with said test to be designed to measure mastery of subject matter in biological and physical sciences presented at the undergraduate level essential for successful graduate study in nutrition.

RESOLVED: That the American Institute of Nutrition, assembled at Chicago, Illinois, in its annual meeting, April 16, 1964, through its appropriate offices and appointed committees recommend to the Civil Service Commission, Washington, D. C., that human and animal nutrition, with its appropriate subdivisions and fields of specialization be designated an *optional field* for employment of research scientists in the different departments of the Federal Government conducting nutrition research and, in addition, work with the said Civil Service Commission and appropriate departments of Government in the development of a classification series to provide for the several grade levels required and, in addition, assist the Civil Service Commision in the establishment of qualification standards for each level and developing a suitable examination for use in recruitment.

B. *Committee on Publication Management:* Dr. W. J. Darby, chairman.

The Journal Management Committee has not met since April, 1963. The Chairman has continued exploratory discussions with other interested publishers relative to support of a new journal owned by the AIN.

It is recommended that essentially the present agreement be extended with Wistar, making the necessary upward adjustment in the budgeted costs for the editorial office.

C. *Public Information Committee:* Dr. Philip L. White, chairman.

The Public Information Committee has responded to inquiries addressed to the Institute. A large number of such queries have been answered by members of my department at the American Medical Association.

The American Institute of Nutrition is listed in a number of career guidance registries. Current listings have been reviewed and kept up to date. The pamphlet "Career Opportunities in Nutrition" is being revised; nevertheless, a great number of the original version have been mailed upon request.

The Public Information Committee screens all nutrition abstracts for possible newsworthiness and reports to the manager of the News Room. The cooperation received from authors, chairmen of sessions and moderators of symposia is very much appreciated. This year, for the first time, news releases were prepared announcing the various AIN award winners and the new Fellows of the Institute. This should help to enhance the awareness of the awards.

The Public Information Committee will provide publicity coverage for the Canadian — U. S. Conference on Nutrition.

D. *Representative to the AAAS Council:* Dr. James M. Orten.

The 130th Meeting of the AAAS was held in Cleveland, Ohio, December 27–30, 1963, with Dr. Alan T. Waterman presiding.

The AAAS continues to increase in size as a "superfederation" of 312 affiliated and associated societies and academies of science, as reported by Dr. Paul M. Gross, chairman of the Board of Directors. The AAAS has a direct active membership of over 90,000, making it the world's largest scientific association. The Council itself totals some 475 members, of which approximately 250 attended the two Cleveland Council meetings. The AAAS Council is concerned wtih problems common to all of the sciences, as for example that of the education of scientists and of prospective scientists at the high school and even elementary school levels. The Council meetings consisted primarily of reports of various committees and discussions of the reports. These are summarized below:

1. *The Committee on Nominations and Elections* reported the election of Dr. Henry Eyring, professor and chairman of the Department of Chemistry at the University of Utah, as president-elect. Drs. David Goddard and A. F. Spilhaus were elected members of the Board of Directors.

2. *The Committee on Council Affairs* reported its study of the method of election of officers by section committees, with approval of the procedure.

3. *The Study Committee on Natural Areas as Research Facilities* submitted a report of 347 pages plus four appendices. The report covers: I. Introduction and Recommendations; II. Bibliography of Research done on Natural Areas; III. Tentative Inventory of Natural Areas in the U. S.; IV. Agencies and Organizations Holding or Administering Natural Areas; V. Preservation; VI. Published Directions for Conduct of Research on Natural Areas with a list of published books available for use in research training utilizing natural areas; VII. Foreign Natural Areas. Copies of the report are available from the AAAS.

4. *The Committee on Affiliation* recommended that the Council grant affiliation to the following four organizations: (1) Society for the Scientific Study of Religion; (2) Society for Economic Botany; (3) American Fisheries Society; and (4) Division of Chemical Literature, American Chemical Society. The Council approved.

5. *Committee on Science in the Promotion of Human Welfare* discussed its study of the air pollution problem and recommended the establishment of a commission to report on the problems and social applications of "air conservation." The scientific background of the race question was also discussed (see November 1 issue of *Science*).

6. *Committee on Public Understanding of Sciences* reported continued use of the medium of television under NSF support, and of seminars and lecture series in bringing scientific research topics to the general public.

7. *Committee on Cooperation among Scientists* report indicated that satisfactory progress was being made in acquainting scientists with problems in other areas.

8. *Reports by the Board of Directors* were presented regarding the selection of new section secretaries and new members-at-large of Section Committees. The Council elected new members of the Committee on Nominations and Elections and new vice-presidents and section chairmen from the slates of nominees presented.

9. *Report of the Executive Officer and Presentation of the 1964 Budget* was given by Dr. Dael Wolfie as follows:

a. During 1963 over 19,000 new members joined AAAS, the largest number for a single year.

b. Receipts of approximately $2,400,500 for 1963 were reported with expenditures of $2,222,510. In addition, the Association expended some $700,000 during 1963 on activities supported by grants from private foundations and government agencies, which included the Traveling Science Libraries, studies of the qualifications of science teachers, Holiday Lecture Series, and a Symposium on the Sciences in Japan presented at Cleveland.

Total income for 1964 was estimated at $2,826,000 and total expenses as $2,719,500, leaving $106,500 as net receipts.

10. *Discussion of scientists' responsibilities* dealt with problems of a scientist's professional responsibilities to his institution or employer, his students or assistants, his colleagues, his sources of research support, and others. These relationships involve questions of ethics and of what constitutes appropriate or good practice.

11. *Under new business,* it was pointed out that the AAAS had adopted a firm policy some years ago that meetings of the Association would be held only in cities in which the segregation of public facilities is forbidden.

The representative of the Alaska Division invited all members to attend the Annual Alaska Science Congress to be held from September 1–4 in College, Alaska.

E. *World Conference on Animal Production:* Dr. L. C. Norris, delegate, and Dr. G. K. Davis, alternate. Both attended the conference in Rome, Italy, September 2–7, 1963.

The purpose of the Conference was to review the present knowledge of animal science and technology and related field problems to arrive at solutions of the problems and thereby increase the production of animal products needed in the human dietary.

The Conference was organized by the European Association for Animal Production, American Dairy Science Association, American Society of Animal Science, Australian Society of Animal Production, Canadian Society of Animal Production, Japanese Society of Zootechnical Science, New Zealand Society of Animal Production and South African Society of Animal Production. The Conference was conducted in collaboration with the FAO, the Soybean Council of America, the Feed Grains Council, the National Renderers' Association and the Foreign Agricultural Service of the USDA.

The program consisted of review papers on animal breeding and genetics, animal physiology

and animal nutrition. The discussions frequently reflected the differences in animal science in the various countries.

Special emphasis was given at the Conference to animal nutrition. Of the 70 papers presented, 47 were either in the field of animal nutrition or closely related to it. Although the primary interest was in increasing the supply of animal proteins throughout the world, there was considerable discussion of the need in some areas to increase first the supply of vegetable protein.

F. *Representative to NRC Divisions and Boards:* Dr. G. F. Combs.

On October 22, 1963, the National Academy of Sciences celebrated its 100th year, on which occasion President Kennedy delivered the convocation address.

The Division of Biology and Agriculture assisted in the XVIth International Congress of Zoology held in Washington, D. C. It also assisted in the following symposia: Synthesis and Structure of Macromolecules; Photosynthesis of Green Plants; Molecular Mechanism in Photobiology; Biological Control of Soil-Bourne Plant Pathogens; and Genetics of Colonizing Species.

In cooperation with other Divisions and NAS-NRC groups, the Division of Biology and Agriculture established an ad hoc committee to determine the interest of U. S. scientists in the International Biological Program, sponsored by the International Council of Scientific Unions. This committee will identify the relevant biological research problems which might be studied more effectively through international cooperation and make recommendations concerning possible modifications of the program which would result in more effective U. S. participation. Dr. George K. Davis is a member of this committee.

The Food and Nutrition Board published NAS Pub. 1100, 1963, entitled *Evaluation of Protein Quality;* NAS Pub. 1146, *Recommended Dietary Allowances,* 1963; and a statement on *Recommendations for Administrative Policies on International Food and Nutrition Programs,* June, 1963.

The 1963 revision of the Dietary Allowances shows increases in the iron allowance for women, and some reductions in the energy, protein, calcium, thiamine, riboflavin, niacin, and ascorbic acid allowances for certain age groups.

Other publications of the Food and Nutrition Board which should be available soon deal with *Problems in Evaluation of Public Health Hazards from Microbiological Contamination of Foods; Dietary Fats in Human Health* (NAS Pub. 1147), and a revision of *The Use of Chemical Additives in Food Processing.*

The Committee on Animal Nutrition of the Agricultural Board has revised the Nutrient Requirements of Beef Cattle, NAS Pub. 1137, 1963, and revised publications concerning rabbits, swine, and sheep and lambs will be available in the next few months. Six of these publications are being translated into Spanish by AID. The Proceedings of the fall meeting of the Agricultural Research Institute are now available. The theme of this meeting was "Agricultural Research in the Space Age."

A report, NAS Pub. 1182, *Federal Support of Basic Research Institutions of Higher Learning* was issued by a committee on Science and Public Policy. This report emphasizes that the health of the research project support system requires, (1) the responsibility of the government for the expenditure of public funds, (2) the independence of universities and, (3) the freedom of the scientist to conduct his research, reach his conclusions in his own way, and make them public.

In March, 1964, the Advisory Committee on Research to the Bureau of Commercial Fisheries held a two-day meeting concerning the development of high quality fish protein concentrates for consumption by humans.

A Working Conference on Space Nutrition and Related Waste Problems was held in Tampa, Florida on April 27–30, 1964, under the auspices of the Space Science Board, NAS and the National Aeronautics and Space Administration. The objectives of this conference were "to collect the physiological, psychological, toxicological and pathological data related to space nutrition, feeding and elimination and to elucidate the man-machine interactions, and their effects on space nutrition and related waste problems for manned space missions of 20 days to 3 years' duration."

G. *International Union of Biological Sciences:* Dr. George K. Davis.

The International Biological Program was approved by the International Union of Biological Sciences. If the report of the committee which worked on the program is approved, a permanent operational committee will be appointed.

The present plan was developed by European scientists. It gives little attention to nutrition and ecology. The U.S. delegates will urge changes in the original plan as proposed by U.S. scientists.

If the program is accepted, the National Academy of Sciences will invite suggestions for committee membership and plans for implementation of the program.

H. *Representative to the Food and Agriculture Organization:* Dr. B. S. Schweigert.

FAO has continued to highlight programs in applied nutrition projects throughout the world and international conferences related to improving the food supply for the nation's expanding population. I detect an increasing awareness by this organization on the interrelationships of various scientific approaches including nutrition and food science, and the contribution it can make to the overall problem of improving the health of the people in developing countries.

I. *Report of the Ad hoc Committee on International Nutrition:* Dr. W. N. Pearson, chairman.

The Committee plans to (1) survey the AIN members to determine their possible association

with international nutrition programs; (2) establish a medium for the publication of news of foreign nutrition; (3) publish a series of solicited review articles that would deal with (a) descriptions (history, physical plants, staff, current interests, etc.) of selected foreign nutrition research centers and (b) activities of various agencies that involve foreign nutrition programs.

The Survey: The AIN members were queried regarding their involvement in international programs. The questionnaire will provide an estimate of the pool of nutritionists in foreign countries who have received training in this country.

A News Medium: News items should be published in a special section of the Journal of Nutrition which would include items of international interest. This would assure coverage far exceeding the membership of the AIN.

Special Articles: It is proposed to publish these in the American Journal of Clinical Nutrition. The authors would have to meet the ground rules laid down by the editorial board of the Journal. Institutions described might include Rowett Research Institute, Dunn Nutritional Laboratory, INCAP, Mysore Food Technology Institute, etc. The foreign nutrition activities of the Kellogg Foundation, USAID, the Columbia Institute of Nutrition, the ICNND, The NRC, PAG, etc. might be described.

AIN Participation in IBP: I believe it would be most unfortunate if the AIN did not participate in some way in the International Biological Program. Although nutrition is not mentioned prominently in the report of the planning committee of this organization, it is obvious that their program demands sound nutrition counsel. This particularly applies to Sections D and E which deal with human adaptability and use and management of biological resources. It is my opinion, therefore, that AIN participation is almost mandatory.

Other Items: (1) The AIN should sponsor the preparation of a nutrition education syllabus for use in all age groups from pre-school children to postgraduates. The authors (all with field experience) would record those basic nutritional principles that would be understood by pre-school children, pregnant women, nurses, etc. This syllabus would be published in several languages and would not be intended for direct use in teaching but rather for adaptation to the specific needs of the country.

(2) The AIN should establish a new award in nutrition for significant contributions outside the area of laboratory research. This award would recognize outstanding achievement in the use of nutritional knowledge. Such an award should be designated the "Conrad Elvehjem Award for Public Service in Nutrition."

J. *Committee on Careers in Nutrition:* Dr. T. B. Van Itallie, chairman.

The Committee suggested:

1. Sponsorship of nutrition clubs (the method of implementing this should be carefully considered). A number of universities have such clubs now. They are oriented primarily to the students majoring in dietetics. Since there are other groups interested in nutrition, the Institute needs to define its goals in this area and determine the group(s) of students at which such clubs would be aimed.

2. The brochure "Careers in Nutrition" should be revised and expanded to include a list of institutions offering graduate work in nutrition with a brief description of the types of programs offered.

K. *Joint Canadian — U.S. Conference on Nutrition:* Dr. H. H. Williams.

Dr. Beveridge resigned as chairman of the committee when he was appointed President of Acadia University. Dr. Williams succeeds him.

The abstracts for the meeting are to be published in the July — August issue of Federation Proceedings. The symposia papers may be published in the Canadian Journal of Physiology and Biochemistry.

The Council authorized a payment of $300 to the Canadian Local Committee for printing, etc. The Council accepted the responsibility of sharing in any financial deficit that might accrue from the Conference. Should a surplus exist at the end of the Conference, AIN will be reimbursed for its expenses; any funds remaining beyond that are to be set aside for future joint conferences.

L. *Report of the Clinical Division:* Dr. J. F. Mueller, Secretary-Treasurer, ASCN.

The ASCN received 36 applications for membership and approved 27. Seven of these already were AIN members.

About twice as many abstracts were received this year as last for the annual meeting. The annual meeting in Atlantic City will include 12 papers. The Constitution and By-laws of ASCN were amended to insure exemption from Federal income tax.

Funds have been provided by the National Dairy Council for a $1000 Award for Meritorious Research in Human Nutrition.

There are 138 active members in ASCN.

It was moved that ASCN members be designated in the Federation Directory as 5C.

ANNUAL DINNER AND PRESENTATION OF FELLOWS AND AWARDS

The annual banquet was held Wednesday, April 15, 1964 at the Sheraton-Blackstone Hotel, with 373 individuals attending. Dr. Goldsmith presided.

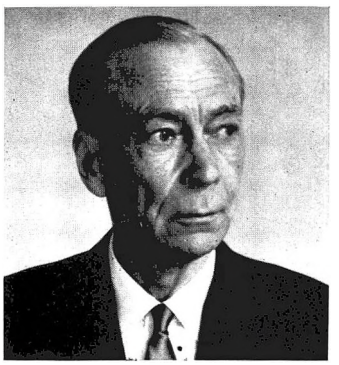
Dr. Icie Macy Hoobler introduced the newly appointed Fellows, whose citations follow:

JOHN BERNIS BROWN

JOHN BERNIS BROWN — "Distinguished teacher and investigator in the field of nutrition; contributor of fundamental knowledge to the chemistry of nutritionally important fats and oils and to their nutritional significance."

HAZEL K. STIEBELING

HAZEL K. STIEBELING — "A contributor of important and significant information to the nutrition of man through her research leadership in the fields of nutritive value of foods, nutritional requirements and the nutrient patterns of families and individuals and the application of such knowledge to programs for improving the nutrition of people; a leader in international programs to improve the diets and nutritional status of people throughout the world."



HENDRIK DAM

HENRIK DAM — "A contributor of fundamental information in the metabolism of fats and in the relation to nutrition of the fat-soluble vitamins; especially vitamin K in its many physiological functions, and vitamin E in its relation to antioxidants, to encephalomalacia, exudative diathesis, and muscular dystrophy."

Osborne and Mendel Award

The 1964 Osborne and Mendel Award of $1000 and a scroll was presented to Dr. L. Emmett Holt, Jr., Professor of Pediatrics, New York University School of Medicine. This was given for Dr. Holt's pioneering contributions to the knowledge of protein and amino acid requirements of infants; his studies of the biological value of proteins used in infant feeding and his investigations of the metabolism and requirements of thiamine, riboflavin and niacin.

Borden Award in Nutrition

The 1964 Borden Award of $1000 and a gold medal was presented to Dr. George K. Davis, Research Professor of Nutrition and Director of Nuclear Sciences at the University of Florida. The award was presented for a series of investigations in biochemistry and nutrition with major emphasis on metabolic and nutritional interrelationships of the trace elements, copper, cobalt, molybdenum and zinc; the utilization and placental transfer of calcium and phosphorus and the application of radioisotopes to the investigation of mineral deficiency diseases in grazing animals. These studies have contributed to a clearer understanding of the relationships between trace element deficiencies of the soil and the nutrition and health of farm animals.

Mead Johnson Award for Research in Nutrition

The 1964 Mead Johnson and Company Award of $1000 and a scroll was awarded to Dr. James Smith Dinning, Professor of Biochemistry at the University of Arkansas School of Medicine. It was presented for his research on the function of vitamin E and related compounds in maintaining normal hemopoietic activity in primates; in particular for his recent series of papers dealing with identification of an anemia associated with vitamin E deficiency in the monkey and man which is responsive to vitamin E and coenzyme Q-chromanol.

L. EMMETT HOLT, JR.

GEORGE K. DAVIS

JAMES SMITH DINNING

AMERICAN INSTITUTE OF NUTRITION

Founded April 11, 1933; Incorporated November 16, 1934; Member of Federation 1940

OFFICERS, 1964–1965

President: R. W. Engel, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia.

President-Elect: J. B. Allison, Research Council, Rutgers University, New Brunswick, New Jersey.

Past President: Grace A. Goldsmith, Tulane University School of Medicine, New Orleans, Louisiana.

Secretary: Olaf Mickelsen, Department of Foods and Nutrjtion, College of Home Economics, Michigan State University, East Lansing, Michigan (1966).

Treasurer: D. V. Frost, Research Division, Abbott Laboratories, North Chicago, Illinois (1965).

Councilors: H. H. Williams (1965), D. M. Hegsted (1966), N. S. Scrimshaw (1967).

COMMITTEES

Nominating Committee: R. E. Hodges chairman; G. F. Combs, Gladys Everson, H. E. Sauberlich, B. S. Schweigert.

Joint Committee on Biochemical Nomenclature (joint with American Society of Biological Chemists): Vernon Cheldelin (1965), Stanley R. Ames (1966).

Committee on Publication Management: W. J. Darby, chairman (1966); P. L. Harris (1966), J. K. Loosli (1966), J. M. Hundley (1965), George Berryman (1965). Ex officio: Olaf Mickelsen, John F. Mueller.

Nominating Committee for Borden Award: L. D. Wright, chairman (1966); R. E. Shank (1965), E. L. Hove (1967).

Nominating Committee for Osborne-Mendel Award: F. W. Hill, chairman (1965); N. S. Scrimshaw (1966), M. O. Schultze (1967).

Nominating Committee for Mead Johnson Award: Carl A. Baumann, chairman (1965); B. Connor Johnson (1966), P. L. Day (1967).

Fellows Committee: James Waddell, chairman (1965); Charlotte Young (1966), Paul E. Howe (1966), S. L. Hansard (1967), Paul E. Johnson (1967).

Public Information Committee: P. L. White (1966), chairman; M. S. Read (1966), Allen D. Tillman (1966), L. J. Teply (1966), J. M. R. Beveridge (1965), Allen L. Forbes (1965), Samuel E. Tove (1965), Martha F. Trulson (1965). Ex-officio: Olaf Mickelsen (1966).

Committee on Honorary Memberships: L. C. Norris, chairman; L. A. Maynard, F. S. Daft.

Auditing Committee: J. H. Roe (1965), Ruth M. Leverton (1965).

Tellers' Committee: C. K. Whitehair (1965), L. R. Arrington (1965).

Symposium Committee: H. H. Williams, chairman; Myron Brin, Leo Friedman, M. K. Horwitt, Lloyd Matterson, Edmund S. Nasset.

Ad hoc Committee on Establishment of an Executive Secretariat: Floyd S. Daft, chairman; George M. Briggs, Willard A. Krehl, E. L. Severinghaus, P. L. White.

Ad hoc Committee on International Nutrition: William N. Pearson, chairman; Guillermo Arroyave, C. F. Asenjo, K. W. King, A. E. Schaefer, W. C. Unglaub, Calvin Woodruff.

Ad hoc Committee on Nutrition Training and Fellowships: L. C. Norris, chairman; James McGinnis, co-chairman; C. O. Chichester, James S. Dinning, Wendell H. Griffith, R. E. Hodges, O. Neal Miller, Clara A. Storvick.

Ad hoc Committee on Nutrition Careers Brochure: F. J. Stare, chairman; James McGinnis, T. B. Van Itallie, C. Edith Weir, E. L. R. Stokstad, Martha Trulson.

Ad hoc Hoblitzelle Award Nominating Committee: L. C. Norris, chairman; R. M. Forbes, J. E. Oldfield, W. H. Pfander, George Wise.

Organizing Committee for Joint Conference with Nutrition Society of Canada: H. H. Williams, chairman; M. O. Lee, D. A. Richert.

Committee on Recommended Constitutional Changes: A. E. Axelrod, chairman; L. M. Henderson, Marian E. Swendseid, U. D. Register, W. J. Visek.

Committee on Experimental Animal Nutrition: G. F. Combs, chairman; E. W. Crampton, F. W. Hill, J. K. Loosli, R. W. Luecke, L. D. Matterson, W. F. Pfander.

Finance Committee: G. M. Briggs (1965), chairman; W. A. Krehl (1966), A. E. Schaefer (1967). Ex officio: D. V. Frost, treasurer, (1965).

Membership: H. P. Sarett (1965), chairman; G. P. Barron (1966), M. L. Scott (1967), K. W. King (1968), Dena C. Cederquist (1969).

U. S. National Committee, IUNS: Paul György (1965), W. M. Beeson (1965), G. F. Combs (1966), Charlotte Young (1966), O. L. Kline (1966), F. S. Daft (1965), C. G. King (1967), W. J. Darby (1967), W. A. Krehl (1967). Also ex-officio (voting) member: R. W. Engel (1965), and ex officio (non-voting) members: T. C. Byerly, R. K. Cannan, Harrison Brown, E .C. Rowan.

Representatives to Other Organizations

Federation Board: R. W. Engel (1966), Grace A. Goldsmith (1965), J. B. Allison (1967).

Federation Advisory Committee: Grace A. Goldsmith (1965).

Editorial Board of Federation Proceedings: P. L. Harris.

Federation Public Information Committee: Philip L. White (1965).

American Association for the Advancement of Science: James M. Orten (1965), delegate; E. W. Crampton (1966), alternate.

Food and Agriculture Organization: B. S. Schweigert (1966).

National Research Council Boards and Divisions: G. F. Combs (1965).

Editorial Board
The Journal of Nutrition

R. H. Barnes, editor (1969); H. H. Williams (1969) associate editor; E. Neige Todhunter, biographical editor (1969); A. E. Axelrod (1965), J. G. Bieri (1966), G. M. Briggs (1967), Harry P. Broquist (1966), G. K. Davis (1965), R. H. Follis, Jr. (1965), R. M. Forbes (1967), R. G. Hansen (1966), L. M. Henderson (1968), F. W. Hill (1968), Jules Hirsch (1967), R. T. Holman (1966), E. E. Howe (1967), Gennard Matrone (1968), E. L. R. Stokstad (1965), Clara A. Storvick (1968).

Officers, American Society for Clinical Nutrition
(A division of the American Institute of Nutrition)

President, W. A. Krehl; Vice-President/President-elect, Charles S. Davidson; Past President, W. Henry Sebrell, Jr.; Secretary-Treasurer, John F. Mueller, Brooklyn-Cumberland Medical Center, 121 DeKalb Avenue, Brooklyn, New York; Councilors: Robert E. Shank, Theodore Van Itallie, Robert M. Kark.

Editorial Board
American Journal of Clinical Nutrition

W. A. Krehl (1967), editor; Robert E. Hodges, associate editor; M. M. Wintrobe, William D. Robinson, Robert E. Olson, D. Mark Hegsted, Donald Watkin, Maurice E. Shils, Margaret Albrink, George Gabuzda, Ernest Beutler, Robert L. Jackson.

Respectfully submitted,

OLAF MICKELSEN, *Secretary*

INVITATION FOR NOMINATIONS FOR THE 1965 HOBLITZELLE NATIONAL AWARD IN THE AGRICULTURAL SCIENCES

The Hoblitzelle Award Committee of the American Institute of Nutrition invites nominations for the 1965 Hoblitzelle National Award in the Agricultural Sciences.

The Hoblitzelle Award, consisting of $10,000, a gold medal and an attesting certificate, is presented in recognition of the outstanding contribution in the agricultural sciences which has been published during the preceding four-year period. The donor of the Award is the Hoblitzelle Foundation of Dallas, Texas. The Award is administered by the Texas Research Foundation, Renner, Texas.

All American scientists working in the United States and its territories, irrespective of creed, color, nationality, sex, age, branch of science, or affiliation with scientific or scholastic organizations, are eligible. Either an individual or team of scientists may be nominated for the Award, but a team must include only those scientists who have contributed the basic ideas. Preferably no more than two scientists should be nominated as a team.

This Award covers research in agronomy, animal science, bacteriology, biochemistry, botany, entomology, genetics, horticulture, nutrition, soil science, veterinary science, zoology, and such other sciences as may be deemed to serve agriculture in their broadest aspects.

The various professional societies related to the agricultural sciences, the Agricultural Research Service and the Agricultural Experiment Stations have been requested to receive and screen nominations for the Award. Each may forward three nominations to the Texas Research Foundation from which the Final Awards Committee will select three candidates. The Hoblitzelle Foundation will select the recipient, or recipients, from these candidates.

The nomination for the Award must be accompanied by a complete set of publications by the scientist covering his scientific accomplishment, and a comprehensive evaluation of the potential significance of the work. The dates of publications for the 1965 Award must fall within the period January 1, 1960 through December 31, 1963. Final date for submission of nominations is November 15, 1964.

In 1953 AIN member Dr. Lorin E. Harris, Utah State University, was corecipient of the Hoblitzelle Award with Dr. C. Wayne Cook for the development of accurate methods of measuring the composition and nutritive value of the diet of animals grazing under actual range conditions.

Hoblitzelle National Award Committee:

L. C. Norris, *Chairman*

R. M. Forbes — W. H. Pfander

J. E. Oldfield — George Wise

Send nominations to:

Dr. L. C. Norris
College of Agriculture
Department of Poultry Husbandry
University of California
Davis, California 95616

Invitation for Nominations for 1965 American Institute of Nutrition Awards

Nominations are requested for the 1965 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination. A bibliography and supporting letters are not to be submitted. (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1964*, to be considered for the 1965 awards.

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

Presentation of awards will be made at the annual dinner at the annual meeting.

1965 Borden Award in Nutrition

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

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis

NOMINATING COMMITTEE:
L. D. WRIGHT, *Chairman*
R. E. SHANK
E. L. HOVE

Send nominations to:

DR. L. D. WRIGHT
Graduate School of Nutrition
Savage Hall, Cornell University
Ithaca, New York

1965 Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
1964 – L. Emmett Holt, Jr.

NOMINATING COMMITTEE:
F. W. HILL, *Chairman*
NEVIN SCRIMSHAW
M. O. SCHULTZE

Send nominations to:
DR. F. W. HILL
Department of Poultry Husbandry
University of California
Davis, California 95616

1965 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 46th birthday during the calendar year in which the Award is given. Selection by the Jury of Award will be based primarily on a single outstanding piece of research in nutrition published in the year preceding the annual meeting or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hogan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
1952 – H. E. Sauberlich
1964 – J. S. Dinning

NOMINATING COMMITTEE:
CARL BAUMANN, *Chairman*
B. CONNOR JOHNSON
P. L. DAY

Send nominations to:
DR. CARL BAUMANN
Department of Biochemistry
University of Wisconsin
Madison, Wisconsin 53706

Invitation for Nominations for 1965 American Institute of Nutrition Fellows

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

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

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

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

Fellows Committee:

JAMES WADDELL, *Chairman*
CHARLOTTE M. YOUNG
PAUL E. HOWE
S. L. HANSARD
PAUL E. JOHNSON

Send nominations to:

DR. JAMES WADDELL
907 Center Road
Westover Hills
Wilmington, Delaware 19806

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

J. B. Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Henrik Dam (1964)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Wendell H. Griffith (1963)
Albert G. Hogan (1959)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Helen T. Parsons (1961)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
Arthur H. Smith (1961)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Robert R. Williams (1958)

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

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

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

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

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

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

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

Committee on Honorary Memberships:

L. C. NORRIS, *Chairman*
L. A. MAYNARD
F. S. DAFT

Send nominations to:

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

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

Kunitaro Arimoto
W. R. Aykroyd
Frank B. Berry
Edward Jean Bigwood
Frank G. Boudreau
Robert C. Burgess
Harriette Chick
F. W. A. Clements
David P. Cuthbertson
Herbert M. Evans
Toshio Oiso
Lord John Boyd Orr
V. N. Patwardhan
Emile F. Terroine
Eric John Underwood
Artturi I. Virtanen

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