Effects of Dietary Safflower Oil or Hydrogenated Coconut Oil on Growth Rate and on Some Blood and Tissue Components of Pigs fed a Fat-free Diet

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ABSTRACT The effects of feeding diets containing no fat, 3% hydrogenated coconut oil (HCO) or graded levels (0.1, 0.5, 1.0 or 3%) of safflower oil (SO) on serum cholesterol, lipids and protein level; on total heart and liver cholesterol and lipid level and on growth rate and skin condition were studied in weanling Yorkshire and Yorkshire × Hampshire pigs. Pigs fed the fat-free diet for 21 weeks developed severe skin lesions not observed in pigs fed HCO diets. Highly significant elevation of serum, liver and heart lipid and liver cholesterol, and a highly significant depression of total serum protein were observed with the feeding of HCO or fat-free diets. Serum cholestrol was significantly increased by HCO as compared with the fat-free diet or diets containing SO during the repletion period. Total heart cholesterol, growth rate and erythrocyte fragility were unaffected by diets. It is concluded that growth rate is not adversely affected in the pig by fat-free diets and that HCO does not produce skin lesions in the pig characteristic of fatty acid deficiency. Of all the correlation coefficients analyzed, only the serum cholesterol and total serum lipids were consistently highly significantly correlated, while serum protein was significantly inversely related to the total liver lipid.

Since the discovery of the need for certain fatty acids in rat diets by Burr and Burr (1) and in swine diets by Witz and Beeson (2), considerable work has been carried on, especially with rats, to elucidate the real functions of these acids. Aaes-Jorgensen (3), Deuel (4), Holman (5) and Sinclair (6) have reviewed the subject in detail. Only in recent years has work in this field with swine been extensive (7–12). The present studies were designed with the following objectives: 1) to re-examine the claim that essential fatty acids are needed for normal growth of young pigs; 2) to determine whether the addition of hydrogenated coconut oil to a fat-free diet aggravates fatty acid deficiency in the pig; and 3) to investigate the effects of a fat-free diet or diets containing hydrogenated coconut oil or safflower oil on erythrocyte cell wall integrity, on lipid and cholesterol levels of swine serum, heart and liver, and on total serum protein level.

EXPERIMENTAL

Thirty-six Yorkshire and Yorkshire × Hampshire female pigs, weighing approximately 10 kg at 5 weeks of age, were allotted at random on the basis of weight and litter to 3 groups of 16, 16 and 4 pigs each. Groups 1, 2 and 3 were given a fat-free diet, a 3% hydrogenated coconut oil (HCO) diet or a 3% safflower oil (SO) diet throughout the first 9-week period. After 9 weeks when severe skin lesions had appeared in most of the pigs fed the fat-free diet, four of those fed the fat-free diet and four fed HCO were necropsied and several tissue samples were taken for histological examinations to be reported separately. The remaining 12 pigs in each group were divided into 6 new groups as follows: four of the pigs fed the original fat-free and HCO diets were selected at random and continued with the same diets; from the remaining sixteen, 4 new

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TABLE 1

Composition of diets

Ingredients	9-week repletion [1]			12-week repletion [2]
	1	2	3	
	%	%	%	%
Glucose [3]	70.0	67.0	67.0	74.0
Casein [4]	20.0	20.0	20.0	16.0
Alfalfa meal [5]	2.0	2.0	2.0	2.0
Hydrogenated coconut oil (HCO) [6]	—	3.0	—	—
Safflower oil (SO) [7]	—	—	3.0	—
Trace mineralized salt	0.5	0.5	0.5	0.5
Vitamin-Zn supplement [8]	0.5	0.5	0.5	0.5
Mineral-vitamin premix [9]	1.1	1.1	1.1	1.1
Limestone	1.0	1.0	1.0	1.0
Dicalcium phosphate	3.3	3.3	3.3	3.3
Potassium phosphate	1.6	1.6	1.6	1.6
Antibiotic [10]	+	+	+	+

[1] Each pig was injected at the start of the experiment and after 4 weeks with 0.5 ml of emulsifiable vitamin A, D, E injectable (product of Chas. Pfizer and Co., Inc., New York) supplying the following in IU/ml: vitamin A, 500,000; vitamin D, 100,000; vitamin E, 50.

[2] Diets 2–6 in the repletion period were prepared by substituting 3% HCO, 30% SO, 0.1% SO, 0.5% SO and 1.0% SO, respectively, for glucose in the basal diet. Diet 7 was a 16% protein corn-soybean-meal type control diet.

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

[4] Crude, 30-mesh, National Casein Company, Riverton, New Jersey.

[5] The alfalfa meal was included as a source of possible unidentified factors that have been proposed as required for normal growth in swine. However, normal growth and identical skin lesions have been subsequently produced in pigs fed a similar diet not containing alfalfa meal.

[6] Hydrol, Durkee Famous Foods, Cleveland.

[7] Pacific Vegetable Oil Corporation, Richmond, California.

[8] Hopro R, Borden Company, New York, supplied the following (units/kg of diet): riboflavin, 4.4 mg; niacin, 13.75; Ca pantothenate, 6.25 mg; vitamin B_{12}, 11.0 μg; vitamin A, 2200.0 IU; vitamin D, 1320.0 IU; $ZnCO_3$, 242 ppm; and BHT, 29.0 ppm.

[9] Supplied the following per kg of total diet: thiamine, 2.2 mg; riboflavin, 4.4 mg; niacin, 35.2 mg; Ca pantothenate, 26.4 mg; pyridoxine, 8.8 mg; folic acid, 2.2 mg; choline, 2200.0 mg; vitamin E, 33.0 IU as *dl*-α-tocopheryl acetate; menadione, 44.0 mg; MgO_2, 880.0 mg; $CuSO_4 \cdot 5H_2O$, 352.0 mg; $FeSO_4 \cdot 7H_2O$, 594.0 mg; $MnSO_2 \cdot H_2O$, 184.8 mg; and $ZnCO_3$, 308.0 mg.

[10] Aurofac 10, American Cyanamid Company, Princeton, New Jersey, supplied 22 mg of chlortetracycline/kg of diet.

groups of 4 were created to include 2 pigs from each of the original fat-free and HCO diets. These 4 new groups were fed graded levels of SO (0.1, 0.5 and 1.0% of the diet) or a corn-soybean meal control diet. The pigs originally fed 3% SO were continued with the same diet for the second phase of the trial. For this repletion period, which lasted for 12 weeks, there were thus 7 treatments consisting of the following: 1) fat-free basal diet; 2) basal diet + 3% hydrogenated coconut oil (HCO); 3) basal diet + 3% safflower oil (2.4% linoleic acid); 4) basal diet + 0.1% safflower oil (0.08% linoleic acid); 5) basal diet + 0.5% safflower oil (0.4% linoleic acid); 6) basal diet + 1.0% safflower oil (0.8% linoleic acid); and 7) corn-soybean meal control diet.

The composition of the fat-free basal diet is shown in table 1.

All diets containing added fat (diets 2 through 6) were prepared by weight for weight replacement of glucose with HCO or SO. The fatty acid composition of these oils which appears in table 2 was determined with Beckman GLC model 2A using 15% ethylene glycol succinate as substrate in columns 2 m long.

Body weight and feed consumption measurements were taken weekly and

TABLE 2

Fatty acid composition of the oils used [1]

Fatty acid designation	Safflower oil	Hydrogenated coconut oil
	% of total fatty acids	
8:0	—	7.8
10:0	—	5.7
12:0	—	44.4
14:0	tr	17.2
16:0	8.8	9.2
18:0	1.5	10.4
18:1	9.3	5.0
18:2	80.2	0.2
18:3	tr	—

[1] Assayed on the Beckman Gas Chromatograph 2A, using columns 2 m long, with 15% ethylene glycol succinate as substrate on 60–80 mesh Chromasorb WDCMS.

blood was drawn from the anterior vena cava at 3-week intervals. Serum protein determinations were made according to the method of Gornall et al. (13), serum, heart and liver lipids extracted according to the method described by Folch et al. (14) and the cholesterol levels by the method described by Mann (15). A test of the erythrocyte cell wall fragility was carried out as described by Wintrobe (16). Data were subjected to the simple 2-way classification of the analysis of variance, using the unweighted squares of means.

RESULTS

Average daily gain, feed efficiency, skin condition and erythrocyte fragility. Table 3 summarizes the results for both depletion and repletion.

There were no statistically significant differences among groups in rate of body weight gain during either the depletion or repletion period. At the end of the first 9-week period pigs fed the fat-free diet showed severe skin lesions. These lesions appeared to resemble those described by Sewell and Miller (9) but appear to be different in some respects from those described in the rat (1) despite the assertion by Witz and Beeson (2) that fat deficiency symptoms in the pig are almost identical to those in the rat. The gross appearance of affected pigs in the present study suggests a close resemblance to the eczema seen in human infants suffering from essential fatty acid deficiency as described by Hansen et al. (17). None of those fed HCO showed skin lesions. After switching some pigs from fat-free to safflower oil or corn-soybean meal control diets, skin lesions disappeared completely with the corn-soybean diet but recovery was slow and incomplete on low levels of SO, the 1% SO showing the best recovery.

Statistical analyses of average feed efficiency ratios shown in table 3 were not possible because the pigs were group-fed. However, pigs fed the corn-soybean control diet had the highest efficiency of feed conversion (3.69 kg feed/kg gain), whereas those fed 3% HCO had the poorest efficiency (5.31 kg feed/kg gain). All diets containing SO ranging from 0.1 to 3% (0.08 to 2.4% linoleic acid) showed similar efficiency ratios.

The results of the erythrocyte fragility test (table 3) also showed no significant differences among treatments.

Serum cholesterol. Table 4 summarizes the results for both the depletion and repletion periods. During depletion, all 3 groups had markedly decreased serum cholesterol levels ($P < 0.01$) at week 3 from the initial value, after which they tended to stabilize. However, the decrease was greatest with the fat-free group. At the end of the depletion period, pigs fed HCO and SO had similar levels which were significantly higher ($P < 0.05$) than those fed the fat-free diet.

During repletion, there were also significant treatment differences as follows: The 3% HCO group which steadily rose and attained the highest overall average level showed a significantly higher level over all other groups ($P < 0.01$ for groups 1, 4, 5, 6 and 7 and $P < 0.05$ for group 3). On the contrary, the fat-free basal group had a significantly depressed serum cholesterol level relative to the HCO group

TABLE 3

Average daily gain, feed efficiency and erythrocyte fragility data (averages for each treatment)

	Diets [1]	Avg daily gain		Feed/gain	Erythrocyte fragility	
		Depletion	Repletion	Repletion	Initial hemolysis	Complete hemolysis
		kg	*kg*		*% saline*	*% saline*
1	Fat-free HCO	0.62	0.62	4.34	0.68	0.42
2	3% HCO	0.69	0.68	5.31	0.63	0.41
3	3% SO	0.73	0.65	4.05	0.70	0.44
4	0.1% SO		0.62	4.20	0.65	0.42
5	0.5% SO		0.67	4.35	0.64	0.40
6	1.0% SO		0.63	4.36	0.64	0.45
7	Corn-soybean diet		0.63	3.69	0.66	0.42

[1] HCO indicates hydrogenated coconut oil; SO, safflower oil.

($P < 0.01$) and the 3% SO group ($P < 0.05$), whereas the differences between this and other treatments receiving lower levels of SO were not significant. Likewise, diet 4 (0.1% SO) produced significantly depressed serum cholesterol. This level of 79.1 mg/100 ml was significantly lower than values for HCO and 3.0% SO ($P < 0.01$) and 0.5% SO ($P < 0.05$).

The overall time trend was a highly significant linear decrease. Highly significant linear interactions were obtained between treatments 1 vs. 2, 4 vs. 2 and 2 vs. 7, indicating that while the serum cholesterol continued to increase with bleeding period for treatment 2 (HCO) the levels fell in a generally linear manner in other treatments.

Total serum lipids. The results for both depletion and repletion are summarized in table 5. The trend here is similar to that described for cholesterol. The initial

TABLE 4

Serum cholesterol levels: depletion and repletion periods (averages)

Diets [1]	Week					Overall means
	Initial	3	6	9	12	
	mg/100 ml					*mg/100 ml*
Depletion						
1 Fat-free [2]	129.9	92.1	91.5	92.5		101.5 [3]
2 3% HCO [2]	135.9	100.3	100.3	105.6		111.8
3 3% SO [4]	130.0	124.5	109.5	106.0		117.5
Repletion [5]						
1 Fat-free	94.8	98.0	81.8	76.5	66.0	83.4 [6]
2 3% HCO	101.0	120.5	107.3	110.0	116.8	111.1 [7]
3 3% SO	106.0	102.0	90.5	83.0	95.8	95.4 [8]
4 0.1% SO	94.5	83.5	74.5	75.8	68.0	79.1
5 0.5% SO	99.3	84.5	91.3	102.5	95.8	94.7
6 1.0% SO	75.3	91.3	77.0	87.8	85.0	83.3
7 Corn-soybean meal	111.3	100.3	70.0	81.0	88.8	90.3

[1] HCO indicates hydrogenated coconut oil; SO, safflower oil.
[2] Sixteen pigs.
[3] Significantly lower than 3% SO ($P < 0.01$) and 3% HCO ($P < 0.05$) during depletion.
[4] Four pigs.
[5] Four pigs/treatment.
[6] Significantly lower than diet 2 ($P < 0.01$) and diet 3 ($P < 0.05$) during repletion.
[7] Significantly higher than diets 1, 4, 5, 6, 7 ($P < 0.01$) and diet 3 ($P < 0.05$) during repletion.
[8] Significantly higher than diet 4 ($P < 0.01$) and diet 6 ($P < 0.05$) during repletion.

TABLE 5

Total serum lipids, depletion and repletion periods (averages)

Diets [1]	Week					Overall means
	Initial	3	6	9	12	
	mg/100 ml					*mg/100 ml*
Depletion period						
1 Fat-free [2]	437	287	282	321		332
2 3% HCO [2]	453	370	389	355		392 [3]
3 3% SO [4]	440	386	379	391		399 [3]
Repletion period [5]						
1 Fat-free	346	321	269	290	273	300 [6]
2 3% HCO	325	410	362	412	430	388 [7]
3 3% SO	391	334	300	289	348	332
4 0.1% SO	307	271	254	276	253	272
5 0.5% SO	353	321	322	322	326	329
6 1.0% SO	274	299	324	319	322	308 [6]
7 Corn-soybean meal	363	320	301	277	326	317

[1] HCO indicates hydrogenated coconut oil; SO, safflower oil.
[2] Sixteen pigs/group.
[3] Significantly higher than fat-free diet ($P < 0.01$) during depletion.
[4] Four pigs in this group.
[5] Four pigs/treatment.
[6] Significantly different from diets 2, 3, 4, 5 ($P < 0.01$) during repletion.
[7] Significantly different from all other diets ($P < 0.01$) during repletion.

levels of serum lipids for all treatments were high but, like cholesterol, dropped to low levels within the first 3 weeks and were maintained for the rest of the depletion period. The decrease was also greatest for the fat-free group and least for HCO and SO, such that at the end of the depletion period, the fat-free group had a significantly lower ($P < 0.01$) overall serum lipid level than HCO and SO. There was no significant difference between the HCO and SO groups in the overall means. There were no significant interactions among treatments, indicating that all had the same falling trend.

Significant differences were also observed in the repletion period. The HCO group showed increased serum lipid from the initial low level to a higher one during the first 3 weeks which was maintained throughout. The fat-free and 0.1% SO groups showed a gradual decrease with time, with some minor variations. Analysis of variance of the data gave highly significant differences between HCO and all other treatments ($P < 0.01$). The 3% SO diet produced a higher overall level than the fat-free and 0.1% SO diets ($P < 0.05$); the 1% SO and corn-soybean meal diets also produced a higher level than did the 0.1% SO diet ($P < 0.05$).

The overall time trend here generally was a highly significant linear type with all treatments except HCO showing falling trends while the HCO increased.

Total serum protein. The results for both depletion and repletion are summarized in table 6. In the depletion period, statistical analysis of the data showed that the 3% SO group had a significantly higher total serum protein level than either the fat-free or the HCO groups which were not significantly different from each other. The overall time trend for the whole depletion period was a highly significant ($P < 0.01$) increase in serum protein level as the depletion period progressed. Since all 3 groups increased, the change was probably associated with the increase that normally occurs with time in pigs of this age (18).

The repletion period produced highly significant differences as follows: 3% SO and corn-soybean meal control were significantly higher than all other groups ($P < 0.01$) for fat-free, HCO, 0.1% SO and $P < 0.05$ for 0.5% SO). In general, the fat-free basal and HCO groups had the lowest serum protein levels, whereas the 3% SO group and the corn-soybean meal control diet had the highest levels. The overall trend showed a highly significant linear increase with time ($P < 0.01$).

Total cholesterol and lipid of the heart and the liver. Table 7 summarizes the results for these parameters. Values are

TABLE 6

Total serum protein: depletion and repletion periods (averages)

Diets [1]	Week					Overall means
	Initial	3	6	9	12	
	mg/100 ml					*mg/100 ml*
Depletion period						
1 Fat-free [2]	5.50	5.74	6.14	6.31		5.92
2 3% HCO [2]	5.63	5.67	5.94	6.24		5.87
3 3% SO [3]	5.35	6.75	6.35	7.50		6.49 [4]
Repletion period [5]						
1 Fat free	6.35	6.35	7.05	7.20	7.27	6.84 [6]
2 3% HCO	6.20	6.62	6.92	6.40	7.27	6.68 [6]
3 3% SO	7.50	7.67	8.32	8.12	8.17	7.96 [7]
4 0.1% SO	6.05	6.60	6.65	7.50	6.92	6.74 [6]
5 0.5% SO	7.07	7.30	7.52	7.82	7.35	7.41
6 1.0% SO	5.77	6.85	7.02	7.10	7.60	6.87 [6]
7 Corn-soybean meal	7.77	7.60	8.02	8.27	8.00	7.93 [7]

[1] HCO indicates hydrogenated coconut oil; SO, safflower oil.
[2] Sixteen pigs/group.
[3] Four pigs in this group.
[4] Significantly higher than fat-free and HCO diets ($P < 0.01$) during depletion.
[5] Highly significant treatment difference ($P < 0.01$).
[6] Significantly lower than diets 3, 7 ($P < 0.01$) and diet 5 ($P < 0.05$) during repletion.
[7] Significantly higher than diets 1, 2, 4, 6 ($P < 0.05$) and diet 5 ($P < 0.05$) during repletion.

TABLE 7

Average total cholesterol and lipids of the heart and liver

	Diets [1]	Heart		Liver	
		Cholesterol	Lipid	Cholesterol	Lipid
		mg/100 g dry matter	*% dry matter*	*mg/100 g dry matter*	*% dry matter*
1	Fat-free	542	24.5	1498 [2]	11.1 [3]
2	3% HCO	525	26.6	1191 [4]	11.6 [3]
3	3% SO	544	24.0	857	6.9
4	0.1% SO	544	25.0	1110	8.6
5	0.5% SO	543	24.6	873	8.3
6	1.0% SO	510	23.9	846	8.1
7	Corn-soybean meal	535	19.7 [5]	799 [6]	6.8

[1] HCO indicates hydrogenated coconut oil; SO, safflower oil.
[2] Significantly higher than diets 3, 5, 6, 7 ($P < 0.01$) and diet 4 ($P < 0.05$).
[3] Significantly higher than diets 3, 7 ($P < 0.01$) and diets 4, 5, 6 ($P < 0.05$).
[4] Significantly higher than diets 3, 5, 6, 7 ($P < 0.05$).
[5] Significantly lower than diets 1, 2, 6 ($P < 0.01$) and diets 4, 5, 3 ($P < 0.05$).
[6] Significantly lower than diet 1 ($P < 0.01$) and diets 2, 4 ($P < 0.05$).

expressed on a dry-weight basis. There were highly significant ($P < 0.01$) treatment differences in the total lipids of the heart. The lowest lipid level of 19.7% was obtained in the corn-soybean control group, this value being significantly lower ($P < 0.01$) than the two highest levels obtained with the fat-free and HCO diets. It was also significantly lower ($P < 0.05$) than the values obtained for all levels of SO. The values obtained for the groups on graded levels of SO (treatments 3–6) were not significantly different, but the means were lower for groups fed 3% and 1% SO than for those receiving 0.1 or 0.5% SO.

The heart total cholesterol content appeared refractory to diet unlike the response in total lipid content. Analysis of variance of these data revealed no significant differences in heart cholesterol due to diet.

The total liver lipid levels were lower than the total heart lipid levels. The highest levels were obtained with HCO (11.6%) and the fat-free diet (11.1%) and the lowest in the corn-soybean meal (6.8%) and 3% SO diet (6.9%). These differences were statistically significant for the HCO and fat-free groups vs. corn-soybean meal and 3% SO ($P < 0.01$) and for HCO and fat-free vs. 0.1%, 0.5% and 1.0% SO ($P < 0.05$).

With respect to the total liver cholesterol, the levels were in all cases higher than the corresponding total heart cholesterol levels. Significant treatment differences were observed as follows: fat-free, HCO and 0.1% SO, with levels of 1498, 1191 and 1110 mg cholesterol/100 g dry liver, respectively, were significantly greater than the levels observed for 0.5, 1.0 and 3% SO and corn-soybean meal ($P < 0.01$). Also, the fat-free group had a significantly higher level than the 0.1 SO group.

DISCUSSION

One of the most controversial issues about the role of essential fatty acids in swine diets is their effect on growth rate. Our results showed that the pigs grew equally well with or without essential fatty acid supplementation, an observation which contradicts early reports (2, 7, 19), but which is supported by a number of more recently published reports (9, 10, 11). Although pigs up to 4 to 5 weeks old as used in the present experiment would be expected to have substantial body storage of essential fatty acids, the pigs in all cases developed severe skin lesions, characteristic of essential fatty acid depletion, yet continued to grow at a rate equal to that of animals receiving control diets.

HCO which contains almost exclusively saturated fatty acids, has been reported to aggravate essential fatty acid deficiency symptoms in the rat (20, 21) and chick,[1] but did not produce skin lesions or reduce growth rate of the pigs in the present study. Howard et al. (11), who used beef tallow at a level of 10% of total ra-

[1] Hopkins, D. T. 1964 The role of linoleic acid in the nutrition of the chick. Ph.D. Thesis, Cornell University, Ithaca, New York.

tion, also reported no detrimental effect on growth rate or on skin condition. Even in the rat, the inclusion of hydrogenated oil has not always caused any deleterious effects (22, 23).

With respect to erythrocyte fragility, MacMillan and Sinclair (24) found that the erythrocytes from rats fed an essential fatty acid-deficient diet were more susceptible to hemolysis than those from rats fed complete diets. Our results with pigs do not support this report. Argument along this line has almost always centered around the speculated membrane function of the polyunsaturated acids. However, Van Deenen et al. (25) showed that cholesterol and phospholipids to which polyunsaturated fatty acids are esterified are present in almost equimolar ratios in almost all mammalian cells and that these components are hardly subject to changes through dietary manipulations. Cholesterol (see review (26)) has a condensing effect on molecular film areas and has a stabilizing effect on molecular arrangements, imparting relatively high rigidity to the membranes from the circulating red cells. These observations, therefore, do not support the concept that a change should be expected in erythrocyte cell wall fragility in essential fatty acid deficiency.

That the feeding of oils high in polyunsaturated fatty acids lowers serum cholesterol in almost all species studied has been well-documented, as reviewed by Kritchevsky (27). This was also true of the pigs in the present study, although it took a long time to depress this significantly. The low levels of total serum cholesterol in fat-free diets and diets very low in SO (0.1 or 0.5%) could be partly explained on the basis of scarcity of exogenous acetate, the main precursor of cholesterol synthesis (although synthesis can still proceed with endogenous and other nonlipid dietary sources) and partly on defective mobilization of the synthesized cholesterol from the liver, the principal site of synthesis. That the low level in the serum is probably due mostly to defective mobilization is indicated by linear and low serum concentration of cholesterol in pigs fed the fat-free or the 0.1% SO diet. For effective mobilization of cholesterol, it has been shown by Schaible (28) and Kelsey and Longenecker (29) that it is preferentially esterified at the hydroxy group on position 3 to the unsaturated fatty acids.

Thus, the defective mobilization of cholesterol from the livers of the pigs fed fat-free or low fat diets could have been due to low levels of circulating essential fatty acids. The high serum cholesterol level obtained by the feeding of hydrogenated coconut oil, despite a correspondingly high liver level in the same pigs is explainable also on the basis of insufficient polyunsaturated fatty acids. In this case, the serum level is high because cholesterol esters were not being excreted in the feces to as great an extent as with the EFA-supplemented (3% SO) or corn-soybean meal control diet. Alfin-Slater et al. (22) observed the same phenomenon of liver and adrenal accumulation of cholesterol in essential fatty acid-deficient rats. They suggested that unsaturated fatty acids enhance the circulation of cholesterol, allowing increased excretion of the sterol as observed in man by Hellman (30) and in rats by Wilson and Siperstein (31).

The precipitous drop in total serum lipids and cholesterol from the initial high weaning levels was probably related to the change from the high fat milk diet to a low fat dry diet at weaning. Kinsell (32) found with humans that milk fat promoted increased serum total lipids and cholesterol as opposed to corn oil. McClellan (33) also noted that in miniature pigs, the preweaning serum cholesterol level was significantly higher than the postweaning level.

The elevated total serum lipids observed with the feeding of hydrogenated coconut oil generally paralleled the increases in total serum cholesterol. This same phenomenon has been observed by Kinsell (32) in humans. He postulated that the increase in total lipids is partly due to an increase in circulating cholesterol and partly due to increases of phospholipids and triglyceride fractions.

Although the increases in cholesterol could be accounted for on the basis of decreased fecal excretion, those of phospholipids and triglycerides have not been satisfactorily explained. The hyperlipemic

effect of hydrogenated fat observed with the pig in this experiment is in accordance with the results (34) in humans. It is noteworthy that this effect is obtained in the pig even with a much lower level of total dietary fat (3%) than in the human studies.

Total serum protein was found to be depressed by the feeding of fat-free and HCO diets, as compared with the levels obtained when SO was present. It is suggested that the decreases could, in part, be due to decreased synthesis by the liver cells of serum protein. The decrease in the synthesis might be related to the accumulation of lipids in the liver cells. All diets had the same total protein. The possibility of reduced absorption of dietary protein appears unlikely, although no measurements were made of nutrient absorption from the gastrointestinal tract. Also uncertain is what fraction of the total serum protein was affected. This could be significant since lipids are known to be associated with apolipoproteins in lipoprotein complexes during transport.

The rather constant heart total cholesterol levels irrespective of diet presents a paradox since the heart, like the liver, tended to have increased total lipids with the feeding of fat-free or HCO diets. This picture does not fit the concomitant rise of total lipids with increasing cholesterol concentrations as observed in the serum and liver. If cholesterol did not increase, then it must have been other lipid components, notably the phospholipids and the triglycerides which accounted for most of the rise in total heart lipids. The significance of this is not known.

Correlation analyses of the data obtained for both depletion and repletion periods are presented in table 8. The only consistently high correlations for serum components were for serum cholesterol and serum lipids. All other comparisons showed erratic behavior, the reasons for which cannot be explained.

Highly significant negative relationships of $r = -0.63$ ($P < 0.01$) and $r = -0.40$ ($P < 0.05$) were observed between serum protein and total liver lipid and cholesterol, respectively. This tends to strengthen the speculation that, with lipid accumulation in the liver, the protein synthesis in the liver was reduced. Also, as expected from the data in table 7, the correlation between the total liver and heart lipids was highly significant, $r = 0.48$ ($P < 0.01$) and that between total liver lipid and liver cholesterol significant, $r = 0.44$ ($P < 0.05$). Heart cholesterol values showed low and nonsignificant correlations with liver cholesterol, liver lipid and total heart lipid ($r = 0.044$; 0.099 and -0.24, respectively).

TABLE 8

Correlation coefficients between serum protein, cholesterol and lipid with time

Periods	Total lipid vs. total cholesterol	Total lipid vs. total protein	Total cholesterol vs. total protein
Depletion			
Initial	0.64 [1]	0.49 [1]	0.47 [1]
Week 3	0.72 [1]	0.24	0.49 [1]
Week 6	0.46 [2]	−0.04	−0.06
Week 9	0.78 [1]	0.35	0.35
Repletion			
Initial	0.75 [1]	0.68 [1]	0.56 [1]
Week 3	0.63 [1]	0.01	0.04
Week 6	0.48 [1]	0.04	0.13
Week 9	0.70 [1]	0.58 [1]	0.24
Week 12	0.75 [1]	0.24	0.14
Overall repletion	0.81 [1]	0.057	0.15
Depletion + repletion combined	0.66 [1]	0.01	0.03

[1] Highly significant ($P < 0.01$).
[2] Significant ($P < 0.05$).

Since the inclusion of 1% safflower oil did not completely correct the skin lesions and the inclusion of 3% HCO did not produce lesions despite the very low linoleic acid content of this diet, it raises the question of whether linoleic acid is needed by the pig for the prevention or cure of skin lesions brought about by feeding fat-free diets.

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Metabolism of the Geometric Isomers of Linoleic Acid in the Rat [1]

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ABSTRACT Studies are reported on the extent and mode of conversion of the geometric isomers of linoleic acid in the rat. Feeding experiments of varying periods were carried out with weanling and adult male rats of the Sprague-Dawley strain in which the geometric isomers of linoleic acid were fed as supplements or administered orally to groups of animals maintained with an otherwise fat-free diet. The fatty acids composition of the liver lipid of individual animals was determined by gas-liquid chromatography of methyl esters. The 20-carbon chain tetraenoic acids were isolated from the pooled methyl esters of each group by a combination of column-argentation chromatography and preparative gas-liquid chromatography, and their structures were determined by a combination of infared spectral analysis and ozonolysis. Conversion of the *trans*-9,*trans*-12-isomer of linoleic acid to the 20:4 species was virtually nil; in fact, this compound appeared to function as an antimetabolite in the conversion of oleic and palmitoleic to 5,8,11-20:3 and 4,7,10,13-20:4 acids, respectively. The conversion of *trans*-9,*cis*-12-linoleic acid to 20:4 acids likewise was very inefficient indicating that a *cis* double bond in the 9-position is required for the interconversion of polyunsaturated fatty acids. Determination of the specific position of the *trans* double bond in the tetraene isolated from animals fed *cis*-9,*trans*-12-linoleate indicated it was converted to 20:4 by the same pathway as all-*cis* linoleic acid. However, its conversion to 20:4 was not nearly as efficient as linoleic acid.

The results of feeding experiments in previous studies (1, 2) from this laboratory indicated that little, if any, *trans*-9, *trans*-12-octadecadienoic acid (*trans*,*trans*-linoleic acid) was converted to polyunsaturated fatty acids in the rat. Evidence was obtained for the conversion of *cis*-9, *trans*-12-octadecadienoic acid (*cis*,*trans*-linoleic acid) to eicosatetraenoic acid (20:4), but the conversion was not as efficient as with *cis*-9,*cis*-12-octadecadienoic acid (linoleic acid). In these studies male rats in an advanced stage of essential fatty acid deficiency were fed the test acids as methyl esters for various periods and the fatty acid compositions of the liver lipids were determined. Recently, Selinger and Holman (3) also showed that feeding *trans*,*trans*-linoleate did not increase the level of polyunsaturated fatty acids in the rat. In earlier experiments in which fatty acid composition was determined by alkali-isomerization, Holman (4) reported that feeding *trans*,*trans*-linoleate to fat-deficient rats resulted in some increase in both tetraene and hexaene content of the body fat. The recent results of Knipprath and Mead (5) indicate that *trans*,*trans*-linoleic acid is converted to an eicosatetraenoic acid containing two *trans* double bonds in the normal rat. In the latter study, experiments with *trans*-9,*trans*-12-octadecadienoic acid-1-^{14}C (*trans*,*trans*-linoleic acid-1-^{14}C) indicated this acid was converted to eicosatetraenoic acid as readily as linoleic acid.

If the conversions of the geometric isomers of linoleic acid proceed to eicosatetraenoic acid by the same pathway as linoleic acid (6), *trans*,*trans*-linoleic acid should give the *cis*-5,*cis*-8,*trans*-11,*trans*-14-isomer of arachidonic acid. The *cis*-9, *trans*-12- and *trans*-9,*cis*-12-isomers of linoleic acid accordingly should be converted to the *cis*-5,*cis*-8,*cis*-11,*trans*-14- and the *cis*-5,*cis*-8,*trans*-11,*cis*-14-isomers, respectively. The objective of the present study was to determine the extent of the conversion of the *cis*,*cis*-, *trans*,*trans*-, *cis*,*trans*- and *trans*,*cis*-isomers of linoleic acid to polyunsaturated acids and to isolate and determine the structures of the eicosatetraenoic acids so as to determine the pathways of the metabolism of these acids in

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the livers in rats raised with otherwise fat-free diets.

MATERIALS

Highly purified methyl linoleate and *trans,trans*-linoleate (linoelaidate) were obtained from the Hormel Institute. Methyl *cis,trans*-linoleate and *trans-cis*-linoleate were prepared from dehydrated ricinoleic and ricinelaidic acids, respectively, by a combination of fractional distillation and crystallization of methyl esters, as described by Jackson et al. (7). The purity of the above compounds was determined by gas-liquid chromatography (GLC) and thin-layer chromatography (TLC) using chromatoplates coated with silica gel G impregnated with silver nitrate. Only traces of impurities could be detected in the methyl linoleate, *trans,trans*- and *cis, trans*-linoleate, their purity being greater than 99%. The preparation of *trans,cis*-linoleate was estimated to be 66% pure by argentation-TLC and GLC. The only impurity detectable in this latter preparation was *trans,trans*-linoleate. Because of the large losses incurred in further recrystallizations of this preparation, it was not further purified. In the experiment in which it was fed, 33% *trans,trans*-linoleate was therefore added to the linoleate fed to animals in the *cis,cis* group.

METHODS

Three experiments were carried out, each using several groups consisting of 4 to 6 male rats of the Sprague-Dawley strain. In the first experiment weanling rats were fed a basic fat-free diet consisting of vitamin-free test casein (24.5%), cellulose [2] (4.0%), minerals [3] (4.0%), sucrose (66.5%) and vitamin mix [4] (1.0%) ad libitum for 21 days and then divided into 3 groups. A control group continued to receive the fat-free diet for the entire duration of the experiment, an additional 130 days. The other 2 groups were fed the basic diet supplemented with 1.0% by weight of linoleate and *trans,trans*-linoleate, respectively, for 117 days. The amount of these test substances was then increased to 6.0% by weight for the remaining 13 days of this experiment. At the conclusion of the experiment, all animals were exsanguinated and the livers and other organs excised, frozen immediately and stored at $-20°$ until the lipids could be extracted.

In experiment 2, weanling rats were fed the basic fat-free diet for 10 months to induce severe essential fatty acid (EFA) deficiency symptoms. The animals were then divided into 4 groups, and while the control group continued to receive the basic diet, the other 3 groups received this diet supplemented with 7.5% by weight of linoleate, *cis,trans*-linoleate or *trans,trans*-linoleate, respectively, for a period of 17 days. The animals were killed and tissues were treated as above.

Experiment 3 was performed using 3 groups of adult rats in which severe EFA-deficient symptoms had also been induced for a period similar to that in experiment 2. All groups were maintained with the basic fat-free diet, but one group received no further supplement and served as the control group, and the other 2 groups were force-fed intragastrically 1 g/rat daily of linoleate or *trans,cis*-linoleate (each containing 33% *trans,trans*-linoleate), respectively, for 9 days. These supplements were administered at equal time-intervals throughout the day in 250-mg doses. Animals were killed and the tissues excised and stored as in the previous 2 experiments.

The liver of each animal was extracted 3 times with 2:1 (v/v), chloroform-methanol, using a Virtis Model 45 homogenizer. The combined extracts were filtered, evaporated to near dryness, and the lipids were taken up in chloroform, leaving most of the non-lipid material as an insoluble residue. The chloroform extract was dried over anhydrous sodium sulfate and the lipid recovered by evaporation of the solvent. In experiment 2, the lipid of the livers of the animals in each group were pooled and the analyses were carried out on the phospholipid fraction isolated

[2] Nonnutritive cellulose: Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[3] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constitutents. Science, 75: 339; obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Percentage composition of vitamin mix: vitamin A conc (200,000 USP units/g), 0.99; vitamin D conc (400,000 USP units/g), 0.055; α-tocopherol, 1.10; ascorbic acid, 9.91; *i*-inositol, 1.10; choline chloride, 16.53; menadione, 0.50; *p*-aminobenzoic acid, 1.10; niacin, 0.99; riboflavin, 0.22; pyridoxine·HCl, 0.22; thiamine·HCl, 0.22; Ca pantothenate, 0.66; biotin, 0.0044; folic acid, 0.020; vitamin B_{12}, 0.0003; and cornstarch, 66.4; obtained as B-Mix 75 from General Biochemicals, Inc.

by acetone precipitation. To obtain essentially complete recovery of the phospholipids the precipitation was carried out at 0°. Since the results on the phospholipids were similar to those obtained for the total lipid in the first experiment, only the total lipid was analyzed in the third experiment.

Lipids were converted to methyl esters by interesterification with dry methanol containing 6% by weight of HCl. The interesterification was carried out by heating the sample with a large excess of the reagent in a glass ampule for 2 hours in a boiling water bath. The methyl esters were recovered by extraction with a 1:1 (v/v) mixture of petroleum ether and diethyl ether.

GLC of the methyl esters was performed at 185°, using an F&M flame ionization instrument, Model 1609, equipped with a 213 × 0.64 cm column packed with 8% ethylene glycol succinate polyester phase [5] on 100–200 mesh Chromosorb W. Fatty acid composition was determined directly from the proportionalities of the peak areas. Detector response for the range of chain lengths of the fatty acids in the samples was so near linear on standard mixtures that no correction factors were used.

A methyl tetraenoate concentrate was prepared from the pooled methyl esters of each group by column chromatography on silicic acid impregnated with silver nitrate by elution in a step-by-step manner with various amounts of diethyl ether in petroleum ether. The 20:4 ester was isolated from this instrument equipped with a thermal conductivity detector and a 183 × 0.64 cm column packed with 20% by weight of diethylene glycol adipate polyester phosphoric acid on 60–80 mesh Chromosorb W.[6] The major portion of this ester was condensed in a 2 to 5 mm ID glass tube that was cooled with dry ice and extended under the surface of a small amount of chlorform in a test tube. Immediately after collection, the condensed material was rinsed into the test tube with chloroform. Finally, the product was recovered by evaporation of the chloroform and its purity checked by analytical GLC as described above. The procedure did not introduce any changes in position or geometry of double bonds as determined by the analysis of samples of all-*cis* methyl arachidonate prepared in a similar way.

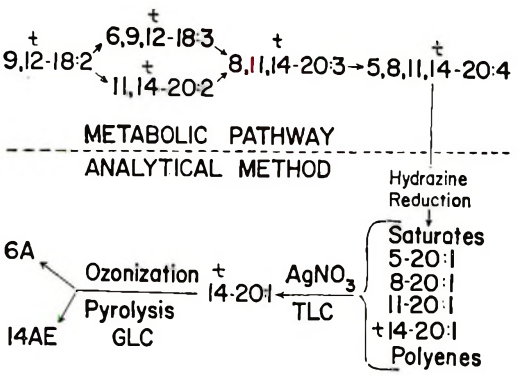

Fig. 1 Scheme showing probable pathway of animal conversion of *cis,trans*-linoleate to *cis*-5, *cis*-8, *cis*-11, *trans*-14-eicosatetraenoic acid (upper line) and method of structural analysis after isolation (lower line). Fragments obtained from resulting *cis*-monoenes are not shown, but would be determined in the same manner.

Tetraenoates isolated from experimental groups were analyzed for *trans* bond content as 10% solutions in carbon disulfide using a Perkin-Elmer Model 237B infrared spectrophotometer. Isolated tetraenoates were also ozonized and the cleavage products analyzed by GLC, according to the method of Nickell and Privett (9), to determine relative proportions of the 5,8,11, 14- and 4,7,10,13-isomers present in each group.

Determination of the precise geometrical structures of the tetraenoic acids was carried out by the method of Privett and Nickell (10) for the localization of the specific positions of the *cis* and *trans* double bonds. The principle of this method is illustrated in figure 1. The sample is partially reduced with hydrazine thereby giving, among the products of the reaction one monoene for each double bond in the molecule. No inversion or shifting of double bonds occurs with this reagent. Thus the double bonds in the monoenes are representative of the position and configuration of those in the original molecule. *trans* and *cis* Monoenes are separated by argentation-TLC and the fragments resulting from ozonolysis of *cis*

[5] EGSS-X, Applied Science Laboratories, State College, Pennsylvania.
[6] Lachat Chemicals, Inc., Chicago.

TABLE 1

Fatty acid analyses on livers of rats fed control and supplemented diets

Exp. no.[1]	Dietary group [2]	18:1	18:2	20:3	20:4
		% by wt	*% by wt*	*% by wt*	*% by wt*
1	ff	35.2 ± 0.3 [3]	1.8 ± 0.2	15.2 ± 1.2	3.2 ± 0.3
	tt	37.1 ± 1.0	11.8 ± 1.0	7.7 ± 0.9	1.6 ± 0.1
	cc	16.0 ± 1.2	20.6 ± 1.1	tr	23.7 ± 1.4
2	ff	24.2 [4]	1.5	18.1	3.1
	tt	33.0	7.3	10.1	1.8
	ct	18.8	2.8	19.0	7.6
	cc	18.6	8.3	4.7	15.2
3	ff	34.4 ± 1.4	2.0 ± 0.2	14.9 ± 0.8	2.4 ± 0.3
	tc	27.7 ± 0.9	14.3 ± 0.9	12.5 ± 0.9	2.2 ± 0.2
	cc	17.5 ± 1.1	20.1 ± 1.0	5.0 ± 0.8	17.8 ± 1.0

[1] Experimental details in text.
[2] ff = fat-free, tt = *trans,trans*-linoleate, ct = *cis,trans*-linoleate, tc = *trans,cis*-linoleate, and cc = linoleate.
[3] Average ± SE of mean.
[4] Values of pooled samples of liver phospholipids.

and *trans* monoenes are analyzed by GLC, giving a way of reconstructing the complete structure of the original tetraenoic acid. Should any *trans,trans* dienes be produced by the procedure from compounds containing two *trans* double bonds, these would be inseparable from *cis* monoenes by argentation-TLC. However, chromatography on silica gel plates may be used to separate the ozonides of monoenes and dienes before cleavage. In this way the structure of *trans,trans* dienes could also be used to confirm the locations of the *trans* double bonds determined by cleavage of *trans* monoenes.

RESULTS AND DISCUSSION

The results of the fatty acid analysis for each group of animals are summarized in table 1. The same general changes in the fatty acid composition occurred in similar dietary groups, compared with the animals receiving only the basic fat-free diet. These may be summarized as follows: 1) the 20:4 and 18:2 were greatly increased and the 18:1 and 20:3 were greatly decreased by feeding linoleate; 2) feeding *trans,trans*-linoleate decreased the 20:4 and 20:3, but increased the 18:1 and the 18:2 (*trans,trans*-linoleic acid); 3) feeding *cis,trans*-linoleate increased the 20:4, decreased slightly the 18:1, and increased the 18:2 (*cis,trans*-linoleic acid); and 4) feeding *trans,cis*-linoleate gave no significant change in the 20:4, decreased the 20:3 and the 18:1 slightly, and increased the 18:2 (*trans,cis*-linoleic acid).

The results shown in table 1 are generally in accord with those obtained in previous experiments (1, 2) with respect to the conversion of *trans,trans* and *cis, trans*-isomers of linoleic acid to higher polyunsaturated fatty acids. Linoleic acid underwent interconversion the most efficiently of the isomers as judged by the increase in 20:4. It also gave the greatest increase in deposition of 18:2. This observation may be explained on the basis that the tissue enzymes can distinguish between the geometric isomers of linoleic acid, and natural *cis,cis*-linoleic acid is conserved because it is essential. In accord with this observation is that by Coots (12) that the *trans* isomers of linoleic acid are catabolized at a greater rate than *cis, cis-linoleic* acid. It may also be significant in this respect as pointed out by Brockerhoff (13) that linoleate is found predominantly in the β-position of triglycerides, whereas the *trans* isomers of linoleic acid are generally esterified predominantly in the 1- and 3-positions of the triglyceride molecule (3, 14–16).

The results in table 1 also show that feeding of *trans,trans*-linoleate suppressed the conversion of oleic acid to 20:3 without giving an increase in 20:4, indicating that much less overall enzymatic interconversion of fatty acids appears to have taken place in the *trans,trans*-linoleate

groups than in the other groups. Since the concentration of the 20:4 was actually less in the livers of the animals receiving *trans, trans*-linoleate than in those fed the fat-free diet it is evident that the conversion of this acid is very inefficient. The conversion of *trans,cis*-linoleate to 20:4 was also very inefficient because, although each of the animals in this group was force-fed 1 g/day for 9 days, the liver 20:4 was not increased over that of the animals in the fat-free group. Since there is an appreciable conversion of *cis,trans*-linoleate to 20:4 but little conversion of either *trans,trans*- or *trans,cis*-linoleate to 20:4, it appears that the *cis* configuration of the double bond in the 9 position is important for the interconversion of polyunsaturated fatty acids.

Although the levels of 20:4 were low in the livers of the animals receiving *trans, cis, cis,trans* and *trans,trans* isomers of linoleate, some conversion of all of these isomers occurred as indicated by an infrared spectral analysis which showed that the isolated tetraene fraction from these groups contained *trans* unsaturation. The tetraene fraction isolated from the livers of animals fed *cis,trans*-linoleate was shown by infrared analysis to contain an appreciable amount of *trans* unsaturation in previous studies (1,2). The infrared spectra of the tetraene fraction of the livers from animals fed *trans, cis*- and *trans,trans*-linoleate in this study are shown in figure 2. The absorption peaks in the region of *trans* absorption (10.3 μ) were small, but by comparison with those obtained from the tetraene of fat-free and linoleate-fed animals appear to be real. The amount of *trans* unsaturation in the tetraene isolated from the livers of the *trans,trans* group and *trans,cis* group were estimated at 9 and 18% (as % methyl elaidate) (8), respectively. When it is considered that the total amount of tetraene in these animals was only approximately 2%, it is further evident that the amount of conversion of these acids was very small.

Normally two-positional isomers of eicosatetraenoic acid, the 4,7,10,13 which arises from palmitoleic acid, and the 5,8, 11,14 or arachidonic acid, may be detected in the livers of fat-free rats (17). To obtain further information on the mode of interconversion of the geometric isomers

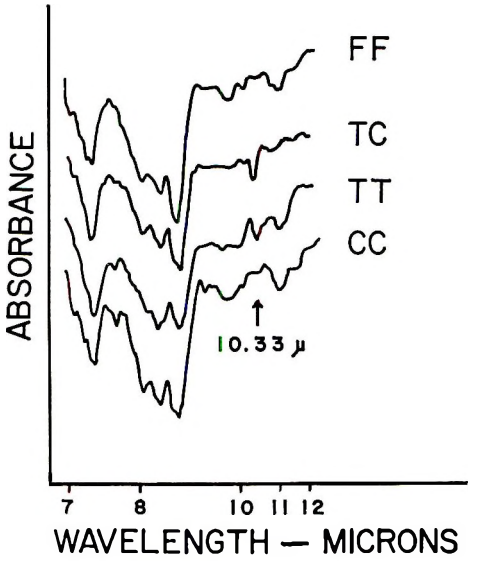

Fig. 2 Infrared spectra. Analysis determined with a 10% carbon disulfide solution of the sample. Samples consist of the 20:4 fraction isolated from the livers of animals fed fat-free diet (FF), and fat-free supplemented with *trans,cis*-linoleate (TC), *trans,trans*-linoleate (TT) and linoleate (CC).

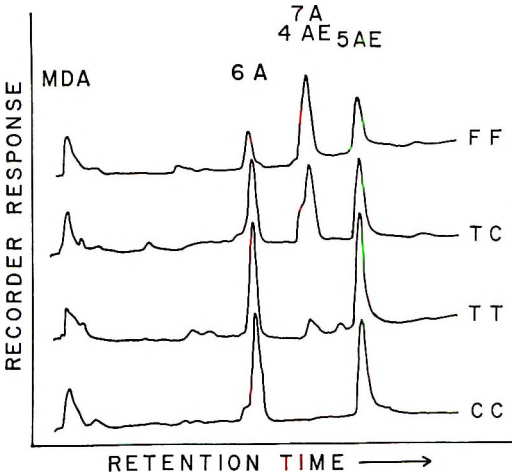

Fig. 3 GLC analyses of aldehyde fragments obtained in the structural analysis of the 20:4 fraction isolated from the livers of the animals fed the fat-free diet (FF) and the fat-free diet supplemented with *trans,cis*-linoleate (TC), *trans, trans*-linoleate (TT) and linoleate (CC). 6A = hexanal, 7A = heptanal, 4AE and 5AE represent the aldester of chain lengths of 4 and 5 double bonds, respectively.

of linoleic acid the relative amounts of these isomers were determined by a simple structural analysis by the method of Nickell and Privett (9). The GLC tracings of the results of these analyses are shown in figure 3. The results are only semiquantitative but they demonstrate distinct differences between the groups receiving the different isomers of linoleate. First, it should be explained that hexanal (6A) and the aldester of 5 carbon atoms (5AE) arise from the 5,8,11,14–20:4, and the peak consisting of a mixture of heptanal (7A) and the aldester of 4 carbon atoms (4AE) arise from 4,7,10,13–20:4. The latter aldehyde and aldester that overlap may be separated and distinguished by an analysis with a polar phase, but since they arise from the same ester in this case a separate analysis was not deemed necessary. The results show that the formation of 4,7,10,13–20:4 was absent from the tetraene of both the linoleate and the *trans,trans*-linoleate groups. In the case of the linoleate group, suppression of the formation of this tetraene apparently results from a competitive inhibition of the type described by Holman et al. (10) inasmuch as linoleate is efficiently converted to arachidonic acid. Suppression of the formation of 4,7,10,13–20:4 by *trans,trans*-linoleate may be the effect of a general suppression of enzyme activity by this isomer of linoleic acid. This effect is also evidenced by the decrease in 20:3. Since the *trans,trans*-linoleic acid is not converted to 20:4 to an appreciable extent but yet apparently combines with the enzyme it may be regarded as prohibiting the interconversion of oleic and palmitoleic acids.

To determine whether the limited conversion of the *trans* isomers of linoleic acid to 20:4 occurred by the same pathway as linoleic acid, an attempt was made to determine the specific positions of the *trans* double bonds in the tetraenes isolated from the livers of the animals in each group by the method of Privett and Nickell (10). The synthetic pathway in the animal including the expected position of the *trans* double bond in the conversion of *cis,trans*-linoleic acid as well as the scheme of analysis are represented diagrammatically in figure 1. The *cis,trans* isomer of linoleic acid was selected for the illustration in figure 1 because it was the only tetraene that could be isolated in sufficient amounts for a complete analysis. The difficulties encountered in this respect may be realized from an examination of figure 1. If the entire 20:4 isolated from the animals fed the *cis,trans*-linoleate consisted of the 5,8,11-*trans*-14 isomer, the *trans*-20:1 produced in the analytical procedure would only make up one-sixteenth of the monoenes, provided it was obtained in the optimal yield (50%) in the hydrazine reduction step. The results of the TLC analysis

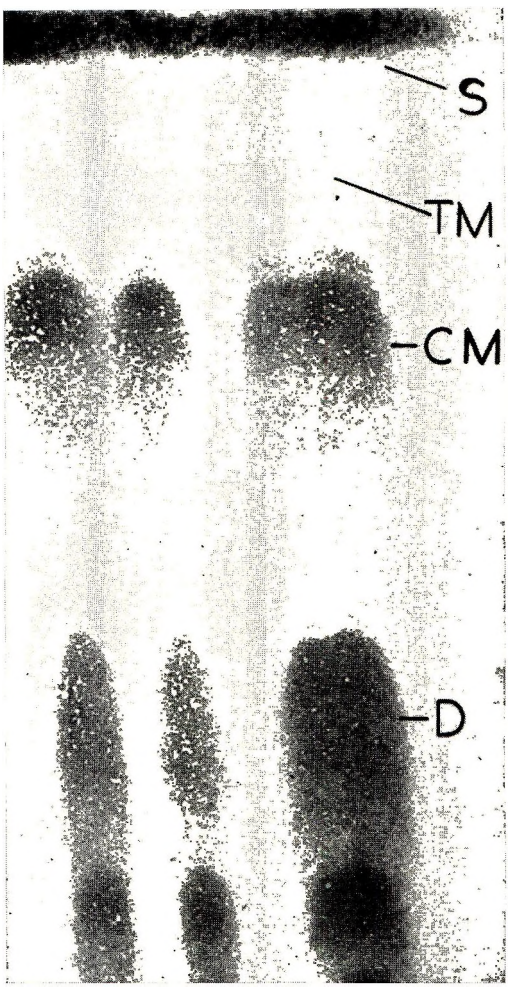

Fig. 4 Thin-layer chromatoplate of the products of the hydrazine reduction of the 20:4 fraction isolated from the liver of the *cis-trans* group of animals. S = saturated, TM = *trans*-monoenes, CM = *cis*-monoenes, and D = dienes.

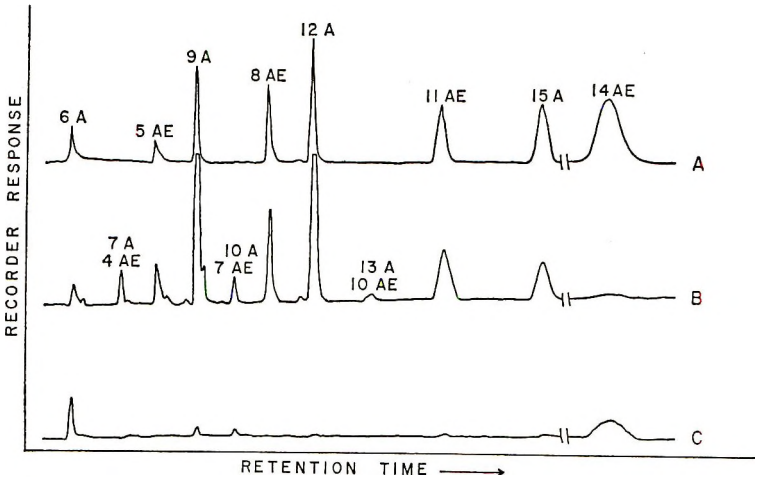

Fig. 5 GLC analysis of the aldehyde fragments produced from the structural analysis of monoenes obtained from the hydrazine reduction of A, arachidonic acid, and B and C the *cis*- and *trans*-monoene fractions, respectively, obtained from the 20:4 of the liver of the animals in the *cis, trans*-linoleate group.

of the products of the hydrazine reduction (fig. 4) show that a small amount of *trans* monoene was present. The structural analysis of this compound (together with a GLC analysis which showed that it was 20:1) showed that the double bond was in the 4-position (fig. 5C). Also shown in figure 5 are the structural analyses of the *cis* monoene fractions isolated from the reduction of arachidonic (fig. 5A) and the 20:4 (fig. 5B) acids isolated from the *cis,trans* group of animals. These results clearly show that not only is the *trans* double bond in the 14-position, but that the bonds in the 5,8,11-position of this isomer have the *cis* configuration. Thus, it may be concluded that the *trans* isomers of linoleic acid are converted to 20:4 by the same pathway as linoleic acid.

ACKNOWLEDGMENTS

The authors wish to acknowledge the technical assistance of Joseph A. Rysavy.

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Effects of Fluoride on Magnesium Deficiency in the Guinea Pig [1]

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ABSTRACT Experiments were conducted with young growing guinea pigs to study the magnesium:fluoride relationship at several dietary levels of magnesium and of fluoride. Addition of fluoride (100 or 200 ppm) to a diet suboptimal in magnesium (0.04%) alleviated soft tissue calcinosis. The effect was evident after 6 and 12 weeks of feeding and appeared to be mediated through a sparing of the magnesium requirement. Growth depression, serum magnesium decrease, and bone magnesium loss were moderated by fluoride addition to the low magnesium diet. The magnesium-sparing and anticalcinatory effects of fluoride were less pronounced in animals fed 0.009% magnesium. A higher level of fluoride (300 ppm) was toxic to guinea pigs fed the low magnesium (0.04%) diet, but not to those fed 0.21% magnesium. Magnesium deficiency was associated with an increase in heart potassium content which was retarded by 100 ppm dietary fluoride. In comparison with data reported previously, species differences are apparent in the magnesium:fluoride interrelationship between the guinea pig, dog and rat.

Magnesium deficiency in the dog, rat, and guinea pig causes a characteristic syndrome which includes decreased growth, a decrease in blood serum magnesium, loss of bone magnesium, and soft tissue calcinosis (1–9). The effect of dietary fluoride on the magnesium deficiency syndrome has been investigated in all 3 species. In the dog, 25 to 200 ppm dietary fluoride reduced or prevented soft tissue calcinosis but had no effect on serum or bone magnesium, serum calcium, serum inorganic phosphorus, or growth (2, 3). In the rat, 200 or 400 ppm fluoride did not alleviate soft tissue calcinosis or any of the other effects of magnesium deficiency (2, 3). In the guinea pig, 450 ppm dietary fluoride for 14 days moderated the decline in serum magnesium in animals fed a low magnesium diet. There was no effect of fluoride on growth of magnesium-deficient guinea pigs. Calcinosis was not observed in this interval (10).

Longer-term experiments were conducted in the present studies to evaluate the effect of fluoride on calcinosis and on certain other criteria of magnesium deficiency in the guinea pig.

EXPERIMENTAL

In a preliminary experiment, young growing guinea pigs of both sexes were fed for 4 to 8 weeks a sucrose-casein diet similar to that formulated by Thompson et al. (10). The levels of calcium, phosphorus and magnesium were varied to select a ration which induced magnesium deficiency with soft-tissue calcinosis but allowed a high rate of survival. Of several combinations tested, a diet with 0.041% magnesium, 0.84% calcium, and 0.84% phosphorus produced in 6 weeks the highest incidence of soft tissue calcinosis which was compatible with a high survival rate.

The following modifications were made in the diet of Thompson et al. (10). The calcium and phosphorus content was adjusted to 0.84% by supplying 29.8 g $CaHPO_4 \cdot H_2O$ and 4.73 g $CaCO_3$/kg diet. Iron was added as ferric citrate at 0.55 g/kg diet. Sucrose was reduced to 41.3% to meet these changes. Changes in mineral additions included (in mg/kg diet) zinc, as $ZnSO_4 \cdot 7H_2O$, 39.8; cobalt, as $CoCO_3$, 0.40; and magnesium, as $MgSO_4$, 1495.

Ascorbic acid was given orally each day in the amount of 8 mg in 0.3 ml of 40% sucrose solution.

In experiment 1, young guinea pigs weighing from 120 to 240 g were allotted to the 8 dietary treatment groups listed in table 1. Each group contained 4 males and

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TABLE 1

Effect of variation in dietary magnesium and fluoride on growth, serum and femur magnesium, heart potassium, and organ calcium in the guinea pig

Dietary treatment		No. animals	Mortality	Body wt gain [1]	Blood serum Mg [1]	Femur Mg [1]	Heart K [1]	Organ Ca [2]			
Mg	F−							Kidney	Heart	Liver [3]	Aorta
%	ppm		no.	g/day	mg/100 ml	% of ash	% dry tissue	mg/100 g dry tissue			
Experiment 1											
0.21	0	8	0	3.72 ± 0.42 [a]	2.89 ± 0.08 [a]			98(1/8)	37(0/8)	11.6(0/8)	
0.21	100	8	0	3.08 ± 0.35 [ab]	2.82 ± 0.11 [ab]			35(0/8)	31(0/7)	10.2(0/8)	
0.21	200	8	0	2.94 ± 0.67 [ab]	2.53 ± 0.08 [b]			48(1/8)	36(0/8)	10.4(0/8)	
0.21	300	8	0	2.90 ± 0.28 [ab]	2.85 ± 0.12 [a]			38(0/8)	40(2/8)	12.2(0/6)	
0.04	0	10	3	1.37 ± 0.30 [c]	1.24 ± 0.14 [c]			134(5/8)	101(3/7)	19.1(2/8)	
0.04	100	8	1	2.45 ± 0.60 [ac]	1.79 ± 0.10 [d]			62(2/8)	34(1/8)	12.8(1/8)	
0.04	200	8	2	2.80 ± 0.26 [ac]	1.60 ± 0.08 [d]			78(2/6)	31(0/6)	11.4(0/5)	
0.04	300	8	4	1.54 ± 0.15 [bc]	1.69 ± 0.17 [d]			144(2/4)	34(0/4)	13.6(0/4)	
Experiment 2											
0.009	0	8	4	0.33 ± 0.28	0.46 ± 0.06 [a]	0.35 ± 0.02 [a]	1.27 ± 0.03 [a]	251(5/5)	313(3/5)	11.1(1/6) [3]	862(4/5)
0.009	100	8	2	1.39 ± 0.45	0.72 ± 0.07 [a]	0.35 ± 0.01 [a]	1.16 ± 0.03 [ab]	375(6/6)	82(3/5)	7.4(2/6) [3]	222(1/4)
0.04	0	10	0	2.74 ± 0.15 [a]	0.98 ± 0.07 [b]	0.39 ± 0.01 [b]	1.18 ± 0.03 [a]	913(8/8)	130(9/9)	48.6(5/10) [3]	420(7/10)
0.04	100	8	1	3.63 ± 0.42 [b]	1.39 ± 0.12 [c]	0.49 ± 0.02 [c]	0.98 ± 0.04 [cd]	396(6/7)	35(1/6)	5.0(1/7) [3]	183(2/7)
0.16	0	8	0	5.20 ± 0.17 [c]	2.58 ± 0.09 [d]	0.65 ± 0.01 [d]	0.96 ± 0.03 [c]	149(6/8)	25(0/8)	5.0(0/8) [3]	74(0/7)
0.16	100	8	0	5.70 ± 0.30 [c]	2.60 ± 0.04 [d]	0.68 ± 0.01 [e]	1.06 ± 0.04 [bd]	83(3/7)	24(0/8)	4.2(0/8) [3]	100(1/8)

[1] Mean ± SE. Any 2 means not having a common superscript letter are significantly different ($P < 0.05$).
[2] Mean followed (in parentheses) by the fraction calcinotic as described in text and in figure 1.
[3] Means calculated on a wet-tissue basis in experiment 2.

4 females with the exception of one group which contained 4 males and 6 females. The low magnesium diet provided 0.041% magnesium as analyzed by atomic absorption spectrophotometry. Additional magnesium was provided to some groups as $MgSO_4$ (0.79%) and fluoride as NaF (0.022%, 0.044% and 0.067%) to provide the amounts specified in table 1. Diet analysis showed that NaF supplements did not provide significant amounts of magnesium.

To improve acceptance, the diets were moistened and "pelleted" by passing through a meat grinder and drying overnight at 60°. The dry diets were stored at −20° until the day of use. Animals were housed (in groups of 4 to 6) in wire-mesh cages. Food and water were given ad libitum. Ascorbic acid was provided as in the preliminary experiment. Animals were weighed weekly and killed on day 42 by a sharp blow on the head and immediate exsanguination. Serum was collected and analyzed for magnesium and calcium by atomic absorption spectrophotometry.[2] Serum inorganic phosphorus was determined by the method of Fiske and Subbarow as described by Hawk et al. (11). Heart, liver, and kidney calcium were determined after drying and dry ashing by atomic absorption spectrophotometry as described previously (3). Results were expressed on a dry-weight basis.

In experiment 2, young male guinea pigs weighing from 155 to 281 g were allotted to 5 groups of 8 and one group of 10 as shown in table 1. The diet was similar to that used in experiment 1 but the basal diet contained only 0.009% magnesium. Additional magnesium was provided to some groups as MgO (0.05% and 0.265%) and fluoride as NaF (0.022%) to provide the amounts specified in table 1.

Ascorbic acid was supplied as before. Chlortetracycline·HCl [3] was given orally as 0.5 mg in 0.3 ml sucrose solution daily.

Analysis of the diet used in this experiment indicated a calcium content of 0.89% and a phosphorus content of 0.74%.

Animals were housed and fed as before. The survivors from the groups fed the lowest level of magnesium (0.009%) were killed on day 28. The remainder of the animals were fed the diets for 12 weeks and killed on day 84. Serum was collected and analyzed for magnesium. Aorta, heart, and kidney calcium were determined and expressed on a dry basis. Liver calcium was expressed on a wet-weight basis.

Femur magnesium analyses and heart potassium analyses were also made, by atomic absorption spectrophotometry, and expressed on a dry-weight basis.

Duncan's new multiple range test (12) was used as a measure of the significance of differences between means other than for organ calcium.

RESULTS AND DISCUSSION

The results of experiments 1 and 2 are given in table 1. Total body weight gain for each animal was divided by the number of days on the experiment to obtain the values expressed in the table. The standard error for some of the organ calcium means was so large that a criterion in addition to mean calcium content for distinguishing between groups was necessary. Figure 1 illustrates the distribution of calcium values in each organ. Data for all animals in the preliminary experiment and in experiments 1 and 2 were pooled to obtain the histograms. These frequency distribution graphs show a peak at the mean calcium content for "normal" guinea pigs, but a wide distribution for calcinotic animals above the "normal." For a particular organ the lowest mean recorded for any group receiving ample magnesium was used as a reference point. Twice this mean was arbitrarily selected as a limit for normal calcium content. The arrows in figure 1 show that this limit also corresponded approximately to the upper limit of the distribution for normal guinea pigs (the peak to the left in each histogram). Calcium values equal to or greater than the limit were classified as calcinotic.

In experiment 1, feeding 0.04% magnesium and no supplemental fluoride resulted in the lowest weight gain and a substantial mortality rate. In this group mean serum magnesium was significantly depressed below that of all other groups and mean organ calcium was high. The incidence of calcinosis in each organ studied was higher

[2] Perkin Elmer Corporation, Model 214, Norwalk, Connecticut.

[3] Aureomycin, American Cyanamid Company, Pearl River, New York.

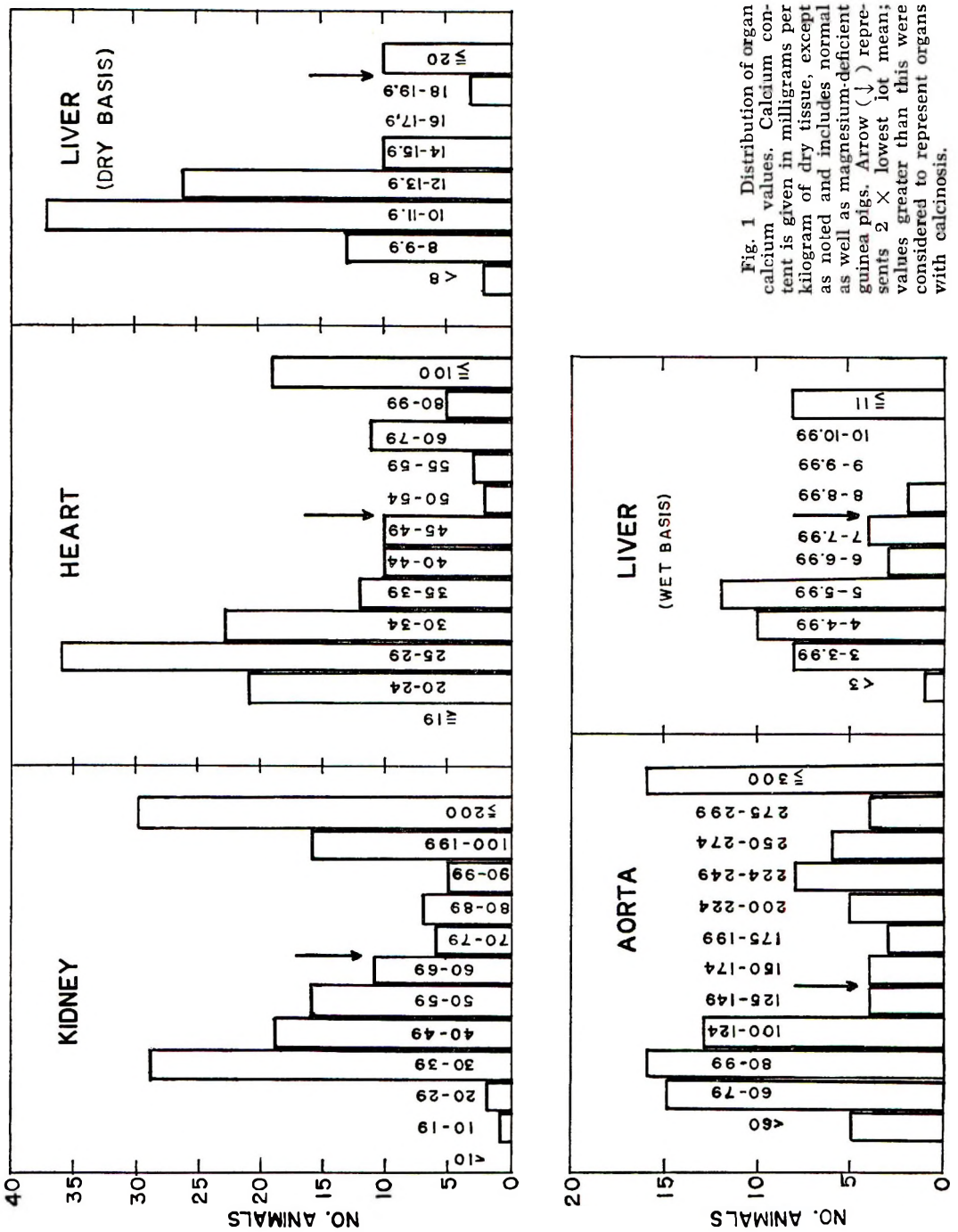

Fig. 1 Distribution of organ calcium values. Calcium content is given in milligrams per kilogram of dry tissue, except as noted and includes normal as well as magnesium-deficient guinea pigs. Arrow (↓) represents 2 × lowest lot mean; values greater than this were considered to represent organs with calcinosis.

than that in any of the groups receiving 0.21% magnesium. Addition of fluoride to the 0.21% magnesium diet had no effect by the criteria studied. However, 100 or 200 ppm fluoride added to the 0.04% magnesium diet reduced the mean organ calcium concentrations and the incidence of calcinosis in kidney, heart and liver; increased body weight gain and serum magnesium significantly; and may have decreased the mortality rate. Supplementation of the 0.04% magnesium diet with 300 ppm fluoride proved toxic as evidenced by an increased mortality, depressed weight gains, and, probably, the observed kidney calcinosis. All 3 levels of supplementary fluoride prevented or reduced the incidence of heart and liver calcinosis due to magnesium deficiency and increased the serum magnesium of the survivors.

Although the data are not recorded here, neither the level of magnesium nor that of fluoride in the diet had an effect on serum calcium or serum inorganic phosphorus.

In experiment 2, the effects of adding 100 ppm dietary fluoride to diets varying in magnesium content were further evaluated. The condition of the animals receiving 0.009% magnesium with or without fluoride deteriorated rapidly. To obtain data on serum magnesium, animals in these 2 lots were killed at 4 weeks on experiment. Magnesium deficiency caused decreased serum and femur magnesium concentrations, organ calcinosis and significantly elevated heart potassium. At 0.009% magnesium, 100 ppm fluoride tended to increase weight gain and serum magnesium, and to reduce heart potassium, but the differences were not significant. Fluoride had little or no effect on the incidence of organ calcinosis, although the mean calcium concentration in 3 out of 4 organs was considerably lower. It appears that, in animals drastically depleted of magnesium, 100 ppm fluoride exerted little protective effect.

In comparison with 0.16% magnesium, feeding 0.04% magnesium for 12 weeks resulted in depressed serum magnesium and weight gain, similar to the effects at 6 weeks of feeding observed in experiment 1. However, the increased length of experiment 2 aggravated organ calcinosis. Femur magnesium was depressed markedly, and heart potassium was significantly increased by feeding 0.04% magnesium instead of 0.16% magnesium. Addition of 100 ppm fluoride to the 0.04% magnesium diet caused a small but significant increase in bone magnesium, a significant elevation of serum magnesium and of body weight gain, a significant decrease in heart potassium, and marked reduction of calcinosis in the heart, liver and aorta. The mean kidney calcium content was lowered by fluoride but the incidence of calcinosis was not noticeably altered.

In experiment 2, feeding 0.16% magnesium resulted in higher body weight gains than feeding of 0.21% magnesium in experiment 1. The possible role of chlortetracycline or other factors in causing this difference was not evaluated.

At the highest dietary magnesium level (0.16%) in experiment 2, fluoride also caused significant effects. It increased femur magnesium and heart potassium slightly but significantly. Also, kidney calcinosis was reduced by fluoride supplementation. It appears that the diet containing 0.16% magnesium without fluoride was not adequate in preventing kidney calcification when fed for 12 weeks.

The observed effect of dietary magnesium level on heart potassium in the guinea pig contrasts with that in the rat (13). In the latter species, magnesium deficiency has been reported to cause a decrease in myocardial potassium of about 10%. In the guinea pig an increase of 20 to 30% in heart potassium was observed, depending on the severity of magnesium deficiency.

It appears that, in the guinea pig, the anticalcinatory effect of fluoride is mediated through a sparing of the magnesium requirement; fluoride not only alleviated the calcinosis but also stimulated growth and increased the concentration of magnesium in blood serum and bone. The correlation between the fluoride-induced moderation of tissue magnesium depletion (serum and femur) with prevention of calcinosis is evident. In experiments 1 and 2, when the tissue magnesium remained at intermediate levels (0.04% magnesium plus fluoride lots), calcinosis was reduced or prevented by fluoride. When tissue magnesium was more thoroughly depleted,

however (experiment 2, 0.009% magnesium lots), fluoride was relatively ineffective in preventing calcinosis. However, in the 0.009% Mg, 100 ppm fluoride lot, fluoride may have retarded the depletion of magnesium, which is consistent with the effect of fluoride in decreasing the mean organ calcium content, yet having no significant effect on the incidence of calcinosis.

Magnesium-fluoride interrelationships in the guinea pig appear to differ from those of the dog and rat (1–3). Fluoride appeared to have a direct influence on magnesium availability or utilization in the guinea pig, whereas in the dog it alleviated the calcinosis without otherwise sparing magnesium and in the rat it had no noticeable effect on magnesium deficiency.

ACKNOWLEDGMENT

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The Physiological Role of Choline in Guinea Pigs

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ABSTRACT The physiological role of choline was investigated by omitting choline from the diet of young guinea pigs. This resulted in a marked decrease in growth, with death of 68% of the animals within 4 to 5 weeks. However, there was no accumulation of liver lipid in contrast with the results of previous studies with rats. With feeding a choline-deficient diet to guinea pigs, the content of various choline derivatives in the liver decreased significantly from the onset of the experiment, along with a decrease of lipid choline in the carcass. These decreases were the major effects of the choline deficiency, with the decrease in liver glycogen preceding that of the carcass lipid. However, injecting the deficient animals with choline chloride during any stage of the experiment reversed these effects without altering the food intake. These observations demonstrate that the various effects described resulted from a deficiency of choline itself. Feeding a choline-deficient diet resulted in an apparent decrease in absorption by the animals. When a decrease in food intake occurred, this produced an acceleration of the effects described. It is postulated that the high mortality in the younger animals might have resulted from severe damage occurring in that period.

It has been reported previously that guinea pigs may have a resistance to choline deficiency because of low choline oxidase activity in their liver (1,2). However, Reid (3) reported that guinea pigs fed a choline-deficient diet suffered severe growth disturbance and had a high mortality. In addition, she found that these effects could not be prevented by adding either methionine, betaine or aminoethanol to the diet. In view of these observations the present study was undertaken in an attempt to clarify the role of choline in the diet. Changes in choline derivatives and glycogen content in the liver of guinea pigs fed a choline-deficient diet are reported, and the physiological role of choline for these animals is discussed.

EXPERIMENTAL

Animals. Guinea pigs weighing 150 to 180 g were fed the diet used by Reid (3), which is listed in table 1. The diet of the control group of animals was supplemented with 0.2% choline chloride. The mortality of the choline-deficient animals was high in comparison with the control group and analytical studies were made only on the survivors. At the end of the experiments the animals were killed by cervical section and exsanguinated. The livers were then resected quickly and prepared for analysis.

Preparation and determinations. For lipid analysis, one gram of the liver was homogenized with 30 volumes of a chloroform-methanol (2:1) mixture, extracted overnight at room temperature, and then filtered. After washing by the method of Folch (4), the evaporated extracts were weighed as total lipid and the analyses were performed. For the analysis of lipid fractions, the extracts were redissolved in chloroform-methanol (2:1).

Lipid phosphorus was determined by the method of Allen (5). After wet-ashing the sample, lipid nitrogen was determined by the indophenol method (6,7). Lipid choline was determined by first hydrolyzing the samples in 4 N HCl [1] at 100° (in a boiling water bath) for 5 hours and then removing impurities by washing the hydrolysate 3 times with 2 volumes of petroleum ether. The aqueous layer was then analyzed for free choline by the periodide method (8) and also by the Reinecke method (9,10). Cholesterol was determined by the Liebermann-Burchard reactions (11,12).

For determination of soluble choline derivatives the liver was homogenized with 10 volumes of 10% trichloroacetic acid

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[1] 3 ml of 4 N HCl solution contained 1 ml of ethanol and the volume was adjusted by adding ethanol every hour. After hydrolysis ethanol was evaporated.

TABLE 1

Composition of diet

	g
Vitamin-free casein [1]	300
Sucrose	103
Starch	200
Cellulose	78
Cellophane	150
Corn oil	73
Salt mix [4]	60
Potassium acetate	25
Magnesium oxide	5
Vitamin mix [2]	34
Folic acid	0.01
Ascorbic acid	2
Vitamin E (20%) powder [3]	0.1
Vitamin A and D mix [5]	0.7
Choline chloride	2

[1] Obtained from Takeda Chemical Corporation, Nagoya, Japan.

[2] Percentage composition of the vitamin mix was: thiamine·HCl, 0.059; riboflavin, 0.059; nicotinic acid, 0.294; Ca pantothenate, 0.235; pyridoxine·HCl, 0.029; menadione, 0.006; biotin, 0.001; folic acid, 0.002; vitamin B_{12}, 0.0002; inositol, 1.176; ascorbic acid, 0.588; and lactose, 97.551 (obtained from Tanabe Pharmacal Corporation, Osaka, Japan).

[3] This powder contained 200 mg of α-tocopherol/g and was obtained from Eizai Pharmacal Corporation, Tokyo, Japan.

[4] Percentage composition of the salt mix was: $CaCO_3$, 29.29; NaCl, 25.06; $ZnCl_2$, 0.02; KI, 0.0005; $CaHPO_4 \cdot 2H_2O$, 0.43; $MgSO_4 \cdot 7H_2O$, 9.98; $CuSO_4 \cdot 5H_2O$, 0.156; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0025; KH_2PO_4, 34.31; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.623; and $MnSO_4 \cdot H_2O$, 0.121 (obtained from Tanabe Pharmacal Corporation).

[5] One gram of the mix contained 50,000 IU of vitamin A and 10,000 IU of vitamin D_2 (obtained from Takeda Pharmacal Corporation, Osaka, Japan).

(TCA) and the homogenate was centrifuged at 600 × *g* for 10 minutes. The supernatant was reserved and the sediment was again homogenized with 10% TCA (10 volumes). This homogenate was centrifuged as before and the supernatant was pooled with that of the first extract. The pooled TCA extract was washed 3 times with ethyl ether. It was then hydrolyzed with 4 N HCl for 5 hours at 100° and free choline was determined by the periodide method (8).

Glycogen was estimated by the anthrone method (13); and protein concentration was determined by the biuret method (14), using albumin as a standard.

Cell particles were prepared according to the method of Schneider (15).

Injection of choline chloride. Choline chloride (0.5% in 50% ethanol, w/v) was dissolved in 11 volumes of 0.9% NaCl. This solution was injected into the test animals intraperitoneally at a concentration of 40 mg/day.

RESULTS

The changes observed in guinea pigs fed a choline-deficient diet for 4 weeks are shown in table 2. Body weight gain for the choline-deficient group was markedly less than that of the control group, and there were few survivors (32%) in the deficient group. Although 3 deaths occurred in the control group, there were no deaths within this group in duplicate experiments (not shown in table 2). In replicate experiments choline-deficient animals died at the same rate as those shown, in agreement with the results of other investigators (1, 3,16).

However, the body weights of the choline-deficient animals reached a maximum and then usually decreased. Furthermore, the animals showing decreased body weight died during the period when their food intake was diminished.

Table 3 lists results of analyses carried out on animals surviving at the end of the fourth week. The experimental animals were divided at random into groups of 4 animals and several components of liver were determined in groups 1 and 2, and the lipid choline content of cell particles was determined in groups 3 and 4 (not shown in table 2). The lipid choline content of the deficient animals decreased significantly as compared with that of the

TABLE 2

Effect of feeding a choline-deficient diet on the growth of guinea pigs

	Control	Deficient
Mean body wt, initial, g	158(19) [1]	162(37)
Body wt gain, 4 weeks, g [2]	119.0 ±22.8 [3] (12)	29.0 ±23.5 (12)
Liver wt, % of body wt	3.82± 0.07 (12)	3.43± 0.16(12)
Liver wt,[4] g	10.1 ± 1.4 (12)	6.6 ± 0.7 (12)
Survival, 4 weeks, %	85(12)	32(12)

[1] Numbers in parentheses indicate number of animals/group.

[2] The difference between 2 groups is statistically significant ($P < 0.01$).

[3] Average ± SE.

TABLE 3

Effect of feeding a choline-deficient diet (4 weeks) on some components of guinea pig liver [1]

Group		Lipid [2]	Lipid P [2]	Lipid N [2]	Lipid choline [2]	Free cholesterol [2]	Soluble choline derivatives [2]	Protein
		mg/g liver	*μmoles/g liver*	*μmoles/g liver*	*μmoles/g liver*	*μmoles/g liver*	*μmoles/g liver*	*mg/g liver*
1	Control	43.1 ± 0.86 [3]	46.7 ± 1.36	42.7 ± 1.43	24.7 ± 0.53	7.19 ± 0.21	5.01 ± 0.32 [4]	262 ± 6.8
2	Deficient	40.5 ± 1.31	40.6 ± 2.20	36.0 ± 2.53	18.8 ± 0.95	6.33 ± 0.35	2.34 ± 0.21 [5]	253 ± 4.9

[1] Each value is a mean of 8 animals except as otherwise noted.
[2] The difference between the 2 means is highly significant ($P < 0.01$).
[3] Average ± SE.
[4] Mean of 4 animals.
[5] Mean of 3 animals.

controls (tables 3 and 4). Total lipid and cholesterol in the liver decreased slightly. This effect differed from the results of experiments with rats (18). Lipid phosphorus and lipid nitrogen also decreased, but at a slower rate than lipid choline. Soluble choline derivatives also decreased significantly, but the protein content per unit of liver weight did not change. At the end of the fourth week the decrease in liver glycogen of the choline-deficient animals was significant, as shown in table 5. Moreover, the carcass lipid of the choline-deficient animals decreased significantly and lipid choline also decreased to about 70% of normal. Liver glycogen values for animals fed ad libitum are shown in table 5. The effect on liver lipids of feeding the choline-deficient diet for 7 and 14 days is shown in table 6. Liver lipid choline and glycogen decreased significantly. In intact animals these lipids decreased only after the animals had been fasted for 24 hours, and then only slightly.

Effects similar to those shown in tables 3, 4 and 6 were obtained on days 9 and 60 of the experiment. Figures 1 and 2 and table 7 summarize the effects of intraperitoneal injection of choline chloride. In the study shown in figure 1 and table 7 the injections were given from day 9. In the injected group, the rate of body weight gain increased significantly and the liver glycogen content was higher than that in the control group. Although the animals showing decreased body weight were selected for injection, a reversal of the effects of choline deficiency was observed after injection of choline chloride. Animals showing decreased body weight at this stage died within a few days after not receiving the injections; and the early deaths were among such animals. As shown in figure 2 a single injection on day 60 was very effective. At the beginning of the second week the lipid choline and glycogen of the liver decreased significantly, but total liver lipid was not significantly decreased (table 6). Furthermore, as shown in table 7, and figures 1 and 2, the food intake of choline-deficient animals was not increased by injection of choline chloride; nevertheless, the increase of both liver glycogen and body weight was significant. Interruption of the injection of choline chloride resulted

TABLE 4

Lipid choline content of liver cell particles of guinea pigs fed a choline-deficient diet for 4 weeks [1]

Group		Mitochondria		Microsomes	
		Lipid choline [2]	Lipid P [2]	Lipid choline [2]	Lipid P
		μmoles/g dry wt		*μmoles/g dry wt*	
3	Control	117 ±4.1 [3]	196±10.4	161±2.5	251±12.5
4	Deficient	71.5±5.8	154±12.2	112±6.7	235±17.8

[1] This value is a mean of eight animals of group 3 and group 4.
[2] The difference between 2 groups is statistically significant ($P < 0.01$).
[3] Average ± SE.

TABLE 5

Liver glycogen, liver lipid and carcass lipid of guinea pigs fed a choline-deficient diet for 4 weeks

	No. animals	Liver glycogen [1]	Liver lipid [1]	Carcass lipid [1]
		mg/g liver	*mg/g liver*	*mg/g carcass*
Control	5	52.6±6.82 [2]	41.6±0.46	60.7±3.10
Deficient	6	10.3±9.11	36.3±0.42	27.5±2.30

[1] The difference between 2 groups is statistically significant ($P < 0.01$).
[2] Average ± SE.

TABLE 6

Effect on liver lipids and glycogen of feeding a choline-deficient diet for one and two weeks [1]

Duration	Group	Body wt gain	Lipid	Lipid P	Lipid choline	Glycogen
days		*g*	*mg/g liver*	*μmoles/g liver*	*μmoles/g liver*	*mg/g liver*
7	Control	38	45.17±1.32 [2]	46.30±1.62	22.8 ±0.46 [3]	64 [3](80–35) [4]
7	Defiicent	2	42.04±1.62	41.84±2.12	19.3 ±0.76	26 (35–18)
14	Deficient	4	43.73±1.42	44.25±1.24	18.06±0.62	

[1] Four male animals/group.
[2] Average ± SE.
[3] The difference between 2 groups is statistically significant ($P < 0.01$).
[4] Range.

in a cessation of body weight gain even though food intake had not decreased appreciably. The injection of choline chloride markedly elevated the rate of body weight gain. As shown in figure 1, stopping the injection of choline chloride resulted in the death of the animal.

DISCUSSION

Even with a high protein diet the effects of choline deficiency in young guinea pigs, such as retardation of growth and high mortality, could not be prevented as shown in table 2; and this is in agreement with the work of other investigators (3,16,17). As shown in table 3, feeding a choline-deficient diet for 4 weeks brought about marked changes in the liver components of guinea pigs; although lipid choline decreased significantly, the total lipid and cholesterol content of the liver did not increase but, rather, decreased slightly, in contrast with results for rats (18). From thin-layer chromatography on silica gel, it was found that most of the lipid choline was contained in the lecithin fraction. The results shown in table 3 suggest that there is insufficient utilization of compounds which can serve as a methyl donor for the synthesis of choline or lecithin in guinea

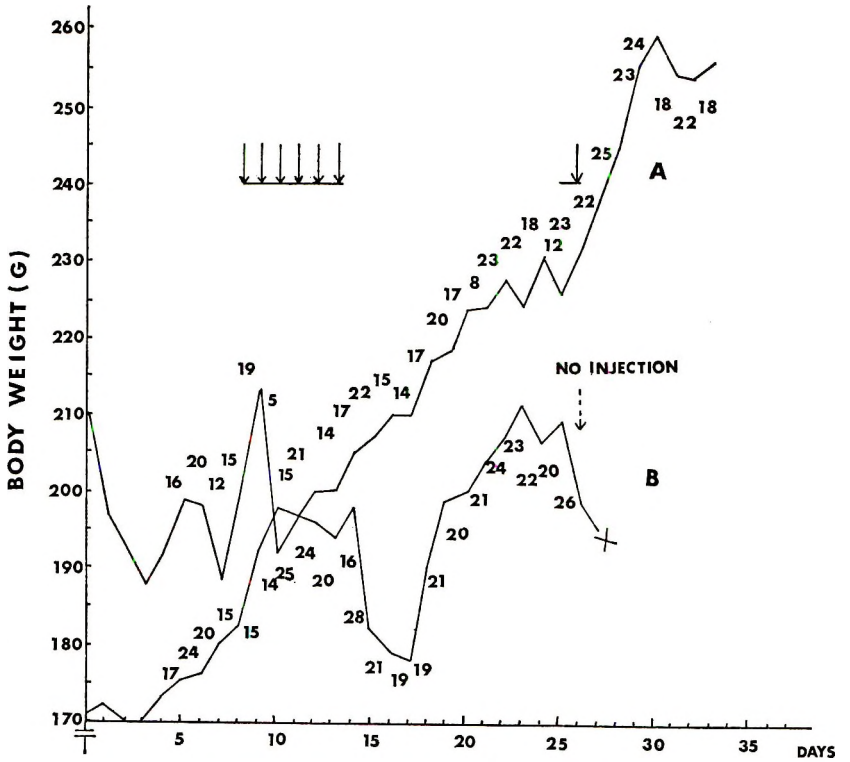

Fig. 1 Effect of intraperitoneal injection of choline chloride on the growth of guinea pigs fed a choline-deficient diet. Forty milligrams/day of choline chloride were injected on the days indicated by arrows; numbers in figure represent grams of diet. Both A and B received injections, but B was not given the final injection. In animals receiving no injection (not shown here) body weight gain was slight after day 10 (see text and table 7).

pigs. Using liver slices, Pilgeram (19) demonstrated a lower synthetic capacity of lecithin from ethanolamine in guinea pigs than in rats. Reid (3) also demonstrated the ineffectiveness of substituting ethanolamine for dietary choline. However, as shown in table 5, the significantly low liver glycogen level in the choline-deficient animals, even when fed ad libiutm, suggests a caloric deficiency, and a decrease in lipid choline might result from this. However, as shown in figures 1 and 2 and table 7, the retardation of growth as well as other effects noted in the animals fed the choline-deficient diet were improved after injections of choline chloride. With feeding a choline-deficient diet the volume of feces increased, along with a decrease in liver glycogen, and in some instances diarrhea resulted. However, injections of choline chloride stopped the diarrhea and decreased the volume of feces. An increase in liver glycogen and weight gain accompanied these improvements.

Such observations suggest that a choline deficiency results in a disturbance of intestinal absorption of nutrients and that the decrease in liver glycogen reflects this. However, from the start of the experiment the choline-deficient group showed slow increases in body weight and the rate of gain was markedly lowered, especially after the second week. A marked decrease in body weight in some animals was also associated with decreased food intake. It was found that the decrease in food intake occurred after a transient period of increased appetite in some instances. The reason for this is not clear but it may have been the result of hypoglycemia. The change in food intake after interruption of the injecting of choline chloride indicates a time relationship between the depression of food utilization and the decrease in food

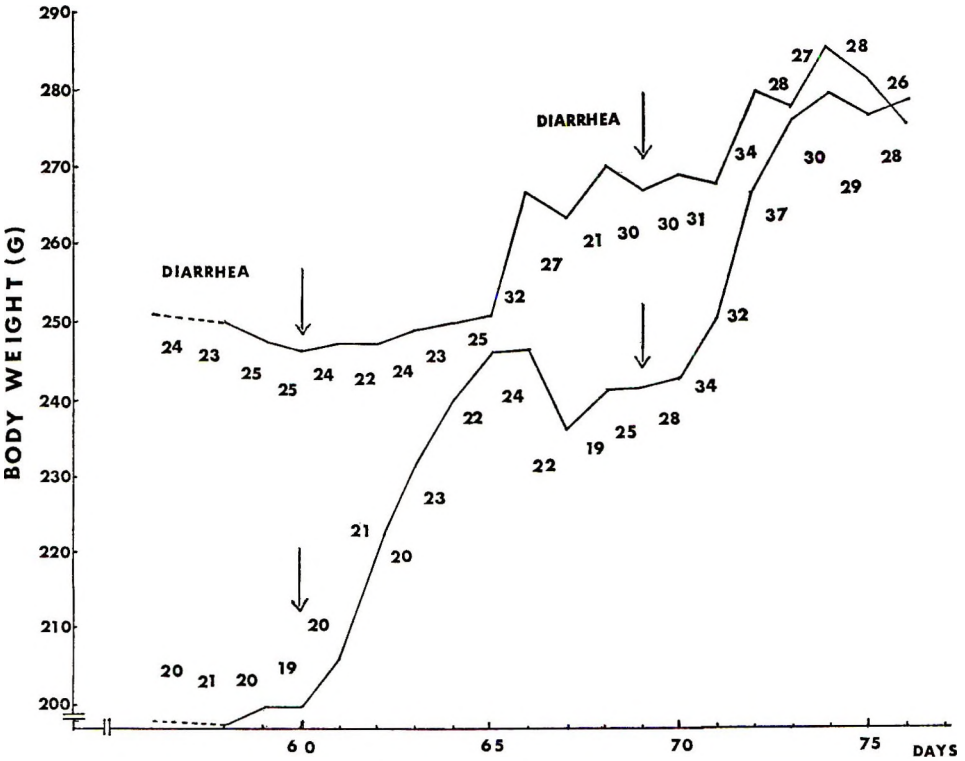

Fig. 2 Effect of intraperitoneal injection of choline chloride on growth of guinea pigs fed a choline-deficient diet for 60 days. Forty milligrams/day of choline chloride were injected on the days indicated by arrows; numbers in figure represent grams of diet.

intake (figs. 1 and 2). Such a relationship was also observed in the early stage of the experiment. A decrease in food intake accelerated the decrease of liver glycogen and body weight which would result from a disturbance in intestinal absorption. This duplication of the 2 mechanisms would be related to the high mortality.

Therefore, since the rate of decrease of food intake varied with individual animals, those animals that suffered most seriously would die at the earlier stage. Concerning the decrease of total lipid and cholesterol in the liver, it was postulated that the decrease in liver glycogen, which resulted from a choline-deficient diet, was accompanied by hypoglycemia, and under such conditions lipogenesis would be significantly depressed.

According to unpublished data,[2] the uptake into lipid from acetate-1-^{14}C was depressed in the choline-deficient animals; the uptake into liver lipid was slightly lower than that for controls, but the uptake into carcass lipid was much lower than for the controls. However, the ^{14}C uptake into long-chain fatty acids in liver slices excised from the choline-deficient animals was greater when the medium contained 5 mM glucose and 2 mM acetate and insulin. This was the case with adipose tissue in rats as reported by Cahill et al. (20).

Thus the low level of liver glycogen and hypoglycemia in the choline-deficient animals results in a depression of lipogenesis and in an increase in lypolysis. On the other hand, since the ^{14}C uptake into fatty acid in adipose tissue was markedly depressed in vitro under the same conditions as for liver, and the uptake was elevated by choline treatment,[3] the slight decrease in liver lipid and marked decrease in carcass lipid would result from both a decrease in liver glycogen content and a

[2] Tani, H., Y. Aoyama, K. Ashida and Y. Kotake, unpublished data.

[3] See footnote 2.

TABLE 7

Effect of injecting choline chloride into guinea pigs fed a choline-deficient diet [1]

Treatment	No. animals [2]	Initial body wt	Body wt			Dietary intake		Total liver lipid choline	Liver wt	Glycogen [4]
			Before injection	3 days after injection	Gain [3]	Before injection	After injection			
		g	*g*	*g*	*g*	*g*	*g*	*μmoles*	*g*	*mg/g liver*
Choline	5	153	147	158	+11	13	13	117.93 ± 0.53 [5]	6.64	53(80–40) [6]
None	4	159	168	157	−11	14	14	106.16 ± 0.48	5.62	20(35–10)

[1] Injections were given every day. Animals were killed 24 hours after last injection.
[2] Male animals.
[3] Body weight of the animals injected increased in all cases, though the animals that lost weight were included in the injected group.
[4] The difference between the 2 means is statistically significant ($P < 0.01$).
[5] Average ± SE.
[6] Range.

depression of lipogenesis based on changes of cell constituents (decrease of choline derivatives). However, the quantitative correlation of changes in lipid content, which resulted from a choline-deficient diet, between the liver and carcass was the same with rats (21), although the level in guinea pigs was lower. Even though the content of most of the choline derivatives was decreased, the decrease in lipid phosphorus was relatively less compared with that of lipid choline (tables 3 and 4). This might also result from insufficient utilization of components acting as a methyl donor in guinea pigs.

The effect of the diet was much more pronounced in the younger than in the older animals and it may depend on the choline requirement and its turnover rate for younger animals. Furthermore, choline, which was taken up, functioned as a pacemaker for growth as shown in figures 1 and 2; and one of its roles may be related to a choline-dependent but unknown mechanism of intestinal absorption. Moreover, since choline derivatives may also be related to other physiological functions as well as to cellular constituents, this decrease would be related to the growth process.

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Effects of Excess Dietary Iodine upon Pullets and Laying Hens [1,2]

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ABSTRACT Iodine as potassium iodide was fed to sexually mature pullets and hens that had completed one year of lay, to study the effects upon egg production, fertility, hatchability, embryonic mortality and incubation time. Dietary intakes at 5 levels from zero to 5000 ppm iodine were fed in a practical-type diet for 6 weeks. Measurement of egg production was continued following the iodine feeding period. Egg production decreased with increasing levels of iodine and ceased with intakes of 5000 ppm. The decreases were greater for hens than for pullets. Production commenced and increased rapidly within one week after change to control diets and was equal to that of control birds during a 27- and 18-week subsequent feeding period for pullets and hens. Molting did not accompany the cessation of lay in pullets but some mature hens molted. Mature ova were present in birds not laying but ovulation did not occur. Weight of eggs produced during the period of iodine feeding was reduced but returned to normal within 3 weeks. Fertility of eggs was not affected but high embryonic death, low hatchability and delayed hatching were observed.

Iodine is recognized as one of the essential elements for poultry and is normally provided in the diet as iodized salt. At the level provided by 0.5 or 1.0% of iodized salt in the diet, no abnormal or harmful effects are produced, but toxic effects have been observed with experimental intakes of excess iodine. Decreased egg production, prolonged hatching, enlarged thyroids of chicks, decreased egg size and appearance of wiry down have been reported (1–3). A more recent study (4) has confirmed these observations and extended them to other effects of iodine. With 5000 ppm iodine as KI in the diet, production ceased within one week and was reduced to 10% at 2500 ppm. Fertility of eggs was not affected, but high embryonic mortality and delayed hatching resulted.

Studies with other species have also demonstrated harmful effects of iodine (5, 6). Rats fed 500 to 2500 ppm iodine were fertile and produced litters, but lactation failed and high mortality of the young resulted. Pregnant rabbits fed 250 to 1000 ppm iodine produced litters but most young died within 48 hours after birth.

The present study was conducted to determine the influence of feeding high levels of iodine upon subsequent egg production and to compare the effects of iodine on pullets and mature hens.

EXPERIMENTAL PROCEDURE

Pullets. One hundred and fifty 27-week-old White Leghorn pullets were assigned at random to 30 replicates of 5 birds each and housed in individual laying cages. Thirty pullets each were fed diets containing zero, 625, 1250, 1875 or 2500 ppm iodine as potassium iodide. Four additional replicate treatments of 5 pullets each were provided with 5000 ppm iodine for 105 days. The basal diet to which selected levels of iodine were added consisted of the following: (in per cent) ground yellow corn, 67.7; soybean meal, 20.6; alfalfa meal, 3.0; ground limestone, 5.8; defluorinated phosphate, 1.96; iodized salt, 0.4; and microingredients, 0.5. The microingredients supplied per kg diet: vitamin A, 4,400 IU; vitamin D_3, 1,540 ICU; and (in milligrams) choline, 500; niacin, 13.2; riboflavin, 4.4; Ca pantothenate, 8.8; ethoxyquin, 12.5; $MnSO_4$, 220; and (in micrograms) vitamin B_{12}, 13.2.

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On the second day after iodine feeding started, pullets were artificially inseminated using pooled semen from normal males. Eggs were collected daily and those produced from day 2 through day 14 after insemination were stored and subsequently incubated. Pullets remaining in production at 35 days were again inseminated and the eggs incubated.

Individual egg production records and weight of eggs were determined through the experimental period and for an additional 27-week period after removal of iodine. Fertility of eggs produced during the period of iodine feeding was determined using the conventional candling technique. During incubation, eggs were candled on days 4, 10 and 18 as a measure of embryonic mortality. Records were also made of hatching time, number hatched, eggs pipped without emerging and those which died in the shell.

Representative one-day-old chicks hatched from eggs produced with the control and each experimental diet were weighed, identified and fed a practical-type chick starter diet. Growth of the chicks as measured by body weight at 2 weeks was determined. Five one-day-old chicks from control pullets and five from those fed 2500 ppm iodine were killed and the thyroids removed, weighed and subsequently examined histologically. At the end of the iodine feeding period, 2 birds from each dietary treatment were killed and the reproductive system was examined.

Mature hens. One hundred mature White Leghorn hens which had been in production for 12 months were used in a concurrent study. Four replicates of 5 hens each received the same basal diet and diets with iodine described for pullets. The same procedures for feeding, insemination and incubation described for pullets were followed with the hens. No eggs, however, were available for incubation at the end of the 6-week period. Interior egg quality was determined for control hens and those fed 2500 ppm iodine at 10 weeks after removal of iodine. Quality was based upon Haugh units calculated from albumin height and egg weight. Statistical calculations were based upon analysis of variance (7).

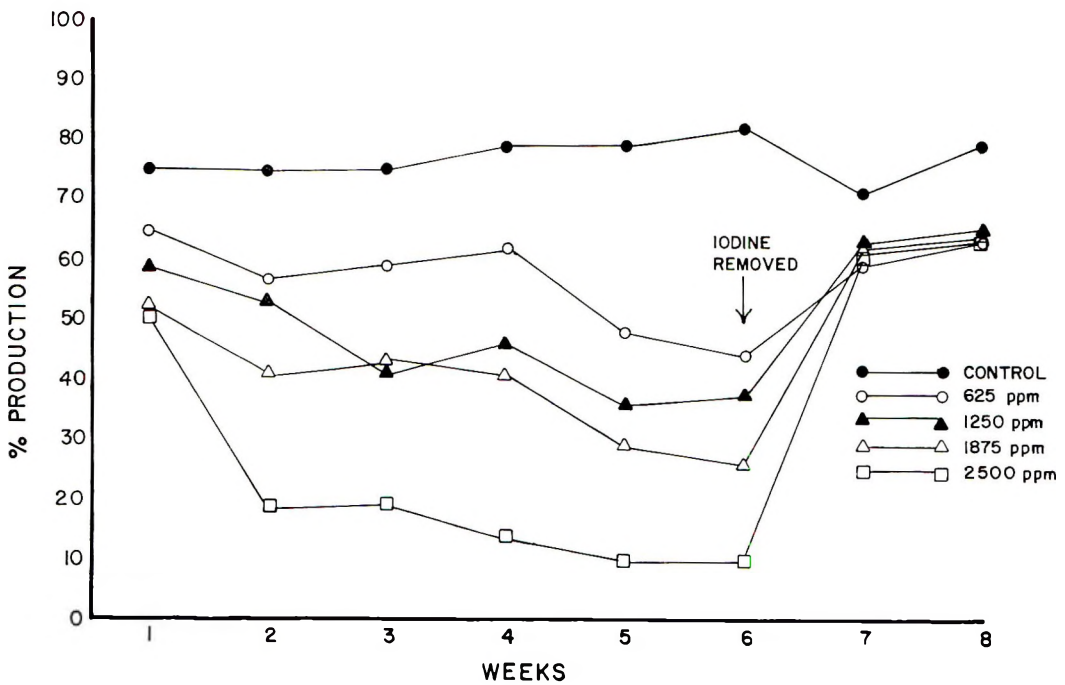

Fig. 1 Egg production of pullets during period of iodine feeding and after removal of dietary iodine.

TABLE 1

Rate of egg production in pullets and hens following the experimental period of iodine feeding [1]

Dietary iodine	Egg production	
	Pullets	Hens
ppm	%	%
0	58.1	37.8
625	59.7	35.0
1250	60.0	40.7
1875	59.1	40.3
2500	56.3	39.3

[1] Production computed as average of 27 weeks for pullets and 18 weeks for hens, beginning 2 weeks after iodine was removed from feed.

RESULTS

Egg production of pullets fed iodine varied inversely with level of iodine beginning during the first week of treatment (fig. 1). With 5000 ppm iodine, virtually no eggs were produced after the second week. Pullets fed the control diet maintained a production rate of 75% or more throughout the 6-week experimental period.

No molting accompanied the cessation of lay, and production increased rapidly after removal of dietary iodine. At the end of the 6-week period, production of pullets fed 2500 ppm iodine was 13% that of controls. By one week after iodine was removed, production equaled approximately 80% that of controls. The rate of production during 27 weeks after removal of iodine was not significantly different from controls (table 1). Two pullets in the group fed 2500 ppm iodine failed to return to production. At the end of the 27-week period, they were killed and eggs with several membranes were found in the isthmus. Tumerous growths present were evidently preventing their passage. This condition was not considered to be caused by iodine and these pullets were not included in the calculation of egg production data. If these 2 pullets had been included,

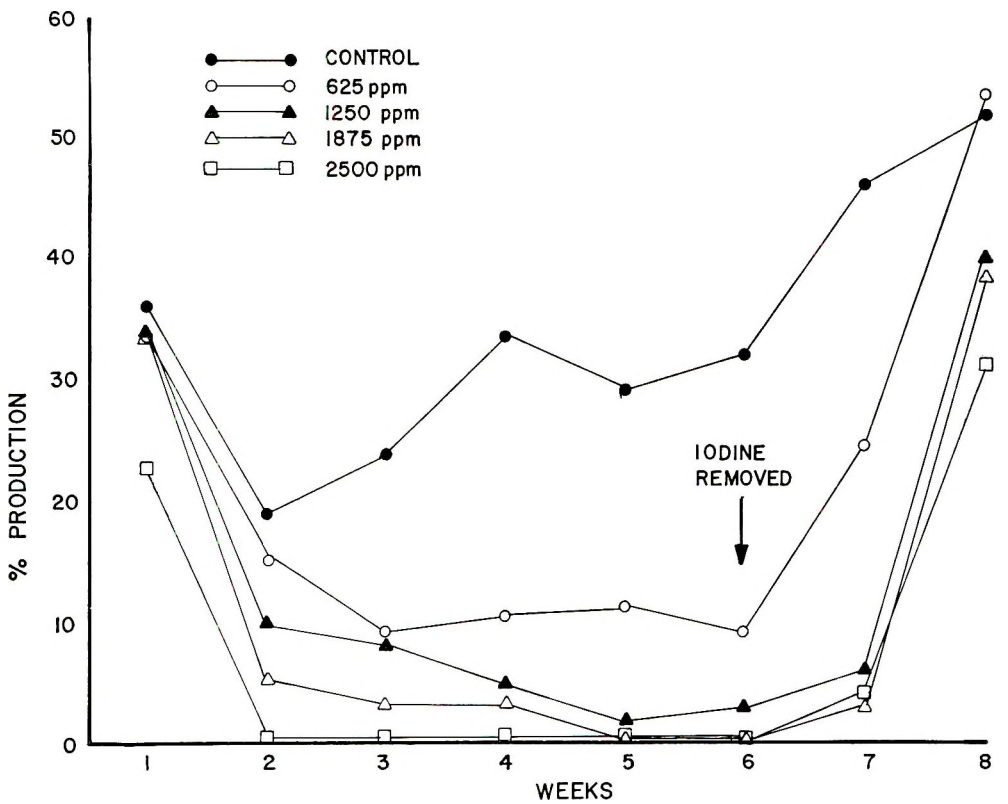

Fig. 2 Egg production of hens during period of iodine feeding and after removal of dietary iodine.

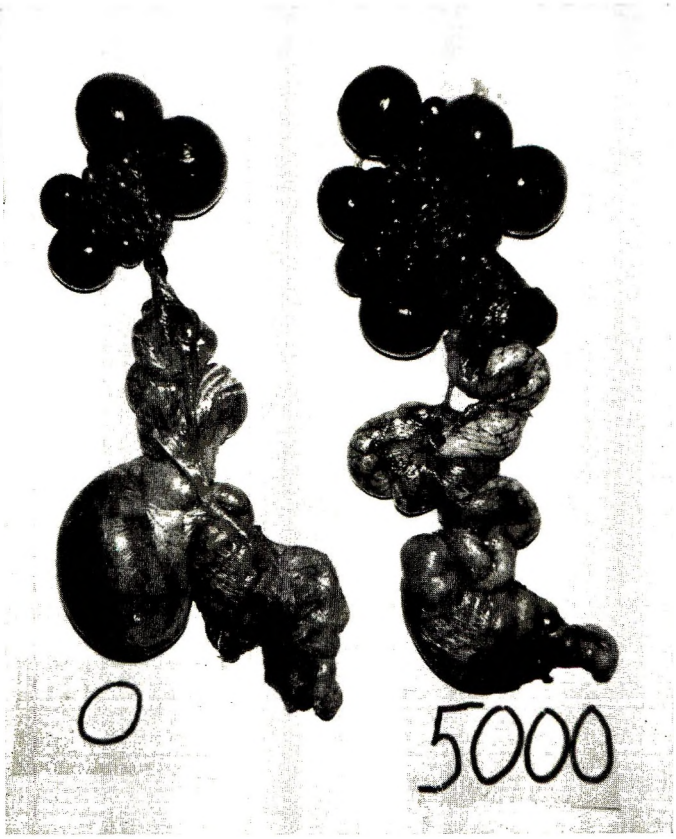

Fig. 3 Reproductive tracts from hens receiving control diet (left) and 5000 ppm iodine for 5 weeks. Note that the ovary was still functioning and ova were present in hen fed 5000 ppm iodine, but hen was not laying.

the rate of production would have been 51.8 instead of 56.3% (table 1).

Pullets provided 5000 ppm iodine were continued for 15 weeks and remained out of production. When returned to the control diet, all except one returned to production within 10 days. The rate of production for 16 weeks, beginning 2 weeks after removal of iodine, was 51.7% compared with 51.6% for the control group.

Mature hens responded somewhat differently to iodine treatment than pullets although the major effects were similar. The hens were removed from floor pens to laying cages at one week before iodine was fed. A small percentage of the hens in each group molted, but the proportion in the control group was less than in the iodine groups. Rates of egg production are shown in figure 2. Sixty-eight per cent of all hens fed iodine were out of production after one week and by 4 weeks, 95% had ceased laying. Only 40% of the control hens were out of production at that time. When the iodine was removed from the diet, they returned to production in a manner similar to that observed for pullets. The rate of production for these hens was equal to that of the controls for an 18-week period beginning 2 weeks after removal of iodine from the feed (table 1).

Physical condition of pullets and hens out of production was such that they would have been judged to be laying. Position of the pelvic bones indicated production and yellow pigment was absent from beaks and shanks. Ovaries of hens not laying contained many ova in various stages of development but ovulation was not occurring. Some ova appeared to be

regressing. The ovary labeled 5000 ppm (fig. 3) was from a hen which had been out of production for 5 weeks.

Average of individual weights of eggs produced by the pullets fed all levels of iodine were significantly less than that of controls (table 2). At 3 weeks after removal of iodine, egg weights were normal. Interior quality of the eggs was improved following the rest induced by iodine in the mature hens. Average Haugh units of eggs from control hens maintained in continuous production was 62.6. The average value for eggs produced by hens at 10 weeks after removal from 2500 ppm iodine was 70.4.

Fertility of eggs produced was not affected by iodine feeding, but hatchability was decreased and embryonic mortality and length of hatching time increased (table 3). The percentage hatchability decreased with increasing levels of iodine and was considerably less with eggs produced near the end of the iodine feeding period. Approximately 80% of all embryonic deaths occurred within the first 4 days of incubation. A number of the embryos survived the incubation period and pipped the shell, but were unable to emerge. Hatching time was delayed by 24 hours or more in 20 to 100% of the eggs hatched from hens fed iodine. Some eggs were hatched following a delay of more than 96 hours.

Thyroid weights of the chicks from hens fed 2500 ppm iodine were 3 times larger than those of controls but no specific differences in microstructure were observed.

TABLE 2

Egg weights from pullets during and following consumption of excess iodine

Dietary iodine	Avg egg wt			
	10 days	3 weeks	6 weeks	3 weeks after return to basal
ppm	*g*	*g*	*g*	*g*
0	55.0 (195) [1]	56.2 (170)	57.2 (157)	57.9(153)
625	52.9 [2](180)	50.9 [2](120)	51.0 [2](92)	57.6(101)
1250	52.1 [2](171)	49.3 [2](106)	50.4 [2](74)	57.9(129)
1875	50.8 [2](146)	46.2 [2](86)	45.8 [2](56)	57.9(141)
2500	53.4 (102)	47.6 [2](32)	48.7 [2](17)	58.6(102)

[1] Figures in parentheses represent number of eggs.
[2] Significantly less than control ($P < 0.01$).

TABLE 3

Hatchability, incubation time and thyroid weight of chicks from pullets fed excess iodine

Dietary iodine	No. fertile eggs set	Embryonic death, 4 days [1]	Hatched [1]	Pipped, not hatched [1]	Delayed hatch [2]	Tyhroid wt
ppm		%	%	%	%	*mg*
			2–12 Days			
0	145	4.8	82.5	1.4	0.8	2.4
625	145	20.7	55.9	5.5	19.8	—
1250	131	30.5	45.8	12.2	25.0	—
1875	109	27.5	39.4	20.2	39.5	—
2500	80	17.5	40.0	13.8	31.3	7.7
5000	20	5.0	0.0	75.0	—	—
			35–45 Days			
0	195	1.5	91.8	1.0	2.2	—
625	94	47.8	12.8	2.1	83.3	—
1250	84	55.9	13.1	4.8	90.9	—
1875	57	59.6	0.0	1.7	—	—
2500	16	43.7	6.2	6.2	100.0	—

[1] Percentage based on number of fertile eggs set.
[2] Delayed by 24 hours or more; percentage based on numbers of chicks hatched.

Hatching weight of the chicks was not affected by iodine, but the chicks from the iodine group appeared weak and did not grow as well as controls. Control chicks averaged 88.5 g body weight at 2 weeks of age and those from pullets fed 2500 ppm iodine averaged 68.7 g.

Relatively few eggs were available for incubation from mature hens and none were produced near the end of the iodine feeding period. Those which were collected early after iodine feeding was started and were incubated, indicated the same effects upon hatchability as observed in pullets.

DISCUSSION

The difference in response of pullets and mature hens to excess dietary iodine is not understood. The change of hens from floor pens to laying cages may have initiated the molting and decrease in production independent of iodine. No molting was observed in mature hens in a former study (4) and did not occur among pullets in the present study. In a subsequent study in progress, high level feeding of iodine has resulted in considerable molting among hens which had been in production for 13 months. The different response observed may have been due to a difference in hormone production at the different age. It is possible that a natural molt and interruption of production was imminent in hens at the time of treatment. A clarification of the mode of action of iodine which permitted formation of ova without release should provide a greater understanding of the ovulation cycle in chickens.

The general absence of molting, physical condition of birds fed iodine and the presence of ova in hens out of production suggest that the action of iodine in causing the cessation of production is different from the action of other substances or methods of forced resting of hens. A specific effect of iodine in causing these and other effects in poultry and in rats and rabbits (5, 6) has not been identified. The rapid return to normal egg production and normal reproduction and lactation in rats and rabbits after removal of iodine suggest some temporary interference with hormone production or action. The presence of many follicles without ovulation in hens suggests an inhibition of luteinizing hormone. Pullets which had received 5000 ppm iodine for 15 weeks laid as well as controls after removal of the iodine. This rest in production did not, however, result in a higher rate of production following the interruption as is observed with some methods of forced resting.

ACKNOWLEDGMENT

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Biosynthesis of Ascorbic Acid in the Acouchi and Agouti [1]

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ABSTRACT The biosynthesis of ascorbic acid has been studied in the acouchi and agouti, 2 members of the Hystricomorph sub-order of Rodentia. Unlike the guinea pig, a member of this same sub-order, the acouchi and agouti are able to produce endogenous ascorbic acid, as demonstrated by studies of homogenates of livers obtained from these animals. Ingestion of a vitamin C-deficient diet by the agoutis produced none of the classical signs of scurvy. In view of these findings, it would be of interest to study other, closely related animals to determine whether they possess the ability to synthesize the vitamin.

Rodentia constitutes the largest order, numerically, of living mammals, containing over half the total species and over a third of all the genera. For purposes of organization, this order is divided into 3 sub-orders: the Sciuromorphs, the Myomorphs and the Hystricomorphs. Included in the last of these sub-groups are such animals as the porcupine, guinea pig, capybara, agouti and acouchi. While various genera of the porcupine family are scattered by nature throughout a great area of the world, the other animals listed are found naturally only in certain sections of Central and South America (1).

One member of the Hystricomorphs, the common guinea pig (*Cavia cobaya*), is well-known to require ascorbic acid. Apparently this rodent, man, some monkeys, the Indian fruit bat (*Pteropus medius*) and the bird, the red-vented bulbul (*Pycnonotus cafer*) (2) are the only animals which have been shown to be unable to synthesize ascorbic acid. It would be of theoretical and perhaps practical importance to know whether other animals closely related to the guinea pig possess this biochemical capability.

Three of the 15 families included by Sanderson (1) in the Hystricomorph suborder grossly resemble the guinea pig and are presumably closely related to it. These are the cavies, the capybaras and the pacagoutis.

The cavies (*Caviidae*) include 4 genera of small animals similar to the guinea pig and the mara (*Dolichotis*) which is much larger and resembles a terrier in size and a small deer in appearance. The common guinea pigs are descendents of the wild species, *Cavia cutleri*, which was apparently domesticated by the Incas or earlier. The various genera of guinea pigs inhabit a large area of South America, ranging from the high Andean mountains to the damp, marshy lowlands of Brazil and Paraguay. The mara is native to the semi-desert areas of southern Argentina and Patagonia.

The capybara (*Hydrochoeridae*) is the largest living rodent, reaching a weight of over 90 kg. It resembles a grossly overgrown guinea pig.

The pacagoutis (*Dasyproctidae*) include the agoutis (*Dasyprocta*) which are common throughout the South American tropics. They have a more delicate build and longer legs than the guinea pig and are about the size of ordinary rabbits (fig. 1). The paca (*Cuniculus*) is a larger guinea pig-like rodent whose habitat ranges from Mexico to Argentina. The animal, which is a common one in its native areas, may attain a length of up to 61 cm. This family also includes the mountain paca (*Stictomys*) found in the highlands of Ecuador and Colombia, which is somewhat smaller than the lowland paca, and the acouchi (*Myoprocta*). The acouchi is slightly taller

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Fig. 1 The agouti.

Fig. 2 The acouchi.

than the guinea pig but more delicately built (fig. 2).

We have obtained limited data demonstrating the ability of the agouti and acouchi to synthesize ascorbic acid. Since it is uncertain whether it will be possible to complete the series in this laboratory, we wish to report the data at this time in the hope that others who have the opportunity may be stimulated to study these animals.

EXPERIMENTAL

One acouchi (*Myoprocta acouchy*), weighing 650 g and 2 agoutis (*Dasyprocta aguti*), weighing 3.3 and 3.4 kg, were obtained from Tarpon Zoo, Tarpon Springs, Florida. When first received, they were fed commercial rabbit ration and cabbage leaves. Six weeks following the surgical procedure outlined below, the agoutis were given a vitamin C-deficient diet [2] for 6 months. Serum ascorbic acid levels were determined at semimonthly intervals for the first 2.5 months of the 6 month period by the method of Roe and Kuether (3).

All 3 animals were subjected to operative biopsy, the acouchi under ether anesthesia, the agoutis under pentobarbital sedation, and approximately 2.5 g of liver were removed. Unfortunately, the acouchi expired during surgery. Five per cent homogenates of the liver were prepared and incubated with L-1,4-gulonolactone (4)[3] and the ascorbic acid produced during the 2-hour incubation was measured (3).

RESULTS

During the 6-month period in which animals were fed the vitamin C-deficient diet, the agoutis increased slightly in weight, from 3.3 to 3.6 kg and from 3.5 to 4.0 kg. Their serum ascorbic acid remained essentially the same over the 2.5-month period during which it was measured, being 2.6, 1.9 and 2.0, 1.8 mg/100 ml at the beginning and at the end of that period, respectively. No evidence of a need for exogenous ascorbic acid could be demonstrated although clear-cut scurvy developed in guinea pigs fed the same diet.

Previous work with the procedures used in this study had demonstrated, in agreement with studies of other workers (4, 5) that guinea pig liver fails to synthesize ascorbic acid. Liver from 2 prosimian species, the tree shrew and slow loris, did, however, produce ascorbic acid under similar analytical conditions (6). Liver tissue from several other primates, the cottontop marmoset, mustached tamarin and mystak tamarin, not previously studied, failed to produce ascorbic acid.[4] In the present investigation, 5 ml of a 5% homogenate of the agouti livers produced from 135 to 195 μg of ascorbic acid during incubation, with average values of 154 and 156 μg for the 2 animals. The liver from the acouchi produced from 258 to 276 μg, average 267 μg.

[2] The vitamin C-deficient test diet (General Biochemicals, Chagrin Falls, Ohio) contained: (in %) skim milk powder (heated 2 hours at 100°), 30; rolled oats (fortified), 39; wheat bran, 20; cod liver oil, 2; cottonseed oil, 8; and sodium chloride, 1.

[3] 10 mg of L-1,4 gulonolactone incubated for 120 minutes at 37° under air in 5 ml of a 5% homogenate in 0.15 M phosphate buffer (pH 7.2) containing 0.13 M sucrose.

[4] Unpublished data.

DISCUSSION

The data are probably sufficient to conclude that the agouti does not require ascorbic acid in the diet, and although no nutritional studies were made, it is unlikely that the acouchi requires this vitamin. It is apparent, therefore, that inability to convert the gulonolactone to ascorbic acid is not characteristic of all members of the family Dasyproctidae. It would be of interest to determine whether the need for ascorbic acid is a common trait of other genera of the same family as the guinea pig or whether this rodent is truly unique.

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Effects of Selenate, Selenite and Tellurite on the Growth and Early Survival of Mice and Rats [1]

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ABSTRACT To evaluate long-term effects of low doses of selenium and tellurium on the growth and survival of small mammals, rats numbering 102 or more were given 2 μg/ml selenium in drinking water, either as Na_2SeO_3 or Na_2SeO_4, and 2 μg/ml tellurium as Na_2TeO_3. Selenate and tellurite were well-tolerated. Selenite was not, males dying rapidly at early ages and females less so. Growth was depressed. Mice numbering 108 or more were given selenite or tellurite, 2 μg/ml of the element in drinking water. Both elements were tolerated. Growth was enhanced in males given selenite, and somewhat depressed in females. At 15 months of age male mice given tellurite showed some increased mortality and body weights were less than those of controls. Species of animal, sex and valence state of selenium were of importance in evaluating effects of this trace element.

Life-term studies on rats and mice exposed to low doses of various trace elements are being conducted in an environment designed to exclude contaminants, the elements being given in drinking water (1–3). The purpose of these experiments is to evaluate possible effects in terms of life-span, longevity, rate of growth and the appearance of diseases or pathological changes hitherto unsuspected to be related to the element given. Currently under investigation are selenium and tellurium. A rather striking toxicity of selenite, but not of selenate, appeared in weanling rats but not in mice shortly after initiation of administration of this element, which deserves reporting.

METHODS

The environment, the low metal diet, the precautions used to avoid contamination and the composition of the drinking water given to animals have been described in detail (1, 2). Besides zinc, copper, manganese, cobalt and molybdenum, the water contained 1 μg/ml chromium as the acetate (4). Three hundred and twenty-six mice of the Charles River CD strain, and 418 rats of the Long-Evans strain were born in our laboratory from random-bred pregnant females purchased from the suppliers.[3] At the time of weaning (20–22 days of age), they were divided as to sex, and littermates divided among groups, 6 mice and 4 rats per cage as follows: 54 male and 56 female mice were given 2 μg/ml selenium as sodium selenite in drinking water; 54 males and 54 females were given tellurium as sodium tellurite in the same dose; an identical number served as controls. Forty-eight male and 53 female rats were given 2 μg/ml selenium as sodium selenite in drinking water; 52 males and 55 females were given 2 μg/ml selenium as sodium selenate; 52 males and 52 females were given 2 μg/ml tellurium as sodium tellurite; 53 males and 53 females served as controls. Animals were weighed weekly at first and later at monthly intervals, the mice in groups of six and the rats in groups of four. The diet contained 1.65 μg/g selenium [4] and 0.22 μg/g tellurium,[5] on a wet basis. Dead animals were autopsied and gross lesions noted. Data were analyzed by Student's *t* test and by chi-square analysis.

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[3] The Charles River Mouse Farms, Inc., Ballardvale Street, North Wilmington, Massachusetts 01887, and Blue Spruce Farms, Inc., Altamont, New York 12009.

[4] Mean of 10 analyses by the colorimetric method of Bornhorst and Mattice (5), using 3.3'-diaminobenzidene.

[5] Analysis by atomic absorption spectrophotometry. Model 303, Perkin-Elmer Corporation, Norwalk, Connecticut 06852.

RESULTS

Effect of selenite and tellurite on growth of mice. Male mice given 2 ppm selenium as selenite in drinking water grew more rapidly than their controls up to 180 days of age (table 1). Female mice given selenite grew less rapidly than their controls after the 90-day interval.

Tellurite-fed mice behaved differently. Significant depression of mean body weights occurred at a year of age in both sexes, and to some extent, in females at two other intervals.

Effect of selenite, selenate and tellurite on growth of rats. Selenite at 2 ppm selenium depressed growth of rats of both sexes (table 2). The same dose as selenate had no effect, except at the 30-day interval. Rats fed tellurite grew as well as the controls.

Effect of selenium and tellurium on mortality. At 15 months of age, no significant difference was observed in survival of mice given selenite compared with that of their controls (table 3). Increased mortality, however, occurred at this interval in males fed tellurite, but not in females.

Different effects were observed in rats fed selenite in drinking water (table 4). After 16 days of exposure to this element, 9 male rats died. Deaths of males continued, and by 35 days (56 days of age) half were dead. The remainder were given the same dose of selenium as selenate in place of selenite; deaths continued at a reduced rate, ceasing 24 days later (80 days of age). Twelve male rats taking selenate remained alive for the next 194 days (9 months of age).

In female rats, selenite was somewhat better-tolerated. After 16 days of exposure, 7 died, after 35 days, 16, and after 100 days, 19. At 9 months of age, 27 of 53 remained alive. No deaths occurred in 107 rats given selenate during the first 9 months of age, nor in 106 controls; there were 3 deaths in 102 rats given tellurite (table 4).

Livers of the rats given selenite and dying were grossly abnormal, with irregular areas of fatty degeneration. Often they were shrunken. Sections showed fatty infiltration and degeneration, nodular hypertrophy and atrophy and hepatic cellular collapse. Other organs appeared unremarkable. Livers of the longest-lived female rats given selenite were less affected than were those of younger animals. Gross abnormalities were found in 15 of 35 livers of mice given selenite, and 12 of 34 livers of mice given tellurite.

TABLE 1

Mean weights of mice given selenite and tellurite in drinking water [1]

Age	Controls	Selenite	Tellurite
days	*g*	*g*	*g*
Males			
30	25.2 ± 0.71 [2]	26.7 ± 0.95	26.2 ± 0.44
60	38.8 ± 0.33	42.1 ± 0.74 [3]	40.9 ± 0.76 [4]
90	44.0 ± 0.75	49.4 ± 1.55 [4]	42.8 ± 1.33
120	48.7 ± 0.64	51.9 ± 0.94 [3]	47.4 ± 0.75
150	52.5 ± 1.02	56.2 ± 1.03 [4]	50.6 ± 1.35
180	51.5 ± 1.38	57.2 ± 0.80 [3]	51.7 ± 1.38
360	57.0 ± 2.09	58.7 ± 1.70	48.4 ± 1.39 [3]
Females			
30	22.2 ± 0.33	20.2 ± 0.41 [3]	21.3 ± 0.33
60	28.5 ± 0.62	30.1 ± 0.61 [5]	31.5 ± 0.50 [3]
90	34.0 ± 0.53	33.2 ± 0.78	35.2 ± 0.53
120	39.4 ± 0.75	35.6 ± 0.89 [3]	37.4 ± 0.82 [5]
150	44.5 ± 0.99	38.6 ± 0.89 [3]	41.3 ± 0.88 [5]
180	44.8 ± 0.72	40.2 ± 0.53 [3]	43.4 ± 1.06
360	53.4 ± 1.55	46.3 ± 1.43 [3]	47.4 ± 1.28 [3]

[1] 2 ppm element.
[2] Mean ± SEM.
[3] Differs from control value, $P < 0.005$.
[4] $P < 0.025$.
[5] $P < 0.05$.

TABLE 2

Mean weights of rats given selenite, selenate, and tellurite in drinking water [1]

Age	Controls	Selenite	Selenate	Tellurite
days	*g*	*g*	*g*	*g*
Males				
30	57 ± 2.8 [2]	35 ± 0.4 [3]	50 ± 0.8 [3]	55 ± 1.1
60	172 ± 4.1	89 ± 1.9 [3,4]	183 ± 6.4	177 ± 2.7
90	234 ± 5.0	200 ± 8.1 [3]	250 ± 4.8	245 ± 3.1
120	269 ± 5.2	224 ± 14.7 [3]	277 ± 6.0	280 ± 4.5
150	312 ± 5.2	278 ± 15.3 [5]	316 ± 7.0	303 ± 5.6
180	348 ± 7.1	299 ± 17.3 [5]	338 ± 9.0	340 ± 4.5
Females				
30	65 ± 4.9	37 ± 0.7 [3]	50 ± 2.0 [3]	55 ± 1.1
60	142 ± 6.9	82 ± 2.7 [3]	137 ± 2.2	143 ± 2.5
90	188 ± 6.0	121 ± 3.9 [3]	178 ± 7.8	185 ± 3.4
120	203 ± 4.3	141 ± 5.2 [3]	207 ± 2.2	197 ± 3.9
150	218 ± 4.9	165 ± 5.0 [3]	212 ± 3.9	219 ± 4.9
180	228 ± 4.2	180 ± 5.5 [3]	213 ± 4.2	227 ± 3.5

[1] 2 ppm element.
[2] Mean ± SEM.
[3] Differs from controls, $P < 0.005$.
[4] Changed to selenate at 56 days of age because of 50% mortality.
[5] $P < 0.01$.

TABLE 3

Survival of mice given selenite and tellurite in drinking water [1]

Age	Control [2]		Selenite [2]		Tellurite [2]	
	No.		No.		No.	
months		%		%		%
Males						
0	54	100	54	100	54	100
3		100		100		100
6		100		98.1		96.3
9		96.3		96.3		92.6
12		87.0		90.7		68.5 [3]
15		75.8		83.6		55.5 [3]
Females						
0	54	100	56	100	54	100
3		100		100		100
6		100		100		98.6
9		96.3		96.4		94.4
12		90.7		76.8		88.9
15		81.5		64.2		77.7

[1] 2 ppm element.
[2] % surviving at interval shown.
[3] Differs from selenite value by χ^2 analysis, $P < 0.01$, and from control, $P < 0.05$.

TABLE 4

Survival of rats given selenite and tellurite in drinking water [1,2]

Age	Males				Females			
	Selenite [3]		Tellurite [3]		Selenite [3]		Tellurite [3]	
	No.		No.		No.		No.	
months		%		%		%		%
0	48	100	52	100	53	100	50	100
1		62.5 [4]		98.1		86.9		98.1
2		41.6 [4,5]		96.3		69.8 [4]		98.1
3		25 [4]		96.3		68.0 [4]		98.1
4		25 [4]		96.3		64.2 [4]		98.1
5		25 [4]		96.3		60.3 [4]		98.1
6		25 [4]		96.3		52.7 [4]		98.1
9		25 [4]		96.3		50.9 [4]		98.1

[1] No deaths occurred in 53 males and 53 female control rats during this period, nor in 52 male and 55 female rats given the same dose of selenium as selenate.
[2] 2 ppm element.
[3] % surviving at interval shown.
[4] Differs from tellurite, control and selenate values, $P < 0.0005$. Values of males and females given selenite differ, $P < 0.01$–0.0005.
[5] Changed to selenate at 56 days of age because of 50% mortality.

DISCUSSION

There has been considerable discussion on the toxicity of selenium, especially in respect to its action as an essential trace element for mammals (6). Usually less attention has been paid to the oxidation state of the selenium used experimentally, sodium selenite generally being given. The present report clearly indicates both a species difference in the toxic effects of selenite, and a difference in respect to sex. From these data, male mice tolerated selenite well, growth being enhanced; females less so, growth being slightly depressed. Male rats tolerated selenite very poorly, females somewhat better. Furthermore, in rats the oxidation state of the selenium was critical. Rats of both sexes tolerated selenate (Se^{6+}) well but not selenite (Se^{4+}). These conclusions apply only to the dosage given in drinking water and may not be applicable to administration of the element in food.

Tellurium, which is not considered to have a physiological function but which is similar in atomic structure to selenium, was well-tolerated by rats in the oxidation state in which selenium was toxic, that is, Te^{4+}. Again, a sex and species difference was observed in that male mice were affected as to early survival and females as to late growth.

The intake of selenium by these rats can be roughly calculated from the average measured daily intake of water, and the estimated intake of food, 6 g/100 g/day. Animals given selenate at 2 ppm selenium drank 10.04 ± 1.66 μg Se/100 g body weight/day; those given selenite drank 12.8 ± 1.20 μg Se/100 g/day. An additional 9.9 μg came from food. Controls consumed 7.2 g H_2O/100 g/day.

Hadjimarkos (7) gave 3 ppm selenium as selenite in drinking water to 15 weanling rats fed laboratory ration for 4 weeks; two died. Water intake was decreased compared with that of controls by 21.5% when his data were corrected on a body weight basis. In the present experiments, intake of water was 11% less by rats given selenite and 20% less by rats fed selenate than that of controls.

Hopkins et al. (8) reported low rates of growth in weanling rats fed various purified diets containing 5 ppm selenium as selenite for 2 weeks, but no depression of growth when a crude ration was fed. The present data suggest that selenite in water is more toxic to young rats than is selenite mixed with food, especially with a crude diet.

Halverson et al. (9) fed rats 10 ppm selenium as selenite or selenate for 3 weeks to study the effects of added sulfate on toxicity. Mortality was higher in the selenite-fed animals, but both forms of selenium depressed growth. Sulfate partly protected only the selenate-fed rats.

Ganther and Baumann (10) compared the effects in rats of 5 ppm selenium added as selenite or selenate to a semipurified diet for 42 to 118 days. Gross liver damage, which was more extensive in selenate-fed animals, appeared. Growth rates were depressed in both selenite- and selenate-fed rats. Although different diets, different strains of rats and different doses of selenium may account partly for these discrepancies, the relative absorption from food or from water may be important.

The diet here used, of seed rye flour, powdered skim milk and corn oil (1), was dissimilar to the "crude" ration used by Ganther and Baumann (10, 11), resembling in some respects their semipurified diet in that it contained low levels of most trace elements and was especially low in cadmium (0.02–0.04 μg/g wet weight) and arsenic (0.05 μg/g). These authors noted that about twice as much selenium injected as selenite was exhaled by rats fed the crude diet as those fed the purified diet, and that arsenite markedly increased the excretion of selenium into the gastrointestinal tract. The selenium-volatizing factor in the crude diet is moderately heat labile and is destroyed by ashing (12). It is possible that the rye-flour diet, because of its low arsenic content and perhaps low content of volatilizing factor, may have promoted accumulation of selenite given in water, but not of selenate.[6]

An element exhibiting innate toxicity which, in small concentrations, is tolerable for growth of small mammals is not

[6] The odor arising from the cages of selenite-fed rats, however, was strong and disagreeable, whereas that from selenate-fed rats was not objectionable. Laboratory personnel were able to distinguish the 2 groups by the odor.

necessarily tolerable for optimal survival. Lead, cadmium, and titanium did not suppress the growth of mice (1) but shortened life-spans and longevity, especially those of males (13). Lead and cadmium somewhat increased the growth rates of rats (2), but shortened life-spans (14). Apparently, tellurium given as tellurite may behave similarly in male mice.

Whereas the differing effects of these elements are confusing, the data indicate the importance of the valence state of selenium when used in biological studies, and the importance of both the sex and the species of the experimental animals exposed to its compounds. Life-term studies on selenate and tellurite in rats and selenite and tellurite in mice will be reported when complete.

ACKNOWLEDGMENTS

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Effect of Dietary Protein Levels, Amino Acid Supplementation and Nitrogen Source upon the Plasma Free Amino Acid Concentrations in Growing Lambs

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ABSTRACT A study involving a growth trial and the examination of plasma free amino acids was carried out using 24 lambs in a 4 × 2 factorialized experiment in which 4 dietary protein levels and the presence or absence of L-lysine and DL-methionine in the diets were treatments. Although only plasma methionine, which decreased with increased dietary protein levels and also with dietary amino acid supplementation, was statistically significantly affected by the treatments, upward trends in most of the plasma amino acid concentrations with increasing dietary protein levels were apparent. Other exceptions to the general trend were lysine and histidine plasma concentrations which did not change appreciably. Supplementation of the diets with 0.24% lysine and 0.19% methionine did not increase the plasma concentrations of lysine or methionine. In a second experiment involving 9 lambs fed a purified diet in which urea was the sole source of nitrogen the plasma amino acid concentrations were considerably lower for most of the amino acids. Exceptions were glycine and serine which were higher in plasma of lambs fed the purified diet. In a third experiment the rumen contents from lambs fed a purified diet with urea as the sole nitrogen source were hydrolyzed and the amino acid content was determined. The amino acid content and amino acid ratio of rumen contents are compared with that of whole egg protein.

While the free amino acid concentrations in blood plasma of monogastric animals have been studied considerably with respect to the effects of dietary protein levels and dietary amino acid supplementation (1–5), few studies have been reported in which the free amino acid concentrations in the blood plasma of ruminants were examined with respect to these variables. It has been reported that the addition of excessive amounts of soybean meal to diets of steers did not affect the total free amino acid concentration or individual free amino acid concentrations in the plasma.[1] Poley and Trenkle [2] reported that certain dietary proteins exhibit an influence upon the plasma free amino acid concentrations in sheep. Beneficial effects on animal performance have been reported when the diets of steers and lambs were supplemented with free lysine [3] or methionine (6–8). However, Gossett et al. (9) and Harbers et al. (10) could detect no increase in the rate of gain of steers or lambs fed supplemental free lysine or methionine in diets of which the nitrogen source was predominantly natural proteins. Purser et al. (11) recently reported that dietary factors have a considerable influence upon the plasma amino acid concentrations of lambs.

The objectives of this study were to determine whether 1) dietary protein level, 2) dietary supplemental methionine and lysine, or 3) a purified diet in which urea served as the sole source of nitrogen influenced plasma amino acid concentrations in the blood of lambs and also to determine the amino acid composition of rumen contents of lambs fed a purified diet.

MATERIALS AND METHODS

Experiment 1. Twenty-four native lambs weighing 18 kg were allotted to the treatments of a 4 × 2 factorially designed experiment in which 4 dietary protein

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TABLE 1

Composition of natural diets

	Diet							
	1	1A	2	2A	3	3A	4	4A
	%	%	%	%	%	%	%	%
Ground corn	53.91	53.91	45.47	45.47	37.03	37.03	28.59	28.59
Soybean meal [1]	17.29	17.29	20.76	20.76	24.22	24.22	27.68	27.68
Cornstarch [2]	3.80	3.80	4.56	4.56	5.32	5.32	6.08	6.08
Corn gluten meal [3]	—	—	4.21	4.21	8.43	8.43	12.65	12.65
Cellulose [4]	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Mineral mix [5]	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Glucose monohydrate	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Vitamin, antibiotic mix [6]	2.00	—	2.00	—	2.00	—	2.00	—
Vitamin, antibiotic, amino acid mix [7]	—	2.00	—	2.00	—	2.00	—	2.00
Protein [8]	13.5	13.5 [9]	16.2	16.2 [9]	18.9	18.9 [9]	21.6	21.6 [9]

[1] Crude protein, 50%.
[2] Pearl grits, A. E. Staley Manufacturing Company, Decatur, Illinois.
[3] Crude protein, 41%.
[4] Solka Floc BW-40, Brown Company, Berlin, New Hampshire.
[5] Composed of: (in per cent) $CaHPO_4 \cdot 2H_2O$, 34.9; KSO_4, 29.7; NaCl, 16.7; Na_2SO_4, 11.6; MgO, 4.53; $FeSO_4 \cdot 7H_2O$, 1.99; ZnO, 0.2067; MnO, 0.2000; $CuSO_4 \cdot 5H_2O$, 0.1567; KI, 0.0367; and $CoSO_4 \cdot 7H_2O$, 0.0033.
[6] Percentage composition: Aurofac (American Cyanamid Company, New York; 1.98 g oxytetracycline and 1.98 mg vitamin B_{12}), 50.0; vitamin mixture (vitamin A, 165,000 IU; vitamin D_2, 16,500 IU; riboflavin, 55 mg; pantothenic acid, 277 mg; niacin, 825 mg; choline chloride, 6.3 g; vitamin B_{12}, 880 μg), 5.0; vitamin E conc (4,400 IU), 2.0; ethoxyquin (Santoquin, Monsanto Company, St. Louis), 2.5; and glucose monohydrate, 40.5.
[7] Same as in footnote 6 except that 9.5% DL-methionine and 24.0% Lyamine-50 (Merck and Company, Rahway, N. J.; 50% L-lysine) were added at the expense of glucose monohydrate.
[8] Calculated; air-dried basis.
[9] Not including the contribution of methionine and lysine added.

levels and the presence or absence of dietary supplemental amino acids were the variables. A 70-day growth study was carried out in which the animals were individually fed ad libitum once daily in cages with expanded steel floors. At the end of this period blood samples were collected for plasma free amino acid analysis.

The composition of the diets is shown in table 1. The 4 dietary protein levels were 13.5, 16.2, 18.9 and 21.6% crude protein on an air-dried basis. The protein levels were increased by adding soybean meal and corn gluten meal to the diet at the expense of corn so that the ratio of soybean protein to corn protein remained constant in all diets. L-Lysine and DL-methionine were added to the amino acid-supplemented diets at 0.24 and 0.19% of the diet, respectively, and thus increased the crude protein content of the diets accordingly. Cellulose [4] served as the roughage source.

On the day of the collection of the blood samples, the lambs were given their daily allotment of feed in the morning and allowed to eat for 2 hours at which time the unconsumed feed was removed. Four hours after the removal of the feed, the blood samples were taken by jugular vein puncture. The heparinized blood samples were centrifuged and the plasma proteins were precipitated with 1.0% picric acid. The excess picric acid was removed by an anion exchange resin. The resulting samples representing 8.33 ml of plasma were concentrated by evaporation and taken up to a 5.0-ml volume with water. The pH was adjusted to 8.0 with sodium hydroxide and the samples were allowed to stand at room temperature for 4 hours to convert cysteine to cystine. After the 4-hour period the pH was readjusted to 2.0 with hydrochloric acid and the sample diluted to 10.0 ml with pH 2.2 citrate buffer. The samples were then frozen until the amino acid analyses were made by automated ion exchange column chromatography.

Experiment 2. Nine native lambs weighing from 25 to 35 kg were individually fed a purified diet for periods of from 40 to 60 days in cages with expanded steel floors. The purified diet given in table 2 was fed twice daily. On the day of blood sampling, the lambs were allowed 2 hours to consume the morning feed

[4] Solka Floc BW-40, Brown Company, Berlin, New Hampshire.

TABLE 2

Composition of purified diet

	%
Cellulose [1]	50.0
Cornstarch [2]	30.0
Glucose monohydrate [3]	5.0
Sucrose	5.0
Urea	4.0
Mineral mix [4]	4.0
Corn oil	2.0
Vitamin mix [5]	+

[1] Solka Floc BW-40, Brown Company, Berlin, New Hampshire.
[2] Pearl grits, A. E. Staley Manufacturing Company, Decatur, Illinois.
[3] Cerelose, Corn Products Company, Chicago.
[4] Same as in table 1 plus sodium selenate, 0.005% and sodium molybdate, 0.004%.
[5] To furnish vitamin A at 2,200 IU/kg diet; vitamin D, 275 IU/kg diet; and vitamin E, 120 IU/kg diet.

offered and the feed was then removed. Four to five hours after the removal of the feed the lambs were bled by jugular vein puncture. The blood samples were processed in a manner identical to that of experiment 1.

Experiment 3. Six Rambouillet lambs weighing approximately 35 kg were fed a purified diet for a period of 50 days at which time rumen samples were collected by stomach tube. The samples were collected 6 hours after the morning feed, were freeze-dried, ground, and sampled. A sample of the dried rumen contents from each animal was then hydrolyzed under vacuum in 6 N hydrochloric acid at 110° for 22 hours; the samples were filtered to remove the insoluble material and evaporated to dryness. After a water-wash and re-evaporation to dryness the samples were taken up to 10.0 ml volume with pH 2.2 buffer and were frozen until the amino acid analyses were made.

RESULTS

Experiment 1. The results of the growth trial are given in table 3. An analysis of variance showed no significant differences in average daily gain or feed conversion due to any of the treatments.

The plasma amino acid concentrations are presented in table 4. Each value represents a pooled sample from 3 lambs. A statistical analysis using the interaction of the protein level treatments with the amino acid supplementation treatments to determine an estimate of error indicated a significant effect ($P < 0.01$) of dietary pro-

TABLE 3

Growth and feed utilization of lambs fed graded protein level diets with and without supplemental amino acids [1]

Diet	1		2		3		4	
Protein, %	13.5		16.2		18.9		21.6	
Amino acid supplementation	−	+	−	+	−	+	−	+
70-day gain, kg	17.3	17.7	17.1	15.5	16.0	15.2	17.6	13.3
Avg daily gain, kg	0.247 ± 0.010 [2]	0.253 ± 0.032	0.245 ± 0.013	0.222 ± 0.015	0.229 ± 0.029	0.217 ± 0.005	0.251 ± 0.012	0.190 ± 0.015
70-day feed consumption, kg	84.8	83.8	84.1	75.1	73.6	71.3	88.3	66.5
Feed/gain	4.90 ± 0.707	4.74 ± 0.245	4.91 ± 0.224	4.84 ± 0.436	4.61 ± 0.574	4.69 ± 0.458	5.01 ± 0.316	5.01 ± 0.245

[1] Three animals/group.
[2] SE.

TABLE 4

Plasma amino acid concentrations of lambs fed natural diets of graded protein levels with and without amino acid supplementation

Diet	1		2		3		4		Mean	SE of estimate
Protein, %	13.5		16.2		18.9		21.6			
Amino acid supplementation	−	+	−	+	−	+	−	+		
	μg/ml plasma		μg/ml plasma		μg/ml plasma		μg/ml plasma			
Threonine	13.6	14.5	17.2	18.8	17.4	17.5	23.0	15.6	17.2	±3.0
Valine	18.4	21.6	29.8	34.5	44.6	32.8	42.6	27.6	31.5	±7.2
Methionine [1,2]	4.6	3.2	3.9	3.0	4.3	2.5	2.1	1.1	3.1	±0.3
Isoleucine	8.8	6.8	10.7	11.8	14.8	9.9	15.7	9.9	11.0	±2.2
Leucine	15.2	11.8	21.2	24.4	33.3	21.4	36.2	20.0	22.9	±6.1
Phenylalanine	15.0	9.7	11.1	14.8	17.8	11.3	16.2	11.1	13.4	±3.3
Lysine	21.0	19.0	18.7	23.9	18.5	16.1	21.5	13.6	19.0	±3.8
Histidine	9.5	5.8	7.6	11.0	6.1	8.2	9.3	6.7	8.0	±2.4
Arginine	20.2	23.0	20.5	25.0	21.0	17.9	25.0	15.2	21.0	±4.6
Serine	9.7	9.7	9.1	11.6	9.6	9.3	12.7	6.8	9.8	±2.5
Proline	12.0	13.3	15.9	16.1	19.3	13.1	19.6	13.9	15.4	±2.8
Glycine	29.0	34.0	33.4	30.6	30.8	32.9	40.9	24.9	32.1	±6.6
Alanine	10.7	12.7	13.8	13.8	11.9	11.5	16.6	10.2	12.6	±2.6
Cystine (half)	4.1	2.3	2.2	3.5	2.0	5.8	5.8	4.2	3.7	±1.9
Tyrosine	23.9	16.5	20.8	22.6	23.9	18.6	29.5	17.6	21.7	±4.0
Glutamic acid	19.2	20.6	25.4	22.2	25.7	19.4	32.0	21.6	23.3	±3.5
Aspartic acid	2.6	1.8	2.2	2.2	4.4	2.6	4.4	2.1	2.8	±0.7

[1] Statistically significant linear effect of protein level at the 1% level.
[2] Statistically significant linear effect of amino acid supplementation at the 1% level.

tein levels and dietary amino acid supplementation only in the case of methionine. The plasma methionine concentrations decreased linearly with increasing dietary protein levels and they also decreased as the result of dietary methionine and lysine supplementation. The data are presented graphically in figures 1 and 2. Although only the changes in methionine concentrations were statistically significant, there was a trend toward an increase in plasma concentrations of most of the amino acids as dietary protein levels of the unsupplemented diets increased. Exceptions to this trend in addition to methionine are lysine, histidine and possibly phenylalanine and cystine. The general trend was considerably different when the amino acid supplemented diets were fed. When these diets were fed the plasma concentrations of most of the amino acids increased to reach a peak with diet 2A and decreased with the higher protein level diets. Methionine, glycine, cystine, and aspartic acid are exceptions.

Experiment 2. Table 5 shows the plasma amino acid concentrations of lambs fed a purified diet. With several exceptions the plasma amino acid concentrations were lower than those from lambs fed the

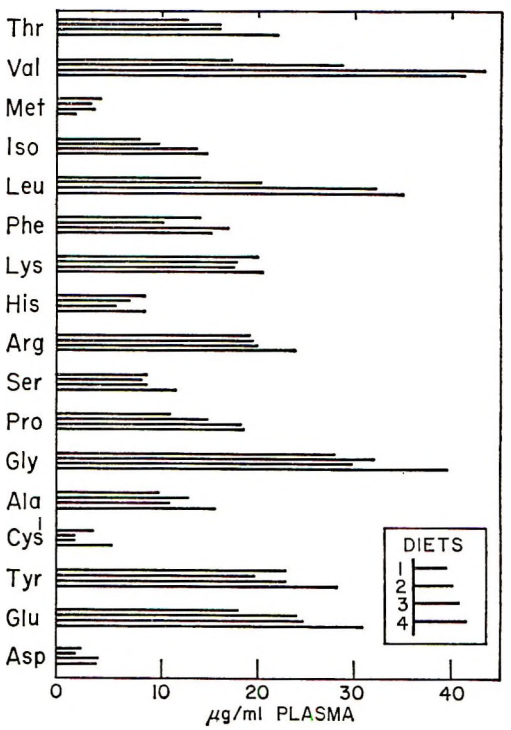

Fig. 1 Plasma free amino acid concentrations of lambs fed diets of graded protein levels. Diet 1, 13.5% protein; diet 2, 16.2% protein; diet 3, 18.9% protein; and diet 4, 21.6% protein.

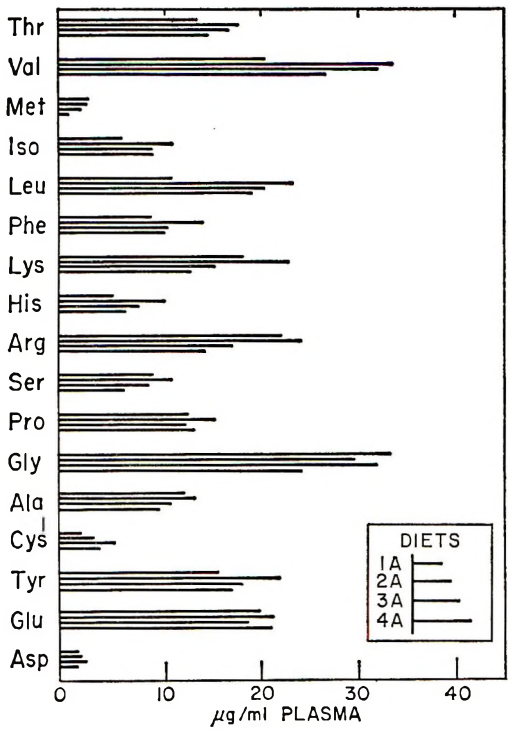

Fig. 2 Plasma free amino acid concentrations of lambs fed amino acid-supplemented diets of graded protein levels. Diet 1A, 13.5% protein; diet 2A, 16.2% protein; diet 3A, 18.9% protein; and diet 4A, 21.6% protein.

natural diets. The exceptions are the histidine and alanine concentrations which are about the same in both types of diets and the serine and glycine concentrations which are higher in plasma of lambs fed the purified diet.

Experiment 3. The amino acid composition of the rumen contents is presented in table 6. Although the absolute amino acid concentrations are somewhat variable between animals, there is little variation in the ratios of the individual amino acids when they are expressed as a percentage of the total essential amino acids. The amino acids hereafter referred to as essential amino acids are those demonstrated to be dietary essentials for the optimal growth of the rat plus cystine and tyrosine which can replace part of the dietary requirements for methionine and phenylalanine, respectively. Tryptophan is not included since it is destroyed during the acid hydrolysis of the protein. The reference to these as essential amino acids for lambs is supported by the work of Downes (12).

The amino acid composition of whole egg protein as compiled by Block and Weiss (13) is presented for comparative purposes. While the ratio of amino acids from microbial protein is similar in many

TABLE 5

Plasma free amino acid concentrations of lambs fed a purified diet

	Amino acid conc					
No. of animals	1	1	2 [1]	2 [1]	3 [1]	Mean [2]
	μg/ml plasma		*μg/ml plasma*			*μg/ml plasma*
Threonine	19.0	11.1	11.8	11.0	8.3	11.2 ± 1.8 [3]
Valine	12.9	9.4	10.8	9.2	9.9	10.2 ± 0.7
Methionine	0.2	1.5	0.6	0.1	1.9	1.0 ± 0.1
Isoleucine	4.4	3.1	4.7	4.1	4.6	4.3 ± 0.3
Leucine	4.3	3.7	5.6	4.8	6.0	5.2 ± 0.4
Phenylalanine	2.1	4.0	4.3	3.3	5.6	4.2 ± 0.6
Lysine	9.1	3.2	6.7	5.8	8.6	8.0 ± 1.1
Histidine	6.1	8.7	8.4	6.2	6.2	7.6 ± 0.6
Arginine	6.2	4.4	7.6	7.1	8.2	7.9 ± 0.7
Serine	14.8	7.6	14.1	22.5	15.0	15.6 ± 2.4
Proline	6.8	8.7	4.3	2.8	9.0	6.4 ± 1.2
Glycine	54.8	85.5	71.6	69.3	62.2	67.6 ± 5.1
Alanine	11.7	7.5	11.9	10.6	14.2	12.9 ± 1.1
Cystine (half)	2.8	2.7	2.1	3.6	2.7	2.8 ± 0.2
Tyrosine	7.8	6.3	6.2	5.6	7.1	6.5 ± 0.4
Glutamic acid	9.6	8.8	10.1	15.6	20.9	14.7 ± 2.3
Aspartic acid	0.8	0.6	0.6	1.1	1.2	0.9 ± 0.1

[1] Pooled samples from animals on same treatment.
[2] Weighted on the basis of number of animals/observation.
[3] SE; each observation given equal weight.

TABLE 6

Amino acid content of rumen hydrolysates from lambs fed a purified diet

	Amino acid content of rumen hydrolysates, sample no.												Rumen content	Whole egg [3]
	1		2		3		4		5		6			
	Amount [1]	Ratio [2]	Amount	Ratio	Amount	Ratio	Amount	Ratio	Amount	Ratio	Amount	Ratio	Mean ratio	Ratio
Lys	6.4	14.1	5.4	14.7	5.2	14.7	4.6	15.3	8.7	14.5	4.5	13.6	14.5 ± 0.2 [4]	11.5
His	1.2	2.6	0.7	1.9	1.0	3.0	0.8	2.5	1.5	2.6	1.0	2.9	2.6 ± 0.2	4.0
Arg	4.3	9.4	3.3	9.0	3.5	10.0	2.8	9.3	5.1	8.6	3.2	9.5	9.3 ± 0.2	11.1
Asp	11.9	26.2	8.6	23.8	9.3	26.5	8.3	24.4	15.8	26.5	8.1	24.3	25.3 ± 0.5	—
Thr	5.6	12.2	4.3	11.9	3.9	11.2	3.3	11.0	7.0	11.8	4.0	11.9	11.7 ± 0.2	8.3
Ser	4.9	10.8	4.4	12.2	4.3	12.3	2.8	9.2	7.0	11.8	4.5	13.5	11.6 ± 0.6	—
Glu	15.0	33.0	11.1	30.5	9.7	27.8	8.9	29.3	15.8	26.6	10.2	30.6	29.6 ± 0.9	—
Pro	4.1	9.0	3.2	8.9	3.5	10.1	2.7	9.0	5.0	8.3	3.1	9.4	9.1 ± 0.2	—
Gly	5.4	11.9	4.1	11.3	4.1	11.6	5.0	16.6	7.2	12.0	3.2	9.6	12.2 ± 1.0	—
Ala	6.9	15.1	5.3	14.6	5.4	15.3	5.3	17.5	9.3	15.6	5.4	16.2	15.7 ± 0.4	—
Cys	0.6	1.3	0.4	1.0	0.2	0.7	0.1	0.4	0.2	0.4	0.4	1.1	0.8 ± 0.2	3.8
Val	6.5	14.2	5.2	14.2	5.0	14.3	4.4	14.4	8.3	14.0	4.7	14.0	14.2 ± 0.1	12.3
Met	2.0	4.3	1.7	4.7	1.6	4.6	1.6	5.2	2.9	4.8	1.6	4.7	4.7 ± 0.1	5.5
Ile	4.8	10.5	3.9	10.6	3.7	10.6	3.3	11.0	6.4	10.7	3.6	10.8	10.7 ± 0.1	11.5
Leu	7.2	15.9	5.6	15.3	5.6	16.1	4.8	15.9	9.7	16.3	5.4	16.3	16.0 ± 0.1	15.6
Tyr	3.0	6.5	2.8	7.6	2.1	6.1	1.9	6.1	4.3	7.2	2.1	6.4	6.6 ± 0.2	6.8
Phe	4.1	9.0	3.3	9.0	3.1	8.8	2.7	9.0	5.4	9.0	2.9	8.6	8.9 ± 0.1	9.6
Total	93.9		73.2		71.3		63.4		119.6		68.0			
Essential amino acids, %		45.5		49.7		49.1		47.8		49.8		49.1	49.0 ± 0.3	60.2

[1] Mg/g sample (air-dried).

[2] Percentage of total essential amino acids. Considered as essential amino acids are: lysine, histidine, arginine, threonine, methionine, cystine, valine, isoleucine, leucine, phenylalanine and tyrosine.

[3] Amino acid composition of hen's whole egg, from Block and Weiss (13).

[4] SE.

respects to that from whole egg protein, several quantitative differences appear. The relative amounts of lysine and threonine are somewhat higher in microbial protein, whereas histidine and the sum of the sulfur amino acids are lower. Another difference exists in that while the essential amino acids account for about 60% of the total amino acids in the egg protein, the essential amino acids of microbial protein account for less than 50% of the total amino acids.

DISCUSSION

Because many known factors influence plasma amino acid concentrations (2, 3, 11, 14–16), and no doubt still others are unrecognized, it is important to evaluate the conditions under which plasma amino acid studies are made. Even with the existence of such factors various methods using plasma amino acid concentrations for determining limiting amino acids in monogastric animals (1, 17–20) have met with moderate success.

In experiment 1 attempts were made to exclude as many variables from the dietary treatments as possible by maintaining an essentially constant energy level in all diets as well as maintaining a constant ratio of corn protein to soybean protein so that the amino acid ratios of all the unsupplemented diets were similar and those of all the amino acid-supplemented diets were also similar to each other. Since the energy content was essentially constant in all diets, the protein-to-energy ratio increased as the protein levels in the diets were increased.

That statistical significance occurred only with methionine, even though definite trends are apparent in other amino acids, probably resulted from the pooling of the plasma samples before the amino acid analysis. Pooling the samples made it necessary to calculate statistical error terms from the interaction of the protein level treatments with the amino acid supplementation treatments. The magnitude of these error terms was such that statistically significant differences did not occur except in the case of methionine.

The decreasing trend peculiar to the plasma concentration of methionine in both the amino acid-supplemented and -unsupplemented diets suggest something unique about this amino acid. A limiting amino acid would probably not accumulate in the plasma as other amino acids would when the dietary protein level is increased. Neither did the plasma concentrations of lysine and histidine increase as the dietary protein levels increased when the unsupplemented diets were fed. Purser et al. (11) implicated lysine and methionine, among others, as possible limiting amino acids in lambs by using a plasma ratio technique. That there was no growth response to the methionine and lysine supplementation of the diets does not rule out these amino acids as limiting amino acids since it is possible that they were catabolized by the rumen microorganisms and thus not available to the host animal. The lowered feed consumption by the lambs fed the higher protein level, amino acid-supplemented diets is probably a factor involved in the different plasma amino acid trends observed when these diets were fed.

The mediocre growth of lambs fed a purified diet with urea as the sole source of nitrogen indicates a nutritional inadequacy in such diets. Although Loosli et al. (21) demonstrated that synthesis of the essential amino acids from nonprotein nitrogen sources does occur in the rumen, the rate at which these amino acids are synthesized may be a factor limiting growth. The lower concentrations of most of the amino acids in the plasma of lambs fed the purified diet relative to those in the plasma of lambs fed the natural diets suggest that amino acid nutrition could be one of the factors limiting growth with the purified diet. Oltjen and Putnam (22) reported greater nitrogen retention when cattle were fed a purified diet with soybean protein as a nitrogen source than when urea was the nitrogen source. They also reported lower plasma amino acid concentrations of some of the essential amino acids when urea was the nitrogen source. It is possible that the elevation of the plasma concentration of glycine, which was quite consistent in the case of lambs fed the purified diet, could be used as an indication of suboptimal amino acid nutrition. This elevation was also noted by Oltjen and Putnam (22).

The amino acid analysis of hydrolyzed rumen contents from lambs fed a purified diet with urea as the sole nitrogen source allows an estimate of the amino acid composition of microbial protein to be made. These data reported in this study represent essentially the amino acid composition of the rumen bacterial population existing under the imposed conditions since microscopic examinations of rumen contents of lambs fed the purified diet showed the absence of a thriving protozoa population. Weller (23) reported that the amino acid composition of the rumen bacterial population of lambs fed a variety of natural diets was essentially unchanged. The same report also indicated that differences in the amino acid composition of rumen bacteria and rumen protozoa do occur — in general a trend for slightly higher amounts of essential amino acids in protozoa protein. Purser and Buechler (24) noted essentially no differences in the amino acid composition of pure cultures of some of the predominant rumen bacteria.

Expressing the ratios of amino acids as a percentage of the total essential amino acids appears to be an appropriate method in the ruminant since normally excessive nonessential nitrogen is available in the form of urea. The comparison of the ratio of amino acids from rumen contents to that of a high quality protein shows both similarities and differences. Of interest are the lower amounts of histidine and sulfur amino acids in the rumen contents. These amino acids also exhibited different plasma concentration trends than most of the other amino acids in experiment 1. However, the comparison of the amino acid composition of rumen contents to that of whole egg could prove to be misleading since the availabilities of the amino acids in the rumen contents are not known. Bergen et al.[5] observed considerable difference in the release of free amino acids from different species of rumen bacteria when they were subjected to a digestion procedure in vitro. The limited availability of any amino acid already present in low amounts would of course tend to further limit the supply of that amino acid.

The lower amount of total essential amino acids in the bacterial protein relative to that of egg protein might suggest that total essential nitrogen is limiting when urea serves as the sole nitrogen source for the ruminant. Duncan et al. (25) measured the rumen volume as well as essential amino acid concentration of rumen contents in calves receiving purified and natural diets. They noted lower absolute amounts of the essential amino acids in the rumens of calves consuming the purified diet with urea as the sole nitrogen source.

Although the evaluation of plasma amino acid concentrations and rumen microbial amino acid content may be useful for the detection of a limiting amino acid(s) in the ruminant, the proof of whether such a condition exists will have to come about by the administration of amino acids so that they are unavailable for intraruminal metabolism in order to insure that these amino acids are available to the host animal.

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Metabolism of DL-1,2-Propanediol-2-^{14}C in a Lactating Cow [1,2]

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ABSTRACT Since propanediol is glucogenic and is extensively used in therapy of bovine ketosis, its metabolic fate was investigated. DL-1,2-propanediol-2-^{14}C with 400 g of carrier propanediol was administered intraruminally to a lactating cow. During the next 24 hours, the percentage of the dose recovered was 43.7 in CO_2, 12.4 in milk, 3.5 to 7 in urine, and less than 0.1 in feces. The propanediol was predominantly absorbed from the rumen without alteration, although some conversion to propionic acid in the rumen was detected. The maximal level of propanediol in milk was 0.04 mg/ml. Distribution of ^{14}C among the carbons of lactose and glutamic acid indicated conversion of propanediol to glucose via carboxylation of pyruvate to oxalacetate. These results demonstrate that propanediol is glucogenic in the classical sense of that term; namely, that it is metabolized via intermediates, probably pyruvate and oxalacetate, which can lead to net synthesis of glucose. The small concentrations of propanediol in peripheral blood and the approximately 2-hour delay in attaining maximal specific activity in CO_2 after attaining maximal specific activity of blood glucose indicated that glucogenesis was primarily hepatic with oxidation primarily occurring in other tissues.

A previous investigation showed that intraruminal doses of 0.9 kg of DL-1,2-propanediol produced maximal levels of propanediol in peripheral blood of 57 mg/100 ml at 2 hours after administration (1). This concentration in blood was only 11% of the ruminal concentration which contrasts sharply with the results from gastric and intravenous doses to nonruminants where peripheral blood concentrations of propanediol were equivalent for both routes of administration in 2 hours (2). Since 1,2-propanediol was resistant to ruminal destruction, it was suggested that the rumen acted as a diluting pool permitting slow absorption (1). Only traces of propanediol appeared in the urine or feces of cows fed 1,2-propanediol for 22 days in increasing amounts to 2.1 kg/day (16.7% of the ration). Maximal concentrations in the blood were attained in 8 days and then declined, which suggested adaptive metabolism (1). Hanslik et al. (2, 3) obtained efficient utilization and apparent adaptive metabolism of 1,2-propanediol in growing rats.

1,2-Propanediol is glycogenic and a precursor of lactic acid in the rat (3, 4). The distribution of ^{14}C in glycogen following tracer doses of propanediol-^{14}C and its continued glycogenesis after enolase inhibition have been considered evidence for propanediol conversion directly to glucogenic triose-phosphates (4, 5). However, the published distributions of ^{14}C in glycogen are consistent with gluconeogenesis via the pyruvate carboxylase-phosphoenol pyruvate carboxykinase route (4–8). Recent reviews on the metabolism of 1,2-propanediol indicate its oxidation to lactaldehyde and then lactic acid by several enzymes with low specificity (7, 8).

The present report describes the fate of a nutrient-size dose of 1,2-propanediol-2-^{14}C in a lactating cow. To clarify the pathway of glucogenesis, milk lactose and glutamic acid were degraded. The radioactivities of rumen fermentation products and of essential amino acids in casein were examined to determine the extent of ruminal metabolism of propanediol.

EXPERIMENTAL

A 653-kg cow in the eighth month of pregnancy and producing 12.2 kg of milk per day was placed in an open-circuit

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respiration apparatus equipped for continuous monitoring of respiratory CO_2 and radioactivity. Its ration was 7.7 kg alfalfa hay plus 12.5 kg of a soybean meal-corn grain mixture (16% crude protein). It had an established rumen fistula with plastic closure through which 400 g of DL-1,2-propanediol-2-^{14}C (217 μCi/g atom carbon) in 1.8 liters of aqueous solution was introduced in a single dose. This solution was manually mixed with the rumen contents. Respiratory CO_2 and radioactivity were measured continuously for 6 hours after which the cow was returned to its stall for feed and water until 18 hours after dosing. Respiration data were again collected during the 18- to 24-hour period of the experiment.

Milk was removed with the aid of oxytocin (15 U, iv) immediately before administration of the propanediol and at 1.1, 3.2, 6.0 and 14.5 hours thereafter. Feces were collected continuously for 24 hours and urine was collected while the cow was in the respiration apparatus. Blood samples were taken from an indwelling jugular catheter, mammary vein, or tail (essentially arterial) at the times indicated in the figures. Since there were no obvious arterio-venous differences for radioactivity or propanediol, the blood samples were pooled. Rumen contents were sampled at the times indicated in figure 1 and 3.6 liters of water were added to the rumen contents 3.5 hours after starting the experiment. A 2.4-g sample of the liver was taken 1.5 hours after administering the propanediol using the biopsy technique of Anderson et al. (9).

Upon collection, all samples were frozen until further processing, and blood samples contained NaF as an additional pre-

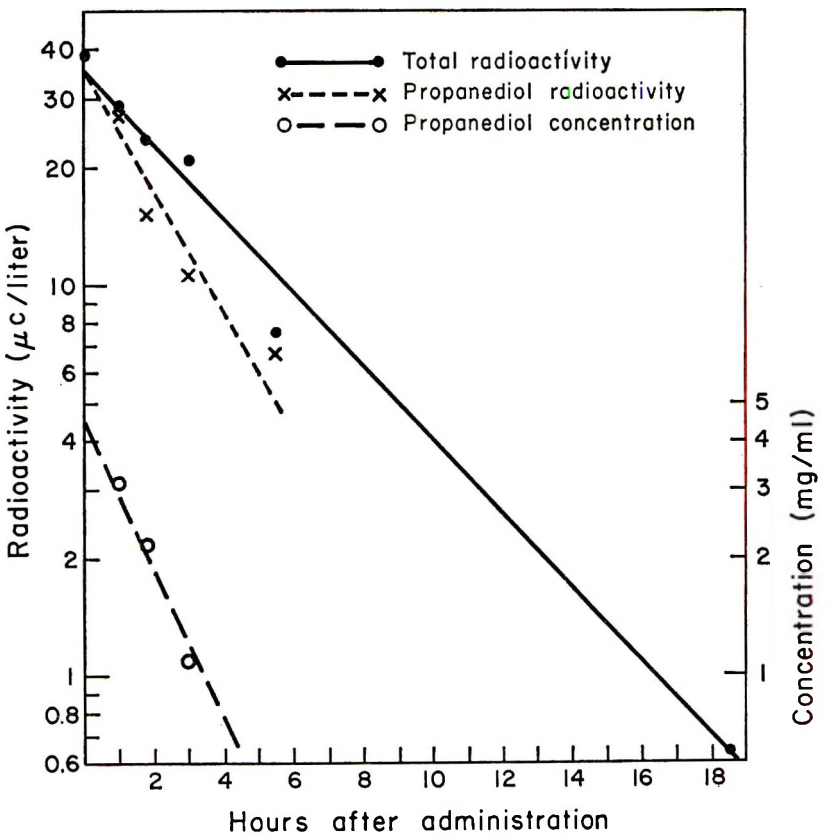

Fig. 1 Disappearance of radioactivity and propanediol from the rumen of a 653-kg cow following administration of 400 g of DL-1,2-propanediol-2-^{14}C (217 μCi/g atom carbon) at zero hours.

servative and anticoagulant. Milk fat, casein and lactose were separated as described by Wood et al. (10) and galactose was isolated as mucic acid as described by Baxter et al. (11). Lactose was assayed by a phenol sulfuric acid procedure (12) and hydrolyzed by autoclaving 1 g with 10 ml 0.5 M HCl in a sealed tube for 2 hours. The hydrolysate was desalted with BioRad AG11A8 ion-retardation resin.[3]

Glucose, derived from lactose, and lactose were fermented to lactic acid with *Lactobacillus casei* (ATCC7469) grown on glucose and lactose, respectively (13). Preliminary experiments showed that the culture fermented only the sugar to which it was adapted. The lactic acid derived from glucose (1.7 mmoles) was oxidized to acetic acid with 1.5 g CrO_3 plus 1 ml H_2SO_4 at 95° for 90 minutes in a volume of about 30 ml H_2O and the SO_2 was collected as described by Bernstein and Wood (13). The lactic acid derived from lactose was recrystallized as zinc lactate before oxidizing to acetic acid with $KMnO_4$ (14). The Schmidt reaction was used to degrade acetic acid (14). Amino acids were isolated from casein and the glutamic acid was degraded step-by-step (15).

Blood glucose was determined by a commercial glucose oxidase procedure [4] before it was isolated as potassium gluconate (16). These isolations were repeated until replicate determinations of the specific activity of blood glucose agreed within 70%.

The liver sample was extracted successively with acetone, ethanol, ether and 10% trichloroacetic acid at room temperature. Carrier glycogen (500 mg) was added to the trichloroacetic acid extract and the glycogen precipitated with an equal volume of ethanol. These liver fractions were dried in a vacuum oven, weighed, and redissolved for determination of radioactivity.

One milliliter of 50% H_2SO_4 was added to each 100 ml of rumen supernatant fluid obtained by centrifugation. Urine samples were concentrated to 50% of their original volume on a steam bath. This procedure gave over 90% recovery of added propanediol. Protein-free filtrates were prepared from blood by adding 2 volumes of 6% $HClO_4$ to 3 volumes of blood. These protein-free filtrates, the rumen samples, urine samples, and the supernatant after removal of casein from milk were subjected to gas chromatography for determination of volatile fatty acids and the propanediol as described previously (1). The carrier gas-flow rate and column length were increased and 86% of the column effluent was diverted to a heated, gas phase proportional radioactivity monitor.[5] Radioactivities of other samples were determined by counting at infinite thickness with a gas-flow, end-window Geiger counter or in a liquid scintillation counter. Internal standards were used to correct for counting efficiency.

RESULTS AND DISCUSSION

The half-life for propanediol in rumen contents was 1.7 hours (fig. 1) which was similar to the 1 hour reported previously (1). The specific activity of propanediol in the rumen during the first 3 hours remained at 217 μCi/g atom carbon. As calculated from figure 1, the 400 g of administered propanediol was distributed in 83 liters, which is a reasonable volume for

[3] Calbiochem, Los Angeles.
[4] Glucostat, Worthington Biochemical Corporation, Freehold, New Jersey.
[5] Nuclear Chicago, Des Plaines, Illinois.

TABLE 1

Concentrations of volatile fatty acids in rumen contents

Time [1]	Proportion of total				Total conc
	C_2	C_3	C_4	C_5 and iso C_5	
hours	*moles/100 moles*				*mM*
1	56	19	13	11	166
2.1	57	23	14	6	183
3	56	25	13	6	168
5	56	25	14	5	59
18.5	59	23	12	6	73

[1] Time after intraruminal administration of 400 g of DL-1,2-propanediol-2-^{14}C (217 μCi/g atom carbon) to a 653-kg cow.

the rumen contents of this cow. The total radioactivity was absorbed more slowly, with a 3.2-hour half-life, indicating retention of some labeled product in the rumen. All of the radioactivity in the 18.5-hour rumen sample was chromatographically identical with propionic acid. Propanediol was absorbed from the rumen at a rate of 41.6%/hour and converted to propionic acid in the rumen at a rate of 19.7%/hour. If this rate is multiplied by the propanediol concentration at 1 hour it can be calculated that 10 μmoles of propionic acid should be formed per ml rumen contents between 1 and 2.1 hours. The actual increase in propionic acid was 11 μmoles which can be calculated from the data in table 1. The small value for total radioactivity at 5 hours may have been due to poor mixing after adding water to rumen at 3.5 hours. These data support the conclusion of Emery et al. (1) that propanediol is primarily absorbed as such from the rumen and also support the partial conversion of propanediol to propionate, as reported by Waldo and Schultz (17).

The propanediol concentration in peripheral blood attained a maximal concentration in 0.5 hours at which time it accounted for 90% of the blood radioactivity (table 2). Glucose gradually accounted for an increasing fraction of the blood radioactivity, attaining a maximum of 50% at 3 hours. After 3 hours, the major portion of blood radioactivity was present in substances other than glucose or propanediol. Blood glucose attained a maximal specific activity of about 61.4 μCi/g atom carbon (fig. 2) between 3 and 5 hours after the administration of propanediol. Since the administered propanediol had a specific activity of 217 μCi/g atom carbon, it can be estimated that about 28% of the blood glucose at 3 hours had been formed from propanediol. Expired CO_2 did not attain maximal specific activity until after 6 hours. The low peripheral blood levels of propanediol and the time delay between the maximal specific activity of blood glucose and expired CO_2 suggests that a major portion of the propanediol was converted to glucose by the liver before oxidation to CO_2. For comparison, glucose injected intraduodenally yielded maximal specific activity for blood glucose within 1 hour and for respiratory CO_2 within 1.5 hours (18). If propanediol had contributed to CO_2 without conversion to some product such as glucose, CO_2 should have reached its maximal specific activity at about 3 hours since the blood was largely cleared of propanediol before this time.

The 24-hour CO_2 excretion contained 43.7% of the administered radioactivity. Assuming that all 3 carbons of propanediol contributed equally to CO_2, 6.9 moles of CO_2 were formed from propanediol out of 140.64 total. Thus, 4.9% of the expired CO_2 came from the propanediol which made up only about 3.3% of the ingested digestible dry matter. Since less than 0.1% of the radioactivity was recovered in feces during the 24-hour period in which 61% of the dose was recovered in other products, propanediol was considered entirely digestible. This conclusion was further supported by the rapid disappearance of radioactivity from the rumen and agrees with data from previous digestion trials (1).

The liver biopsy taken at 1.5 hours contained 0.11 μCi/g. This radioactivity was

TABLE 2

Distribution of radioactivity and concentration of metabolites in blood

Time [1]	Propanediol	Glucose	% of blood radioactivity		
			Propanediol	Glucose	Unidentified
hours	*mg/ml*	*mg/100 ml*	%	%	%
0.5	0.32	66	90	10	0
1.0	—	61	—	13	—
2.0	0.16	82	42	34	24
3.0	0.05	80	21	50	29
3.5	—	80	—	30	—
6.0	0.04	71	16	37	47
18.0		117	—	21	—

[1] Time after intraruminal administration of 400 g of DL-1,2-propanediol-2-^{14}C (217 μCi/g atom carbon) to a 653-kg cow.

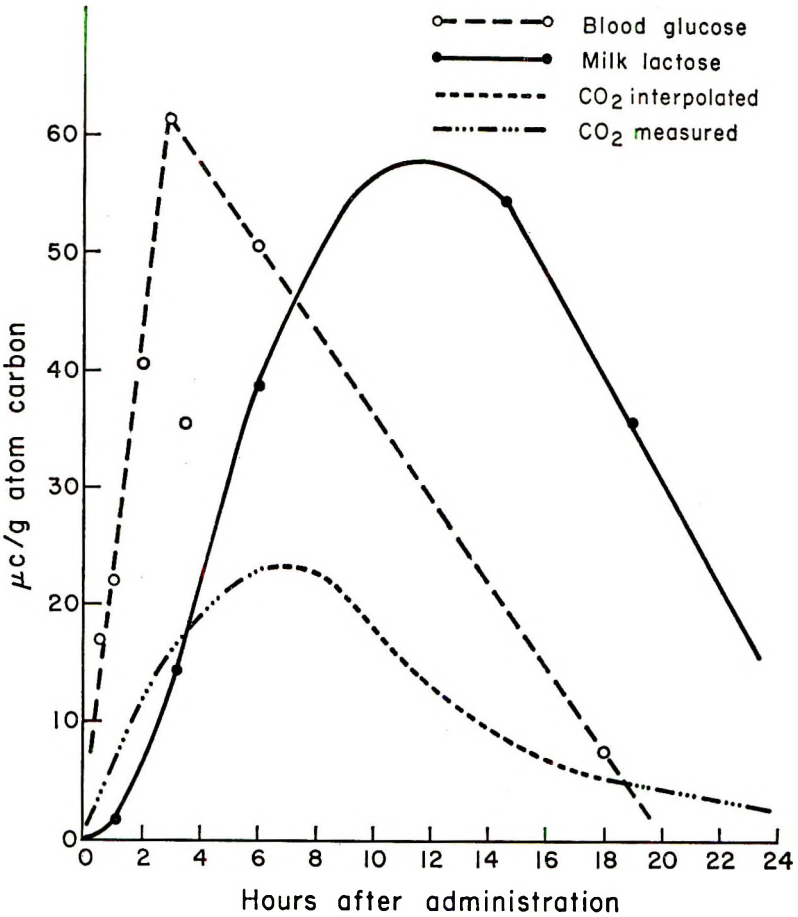

Fig. 2 Specific activities of blood glucose, milk lactose and respiratory CO_2 as a function of time after intraruminal administration of 400 g of DL-1,2-propanediol-2-^{14}C (217 μCi/g atom carbon) to a 653-kg cow at zero hours.

distributed 18% in glycogen, 2% in lipid, 7% in protein residue, and 73% in a cold trichloroacetic acid-soluble fraction, which was not hydrolyzed glycogen because the carrier glycogen was completely recovered. Propanediol at a concentration of 9.5 mg/g liver would account for this trichloroacetic acid-soluble radioactivity but propanediol should have been extracted with the lipid in ethanol-acetone. This trichloroacetic acid-soluble radioactivity may represent metabolic intermediates between propanediol and glucose.

Glycogen accounted for 5.9% of the dry weight of the liver sample. Its specific activity of 0.01 μCi/g was much lower than the 0.03 μCi/g found in blood glucose at the corresponding time. Moreover, liver glycogen could not have contained more than 0.2% of the ^{14}C which had been absorbed at this time. This excludes glycogen as a storage pool for the propanediol activity in this experiment but does not exclude a flux through a small portion of the liver glycogen.

Urine collected during this experiment (3.3 liters) accounted for 3.5% of the ^{14}C dose. However, urine was not collected during a 12-hour period in the middle of the experiment and previous experience suggests about 7 liters should have been excreted during the 24-hour experiment. A more reasonable figure for urine excretion of ^{14}C might be 7% of the dose. Urine collected at 0.4, 2.5 and 5.2 hours contained 0.46, 7.8 and 5.8 mg of free pro-

panediol per ml which accounted for 80, 42 and 57% of the radioactivity in these samples, respectively. Miura (19) reported that propanediol was excreted in urine as a glucuronide. Although the blood (table 2) and urinary radioactivity in unidentified products could have been conjugated propanediol, these products probably were intermediates between glucose and CO_2. Supporting this conclusion, the proportion of radioactivity in unidentified products was small until glucose approached maximal specific activity from which time this proportion increased continuously.

Lactose accounted for 64 to 93% of the radioactivity in milk as shown in table 3. The maximal specific activity of milk lactose was 54.5 μCi/g atom carbon which approached the 61.4 μCi/g atom carbon measured in blood glucose (fig. 2). This close agreement between the specific activities of lactose and blood glucose precluded cycling of glucose through other tissues before conversion to lactose as could be predicted from the conclusion of Bartley and Black (18) that the mammary gland has first priority on blood glucose. Propanediol accounted for only 2.3% and 0.05% of the milk radioactivity at 1.1 and 6.0 hours, respectively. These radioactivities correspond to 1 and 4 mg propanediol per 100 g milk. On the basis of the average amino acid composition of casein and the specific activities of the amino acids isolated from the 6-hour casein sample, 8% of the milk radioactivity was in casein. Casein plus lactose accounted for 76% of the total 6-hour milk radioactivity. Similarly, less than 2% of the 14.5-hour milk radioactivity was present as milk-fat. But, blood acetate is a good carbon precursor for milk fat and hence any acetate derived from propanediol must not have equilibrated with the blood acetate pool. Acetyl-CoA formed in the liver would not be expected to enter the blood acetate pool. The cumulative excretion of radioactivity in milk was 12.4% of the dose. Isotope excretion in milk remained high at termination of the experiment (table 3) and milk was probably a major source of isotope excretion during the next 24 hours.

The specific activities of amino acids from casein reported in table 4 are similar to those found by Black et al. (20), 3

TABLE 3

Distribution of radioactivity in milk

Time [1]	Milk		% of milk radioactivity	
	Amount	Activity	Propanediol	Lactose
hours	*kg*	*μCi/kg*	%	%
1.1	0.68	3.3	2.30	80
3.2	0.95	22.0	—	93
6.0	1.13	69.8	0.05	68
14.5	2.63	86.7	—	66
19.0	2.18	59.4	—	64

[1] Time after intraruminal administration of 400 g of DL-1,2-propanediol-2-^{14}C (217 μCi/g atom carbon) to a 653-kg cow.

TABLE 4

Specific activity of the amino acids from the 6-hour casein [1]

Essential amino acids		Nonessential amino acids	
	μCi/g atom C		*μCi/g atom C*
Methionine	0.04	alanine	24.83
Lysine	0.08	serine	7.93
Leucine	0.08	glutamic acid	13.46
Isoleucine	0.20	aspartic acid	7.96
Valine	0.08	glycine	4.83
Histidine	0.36	proline	0.55
		arginine	0.67

[1] Casein isolated from the milk formed between 3.2 and 6.0 hours after intraruminal administration of DL-1,2-propanedicl-2-^{14}C (217 μCi/g atom carbon).

hours after intravenous injection of uniformly labeled glucose. To whatever extent propanediol entered the carbon pools of the rumen bacteria, it would be expected to contribute ^{14}C to the essential amino acids synthesized by these bacteria (19). The low specific activities of essential amino acids relative to the nonessential amino acids is further evidence for a lack of significant propanediol fermentation in the rumen.

Alcohol dehydrogenases which oxidize DL-propanediol to lactaldehyde with reduction of NAD have been isolated from rat and rabbit liver (7, 8). These enzymes had Michaelis constants for propanediol of about the same size as the maximal blood concentration of 4.2 mM observed in this study. The equilibrium constants and low activities of these enzymes were also unfavorable for rapid propanediol metabolism. The characteristics of the propanediol, NADP specific dehydrogenases which have been isolated from several tissues were even less favorable for propanediol utilization (21, 22). These enzymes might account for the slow rate of propanediol metabolism observed in this trial and by Hanzlik et al. (2) following a single dose but would not appear adequate to explain the efficient metabolism of the large amounts used in feeding trials (1, 2). Lactaldehyde is as glycogenic as lactic acid and enzymes accounting for its rapid utilization have been characterized (5, 8). With these enzymes, propanediol-2-^{14}C could proceed to pyruvate-2-^{14}C via lactaldehyde and lactic dehydrogenase. Pyruvate-2-^{14}C could then be carboxylated to oxalacetate as discussed by Utter et al. (6) and, via the tricarboxylic acid cycle, yield glutamate labeled in carbon 3. Equilibration of the oxalacetate with fumarate would cause equal labeling in carbons 2 and 3 and complete equilibrium within the tricarboxylic acid cycle would yield about 50% as much activity in carbon 1 as in carbons 2 and 3 but no labeling in carbons 4 or 5 (23, 24). If pyruvate-2-^{14}C is decarboxylated and enters the cycle as acetate-1-^{14}C, the label appears as glutamate-5-^{14}C. During gluconeogenesis through phosphoenolpyruvate, glucose would be labeled in the 1-, 2-, 5- and 6-position to twice the extent of the labeling in the 3- and 4-position, assuming complete equilibration with the tricarboxylic acid cycle. This model accounts for the intramolecular ^{14}C distribution in glutamate and sugars presented in table 5, if it is assumed that about one-third of the carbon from propanediol entered the tricarboxylic acid cycle as acetate.

Results for the glucose portion of lactose (table 5) were considered less reliable than the results for lactose because glucose standards degraded simultaneously showed about 10% randomization. Glucose standards degraded simultaneously with lactose showed less than 3% randomization. The slightly greater specific activity for lactose compared with its glucose moiety suggested preferential labeling of galactose. However, mucic acid prepared from the galactose moiety had a specific activity of 31 μCi/g atom carbon compared with 38 for the lactose prepared from the 6-hour milk sample and it was concluded that within experimental error glucose and galactose were equally labeled. Although it has been shown that lactaldehyde can combine with triosephosphate to form glycogenic methylpentoses such as fucose, it is doubtful if such a pathway would yield the labeling patterns shown in table 5 (5,25,26). Dilution of glutamic acid carbon

TABLE 5

Distribution of radioactivity in glucose from lactose, and glutamic acid from the 6-hour milk sample

Compound	Carbon no.					
	1	2	3	4	5	6
	μCi/g atom carbon					
Glucose [1]	47.3	49.1	15.4	15.4	49.1	47.3
Lactose [2]	54.1	55.9	10.0	10.0	55.9	54.1
Glutamic acid	10.8	16.6	18.6	4.8	15.2	—

[1] Carbons 1 and 6, 2 and 5, 3 and 4 were determined together.
[2] Glucose and galactose fermented together and carbon pairs degraded as in footnote 1.

by entry of acetate and other tricarboxylic acid cycle intermediates would be expected to cause a lower specific activity in glutamate than in glucose (or lactose) if most of the gluconeogenesis proceeded via oxalacetate without traversing the cycle to glutamate. Glutamic acid would have been much lower in specific activity than lactose if glucogenesis had proceeded by any pathway resulting in direct transfer of propanediol to a triose-phosphate intermediate of glycolysis since glutamate would be further diluted by the flux of carbon through glycolysis (23). To the extent that propanediol was converted to propionic acid in the rumen, some randomization between carbons 2 and 3 of propionic acid would have occurred and the labeling of lactose or glutamic acid would not have differed perceptibly from the model presented above.

These data support the concept of propanediol absorption from the alimentary tract with some ruminal conversion to propionic acid. Absorption of propanediol was apparently followed by nearly complete conversion to glucose predominantly in the liver and via the established pathway of pyruvate carboxylation (6).

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Enzymatic Determination of the Protein Quality of Individual Rumen Bacteria [1]

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ABSTRACT Protein quality of individual rumen bacteria was studied using an in vitro enzymatic digest system, and a protein quality index (NPU_{enz}) was computed from egg ratios. Depending upon the individual strain the NPU_{enz} values ranged from 37 to 80, whereas control digests with casein gave NPU_{enz} values of 86.5 and 73.6. The proportion of the essential amino acids being released as free amino acids during the digest varied between 2.5% and 52.6%. Furthermore, the distribution of the essential amino acids within this fraction varied markedly also. It is suggested that modification of the bacterial population may be an important factor with respect to the nitrogen status of the animal and its response to dietary changes.

Microbial protein may constitute a major portion of the nitrogen-containing substances that arrive in the lower digestive tract of the ruminant. Thus, Weller et al. (1) observed that 80% to 90% of omasal nitrogen content was microbial nitrogen, with ruminal bacteria accounting for 55% to 85% of this nitrogen. The quality of this crude microbial protein has been investigated by a number of workers (2–6) and biological values, determined with rats, ranging from 66 to 88 have been reported. Recently it was reported (7) that the amino acid compositions of acid hydrolysates of 22 individual strains of rumen bacteria were essentially similar to each other, and it was suggested that little variation in the protein quality of the bacterial protein fraction would occur as a result of variations in the composition of the population, unless differences in digestibility or amino acid availability between individual strains were important factors. A digestibility difference between species has been suggested by Pounden et al. (8). Thus, assessment of the protein quality of individual strains of rumen bacteria based on amino acid compositions or chemical scores appear to be of little value.

The present work was undertaken to determine the protein quality of individual strains of rumen bacteria, using a modification (9) of an in vitro enzymatic digest method (10). This method was chosen since it correlated well with bioassays; the numerical values are in the same range as for bioassays and only small samples of test protein sources are needed.

METHODS

Two experiments were performed; in experiment A the protein quality of the strains listed in table 1 was determined, and in experiment B the protein quality of strains listed in table 2 was determined. The bacteria were grown in batch cultures

TABLE 1

Duplicate enzymatic protein quality determinations of cellulolytic rumen bacteria (exp. A)

Protein source	Strain	Pepsin pancreatin digest index [1]	Amino acid showing lowest egg ratio
Bacteroides succinogenes	S85	74.0	Leu and Met
	S85	63.2	Leu and Met
B. succinogenes	B21a	67.7	Leu and Met
	B21a	67.5	Leu and Met
Ruminococcus flavefaciens	C1a	63.8	Met
	C1a	63.1	Met
R. flavefaciens	C94	81.0	Met
	C94	79.5	Met
Arithmetic mean		69.9	—
Casein		86.5	—

[1] Calculated according to ref. (9).

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TABLE 2

Enzymatic protein quality determinations of rumen bacteria (exp. B)

Protein source	Strain	Type	Pepsin pancreatin digest index (NPU_{enz}) [1]	Essential amino acid index [2]	Biological value [3]	Digestibility [4]	Amino acid showing lowest egg ratio
Succinivibrio dextrinosolvens	24	non-cellulolytic	49.7	64.4	53.4	93.1	Leu
Bacteroides amylophilus	70	non-cellulolytic	55.7	81.1	82.3	67.7	Phe and Tyr
Peptostreptococcus elsdenii	B159	non-cellulolytic	50.3	72.6	69.8	72.1	Val and Met
Selenomonas ruminantium	GA192	non-cellulolytic	54.2	79.4	65.0	83.4	Leu
Bacteroides ruminocola	H8a	non-cellulolytic	80.5	79.8	109.8	73.3	Val
Bacteroides succinogenes	A3c	cellulolytic	69.0	73.1	111.3	62.0	Leu and Met
Ruminococcus flavefaciens	B1a	cellulolytic	37.7	75.9	85.4	44.2	Val
Ruminococcus flavefaciens	B34b	cellulolytic	58.9	74.5	68.6	85.9	Lys
Bacteroides fibrisolvens	H10b	cellulolytic	69.5	73.3	82.1	84.7	Val
Ruminococcus albus	7	cellulolytic	50.1	62.4	71.2	70.4	Lys
Arithmetic mean		non-cellulolytic	58.1	75.5	76.1	77.9	—
Arithmetic mean		cellulolytic	57.0	71.8	83.7	69.4	—
Arithmetic mean		all 10 strains	57.6	73.7	79.9	73.7	—
Casein			73.6	—	76.8	95.8	Met

[1] Calculated according to ref. (9).
[2] Calculated according to ref. (12).
[3] $BV = \frac{NPU_{enz}, \text{ i.e., PPDR-I}}{\text{Digestibility}}$.
[4] Calculated according to a modificaiton of AOAC method 22.025 (ref. 13).

at 39° for 24 hours and were subsequently harvested by centrifugation at 45,000 × *g* for 10 minutes. The harvested bacteria were resuspended in H_2O, homogenized and made to volume. A portion of this suspension was hydrolized and analyzed for amino acids as described previously (7). A 1:10 and a 1:100 dilution were used to obtain an approximate protein determination of the bacterial suspensions (11).

Fifty milligrams of casein, egg and bacterial protein were digested in vitro with pepsin for 3 hours at 37° and with pancreatin for 24 hours at 37° (9), after which time a subsample was removed from each digest mixture, deproteinized with 5% sulfosalicylic acid, filtered through no. 50 Whatmann paper and the filtrate analyzed for free amino acids. The remaining contents in each of the digest flasks were centrifuged at 25,000 × *g* for 10 minutes, the residues were resuspended in a known volume of H_2O and aliquots of these suspensions were acid-hydrolyzed and analyzed for amino acid content. All amino acid analyses were performed with a Technicon Auto Analyzer using norleucine as an internal standard.

The terminology used to refer to the various digest fractions is as follows: 1) total amino acid: the amino acid composition of the acid hydrolysate of the protein source (bacteria, egg and casein); 2)

free amino acids: the amino acids that were completely released from the protein source during the enzymatic degeneration; 3) soluble amino acids: the amino acids in the proteins and peptides which were either solubilized or partially degraded by the enzyme; and 4) insoluble amino acids: amino acids in the sediment of the digest mixture after centrifugation at 25,000 × *g* (that is, undigested protein).

Pepsin pancreatin digest residue indexes (PPDR-I) were calculated according to the egg ratio method of Sheffner et al. (10) as modified by Akeson and Stahmann (9), and essential amino acid indexes (EAA-I) were calculated according to Oser (12). An average value of 86% amino acid-N of total nitrogen was used to correct for NPN (7), in the calculation of the EAA-I. Digestion coefficients were estimated by modifying the pepsin digestibility method of proteins (13) according to the formula.

$$\text{Digestibility} = \frac{\text{Total amino acid} - \text{insoluble amino acid}}{\text{Total amino acid}}$$

By using these digestion coefficients and by assuming the PPDR-I's to be equivalent to net protein utilization (NPU) values, biological value (BV) for the strains in experiment B were estimated according to the formula:

$$\text{BV} = \frac{\text{PPDR-I}}{\text{Digestibility coefficient}}$$

RESULTS

In this investigation the protein quality of the bacterial strains was compared with the protein quality of egg. The essential amino acid (EAA) composition of whole egg and 22 strains of rumen bacteria is shown in figure 1. Egg had a higher percentage of valine and leucine, whereas the bacteria had a higher percentage of threonine, phenylalanine plus tyrosine and lysine.

Experiment A was designed to gain information on the variability of the PPDR-I method per se when used with bacterial cells. The data (table 1) showed that PPDR-I values are readily reproducible, for out of 8 determinations (4 pairs), 3 pairs had virtually identical values.

In table 2 are listed the PPDR-I, EAA-I and those amino acids showing the lowest

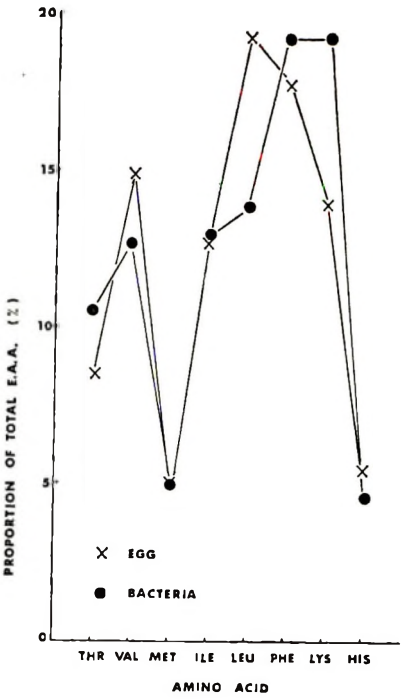

Fig. 1 Essential amino acid composition of acid hydrolysates of egg and rumen bacteria.

egg ratios for each of the protein sources. Digestion coefficients and BV are presented only for the strains studied in experiment B. In experiment B, 5 strains were noncellulolytic, the rest of the strains for both experiment A and B were cellulolytic.

Bacteroides succinogenes, strains A3C, S-85, and B21a showed similar indexes of protein quality, that is 69.0, 68.6, and 67.6, respectively, whereas the PPDR-I of *Ruminococcus flavefaciens*, strains B1a, B34b, C1a and C94 were more variable; the values ranged from 37.7 to 80.0.

The PPDR-I values for casein were also determined and these values provide a positive control. In experiment A a PPDR-I of 86.5 was found for casein, whereas in experiment B a PPDR-I of 73.6 was determined. The value for casein in experiment B was the average of 3 determinations, whereas the value in experiment A was derived from a single determination. The higher PPDR-I for casein in experiment A can be attributed in part to a large fraction of the total amino acids being released into the free amino acid fraction; this was an unusual occurrence and no explana-

tion for this is available at present. The values for casein, however, are comparable to those of 76 and 78 found by Akeson and Stahmann (10). This indicated that at least for casein, the enzymatic digest method as applied in this laboratory gave PPDR-I's comparable to those determined in other laboratories.

The EAA-I are listed for all the strains studied (table 2). These data showed that the EAA-I values were much less variable from strain to strain than were the PPDR-I; this was expected since the individual strains have similar total amino acid compositions (7). Furthermore, the data show that the PPDR-I and EAA-I did not rate the protein quality of the strains in an identical fashion. Thus, *Bacteroides amylophilus*, strain 70, was ranked first by the EAA-I but was ranked ninth by the PPDR-I, whereas strain C-94 was ranked first by the PPDR-I, and ninth by the EAA-I.

The BV for the strains in experiment B was calculated as described above. On the basis of the arithmetic means the cellulolytic strains had a higher BV than the non-cellulolytic strains 83.7 vs. 76.1. The BV of all the strains together showed an average of 79.9 and this compared favorably with values derived for rumen bacteria with rat feeding trials by McNaught et al. (3). The average digestibility of the cellulolytic strains was lower than for the non-cellulolytic strains, 69.4% and 77.9%, respectively, but the mean digestibility of 73.7% of all strains compared closely to the value of 73.3% found by McNaught et al. (3) in rat feeding trials.

Table 3 shows the distribution of EAA's in the 3 digest fractions (as defined above) expressed as a percentage of the EAA's in the original undegraded protein source. These data did not show a general pattern which would divide the non-cellulolytic and

TABLE 3

Distribution of total essential amino acids in the respective digest fractions (exp. B) [1]

Bacteria	Strain	Type	Fractions		
			Free amino acids	Soluble amino acids	Insoluble amino acids
			%	%	%
Succinivibrio dextrinosolvens	24	non-cellulolytic	17.5	75.6	6.9
Bacteroides amylophilus	70	non-cellulolytic	25.1	42.6	32.2
Peptostreptococcus elsdenii	B159	non-cellulolytic	16.9	55.2	27.9
Selenomonas ruminantium	GA192	non-cellulolytic	13.3	70.1	16.6
Bacteroides ruminocola	H8a	non-cellulolytic	24.5	48.8	26.7
Bacteroides succinogenes	A3c	cellulolytic	23.8	39.0	37.3
Ruminococcus flavefaciens	B1a	cellulolytic	2.5	41.7	55.8
Ruminococcus flavefaciens	B34b	cellulolytic	19.6	66.3	14.1
Bacteroides fibrisolvens	H10b	cellulolytic	52.6	32.1	15.3
Ruminococcus albus	7	cellulolytic	22.0	52.1	25.9
Casein			21.3	74.5	4.2

[1] Values expressed as % by weight of total essential amino acids in the digest.

cellulolytic strains into separate groups, or which would place various species (as *Bacteriodes* and *Ruminoccocci*) into distinctive categories, but many variations between individual bacteria occurred. Most noteworthy was the low value of 2.5% of the total EAA's in the free amino acid fraction for strain B1a and the high value of 52.6% in the free amino acid fraction for strain H10b.

Although strains 70, H8a, A3c, and 7 had similar percentages of EAA in the free amino acid fraction this observation was not necessarily reflected by similar PPDR-I values for these strains although this was the case with strain B1a. Except for strains B1a and H10b the soluble amino acid fraction contained the greatest fractions of the EAA's.

Figures 2, 3, and 4 show the EAA pattern of the total acid hydrolysates and of the 3 digest fractions for 3 groups of bacteria. Based upon the relative proportion of leucine and lysine, 3 patterns of EAA composition were apparent in the free amino acid fractions. These were: a) lysine present in a greater quantity than leucine (strains GA192, B159, 24, and 70) (fig. 2); b) lysine and leucine present in approximately equal quantities (strains H8a, B1a, A3c, and H10b) (fig. 3); and c) leucine present in a greater quantity than lysine (strains 7 and B-34b) (fig. 4). Pattern C was that found for the EAA composition in the free amino acid fraction of egg. In all cases, threonine was released to the free amino acid fraction to a very small extent; furthermore, threonine did not influence any of the PPDR-I values.

The data in figures 2, 3 and 4 show that the EAA pattern of the insoluble amino acid fraction closely resembled the EAA pattern of the acid hydrolysate,

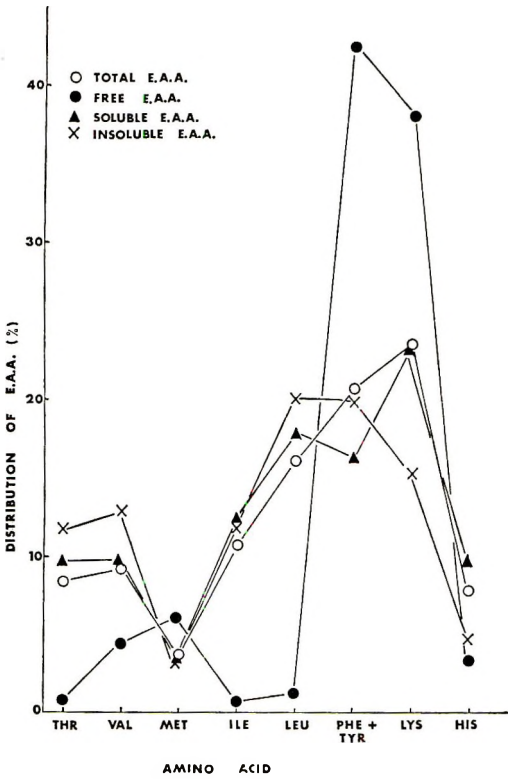

Fig. 2 Essential amino acid composition of the acid hydrolysate and of the 3 enzymatic digest fractions of *S. ruminantium* strain GA192. Similar compositions (pattern A, see Results) were obtained with *P. elsdenii* strain B159, *S. dextrinosolvens* strain 24 and *B. amylophilus* strain 70.

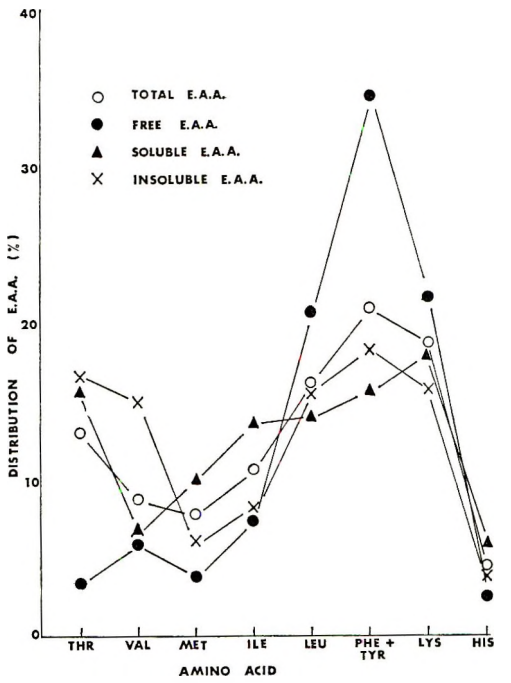

Fig. 3 Essential amino acid composition of the acid hydrolysate and of the 3 enzymatic digest fractions of *B. ruminocola* strain H8a. Similar compositions (pattern B, see Results) were obtained with *R. flavefaciens* strain B1a, *B. succinogenes* strain A3c and *B. fibrisolvens* strain H10b.

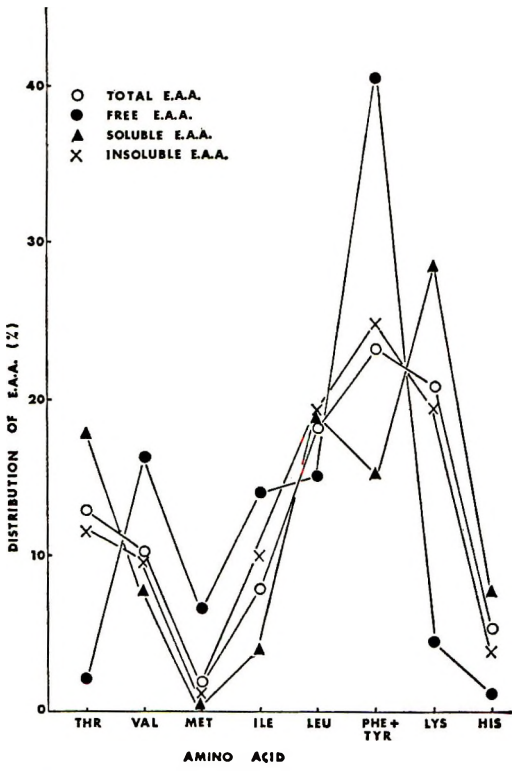

Fig. 4 Essential amino acid composition of the acid hydrolysate and of the 3 enzymatic digest fractions of *R. albus* strain 7. A similar composition (pattern C, see Results) was obtained with R. *flavefaciens* strain B34b.

but that the EAA pattern in the free amino acid fraction did not reflect the pattern for the acid hydrolysate. Histidine tended to be an exception. This means that the EAA pattern of the soluble amino acid fraction was inversely related to the EAA pattern of the free amino acid fraction.

DISCUSSION

In these experiments, strains of rumen bacteria were evaluated for protein quality using whole egg protein as a standard reference. Egg protein is universally accepted as the protein source of optimal quality for monogastric animals. However, it has not been clearly established that egg protein presented to the ruminant postruminally represents the protein source of optimal quality for the ruminant, but reports which indicate otherwise have not appeared either. On this basis and from the data presented, the rumen bacteria studied appear to be a protein source of a quality comparable to that of casein.

The generally accepted hypothesis that protein quality cannot be judged directly and solely from amino acid compositions has led many investigators to feel that the quality of any protein source must ultimately be determined with bioassay methods, whereas the EAA-I (12) and the score method (14) should be used only for screening purposes.

The in vitro digest method represents a compromise between bioassay procedures and evaluation based only on amino acid composition. Large differences in protein quality between the various bacterial strains as determined by the PPDR-I were apparent, but these differences were completely masked when EAA indexes were compared. Thus additional nutritional and physiological variables are considered which make the determination a closer approximation to the physiological system. Some further advantages of the digest system are that the protein quality values determined by its application are numerically equal and correlate highly with values from bioassays, and that only small quantities of protein are needed for the evaluation.

For the results to be meaningful they must approximate protein quality values of rumen bacteria as determined with bioassays. The estimated BV and digestion coefficients agreed closely with bioassay values (3) and thus are an indication of the verity of the values determined in vitro, reported in this paper. Sheffner et al. (9), who used only pepsin in their in vitro digest method, equated the pepsin-residue digest index with NPU, whereas Akeson and Stahmann (10) referred to the PPDR-I as a BV expression. However, this latter relationship does not appear to be well justified since values derived from the in vitro system depend in part upon the digestibility of the protein source as do NPU values but not BV.

The calculated PPDR-I is largely influenced by the quantitative distribution of the essential amino acids in the digest fractions (of which our digestibility value is an estimate) and also by the qualitative distribution of the amino acids in these fractions. The results presented here sug-

gest that differences between the bacteria arise from variability within both of these factors. Thus large differences in digestibility values are indicated in table 2 and marked differences in amino acid distribution, particularly for leucine and lysine, are apparent from the results shown in figures 2, 3, and 4.

If histidine is regarded as a nonessential amino acid the data of Akeson and Stahmann (15) (table 4) show the amino acid with the lowest egg ratio in the free amino acid fraction to be the limiting amino acid for the respective proteins as reported in the literature.

On this basis lysine was indicated as the limiting amino acid for only two of all the organisms tested, whereas previous work (16, 17) suggests that lysine was the limiting amino acid in defaunated ruminants (that is, for the protein that reached the intestine). Four factors may be involved in this apparent discrepancy. First, ruminants may have a proportionally higher lysine requirement than monogastrics; second, the strains of bacteria present in the rumen of the sheep in the previous work (16, 17) were not necessarily the same qualitatively or quantitatively as those presently studied; third, the analytical methods used did not distinguish between D- or L-isomers of lysine (D-lysine is present in significant quantities in bacteria, but is unavailable to the host). However, this source of error may not be as great as it appears, as the D-isomer is presumably largely associated with the cell wall and it appears likely that this fraction would occur mainly in the insoluble amino acid fraction which would be unavailable for digestion in any case. Finally, the protein of the rations fed to the defaunated sheep (17) was primarily corn protein (as zein and gluten) which is deficient in lysine. Furthermore zein is resistant to degradation in the rumen and could therefore profoundly modify the amino acid composition reaching the lower gut, since the protein presented to the animal for absorption may consist of undegraded and partially degraded food protein in addition to microbial protein.

TABLE 4

Egg ratios of the free amino acid fraction of four proteins

Amino acid	Protein			
	Lact-albumen	Casein	Soy	Gluten
Thr	100	86	93	71
Val	100	70	96	100
Met	83	66 [2]	45 [2]	64
Ile	82	70	100	100
Leu	100	100	100	100
Tyr and Phe	68 [2]	96	100	98
Lys	100	100	100	52 [2]

[1] Calculated from the data of Akeson and Stahmann (15).
[2] Lowest egg ratio and limiting amino acid provided histidine is not included.

Data indicating the relative contribution of individual strains of bacteria to the total protein supply of the host animal are not available. However, the present results indicate clearly that factors modifying the population composition may at the same time profoundly modify the quality of the protein presented to the host for digestion and absorption. For example, any factor favoring the predominance of strain B1a would depress protein quality, whereas factors favoring strain H8a would enhance protein quality.

There appears little doubt that despite the similarity of the amino acid compositions of the acid hydrolysates of individual strains of rumen bacteria, marked differences occur between the bacteria with respect to their protein quality. Furthermore, it appears that these differences may arise from differences in both total digestibility and the pattern in which amino acids are released from the bacterial protein. Consequently, it appears that a modification of the ruminal population composition must be considered as a possible factor in explaining changes in nitrogen utilization or amino acid patterns resulting from dietary modifications. Changes in amino acid availability to the host animal appear likely to arise from this type of variation as much as from changes in the synthesis of specific amino acids by specific organisms.

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Arginine Deficiency in Two Strains of Chickens Selected for Differences in Dietary Requirements of Arginine [1]

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ABSTRACT The factors affecting the response to an arginine deficiency in 2 strains of chicks selected for high (HA) or low (LA) requirement of arginine were investigated in several experiments. For maximal growth chicks from the HA strain required more than 0.6% arginine added to a basal diet containing casein, whereas only 0.3% appeared to be needed by chicks of the LA strain. The 2 strains differed considerably more in response to an arginine deficiency when a diet containing casein was fed than when an arginine deficiency was produced with a diet containing crystalline amino acids. Force-feeding an arginine-deficient casein diet to chicks of the HA strain in an amount consumed ad libitum by chicks of the LA strain produced enlarged and fatty livers, whereas force-feeding a similar quantity to chicks of the LA strain produced no detrimental effects. The arginine content of plasma and protein-free extracts of liver and muscle was not appreciably different when the 2 strains were fed diets deficient or adequate in arginine. Lysine levels in these tissues were consistently higher in chicks of the HA strain compared with the LA strain, at all levels of arginine fed. The data suggest that the inability to metabolize high levels of dietary lysine by chicks of the HA strain may increase their arginine requirement.

Arginine metabolism in birds is considerably different from that in mammals. The enzymes concerned with arginine synthesis in mammals, normally present in rat liver, are absent from avian liver although small amounts of some are present in kidney and other extra-hepatic tissues of chicks (1, 2). Thus arginine is not synthesized by birds, and is an absolute dietary requirement. In spite of this, considerable variation in requirement has been observed (3, 4), some of which is apparently under genetic control. Nesheim and Hutt (5) noted that 3 strains of White Leghorn chickens differed in their dietary arginine requirement, and sire families within the strains also showed considerable variation in response to an arginine deficiency. Griminger and Fisher (6) observed similar variation among offspring of several hens.

Recently, Hutt and Nesheim (7) reported the results of a specific selection program aimed at producing 2 strains of chickens differing in their quantitative dietary requirement of arginine. The experiments described in the present paper were designed to characterize the marked differences in response of these strains to an arginine deficiency.

EXPERIMENTAL PROCEDURES

The chicks used were from strains selected for a high requirement (HA) or a low requirement (LA) for arginine (7). The experiments to be described were conducted over a 3-year period and involve chicks from different selected generations of the breeding program. The generation used will be indicated with each experiment, and the nomenclature for each selected generation is that of Hutt and Nesheim (7). Growth experiments took place in thermostatically controlled, electrically heated battery brooders with raised wire floors. The basic experimental diets are included in table 1. The actual diets used, and the modifications made are described for each experiment.

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TABLE 1
Basal diets

	A	B	C	D
	g/100 g	*g/100 g*	*g/100 g*	*g/100 g*
Casein	25.00	—	—	27.00
Amino acid premix	—	23.68 [1]	28.78 [2]	—
Glucose	59.02	—	—	—
Sucrose	—	47.66	42.56	43.82
Cellulose	3.00	4.00	4.00	4.00
Corn oil	4.00	15.00	15.00	15.00
Glycine	1.00	—	—	1.00
Methionine	0.40	—	—	0.40
Vitamin premix [3]	1.22	1.20	1.20	1.22
Mineral premix	6.36 [4]	8.46 [5]	8.46 [5]	6.36 [4]

[1] Supplied the following amino acids in g/100 g diet: L-arginine·HCl, 0.73; L-histidine·HCl, 0.62; L-lysine·HCl, 1.40; L-tyrosine, 0.63; L-tryptophan, 0.23; L-phenylalanine, 0.68; L-cystine, 0.35; DL-methionine, 0.55; L-threonine, 0.85; L-leucine, 1.20; L-isoleucine, 0.80; L-valine, 1.04; L-glutamic acid, 12.0; glycine, 1.60; and L-proline, 1.00.

[2] Supplied the following amino acids in g/100 g diet: L-arginine·HCl, 1.0; L-histidine·HCl, 0.89; L-lysine·HCl, 2.22; L-tyrosine, 1.12; L-tryptophan, 0.24; L-phenylalanine, 1.19; L-cystine, 0.06; DL-methionine, 1.15; L-threonine, 0.89; L-leucine, 2.20; L-isoleucine, 1.36; L-valine, 1.63; L-glutamic acid, 4.93; glycine, 2.39; L-proline, 2.95; DL-serine, 1.5; L-alanine, 1.46; and L-aspartic acid, 1.60.

[3] For composition see Nesheim et al. (8).

[4] Supplied the following in g/100 g diet: $CaHPO_4 \cdot 2H_2O$, 1.80; $CaCO_3$, 1.90; KH_2PO_4, 1.40; $NaHCO_3$, 0.88; $MnSO_4 \cdot H_2O$, 0.0333; $FeSO_4 \cdot 7H_2O$, 0.0333; $MgSO_4$, 0.30; KI, 0.00026; $CuSO_4 \cdot 5H_2O$, 0.00167; ZnO, 0.00623; $CoCl_2 \cdot 5H_2O$, 0.00017; $NaMoO_4 \cdot 2H_2O$, 0.00083; and Na_2SeO_4, 0.0001. When this mineral mixture was used, NaCl was added to diets not containing adequate amounts of chloride from amino acid hydrochlorides.

[5] Supplied the following in g/100 g diet: $CaHPO_4 \cdot 2H_2O$, 3.89; $CaCO_3$, 0.75; $KHCO_3$, 1.90; $NaHCO_3$, 1.60; $MnSO_4 \cdot H_2O$, 0.0333; $FeSO_4 \cdot 7H_2O$, 0.0333; $MgSO_4$, 0.25; KI, 0.00026; $CuSO_4 \cdot 5H_2O$, 0.00167; $ZnCO_3$, 0.0115; $CoCl_2 \cdot 5H_2O$, 0.00017; $NaMoO_4 \cdot 2H_2O$, 0.00083; and Na_2SeO_4, 0.00001.

Amino acid analyses were made with a Technicon amino acid analyzer [4] using 21 hours for complete analysis. Samples of blood plasma were freed of protein by precipitation with sulfosalycylic acid. Two milliliters of plasma were treated with 4 ml of 4% sulfosalycylic acid. The precipitated protein was removed by centrifugation and the supernatant was used for analysis. Norleucine was used as an internal standard for each run. Free amino acids were also determined in liver and muscle homogenates following precipitation of proteins with sulfosalycylic acid. For muscle analysis, 1.25 g of gastrocnemius and associated muscle were homogenized with 15 ml of 4% sulfosalycylic acid in an Omni-Mixer.[5] The homogenate was diluted to 25 ml and the protein was separated by centrifugation. Liver analyses were carried out in the same manner except that 3 g of liver were homogenized with 10 ml of 4% sulfosalycylic acid before dilution to 25 ml. One or two milliliters of the supernatant were applied to the column.

EXPERIMENTAL RESULTS

The differentiation of the HA and LA strains was primarily on the basis of ability to grow to 4 weeks of age with an arginine-deficient diet. An experiment was carried out to determine whether the difference in growth rate of the 2 strains was reflected in a difference in actual quantitative dietary requirement for arginine. Chicks from the S-2 generation were used. Levels of 0.15, 0.30, 0.60 and 1.2% L-arginine, as the hydrochloride, were added as modifications to basal diet A, table 1, and the experimental diets were fed to 3 groups of 8 male chicks from each strain, from hatching to 4 weeks of age.

The results of this experiment are shown in figure 1. The 2 strains of chicks showed a marked difference in their response to the increments of dietary arginine. A level of 0.3% added arginine appeared to be as adequate as higher levels for chicks from the LA strain, whereas chicks from the HA strain needed at least 0.6% and probably more supplementary arginine to grow normally. The response to each increment of arginine by the HA strain appeared to be somewhat less than the response observed in chicks from the LA strain.

[4] Technicon Company, Chauncey, New York.
[5] Servall, Ivan Sorvall, Inc., Norwalk, Connecticut.

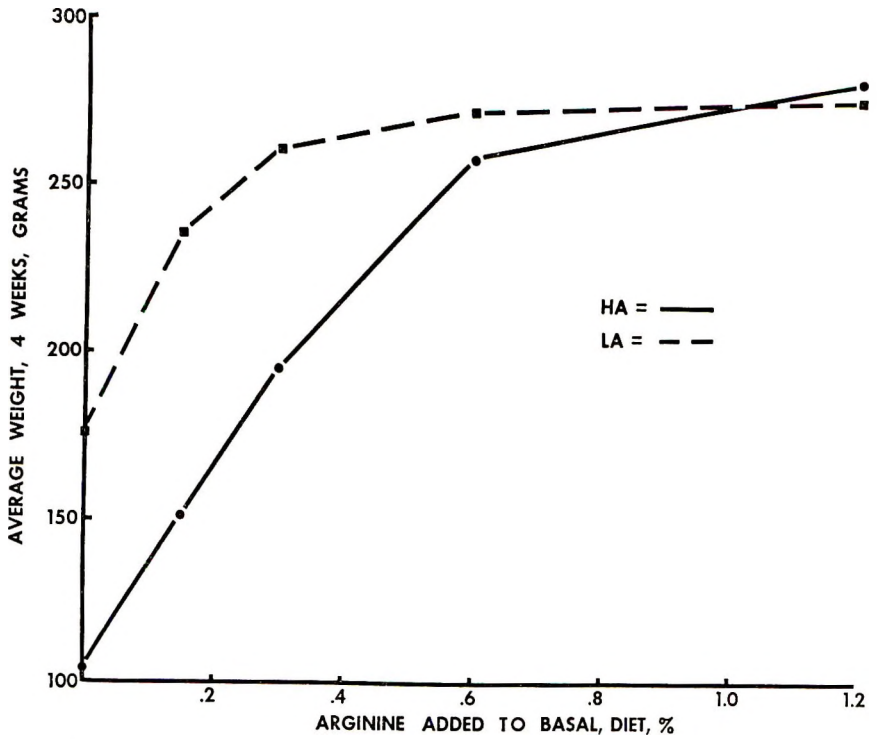

Fig. 1 The response of the LA and HA strains to levels of dietary arginine. Each point is the average weight at 4 weeks of age of 3 replicates consisting of 4 males and 4 females each. The basal diet contained casein as the source of protein (diet A, table 1).

The HA and LA strains were developed under conditions where the arginine-deficient test diet was made up of casein as the source of protein. This protein is known to increase the arginine requirement of chicks (3, 9). In several experiments, the 2 strains were studied for their response to an arginine deficiency using diets containing crystalline amino acids in place of casein in the deficient diet. The basal diet is shown in table 1 (diet B). The amino acid mixture is the same as that developed by Dean and Scott (10). The chicks in these experiments were fed to 7 days of age a standard purified diet containing isolated soybean protein (11). Following the preliminary period, the chicks with either high or low extremes of weight were discarded and experimental pens were made up so that the weight distribution of chicks within a pen was similar. The pen of chicks was then considered as the experiment unit.

The results of 2 experiments with the HA and LA strains from the S-1 generation are shown in table 2. The basal level of arginine used was 0.7% of the diet in experiment 1 and 0.6% in experiment 2. In the control diet the level of arginine was 1.1%, the level found by Dean and Scott to be sufficient to obtain maximal growth with this amino acid mixture. Results of these 2 experiments showed that the 2 strains did not differ significantly in their response to an arginine deficiency when the amino acid diet was fed.

The response of the HA and LA strains to an arginine-deficient amino acid mixture was studied in a further experiment shown in table 3. In this experiment, a direct comparison was made of the response of the 2 strains to an arginine deficiency produced with a diet containing casein or the amino acid mixture. The chicks used were hatched from proven-sire matings which had been selected by progeny tests to produce offspring for the S-2 generation. Thus there had been additional selection for a high or low arginine requirement with these chicks compared with those

TABLE 2

Response of HA and LA strains to arginine deficiency with diet containing crystalline amino acids (Diet B, table 1)

Arginine level	Gain [1]		Feed consumption/ chick	
	Strain HA	Strain LA	Strain HA	Strain LA
% of diet	*g*	*g*	*g*	*g*
	Experiment 1 (7–19 days of age) [2]			
0.70	51 [a]	57 [a]	129	151
1.10	95 [b]	89 [b]	171	189
	Experiment 2 (7–25 days of age) [3]			
0.60	40 [a]	50 [a]	173	169
0.85	113 [b]	113 [b]	236	243
1.10	148 [c]	141 [bc]	262	258

[1] Values within an experiment followed by same superscript letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test (12).
[2] Values shown are averages of 3 replicates consisting of 3 male and 2 female chicks each.
[3] Values shown are averages of 3 replicates of 3 male and 3 female chicks each.

TABLE 3

Comparison of response to arginine deficiency by HA and LA strains in diets containing casein or crystalline amino acids

Amino acid source	Avg body wt gain, 1–3 weeks of age [1,2]		Feed consumption/ chick	
	Strain HA	Strain LA	Strain HA	Strain LA
	g	*g*	*g*	*g*
Casein (0.8% arginine) (diet A, table 1)	45 [a]	115 [cd]	152	225
+1.20% L-arginine	128 [de]	138 [e]	241	240
Crystalline amino acid mixture (diet B, table 1) containing:				
0.60% L-arginine	46 [a]	69 [b]	147	178
0.85% L-arginine	77 [b]	102 [c]	181	214
1.10% L-arginine	101 [c]	126 [de]	183	244

[1] Each value shown is average of 3 replicates, each consisting of 4 male and 4 female chicks.
[2] See footnote 1, table 2.

used in previous experiments. In this experiment chicks from the 2 strains receiving the arginine-deficient diet differed in growth rate and feed consumption during the period from 1 to 3 weeks of age. Chicks from the LA strain receiving the amino acid diet grew more rapidly with the deficient levels of arginine than chicks from the HA strain. It appeared that 1.1% L-arginine may not have been sufficient for maximal growth of the HA strain with the amino acid diet. The difference between the 2 strains was much greater when the diet containing casein was fed than when the diet with the crystalline amino acid mixture was used to produce the arginine deficiency.

The comparison between the diet containing casein and diets containing amino acids was not strictly between the 2 amino acid sources in the above experiments, since levels of fat and the type of carbohydrate also were different in the diets compared. These differences were eliminated in a similar experiment conducted with the S-4 generation (table 4).

The diet containing casein as the arginine-deficient protein was compared with one containing the amino acid mixture used in the previous experiments, and with an amino acid mixture simulating the amino acid composition of a diet containing casein (diet C, table 1). Chicks from the HA strain grew more slowly than those from the LA strain with all arginine-deficient diets. Again, the greatest difference between the 2 strains was observed with the diet containing casein. Although the

TABLE 4

Response of chicks from HA and LA strains to 3 arginine-deficient diets

Source of amino acids	Avg body wt gain,[1,2] 7–21 days of age	
	Strain HA	Strain LA
	g	*g*
Intact casein	106	151
+1.0% arginine (diet D, table 1)	154	175
Amino acid diet (0.6% arginine)	115	133
+0.65% arginine (diet B, table 1)	168	174
Simulated casein (0.83% arginine)	107	131
+1.0% arginine (diet C, table 1)	165	172

[1] Represents average gain of 3 groups of 5 chicks for period shown.

[2] Analysis of variance of the factorially arranged treatments indicated the following: Significant effects ($P < 0.05$) were found for strain, arginine level, and for the interactions, strain × arginine level and strain × type of diet.

amino acid diet simulating casein had more arginine in the basal amino acid mixture than in the other amino acid diet, the arginine deficiency was just as severe. The difference between the strains was somewhat greater when the diet contained intact casein than when the diet was simulated with amino acids. It did appear, however, that the 2 strains differed under conditions of arginine deficiency even with the amino acid diets, although the degree of the differentiation between the 2 strains appeared to depend upon the type of arginine-deficient diet.

CONTROLLED FEEDING EXPERIMENTS

Food intake is markedly different between chicks from the HA and LA strains when they receive an arginine-deficient diet. Although the low feed consumption by chicks from the HA strain is most likely a consequence of arginine deficiency, it was considered possible that the differences in food intake between the 2 strains could be a reflection of a defect in the food intake control mechanism. The arginine deficiency could conceivably result in an exaggerated decrease in feed intake by the HA strain compared with that observed with the LA strain. Therefore, a feeding experiment was conducted in which the chicks from the LA strain were restricted in feed intake to the amount consumed ad libitum by the HA strain fed the arginine-deficient diet. The paired feeding was carried out by feeding the pens of chicks from the LA strain the amount of feed consumed by the pens of chicks from the HA strain the previous day. The paired feeding was on a group basis, and the average response of the groups of chicks was used to assess the experimental results (table 5). Chicks from the HA strain voluntarily consumed only about 64% as much feed as chicks from the LA strain. Restriction of feed intake of chicks from the LA strain also restricted weight gains nearly the same as for the HA strain. Eliminating differences in feed intake appeared to overcome any differences in amino acid utilization, possibly because of measurably reduced energy consumption.

An additional experiment was conducted in which chicks from the HA strain were force-fed the quantity of feed which the LA strain voluntarily consumed. The basal diets were pelleted in a California laboratory pellet mill. The chicks were force-

TABLE 5

Effect of pair-feeding an arginine-deficient diet to HA and LA strains

Diet	Avg body wt gain,[1] 7–28 days of age		Feed consumption/ chick	
	Strain HA	Strain LA	Strain HA	Strain LA
	g	*g*	*g*	*g*
Basal diet (diet A, table 1)	86	187	250	389
Basal diet pair-fed to HA strain	—	99	—	252
Basal diet + 1.2% L-arginine	269	255	454	453

[1] Each value represents average gain of 3 groups of 10 male chicks/treatment. Paired feeding began at 7 days following hatching. All chicks were fed the supplemented diet until they were 7 days of age.

TABLE 6

Effect of force-feeding an arginine-deficient diet to the HA and LA strains

	Avg body wt gain,[1] 13 days' feeding		Feed consumption/ chick		Adjusted liver wt [2]		Liver lipid [3]	
	Strain HA	Strain LA	Strain HA	Strain LA	Strain HA	Strain LA	Strain HA	Strain LA
	g	*g*	*g*	*g*	*g*	*g*	*g/100 g dry wt*	
Basal diet, ad lib. (diet A, table 1)	45 ± 14	115 ± 18	147	197	4.8	5.4	21 ± 1.2	27 ± 4.2
Basal diet, force-fed	73 ± 2	102 ± 6	204	205	7.8	6.3	54 ± 16.7	28 ± 13.6
Basal diet + 1.2% arginine, ad lib.	127 ± 15	141 ± 27	223	237	4.6	4.8	22 ± 2.3	20 ± 1

[1] Values are averages for 10 chicks/treatment with associated standard error except for force-fed treatments, where 5 chicks of each strain were used.
[2] Liver weight was adjusted for differences in final body weight by analysis of covariance. Significant difference in adjusted liver weight ($P < 0.05$) is 1.7 g by Tukey's HSD (12). Five livers were taken from each treatment.
[3] Values shown are averages for 5 livers/treatment with associated standard error.

fed by feeding the pellets by hand 3 times a day. The amount to be fed was based on the average amount consumed ad libitum by a group of chicks from the LA strain the previous day. Five chicks from each strain (S-3 generation) were force-fed the arginine-deficient diet for a 14-day period. Controls fed the deficient or arginine-supplemented diets ad libitum were also included in this experiment. The results of this study are shown in table 6.

Chicks from the HA strain that were forced to consume the same quantity of feed as those from the LA strain gained less weight during the feeding period, and developed extremely enlarged and fatty livers. Liver weights were increased in both strains by the force-feeding but liver fat was increased only in chicks from the HA strain. At the end of the 14-day feeding period the chicks of the HA strain were in poor condition and obviously did not tolerate the force-feeding of the arginine deficient diet as well as those from the LA strain. Although the chicks from the HA strain force-fed the basal diet gained more weight during the experimental period than those consuming the diet ad libitum, this experiment shows that the HA strain was unable to tolerate the deficient diet as well as the LA strain.

Amino acid analysis of plasma and other tissues. Several body pools of amino acids were analyzed to determine possible differences in circulating levels of amino acids, particularly arginine and others that may affect its metabolism.

In the first experiment, 3 pens of chicks from the LA and 3 from the HA strains (S-2 generation) were fed deficient (basal diet A, table 1) or adequate (1.2% supplementary arginine) diets from hatching to 4 weeks of age. The chicks were fed ad libitum until the day blood samples were obtained, and fasted for 4 hours prior to obtaining the samples. Plasma obtained

TABLE 7

Free amino acids in plasma of HA and LA chicks fed arginine-deficient or supplemented diet [1]

Amino acid	Level of dietary arginine, %			
	2.0		0.8	
	Strain HA	Strain LA	Strain HA	Strain LA
	μg/ml		*μg/ml*	
Aspartic acid	19	20	27	21
Threonine + asparagine	89	70	254	287
Serine + glutamine	99	89	107	120
Glutamic acid	46	38	44	43
Proline	126	95	135	136
Glycine	60	50	55	66
Alanine	61	54	54	70
Valine	41	33	45	50
Cystine	23	21	35	36
Methionine	40	30	24	33
Leucine	21	16	21	22
Isoleucine	32	24	26	33
Tyrosine	39	32	40	57
Phenylalanine	19	14	18	21
Lysine [2]	144	64	150	101
Histidine	28	16	25	32
Arginine	57	52	15	17
Ornithine	21	12	5	3

[1] Values are for analysis of pooled samples of blood from 3 pens of chicks fed each diet. Each pen contained 8 chicks.
[2] There is a significant strain difference in lysine content ($P < 0.05$) by analysis of variance.

from pooling 2 ml of blood from each of 8 chicks in a pen was analyzed (table 7). Although some amino acids, notably glutamine, asparagine, serine and threonine, were influenced by type of diet, the major strain difference observed was in plasma lysine level.

The arginine content of plasma reflected the difference in arginine content of the diets, but at each dietary level there was little difference between the strains. There was, however, a striking difference in plasma lysine content. Chicks from the HA strain had a significantly higher level than chicks from the LA strain. Ornithine levels in plasma were also higher in chicks of the HA strain than in those of the LA strain at both levels of dietary arginine.

Free amino acid levels in plasma, muscle and liver were measured under conditions of controlled feeding in a second experiment. Sixteen chicks, 8 from each strain (S-3 generation), were fed diet A (table 1) supplemented with 1.2% arginine, from hatching to 2 weeks of age. For an additional 2 weeks, 4 chicks of each strain were then fed the basal diet unsupplemented, and 4 continued to receive the diet adequate in arginine. At the end of the 2-week period, the chicks were fasted for 4 hours, and each hour for 6 hours were hand-fed 1/24th of the feed consumed the previous day. One hour after the last feeding, a blood sample was obtained from each chick and amino acid analysis was conducted on the individual plasma samples. At the same time a muscle sample was obtained from each chick, and this was treated for amino acid analysis as indicated previously. A separate series of chicks, treated exactly as described above, was used to obtain protein-free filtrates from liver for analysis for free amino acids.

The results of these experiments are shown in table 8. Only the levels of the basic amino acids are shown since there were no consistent strain differences for the other amino acids, although some were affected by diet. The levels of arginine in plasma, muscle and liver were affected by diet, but the 2 strains did not differ appreciably in arginine levels in the plasma and tissues shown. Again, the most striking difference was in lysine level.

TABLE 8

Basic amino acids in plasma, liver and muscle of chicks from the HA and LA strains fed arginine-deficient or supplemented diets

Amino acid	Level of dietary arginine, %			
	2.0		0.8	
	Strain HA	Strain LA	Strain HA	Strain LA
	μg/ml plasma or /g tissue [1]			
	Plasma [2]			
Lysine	91.4	57.3	113.2	94.4
Histidine	18.8	20.1	19.1	26.8
Arginine	45.4	48.9	17.4	20.1
Ornithine	14.1	13.6	5.9	6.1
	Liver [2]			
Lysine	82.2	54.7	218.9	79.5
Histidine	84.3	64.4	78.8	101.9
Arginine	47.8	40.6	29.0	23.2
Ornithine	16.0	14.7	9.9	8.0
	Muscle [2]			
Lysine	122.6	54.9	60.1	40.2
Histidine	10.1	14.6	8.4	13.8
Arginine	53.2	39.0	25.2	19.8
Ornithine	10.7	7.9	2.4	3.5

[1] Each value represents a single analysis of 4 samples/treatment.
[2] The only significant strain difference is for lysine level ($P < 0.05$) by analysis of variance.

Chicks of the HA strain had consistently higher lysine levels in plasma, muscle and liver than chicks of the LA strain. This was true at both levels of dietary arginine. No difference between the 2 strains was observed in histidine content of the plasma or tissues studied.

DISCUSSION

The development of the 2 strains of chickens used in these experiments has made it possible to study the variations in arginine requirements that have been observed by a number of investigators. It is quite likely that most populations of chickens have representatives which are similar to the chicks from the present LA and HA strains. Depending upon the genetic background of the chickens, the response to an arginine-deficient diet may be quite different. Our experience with several commercial strains of White Leghorn chickens suggests that the general response to an arginine deficiency in these strains resembles that of the HA strain more closely than the LA strain. However, within these strains there are individuals that respond to an

arginine deficiency in the manner of the LA strain.

The 2 strains appear to have a marked difference in arginine requirement which is shown by the contrasting response to an arginine deficiency. The differing effects of force-feeding an arginine-deficient diet to the 2 strains were particularly striking. Chicks from the HA strain force-fed the deficient diet showed a response in liver size and liver lipid similar to that observed by Sidransky and Baba (13) from force-feeding amino acid-deficient diets to rats. The deficient diet was tolerated much better by chicks from the LA strain, showing little detrimental effect. For the first few days of forced feeding the HA strain gained nearly as well as the LA strain, but the differences then showed up quickly.

The type of arginine-deficient diet certainly has an influence on the degree of difference in response between the strains. When a diet was fed containing casein as a source of protein, the 2 strains differed markedly in response, whereas a much smaller difference was observed when the arginine-deficient diet was produced by using crystalline amino acids. This suggests that the differences between the 2 strains may be a function of their ability to tolerate the amino acid imbalances present in casein which appear to be responsible for increasing the arginine requirement of chicks when fed such a diet. Snyder et al. (3) and Krautmann et al. (9) showed that the arginine requirement of chicks was considerably greater when diets contained casein than when other proteins were used to produce the arginine deficiency. Klain et al. (14) showed that the arginine requirement of chicks was higher when an amino acid mixture simulating casein was used compared with the requirement when an amino acid mixture with a considerably different composition was used. In these experiments the amino acid diet simulating casein did not have the same effect on the strains as the casein diet. Perhaps this is due to some difference in assumed or actual amino acid content of the casein, or in availability of some amino acids.

The lysine content of casein has been particularly implicated in the high arginine requirement of chicks fed diets containing this protein. Snetsinger and Scott (15), Jones (16), Smith and Lewis (17), Boorman and Fisher (18) and O'Dell and Savage (19) have all published evidence showing that high levels of dietary lysine increase the arginine requirement of chicks. Although Fisher presented evidence that other amino acids may also produce growth depressions that can be partially reversed by feeding arginine, the effect of lysine in increasing arginine requirement appears to be considerably greater than for any other amino acid studied.

The analyses conducted on various amino acid pools showed that there was very little difference in the arginine content of plasma or muscle and liver between the 2 strains of chicks. A major, consistent difference observed, however, was the level of lysine in tissue fluids. Thus it is conceivable at least that the differences in arginine requirement may in part be due to differences in lysine metabolism between the 2 strains. The possible differences in ability of the HA and LA strains to metabolize lysine, and possible effects on their arginine requirements by dietary lysine are under investigation.

These experiments show that 2 strains of chickens that differ quite markedly in arginine metabolism have been developed with a relatively short period of intensive selection. The differences in ability of these 2 strains to grow with an arginine-deficient diet appear to be due to true differences in the efficiency with which dietary arginine is used for growth. The development of these strains has made it possible to study factors influencing arginine metabolism that would be much more difficult to study with an unselected group of chickens.

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Biotin Deficiency and Orotic Acid Fatty Liver in the Rat [1]

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ABSTRACT To determine whether biotin deficiency would influence the development of fatty liver due to orotic acid, female rats were fed ad libitum a basal or biotin-deficient diet for 10 weeks. The animals were then force-fed an adequate amount of basal or biotin-deficient diet containing 1% orotic acid for 3 or 6 days. All animals force-fed the orotic acid-containing diets developed fatty livers. These results indicate that biotin deficiency has no inhibitory effect on fatty liver induction by orotic acid.

Rats fed a purified diet containing orotic acid rapidly develop fatty liver (1–3). In studies on lipid synthesis, liver slices of rats fed orotic acid have been observed to incorporate more acetate-2-^{14}C into fatty acids than comparable liver slices of control animals (4). Also, the addition of orotic acid in vitro to liver slices of normal rats caused a stimulation of incorporation of ^{14}C-labeled acetate into fatty acids (4).[2] However, the site and mode of action of orotic acid on fatty acid synthesis in the liver is not clear.

Marchetti and Puddu (5) have reported that the amount of biotin in the livers of orotic acid-fed rats was lower than in controls and that in biotin-deficient rats the development of orotic acid fatty liver was inhibited. They suggested that the site of action of orotic acid upon the fatty acid synthetic system could be at the stage of acetyl CoA-carboxylase reaction. Since it is known that biotin-deficient animals consume less diet than controls (6), it seemed possible that the decreased intake of diet containing orotic acid could account indirectly for the failure to observe fatty liver. The importance of both the quantity and quality of diet intake along with orotic acid in the induction of fatty liver has been reported previously (3).

Our present study was conducted to test whether the amount of diet intake was an important factor in the reported effect of biotin deficiency on orotic acid fatty liver. Biotin deficiency was induced in rats by feeding ad libitum a biotin-deficient diet for 10 weeks. These animals were then force-fed on adequate amount of biotin-deficient, orotic acid diet for 3 or 6 days. Our results show that biotin deficiency has no direct inhibitory effect on fatty liver induction by orotic acid.

MATERIALS AND METHODS

Female rats of the Sprague Dawley [3] and Wistar [4] strains were used. In the first experiment the Sprague Dawley rats weighed on the average 117 g and in the second experiment the Wistar rats weighed 57 g. In each experiment the animals were divided into 2 groups: rats fed the basal diet and rats fed the biotin-deficient diet. The basal diet was similar to that used in an earlier study (3) except that it contained autoclaved egg white (spray-dried).[5] It was composed of 20% vitamin-free casein,[6] 11% autoclaved egg white, 5% corn oil, 4% salts, 5% vitamin-sucrose mixture, and 55% sucrose. This diet contained 20 μg biotin/100 g diet. The biotin-deficient diet was the same as the basal diet except that no biotin was added and 11% raw egg white (spray-dried) was used. Animals were weighed at onset and at weekly intervals. Animals were housed in individual cages and had free access to diet and water for 10 weeks. Thereafter, the 2 groups were further subdivided ac-

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[2] Sarma, D. S. R., and H. Sidransky, unpublished data.
[3] Sprague-Dawley, Madison, Wisconsin.
[4] Hilltop Lab Animals, Inc., Scottdale, Pennsylvania.
[5] Egg white solids spray-dried, General Biochemicals, Chagrin Falls, Ohio.
[6] "Vitamin-Free" Casein, Nutritional Biochemicals Corporation, Cleveland.

cording to the diet that was force-fed for 3 or 6 days. Animals fed the basal diet were divided into: group 1, basal diet, and group 2, basal diet containing 1% orotic acid. Animals fed the biotin-deficient diet were divided as follows: group 3, biotin-deficient diet, group 4, biotin-deficient diet containing 1% orotic acid, and group 5, the same diet as for group 4 but in addition the animals received 250 μg *dl*-biotin subcutaneously during the first day of force-feeding. For force-feeding, the diets were blended with distilled water so that each milliliter of diet mixture contained 0.7 g diet. Animals were tube-fed 3 times daily, at 9 AM, 1:30 PM and 6 PM, similar to the procedure described previously (3). Animals force-fed for 3 days received an average diet intake of 0.6 g/10 g body weight, and animals force-fed for 6 days received an average of 0.8 g/10 g body weight. Rats were killed by exsanguination following ether anesthesia. The livers were weighed and portions were taken for lipid and biotin determinations. Liver lipid was assayed as described previously (3) and liver biotin was assayed microbiologically using *Lactobacillus arabinosus* 17/5 ATEC 80/4 (7).

RESULTS AND DISCUSSION

In the first experiment the rats fed the biotin-deficient diet gained 98 g during 10 weeks and weighed 216 g, whereas those fed the control basal diet gained 120 g and weighed 237 g. In the second experiment the rats fed the biotin-deficient diet gained 135 g in 10 weeks and weighed 192 g, and the controls gained 185 g and weighed 241 g. In both experiments the rats fed the biotin-deficient diet began to gain less weight than the controls beginning at the fifth week.

In the first experiment all animals were killed after 3 days of force-feeding. Since the results of this experiment were similar to those of the animals force-fed for 3 days in the second experiment, the results were combined (table 1). Rats pre-fed the biotin-deficient diet ad libitum and then force-fed the biotin-deficient diet with or without orotic acid (groups 3, 4 and 5)

TABLE 1

Liver weight, lipid and biotin of rats fed ad libitum for 10 weeks and then force-fed for 3 or 6 days basal or biotin-deficient diet with orotic acid

No.	Group [1] Diet		No. of rats	Liver		
	Ad libitum	Force-fed		Wt	Lipid	Biotin
				g/100 g body wt	*mg/liver/100 g body wt*	*mμg/liver/100 g body wt*
			3-day experiments			
1	Basal	basal	5	2.73 ± 0.09 [2]	139 ± 5	2566 ± 412
2	Basal	basal + orotic acid	4	3.63 ± 0.08 [3]	220 ± 16 [3]	2315 ± 676
3	Biotin-deficient	biotin-deficient	8	3.42 ± 0.15 [3]	222 ± 28 [5]	989 ± 196 [3]
4	Biotin-deficient	biotin-deficient + orotic acid	11	4.10 ± 0.11 [3]	308 ± 31	1052 ± 102
5	Biotin-deficient	biotin-deficient + orotic acid + biotin [4]	9	3.89 ± 0.12 [5]	251 ± 33	4901 ± 300 [3]
			6-day experiments			
1	Basal	basal	3	3.49 ± 0.14	308 ± 23	3840 ± 403
2	Basal	basal + orotic acid	4	4.31 ± 0.13 [3]	530 ± 67 [5]	2075 ± 94 [3]
3	Biotin-deficient	biotin-deficient	6	4.06 ± 0.10 [5]	388 ± 39	1486 ± 222 [3]
4	Biotin-deficient	biotin-deficient + orotic acid	6	5.29 ± 0.10 [3]	864 ± 180 [5]	1196 ± 129
5	Biotin-deficient	biotin-deficient + orotic acid + biotin [4]	7	4.80 ± 0.15 [3]	627 ± 58 [3]	6026 ± 388 [3]

[1] For statistical analysis, group 2 compared with group 1; group 3, with group 1; group 4, with group 3; group 5, with group 3.
[2] Mean ± SE.
[3] $P < 0.01$.
[4] Biotin, 250 μg subcutaneously × 1.
[5] $0.05 < P > 0.02$.

gained an average of 4.0 g in 3 days and 14.7 g in 6 days, whereas rats pre-fed the basal diet and then force-fed the basal diet with or without orotic acid (groups 1 and 2) gained an average of 4.1 g in 3 days and 6.3 g in 6 days. Rats force-fed diet with or without orotic acid gained the same amounts.

Table 1 summarizes the results for liver weight, lipid and biotin content of the 5 groups of animals in the 3- and 6-day experiments. The livers of rats fed the biotin-deficient diet (group 3) were heavier and contained somewhat more lipid than the livers of rats fed the basal diet (group 1). Rats fed the basal or biotin-deficient diets supplemented with 1% orotic acid (groups 2 and 4) had heavier livers which contained more lipid than comparable control animals (groups 1 and 3). In the 3-day experiments the increase of liver lipid due to orotic acid was 58% with the basal diet and 39% with the biotin-deficient diet, whereas in the 6-day experiment the increases were 72% and 123%, respectively. Rats fed the biotin-deficient diet supplemented with orotic acid that received a subcutaneous injection of biotin (group 5) also developed an enlarged liver with increased lipid, especially in the 6-day experiment. However, this increase was less than that observed in animals not receiving biotin (group 4). This can probably be related to a reversal of the increase noted in the biotin-deficient animals (group 3) over that in control animals (group 1). Rats fed the biotin-deficient diet had a marked depletion (60%) of liver biotin levels. It is of interest that rats pre-fed the basal diet and afterwards force-fed the basal diet supplemented with orotic acid showed a decrease (10–46%) in liver biotin. The latter result is in agreement with the results of Marchetti and Puddu (5). These workers did not report the liver biotin content in their biotin-deficient rats in that study. However, in a more recent paper (8), in which they studied biotin deficiency in relation to ethionine fatty liver, they reported liver biotin levels of 470 mμg/g liver in rats fed a biotin-deficient diet. The liver biotin levels in our biotin-deficient animals were even lower (224–313 mμg/g).

The results of our present study indicate that biotin deficiency per se does not influence the development of fatty liver due to orotic acid. The discrepancy between our results and those of Marchetti and Puddu (5) can probably be explained by differences in consumption of diet and of orotic acid. The quantity as well as quality of diet consumed has been found to be of importance in the induction of fatty liver due to variety of experimental conditions (3, 9–11). Therefore, diet consumption must be considered as an influencing factor in the pathogenesis of nutritionally induced or related fatty liver.

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Effect of Nonspecific Nitrogen Supplementation on Minimum Corn Protein Requirement and First-limiting Amino Acid for Adult Men [1]

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ABSTRACT The minimum amount of corn protein and first-limiting amino acid in corn protein for maintenance of nitrogen equilibrium of human adults were investigated at a high constant level of total nitrogen intake. Nitrogen balances of adult men fed various proportions of corn protein nitrogen to glycine-diammonium citrate nitrogen at a total nitrogen intake of 12.45 g/day indicated a minimum corn protein requirement of between 4.0 and 5.0 g nitrogen/day under these conditions. When a slightly inadequate amount of corn protein as individually determined for each subject was fed under conditions of constant total nitrogen intake (12.45 g/day) only the inclusion of those supplements containing lysine resulted in the re-establishment of nitrogen retention. Thus, lysine is the first-limiting amino acid in corn for nitrogen equilibrium in human adults under the condition of high total nitrogen intake.

Comparison of the corn protein amino acid pattern (1) with the Rose (2) minimal requirement pattern indicates that 6.0 g corn protein nitrogen should meet the requirements of adult men for all the essential amino acids except tryptophan for which only a slight deficit is shown. Nevertheless, as reported by Truswell and Brock (3) and by Kies et al. (4), this level of intake will not support nitrogen equilibrium in adult men. Apparent nitrogen retention can be established by supplementing the 6.0-g corn nitrogen diet with lysine at the same level of total nitrogen intake (3) by increasing the intake of corn protein to provide 8.0 g nitrogen/day (4), or by supplementing the 6.0-g corn protein diets with various purified or intact sources of nonspecific nitrogen so as to increase the total nitrogen content of the diet (4, 5). When the latter method using a mixture of glycine and diammonium citrate as the supplementary source of nitrogen was used, high levels of nitrogen retention could be achieved, the maximum being at a total nitrogen intake of 12 g/day (6.0 from corn and 6.0 from glycine and diammonium citrate). The amount of corn protein needed for nitrogen equilibrium is probably reduced under conditions of high total nitrogen intake; however, the extent of this sparing effect has not been demonstrated. In addition, while lysine rather than tryptophan has been demonstrated probably to be the first limiting amino acid in corn protein for nitrogen retention of children (6–8) and of adults (3), these evaluations were not carried out under conditions of high total nitrogen intake.

The objectives of the present project were to determine the minimal amount of corn protein necessary for maintenance of nitrogen equilibrium of adult men under conditions of a high, constant total nitrogen intake and to determine the first-limiting amino acid in corn protein for maintenance of nitrogen equilibrium of human adults under the same conditions of high total nitrogen intake.

EXPERIMENTAL

The 80-day study consisted of 2 parts: part A composed of a 5-day preliminary period and 5 experimental periods of 5 days each, and part B composed of 10 experimental periods of 5 days each. Part B was conducted immediately following part A.

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Part A. During the initial 2 days of the part A preliminary period, nitrogen intake per subject per day was 1.45 g of which 1.00 g was from white, degerminated corn meal [3] and the remainder from the basal diet (table 1). This preliminary use of a very low protein diet has been found in previous experiments to speed the adjustment of subjects to experimental diets. Total nitrogen intake during the last 3 days of the preliminary period was 12.45 g per subject per day; 6.00 g from white, degerminated corn meal, 6.00 g from an isonitrogenous mixture of glycine and diammonium citrate, and 0.45 g from the basal diet. Objectives of this period included adjustment of the subjects to the level of total nitrogen intake to be used during the experimental periods of both parts A and B, determination of individual caloric requirements for weight maintenance, and introduction of subjects to their duties and responsibilities.

Total nitrogen intake during the 5 experimental periods of part A was maintained constant at 12.45 g nitrogen per subject per day from corn meal, glycine and diammonium citrate, and the basal diet (table 1). During the first 4 experimental periods which were arranged at random for each subject, the corn meal provided 6.00, 5.00, 4.00, and 3.00 g nitrogen/day, respectively, and the glycine-diammonium citrate mixture correspondingly provided 6.00, 7.00, 8.00, and 9.00 g nitrogen/day. Thus, total nitrogen intake was maintained constant. During the final

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

TABLE 1

Diet plan

Diet	No. days	Corn [1]	DAC + glycine [2]	Amino acid supplement [3]	Total N intake [4]
		g N/day	*g N/day*		*g/day*
Part A:					
Adjustment 1	2	1.0	0	none	1.45
Adjustment 2	3	6.0	6.0	none	12.45
1	5	6.0	6.0	none	12.45
2	5	5.0	7.0	none	12.45
3	5	4.0	8.0	none	12.45
4	5	3.0	9.0	none	12.45
5	5	4.5	7.5	none	12.45
Part B:					
6	5	varied [5]	varied [5]	tryptophan	12.45
7	5	varied [5]	varied [5]	threonine + isoleucine	12.45
8	5	varied [5]	varied [5]	isoleucine	12.45
9	5	varied [5]	varied [5]	sulfur-containing	12.45
10	5	varied [5]	varied [5]	lysine	12.45
11	5	varied [5]	varied [5]	lysine + tryptophan	12.45
12	5	varied [5]	varied [5]	sulfur-containing + tryptophan	12.45
13	5	varied [5]	varied [5]	negative control	12.45
14	5	varied [5]	varied [5]	positive control 1	12.45
15	5	varied [5]	varied [5]	positive control 2	12.45

[1] White degerminated unenriched corn meal having a nitrogen content of 0.0127 g N/g meal was used. Corn meal to cornstarch proportions were adjusted as necessitated by the experimental plan but always totaled 472.5 g so as to provide for a constant caloric intake.

[2] An isonitrogenous mixture of glycine and diammonium citrate (DAC) was used in amounts to maintain total nitrogen content of the diets constant.

[3] Essential amino acid supplements were added in the amounts and proportions present in 0.50 g corn protein nitrogen. Positive control 1 consisted of all the essential and nonessential amino acids present in corn protein; positive control 2 consisted of only the essential amino acids present in corn protein. The negative control consisted of only glycine and diammonium citrate.

[4] Total nitrogen intake figures include the 0.45 g N/day provided by the basal diet. The basal diet consisted of 150 g applesauce, 3.5 g dry boullion powder, 50 g cabbage, 100 g stewed tomatoes, 60 g jelly, 20 g hard candy, 100 g pears, 100 g peaches, 10 g instant decaffeinated coffee (dry). A vitamin supplement provided 5000 IU vitamin A, 400 IU vitamin D, 2 mg thiamine, 2.5 mg riboflavin, 50 mg ascorbic acid, 1 mg pyridoxine, 1 μg vitamin B_{12}, 20 mg niacin, and 1 mg Ca pantothenate. A mineral supplement provided the following: (g/subject/day) Ca, 1.00; P, 1.001; Mg, 0.199; Fe, 0.015; Cu, 0.002; I, 0.00015; Mn, 0.002; and Zn, 0.0009.

[5] Daily nitrogen intake from corn was maintained at a level 0.50 g less than that necessary for nitrogen equilibrium as determined for each subject during part A. Corn meal provided 4.0 g N/day for subjects 70, 71, 74, 75, 76, 79 and 4.5 g N/day for subjects 73, 78. DAC-glycine nitrogen was given in amounts necessary to maintain total nitrogen intake constant at 12.45 g/day.

experimental period, corn meal provided 4.5 g nitrogen and the diammonium citrate mixture, 7.5 g daily since on the basis of performance in previous periods, it appeared that individual subjects required between 4.0 and 5.0 g corn meal nitrogen for maintenance of nitrogen balance. This procedure was included to allow for the establishment of the minimum corn protein requirement for each individual to within 0.50 g corn nitrogen.

Part B. During the 10 experimental periods composing part B, daily nitrogen intake from corn was maintained at a level 0.50 g less than that necessary for definite nitrogen equilibrium as individually determined for each subject during part A (fig. 1 and table 2). As in part A, the basal diet provided 0.45 g nitrogen/day (table 1), and an isonitrogenous mixture of glycine and diammonium citrate was used to maintain the nitrogen content of all diets constant at 12.45 g/day. Various individual amino acids in purified form suspected to be the first-limiting amino acid were added in the amounts found in 0.50 g corn protein nitrogen during the 10 experimental periods. These were selected on the basis of results of several other investigations (3, 6–8) and by comparison of the essential amino acid pattern of corn with the FAO pattern (1, 9), and with the essential amino acid pattern of egg protein (1). That amino acid which when added to the inadequate corn diet, resulted in the re-establishment of nitrogen equilibrium was to be designated as the first-limiting amino acid in corn protein. A modified Latin square design was used in arrangement of experimental periods.

Diets. Caloric intake for each individual subject was kept relatively constant at the amount required for weight maintenance (table 3) by adjusting the intake of cornstarch [4] and fat; however, fat always provided 20% of the calories. Butterfat was the primary dietary fat source for subjects 70, 71, 73, and 74 while corn oil [5]

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

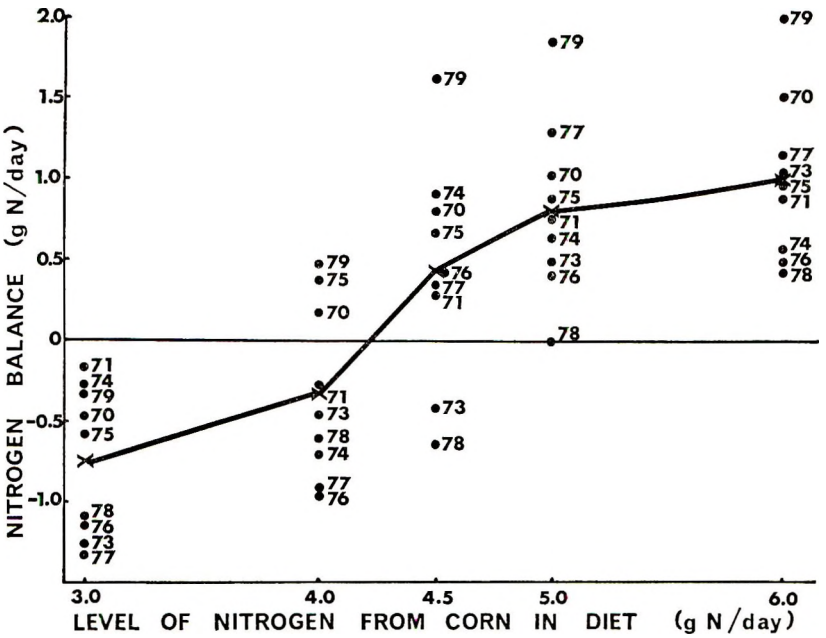

Fig. 1 Effect of several levels of dietary corn protein on nitrogen balances of adult men when total nitrogen intake was maintained constant at a high level (12.45 g N/day) by additions of glycine and diammonium citrate. Dots represent average nitrogen balances of each individual for the 5 days composing each experimental period. Crosses represent mean balances of all subjects at each intake level.

TABLE 2

Effect on nitrogen balances of purified amino acid additions to suboptimal corn protein diets [1] of human adults

Subject no.	Mean N balances [2] of subjects while receiving purified N supplement [3]									
	Positive control 1	Positive control 2	Lysine	Lysine + tryptophan	Negative control	Tryptophan	Isoleucine	Sulfur-containing	Isoleucine + threonine	Tryptophan + sulfur-containing
	g/day		*g/day*		*g/day*		*g/day*		*g/day*	
70	0.13	0.07	0.35	0.36	−0.37	−0.08	−0.17	−0.45	−0.17	−0.91
71	0.33	0.36	0.92	0.63	0.01	−0.34	−0.04	−0.14	−0.04	−0.31
73	0.20	−0.36	0.79	0.66	−0.21	−0.31	−0.36	−0.36	−0.36	−0.35
74	0.89	0.48	0.70	0.48	−0.14	0.23	0.32	0.05	0.32	−0.05
75	−0.10	−0.23	0.01	−0.12	−0.52	−0.36	−0.59	−0.21	−0.59	−0.59
76	0.96	0.19	0.21	0.35	−0.56	−0.19	−0.83	−0.12	−0.83	−0.55
77	0.78	0.23	0.51	0.44	−0.52	−1.66	−0.13	−0.03	−0.13	−0.17
78	0.87	1.64	0.99	1.13	−0.56	−0.31	0.20	0.05	−0.20	0.67
79	0.57	1.69	0.60	0.88	0.27	0.12	0.04	−0.19	0.04	−0.14
Mean	0.51	0.45	0.56	0.54	−0.29	−0.32	−0.22	−0.15	−0.22	−0.27

[1] Cornmeal nitrogen provided 4.0 g N/day for subjects 70, 71, 74, 75, 76, 79, and 4.5 g N for subjects 73 and 78. An isonitrogenous mixture of glycine and diammonium citrate maintained total nitrogen content of diets constant at 12.45 g N/day.

[2] Mean of the nitrogen balances for the 5 days composing each experimental period.

[3] Essential amino acids were added in amounts and proportions as found in 0.50 g corn protein nitrogen. Positive control 1 consisted of all of the essential and nonessential amino acids in the proportions present in corn protein; positive control 2 consisted of just the essential amino acids present in corn protein; and the negative control consisted of only glycine and diammonium citrate. In each case, a mixture of glycine and diammonium citrate was added to make all supplements isonitrogenous.

TABLE 3

Age, height, weight and caloric intakes of subjects

Subject no.	Age	Height	Weight		Calories
			Initial	Final	
	years	*cm*	*kg*	*kg*	*kcal/kg body wt*
70	40	180	82.3	83.2	40.9
71	30	188	85.4	85.5	50.0
73	30	185	85.9	85.5	50.0
74	27	178	75.0	76.2	45.0
75	36	173	86.8	87.6	38.0
76	26	165	74.5	75.0	45.2
77	24	175	73.2	73.0	50.0
78	44	170	72.3	72.2	50.0
79	24	176	80.4	79.0	50.0

was given to subjects 75, 76, 77, 78, and 79. Vitamin and mineral supplements were also given (table 1).

The principal dietary item was a corn meal biscuit composed of 473 g of corn meal or corn meal plus cornstarch (relative proportions were determined by the nitrogen intake from corn dictated by the experimental plan), 3.6 g mineral mix (table 1), 10 g calcium phosphate baking powder, 5.0 g cellulose flakes, and 60 g fat. These items were mixed together with a minimal amount of water, divided into 3 equal portions, and baked for consumption at the 3 daily meals. A cornstarch-fat wafer was used to provide extra calories for those individuals requiring a higher caloric intake. Nonprotein, low calorie soft drinks [6] were allowed ad libitum. Other items making up the diet and listed in table 1.

Daily allotments of diammonium citrate, glycine, and other purified amino acids were given to the subjects in water suspensions or solutions. These were equally divided among the three daily meals.

Subjects. The same 9 men (table 3) were subjects for both parts A and B of the study. All were inmates of the Nebraska Penal and Correctional Complex for Men. During the study, they were housed together in a separate part of the institution where they ate all meals, slept, and spent free time. Physical examinations conducted by the institution's physician at the beginning and end of the study, as well as his observations throughout the study, attested that all were in good health.

Analyses. The nitrogen balance technique was used as the criterion of evaluation of the experimental diets. Nitrogen determinations by the boric acid modification of the Kjeldahl method (10) were made on the samples of food, amino acids, diammonium citrate and glycine solutions, urine and feces. These materials were collected for analysis according to methods described previously (11). Daily urinary creatinine excretions were determined using the procedure described by Folin (12).

RESULTS

Part A. The average nitrogen balances of the 9 subjects while receiving the various experimental diets composing part A of the study are shown in figure 1. During the first 4 experimental periods when corn protein provided 3.0, 4.0, 5.0, or 6.0 g nitrogen, all subjects were in positive balance while receiving 5.0 or 6.0 g corn nitrogen but were in negative balance when receiving the 3.0 g corn nitrogen amount. Of the 9 subjects, 6 were in negative balance and 3 were in positive balance while receiving the 4.0-g corn nitrogen diet. However, the 3 subjects who were in positive balance on this level of intake showed substantial decrease in nitrogen retention between the 5.0- and 4.0-g corn nitrogen intake levels and also were in negative balance during some periods of part B when 4.0 g nitrogen from corn was fed. This indicates that the observed nitrogen retentions on the 4.0-g level for these subjects were probably apparent rather than real. Thus, the minimum corn protein requirement is probably between 4.0 and 5.0 g corn protein nitrogen

[6] Non-protein, low calorie soft drinks used in this study were cooperatively supplied gratis by the Pepsi Cola Company, New York, and by the Pepsi Cola Bottling Company, Lincoln, Nebraska.

under conditions of high total nitrogen intake. When an intermediate level of corn protein nitrogen (4.5 g nitrogen per day) was fed in order to more closely define this requirement, results as shown in figure 1 indicated a minimum corn protein requirement under conditions of high total nitrogen intake of between 4.0 and 4.5 for subjects 70, 71, 74, 75, 76, and 79 and between 4.5 and 5.0 for subjects 73 and 78.

Part B. Average nitrogen balances of the 9 subjects during part B of the study are shown in table 2. Results indicated that positive nitrogen balances of subjects fed a slightly inadequate level of corn protein nitrogen could be re-established with lysine-containing additions to the diet but not with non-lysine-containing supplements. Analysis of variance of these differences gave *F* values which were significant at greater than the 0.05% level of probability.

DISCUSSION

Nitrogen equilibrium of subjects receiving inadequate amounts of corn protein could be re-established in the present study only by inclusion of lysine-containing supplements. Thus, lysine is indicated to be the first-limiting amino acid in corn protein for maintenance of nitrogen equilibrium in human adults when total nitrogen intake is high. Lysine has also been found to be the first-limiting amino acid in corn for adults (3) and children (6–8) when high total nitrogen intake levels were not maintained.

However, comparison of results of the present study with earlier research (4) indicates that minimum requirements of human adults for protein as provided by corn can be substantially reduced by additions of "nonspecific nitrogen," apparently, to provide an optimal intake of total dietary nitrogen. ("Nonspecific nitrogen" has previously been defined as nitrogen from any metabolically usable, nontoxic source.) In the present study, subjects maintained on high levels of total nitrogen intake had minimum requirements between 4.0 and 5.0 g corn protein nitrogen. However, when total nitrogen intake was not maintained at the high level with "nonspecific nitrogen" supplements, other subjects demonstrated minimum corn protein nitrogen requirements of between 6.0 and 8.0 g nitrogen/day for maintenance of nitrogen equilibrium (4).

Since increased nitrogen retention resulted from additions of lysine supplements to corn meal diets of men maintained at the 6.0-g nitrogen intake level in studies by Truswell and Brock (3), the lowering of the minimum corn protein requirement observed in the present study was probably at least in part due to a lowering of the lysine requirement under the condition of high total nitrogen intake. In an earlier study from this laboratory (4), supplements not containing lysine were as effective as those containing lysine in promoting nitrogen retention when added in amounts to increase the total nitrogen intake of men from 6.0 g/day (as provided by corn) to 8.0 g. Since the lysine content of these supplements was low (lysine as contained in 2.0 g corn protein nitrogen), it is possible that the influence of increase in the total nitrogen intake was greater and overshadowed that resultant from the low-level lysine supplementation. However, it is difficult to understand why a cumulative effect was not obtained since, theoretically, both effects were in the same direction. In addition, it has been shown in a later study (5) that greater nitrogen retention can be obtained by men when the same corn diet is supplemented with higher levels of a non-lysine-containing nitrogen source (glycine and diammonium citrate).

Neither site nor mode of the apparent sparing effect caused by the inclusion of high level nonspecific nitrogen supplementation on the human requirement for corn protein has been defined or is easily explainable. Results of several other studies suggest that a similar sparing effect on the adult and infant requirements for milk (13) and egg (14) protein may result with nonspecific nitrogen supplementation. A possible effect of level of total nitrogen intake on intestinal absorption of the amino acids is an explanation easier to rationalize than explanations involving possible influence on cellular protein metabolism or protein synthesis by intestinal microorganisms (as is true in ruminant animals).

While recent results of Fisher et al. (15) indicate a lowering of the tryptophan re-

quirements of humans with increased levels of total nitrogen intake, it is unknown whether all essential amino acid requirements are affected in the same direction or to the same degree. It is possible that level of total nitrogen intake could affect not only the minimum requirement of a protein but also the order in which the component amino acids become limiting for nitrogen retention. Thus, while lysine has been demonstrated to be the first-limiting amino acid in corn protein under conditions of optimal or limited total nitrogen intake, it does not necessarily follow that the order in which the other amino acids become limiting is in the same pattern.

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Influence of Vitamin A Deficiency on Testes, Bursa Fabricius, Adrenal and Hematocrit in Cockerels [1]

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ABSTRACT Additional physiological measurements were studied to aid in understanding the unique observation that marginally vitamin A-deficient cockerels had increased testes size. One-day-old Single Comb White Leghorn cockerels were fed either a vitamin A-deficient or -adequate (6600 USP units/kg) ration. Hematocrits were greater from vitamin A-deficient birds at 6, 9, 10 and 11 weeks of age. Combs, testes, bursae and adrenals were removed and weighed, and the latter 3 organs corrected for body weight. Bursae weights from vitamin A-supplemented birds were larger from 6 through 11 weeks of age. Vitamin A-deficient birds had heavier testes and heavier combs at 8 and 10 weeks of age. Histological examination of the testes from 11-week-old vitamin A-deficient birds showed mature sperm. No sperm were observed in the testes of control birds at this age. Corrected adrenal weights were heavier in the deficient birds at 9, 10 and 11 weeks of age. No differences in oxygen consumption were found between treatments measured at 3, 5 and 11 weeks of age. Increased testis weight, adrenal weight, comb size, red blood cell volume and decreased bursa weight suggested higher androgen secretion by vitamin A-deficient birds.

During an investigation of vitamin A deficiency in Single Comb White Leghorn cockerels, it was observed unexpectedly that marginally deficient birds had much larger testes than those that had received adequate vitamin A. This result was in conflict with the work of Lowe (1), who reported a marked retardation of sexual development in vitamin A-deficient male fowl. However, in 1938 Burrows and Titus (2) reported that cockerels fed a vitamin A-deficient diet for 4.5 months maintained a higher level of semen production than that maintained by controls.

In a review, Johnson and Wolf (3) stated that glucocorticoid production was inhibited in vitamin A-deficient rats. The severity of the deficiency determined which steps were blocked in adrenal glucocorticoid production. They noted that 17-hydroxydeoxycorticosterone accumulated in the adrenals of vitamin A-deficient rats. No suggestions were made as to alternate pathways in steroid production resulting from the metabolic block. Reduced adrenal glucocorticoid production was also indicated by Glick (4) who reported that vitamin A-deficient chicks were unable to increase blood heterophil counts in response to adrenocorticotrophic hormone (ACTH) injection.

Mayer and Truant (5) reported that vitamin A deficiency in rats produced a castration-like effect which was due to lack of circulating androgens, not due to lack of target organ response to androgens. Therefore, it seemed important in our study to measure androgen production even if indirectly.

Glick (6) reported that the bursa of Fabricius in fowl was lymphoid tissue; its size was partly dependent on body weight increases, and its involution was accompanied by an increase in rate of testis growth. Therefore, the bursa, like the thymus, should provide a measure of androgen production.

Vitamin A deficiency in both the pig (7) and rat (8) has been shown to influence the thyroxin secretion rate and metabolic rate.

The present research was designed to investigate the effect of vitamin A deficiency on the size of the bursa, comb, adrenal, testes, packed red blood cell volume and oxygen consumption in growing cockerels. The objective was to determine why marginal vitamin A deficiency increased testes size.

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TABLE 1

Vitamin A-supplemented diet

	%
Ground milo	65.80
Soybean oil meal, solvent process (50%)	29.20
Dicalcium phosphate	2.70
Limestone	0.80
Salt, iodized	0.50
DL-Methionine	0.22
Vitamins added/kg ration	
	IU
Vitamin A palmitate	6600
Vitamin D_3	990
α-Tocopheryl acetate	12
	mg
Choline	1980
Riboflavin	2.54
Vitamin K	1.16
	μg
Vitamin B_{12}	11.10
Minerals added/kg ration	
	mg
Manganese, as managanese sulfate	80
Zinc, as zinc carbonate	31

METHODS

Two experiments were conducted in which one-day-old Single Comb Leghorn cockerels, obtained from a commercial source, received either a vitamin A-deficient ration or the same ration[2] supplemented with 6600 USP units vitamin A palmitate/kg of diet (table 1).

Chicks were reared in electrically heated batteries with raised wire floors until 5 weeks of age, when they were transferred to growing batteries. They received continuous fluorescent lighting with feed and water ad libitum. Chicks were weighed individually each week. The vitamin A-deficient cockerels used in these experiments were considered to be marginally vitamin A-deficient as they continued to gain in weight. In the first and second experiments, 97.5 and 93%, respectively, of the total number of birds fed the vitamin-deficient ration were acceptable for physiological measurements, whereas 100% of the controls were acceptable. The remainder of the vitamin A-deficient birds either died before termination of the experiment or appeared morbid.

Differences between treatment means were analyzed for statistical significance by the *t* test (9).

Experiment 1. Oxygen consumption was measured to determine whether vitamin A deficiency was altering metabolic rate. Oxygen consumptions were determined on control and deficient cockerels using a minute oxygen uptake spirometer. Six birds were selected at random from each treatment when they were 3, 5 and 11 weeks of age. Oxygen consumption was determined for at least 8 minutes/bird/age.

Seven cockerels selected at random at 7, 8, 10 and 11 weeks of age from each treatment were weighed and killed with chloroform. Testes, combs, and bursae of Fabricius were removed and weighed. Testes from 11-week-old cockerels were fixed in Bouin's solution, sectioned at 7 μ, and stained with hematoxylin and eosin. The testes were examined histologically and scored upon their degree of spermatogenesis. Ten tubules of each testis were individually rated as follows: zero, only spermatogonia present; 1, primary spermatocytes present; 2, secondary spermatocytes present; 3, spermatids present; and 4, mature spermatozoa present. The degree of maturation of 10 tubules from each testis was converted to a percentage for each score, and the average score of the 7 testes per treatment was determined.

Experiment 2. Before slaughter, blood was taken from the wing vein for determination of red blood cell volume. Hematocrits were obtained by a microcapillary method and centrifuged for 10 minutes. Six cockerels/treatment/week were killed by exsanguination at 6 through 11 weeks of age. The testes, bursae of Fabricius, and adrenals were weighed immediately upon removal.

RESULTS

The general signs of vitamin A deficiency were red-blue combs at 5 weeks of age, and some ataxia. The majority of deficient birds at this time also displayed drying of the mucosal surfaces of the mouth to which feed adhered. The ureters contained excessive quantities of urates.

Experiment 1. Table 2 shows mean body, testes, comb and bursa weights, and

[2] Appreciation is expressed to The Ray Ewing Company, Division of Hoffmann-LaRoche; Pfizer, Inc.; Commercial Solvents Corporation; Dawes Laboratories, Inc.; and Merck and Company for vitamins used in these experiments.

TABLE 2

Effects of vitamin A deficiency on body size, testis and bursa size in growing cockerels [1]

	Age in weeks			
	7	8	10	11
Vitamin A-supplemented cockerels				
Body wt, g	708 **	760 **	1021 **	1219 **
Testes, g	0.48	0.47	0.35	1.00
Testes, % body wt	0.06	0.06	0.03	0.04
Bursa, g	3.04**	3.23**	3.10**	3.54**
Bursa, % body wt	0.41**	0.43**	0.30**	0.30**
Comb wt, g	— [2]	8.3	6.8	14.3
Vitamin A-deficient cockerels				
Body wt, g	490	628	832	1015
Testes, g	0.28	1.55**	2.28**	2.87**
Testes, % body wt	0.05	0.24**	0.27**	0.12**
Bursa, g	0.89	1.00	1.15	1.59
Bursa, % body wt	0.17	0.16	0.14	0.16
Comb wt, g	— [2]	9.7	9.0	14.0

[1] Average values from 7 cockerels/treatment.
[2] Comb weights not determined.
* $P < 0.05$, significantly heavier than other treatment.
** $P < 0.01$, significantly heavier than other treatment.

bursa and testes weights as a percentage of body weights. The testes of vitamin A-deficient cockerels were heavier at 8, 10 ($P < 0.01$) and 11 ($P < 0.05$) weeks of age. Testes weights corrected for body size were significantly heavier ($P < 0.01$) in the deficient birds at 8, 10 and 11 weeks of age. Histological examination of testes from deficient birds showed either mature tubules or more mature tubules than control birds (table 3).

Both bursa weight and bursa weight as a percentage of body weight were smaller ($P < 0.01$) in vitamin A-deficient birds at all ages studied (table 2). Although deficient birds tended to have larger combs at 8 and 10 weeks of age than controls, individual variation negated statistical differences.

There were no significant differences in oxygen consumption between deficient and vitamin-supplemented birds. The mean oxygen uptake in cm^3/g body weight/minute at 3, 5 and 11 weeks of age was: 0.034, 0.029, 0.021 for deficient birds, and 0.038, 0.033 and 0.020 for control birds.

Experiment 2. The mean body weights, packed erythrocyte volume, testes weights, bursa weights, adrenal weights, and the adrenal, testes and bursa weights as percentage of body weight are shown in table 4.

Vitamin A-deficient birds had heavier testes weights ($P < 0.05$) at 9 weeks of age than the controls. Testes weights as a percentage of body weight were larger ($P < 0.05$) at 6 and 9 weeks of age in the deficient cockerels. In addition, their mean testes weights tended to be greater at 10 and 11 weeks than the controls. At each age studied, bursa weights and adjusted bursa weights of control cockerels were greater ($P < 0.01$). Deficient cockerels had greater mean red blood cell volume at 6, 9, 10 and 11 weeks of age ($P < 0.01$) than the controls. Although the adrenals were heavier ($P < 0.01$) in the vitamin A-supplemented birds at all ages, weights corrected for body size in the deficient birds at 9, 10 and 11 weeks of age were greater ($P < 0.05$).

TABLE 3

Effect of vitamin A deficiency upon maturation score [1] *of testes development at 11 weeks of age*

With vitamin A		Without vitamin A	
Score		Score	
	% of tubules		*% of tubules*
0	56.4	0	0.0
1	22.2	1	1.5
2	11.4	2	10.0
3	10.0	3	21.5
4	0.0	4	67.0
Avg 0.75		3.5	

[1] Score 0, only spermatogonia present; 1, primary spermatocytes present; 2, secondary spermatocytes present; 3, spermatids present; and 4, mature spermatozoa present.

TABLE 4

Effect of vitamin A deficiency upon sequential body weight, hematocrit, testis, bursa, and adrenal[1]

	Age in weeks					
	6	7	8	9	10	11
Vitamin A-supplemented cockerels						
Body wt, g	536 **	718 **	903 **	962 **	1141 **	1251 **
Hematocrit, %	33.0	35.9	38.2	34.7	35.3	32.9
Testes, g	0.14	0.23	0.33	0.34	0.61	0.90
Testes, % body wt	0.03	0.07	0.04	0.03	0.05	0.07
Bursa, g	2.36 **	3.52 **	4.23 **	2.69 **	4.29 **	4.56 **
Bursa, % body wt	0.43 **	0.49 **	0.47 **	0.28 **	0.37 **	0.36 **
Adrenal, g	0.11 **	0.11 **	0.09 *	0.12 *	0.15 **	0.12 *
Adrenal, % body wt	0.021	0.015	0.010	0.013	0.013	0.009
Vitamin A-deficient cockerels						
Body wt, g	420	462	580	628	677	810
Hematocrit, %	37.9 **	38.0	42.1	41.4 **	40.9 **	38.3 **
Testes, g	0.17	0.21	0.24	0.61 *	0.81	1.29
Testes, % body wt	0.04 *	0.04	0.04	0.09 *	0.12	0.16
Bursa, g	0.92	0.78	0.98	1.18	1.41	0.91
Bursa, % body wt	0.22	0.17	0.17	0.18	0.21	0.11
Adrenal, g	0.08	0.07	0.07	0.10	0.10	0.10
Adrenal, % body wt	0.018	0.016	0.012	0.019 *	0.016 *	0.012 *

[1] Average values from 6 cockerels/treatment.
* $P < 0.05$, significantly greater than other treatment.
** $P < 0.01$, significantly greater than other treatment.

DISCUSSION

No differences in oxygen consumption between the control and vitamin-deficient cockerels indicated similar metabolic rates. Since the deficient birds were only marginally vitamin A-deficient, their thyroid secretion rate may not have been influenced by the deficiency. These results are not in accord with those of other species (7, 8).

Glick has shown that bursa size decreases concomitant with testes growth (6) or in response to injections of testosterone or cortisone (10). Involution of the bursa could be induced by injecting cortisone, hydrocortisone, 11-deoxycorticosterone, corticosterone, 11-deoxy-17-hydroxycorticosterone, α-methyl-17-α-hydroxyprogesterone acetate, 17-α-ethyl-19-nortestosterone, testosterone, and estradiol. Injections of progesterone, 17-α-hydroxyprogesterone, 17-α-hydroxyprogesterone caproate, and 17-α-hydroxyprogesterone acetate were ineffective (11).

Adrenal glucocorticoid production in the vitamin A-deficient rat was inhibited (3, 4) with the adrenal accumulating 17-hydroxy-11-deoxycorticosterone (3). Also, Turner (12) described the human adrenogenital syndrome in which there was adrenal accumulation of 17-hydroxyprogesterone and 17-hydroxy-11-deoxycorticosterone. He stated that since the adrenal did not add enough cortisol to the circulation to inhibit pituitary release of ACTH, the adrenals responded to continuing high titers of ACTH by producing androgens. This may be the case in the vitamin A-deficient cockerel, where the main glucocorticoid, corticosterone (13, 14), was not produced in quantities sufficient to suppress pituitary release of ACTH. The increased adrenal weight, as a percentage of body weight, in the vitamin A-deficient birds may be due to hypertrophy of the gland from increased ACTH levels.

ACTH injections into young male chickens produced a regression in spleen and bursa weights (15). When Dulin (16) injected ACTH into either capons or cockerels, body weight and adrenal weight were depressed. However, comb and testes weights were increased in cockerels, but comb weight of capons was decreased. He suggested that ACTH stimulated gonadotrophin production.

Although the vitamin A-deficient cockerels showed a trend toward larger comb size, individual variation and small group numbers precluded significance. The poorer condition of these birds may have affected their comb development.

Further indirect evidence of possible increased androgen levels in the vitamin A-deficient cockerels was indicated by their higher red blood cell volume. Chicken red blood cell numbers and volume were increased by circulating androgens (17, 18).

Vitamin A-deficient birds were characterized by significantly smaller bursae of Fabricius and heavier testes showing a greater degree of spermatogenesis. Although the vitamin A-deficient cockerels in the second experiment did not show as great testes development as in experiment 1, a similar trend was observed. This difference may have been due in part to greater debility of the deficient birds in experiment 2, which may have had smaller liver vitamin A reserves at the onset.

The results of these experiments suggest the possibilty of increased androgen production in vitamin A-deficient cockerels as evidenced by increased comb size, testes size, smaller bursa of Fabricius, and greater packed red blood cell volume. It is hypothesized that marginal vitamin A deficiency blocks the normal production of glucocorticoids, with the precursors being shunted into androgen-producing pathways. Higher blood titers of ACTH result due to lack of glucocorticoids. Increased ACTH stimulus to the adrenals could result in higher androgen synthesis. Continued experimentation is essential to test this hypothesis.

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Effects of Dietary Calcium upon Lipid Metabolism in Rats Fed Saturated or Unsaturated Fat [1]

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ABSTRACT To study the effects of varying the degree of saturation of dietary fat upon the hypolipemic action of elevated dietary calcium, mature male rats were fed cocoa butter or corn oil with 0.08, 0.2 and 1.2% dietary calcium for 3 weeks. The previously reported hypocholesterolemic and hypotriglyceridemic effects of calcium were observed with both fats. Corn oil significantly elevated serum bile acids. At the 0.08% calcium level, corn oil caused a significantly increased deposition of lipid in the liver and triglycerides in the lung as compared with cocoa butter. Increased dietary calcium tended to reverse this deposition. Although fecal total lipids and bile acids increased significantly with both fats at the 1.2% calcium level, fecal lipid phosphorus and 3-β-hydroxysterol excretion increased only in the presence of cocoa butter. Corn oil caused the excretion of significantly less fecal total lipids than cocoa butter. Calcium was hypolipemic in rats fed either fat; however, the effects of calcium were more pronounced in the presence of saturated fat.

Studies reported from our laboratory have shown a decrease in serum lipids and increase in fecal lipids after increase in dietary calcium in man and rats. The literature on these observations has been reviewed previously (1). In the rat study reported (2), the source of fat used was edible-grade beef tallow. The work reported here was conducted to determine whether there was any difference in the hypolipemic and steatorrheic effects of calcium in the presence of a highly saturated fat (cocoa butter) as compared with a highly unsaturated fat (corn oil). An abstract of this work has been published elsewhere.[3]

EXPERIMENTAL

Forty-eight male 400-day-old albino rats of the Holtzman strain, mean weight 515 g, were distributed at random into 6 groups and fed a corn-soya basal diet containing 18% added fat as USP cocoa butter or table-grade refined corn oil, and 2% added cholesterol. The composition of this diet has been reported previously (2).

The basal diet assayed 0.08% calcium and 0.35 phosphorus. Calcium, in the form of USP calcium carbonate, was added to the basal diet in place of an equal weight of washed sand to obtain two additional calcium levels of 0.2 and 1.2%, which provided approximately 2.5 and 15 times the reported requirement of the adult rat (3). Replacement of sand in the diet with calcium carbonate made it possible to maintain diets isocaloric, isonitrogenous and equal in density. In addition, the sand was used as an indigestible fecal marker for calculation of feed consumption. All diets were calculated to contain 15.6% protein, 20.3% fat, and 1.18% fiber.

The rats were housed two in a cage and 4 cages were used per diet. During the second week of the experiment, feces were collected daily for 3 days and stored in a freezer until they were assayed. The pooled feces from each cage were assayed for lipids. At 21 days the rats were weighed, anesthetized with sodium pentobarbital and exsanguinated by heart puncture. Food was removed 18 hours before

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[3] Fleischman, A. I., H. Yacowitz and M. L. Bierenbaum 1966 Effects of dietary calcium on lipid metabolism in rats fed saturated and unsaturated fat. Federation Proc., 25: 367 (abstract).

TABLE 1

Effects of dietary calcium on body weight, serum, fecal and tissue lipids after 3 weeks [1]

Dietary fat	Cocoa butter			Corn oil		
Calcium level in diet, %	0.08 (1)	0.2 (2)	1.2 (3)	0.08 (4)	0.2 (5)	1.2 (6)
Mean wt, initial, g	502 ± 8 [2]	521 ± 9	521 ± 11	541 ± 16	509 ± 17	514 ± 14
Mean wt, final, g	527 ± 10	542 ± 9	528 ± 10	570 ± 19	554 ± 15	534 ± 25
Mean wt gain, g	25	21	7	29	45	20
Estimated mean feed consumption,[3] g/rat/day	18.6	22.9	—	20.8	21.3	—
Serum:						
Total lipids, mg/100 ml	600 ± 89	567 ± 17	300 ± 40 ** [4]	438 ± 44	444 ± 28	400 ± 27
Phospholipids, mg/100 ml	172 ± 4	140 ± 1 **	119 ± 3 **	113 ± 5	104 ± 6	92 ± 9
Total cholesterol, mg/100 ml	168 ± 7	135 ± 2 *	93 ± 2 **	126 ± 1	93 ± 6 *	73 ± 2 **
Triglycerides, mg/100 ml	126 ± 16	106 ± 8	66 ± 3 *	126 ± 5	94 ± 8	70 ± 5 *
Calcium, mEq/1000 ml	5.75 ± 0.05	5.63 ± 0.03	5.60 ± 0.10	5.43 ± 0.13	5.63 ± 0.08	5.55 ± 0
Total bile acids, mg/100 ml	1.41 ± 0	1.62 ± 0.19	1.71 ± 0.09	3.37 ± 0.19	3.37 ± 0.18	3.69 ± 0.95
Feces:						
Wet feces wt, g/rat/day	7.88 ± 0.43	7.54 ± 0.75	9.21 ± 0.10	8.13 ± 0.24	6.79 ± 0.56	8.29 ± 1.12
Dry feces, g/rat/day	3.83	4.49	6.56	3.46	3.78	4.69
Total lipid, mg/rat/day	962 ± 68	1189 ± 146	1986 ± 78 **	670 ± 33	781 ± 66	978 ± 44 **
3-β-Hydroxysterol, mg/rat/day	355 ± 15	440 ± 34	506 ± 13 **	408 ± 26	407 ± 17	418 ± 26
Phospholipid, mg/rat/day	11.3 ± 0.2	12.1 ± 1.0	15.9 ± 0.5 **	9.9 ± 0.4	7.3 ± 1.2	8.0 ± 1.9
Fecal bile acids, mg/rat/day	10.2 ± 1.1	17.1 ± 0.1 **	21.7 ± 0.5 **	6.26 ± 1.2	25.0 ± 1.4 **	23.2 ± 0.6 **
Calcium, mg/rat/day	33 ± 4	53 ± 6 *	296 ± 25 **	21 ± 2	62 ± 7 *	357 ± 67 **
Tissues:						
Liver						
Mean liver wt, g	17.4 ± 0.77	16.4 ± 0.35	15.9 ± 1.38	19.3 ± 0.71	19.2 ± 0.65	16.6 ± 1.04
Total lipid, g/liver	1.83 ± 0.07	2.06 ± 0.12	1.65 ± 0.16	3.05 ± 0.17	2.87 ± 0.26	2.48 ± 0.32
Total cholesterol, mg/liver	511 ± 36	639 ± 26	540 ± 47	805 ± 82	691 ± 60	818 ± 104
Triglycerides, mg/liver	395 ± 105	389 ± 39	275 ± 63	673 ± 53	397 ± 82 *	234 ± 71 **
Lung						
Mean lung wt, g	2.01 ± 0.09	1.99 ± 0.08	1.92 ± 0.12	1.95 ± 0.12	2.08 ± 0.05	1.98 ± 0.12
Total lipids, mg in lungs	67.8 ± 1.7	66.0 ± 2.9	65.4 ± 7.8	92.4 ± 8.1	76.8 ± 10.9	75.4 ± 9.4
Cholesterol, mg in lungs	10.5 ± 0.3	11.7 ± 0.5	12.2 ± 0.6	13.5 ± 1.1	14.6 ± 0.6	14.1 ± 1.3
Triglycerides, mg in lungs	13.7 ± 1.6	15.9 ± 3.0	12.9 ± 4.3	24.3 ± 5.2	21.0 ± 5.3	12.6 ± 4.7

[1] Eight rats/group.
[2] Mean ± SD.
[3] Computed from sand in feces.
[4] Significance computed by analysis of variance technique and designate differences between calcium levels for animals fed the same fat.
* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

death, with fasting time comparable for all groups.

Methods used for the analysis of serum, tissues and feces have been reported previously (2), except for serum calcium which was analyzed by flame photometry, and serum bile acids, by spectrophotometry (4, 5).

RESULTS AND DISCUSSION

The weight gain in all groups was comparable except for the group fed 1.2% calcium and cocoa butter, where the weight gain was somewhat lower. This can be attributed to the significantly greater fecal fat excretion noted in the rats fed 1.2% calcium and cocoa butter ($P < 0.01$). There is, at present, no explanation for the somewhat greater weight gain in the group fed 0.2% calcium and corn oil. Due to the large standard deviations in the weights, the differences in weight gain were not statistically significant. Feed consumption could not be calculated at the 1.2% calcium level since the indigestible sand marker was not included in these groups; however, the total feed used (wasted and consumed) was comparable in all groups.

At the 1.2% calcium level, serum total lipids and phospholipids were significantly lower with the cocoa butter diet ($P < 0.01$), but were not significantly changed with the corn oil diet. This same calcium level resulted in a significant lowering of total cholesterol ($P < 0.01$) and triglycerides ($P < 0.05$) with both dietary fats. Rats fed corn oil had lower serum cholesterol and phospholipid levels than rats fed cocoa butter at comparable calcium levels ($P < 0.01$). Serum total bile acids were not altered by dietary calcium levels but were significantly higher in rats fed corn oil, than in those fed cocoa butter ($P < 0.01$). This is in agreement with the results of Byer and Friedman (6) showing greater bile acid synthesis in rats fed unsaturated fats. The effects of 1.2% dietary calcium on the serum lipids of rats fed cocoa butter were sufficiently great to reduce the lipid levels to approximately those of rats fed the comparable corn oil diet. These hypolipemic effects of increased dietary calcium are in agreement with previously reported human and rat studies (1, 2).

In a previously reported rat experiment using beef tallow (2), 0.2% calcium was more hypolemic than in the present cocoa butter study. These differences in the hypolipemic effects of calcium may be due to the differences in fatty acid chain length of the 2 fats.

Serum triglycerides, in contrast with serum cholesterol, were the same with both dietary fats and responded similarly to calcium supplementation. This agrees with work reported in humans by Fleischman et al. (7), in which serum triglyceride levels were found to be independent of the nature of the dietary fat.

Fecal dry matter excretion increased with increased dietary calcium levels. Fecal total lipids were significantly increased ($P < 0.01$) at the 1.2% calcium level with both dietary fats, whereas fecal 3-β-hydroxysterols and lipid phosphorus, as phospholipids, were increased significantly ($P < 0.01$) only with the cocoa butter diet. Total lipid excretion was greater with the cocoa butter diet than with the corn oil diet. These differences were 44, 52 and 103% greater fat excretion with the cocoa butter diet at the three calcium levels ($P < 0.01$). Fecal bile acids increased significantly ($P < 0.01$) with increased dietary calcium in the presence of either fat, thus confirming previous results with beef tallow (2). Calcium excretion in the feces increased significantly with increased dietary calcium levels and the amounts excreted were similar with both fats. Calcium decreased fat absorption; however, the type of dietary fat did not influence calcium utilization under these conditions. Rats fed corn oil excreted significantly less total lipids ($P < 0.01$) and more bile acids ($P < 0.05$) at the 0.2% calcium level, than comparable rats fed cocoa butter.

Liver weight, total lipid and cholesterol were not significantly changed by increased dietary calcium levels. Liver triglycerides, however, were significantly lowered at the 0.2 and 1.2% calcium levels in rats fed corn oil ($P < 0.05$ and $P < 0.01$), respectively, but not in those fed cocoa butter. Livers of rats fed corn oil, at the 0.08% calcium level showed higher total lipids ($P < 0.01$), higher cho-

lesterol and higher triglycerides than similar rats fed cocoa butter.

Lipids in the lungs showed no significant changes with dietary calcium; however there was a trend toward lowered triglycerides in the groups fed increased dietary calcium in the presence of corn oil. At the 0.08% calcium level, lung triglycerides were higher in the corn oil group than in the cocoa butter group.

Based on these data, calcium is hypolipemic in rats fed either highly saturated or highly unsaturated fat; however, the influence of increased dietary calcium is more pronounced in the presence of saturated fat.

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Effect of Diethylstilbestrol on Energy and Protein Utilization by Chicks Fed a Diet High in Fat Content

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ABSTRACT Comparative studies were made of the effect of diethylstilbestrol on the utilization of dietary energy and protein by chicks fed diets containing glucose or corn oil as the major energy component. The metabolizable energy value of the high fat diet was decreased, but metabolic efficiency of energy utilization increased, by treatment of chicks with diethylstilbestrol. Restriction of energy intake amplified the depressive effect of diethylstilbestrol on metabolizable energy yield, and in itself caused a depression in metabolizable energy value of the high fat diet. Diethylstilbestrol treatment of chicks markedly decreased nitrogen retention at restricted levels of energy intake when the high fat diet was used. None of these effects was observed when glucose was the major dietary energy component. It is concluded that the effect of estrogens on certain metabolic processes is influenced by the form in which dietary energy is supplied.

In most investigations concerned with the effects of estrogens on efficiency of energy utilization by the chick, diets relatively low in fat content have been used (1–3). Under such conditions Hill et al. (3) reported that metabolizable energy and net energy yields of diets were not altered when male chicks were injected subcutaneously with diethylstilbestrol. Although these results demonstrated a marked effect of diethylstilbestrol on appetite regulation and nitrogen metabolism, no changes from the usual pattern of energy metabolism were noted.

More recently it has been shown that the efficiency with which chicks convert dietary energy into tissue growth may depend on the source of dietary energy (4). When fats containing highly unsaturated fatty acids are substituted for part of the carbohydrate in chick diets, metabolic efficiency of energy utilization is increased (5). In the present study results were obtained which show that the effect of diethylstilbestrol on certain parameters of metabolism in the chick may be influenced by the quantity of dietary energy supplied as fat.

MATERIALS AND METHODS

The composition of the low fat basal diet is shown in table 1. This diet was formulated to contain adequate levels of all nutrients known to be required by the chick and contained unidentified growth factor sources as well as 2.5% added corn oil supplied in the constant ingredients premix. The high fat diet was formulated by substitution of 15.6% corn oil for 37.7% glucose in the basal diet on a metabolizable energy basis using values of 3.64 kcal/g for glucose (6) and 8.8 kcal/g for corn oil (7). By this method of substitution corn oil replaced an equal number of glucose calories and the proportion of constant ingredients to metabolizable energy was maintained identical in the 2 diets even though the caloric densities differed. On the basis of 100 parts of diet this was equal to the addition of 20% corn oil. The final formulation of this diet was glucose 18.1%, constant ingredients premix 61.9% and corn oil 20%. Crude protein and ether extract content of the basal and high fat test diets was 28.3 and 3.0%, and 36.3 and 23.9%, respectively.

Crossbred Rhode Island Red × Barred Plymouth Rock male chicks were fed the basal diet ad libitum during a pre-experimental period from one day to 2 weeks of age. They were then divided into lots of

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TABLE 1

Composition of basal diet [1]

	%	%
Glucose [2]		51.8
Constant ingredients premix		
Soybean oil 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, refined	2.5	
Chromium oxide mix (30% Cr_2O_3)	1.0	
Antioxidants [3]	0.02	
Vitamin mixture [4]	0.7	
Mineral mixture [5]	4.0	
Total		48.2

[1] Dry-matter basis.
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] Equal quantities of butylated hydroxytoluene and diphenyl-*p*-phenylenediamine.
[4] Vitamin mix supplied (in mg/kg diet): nicotinic acid, 30; Ca D-pantothenate, 25; riboflavin, 10; pyridoxine·HCl, 10; thiamin·HCl, 5; folic acid, 2; menadione, 1; biotin, 0.4; choline chloride, 1400; vitamin B_{12}, 0.011; *d*-*α*-tocopheryl acetate, 22; chlortetracycline, 5.5; vitamin A, 10,000 IU, and vitamin D_3, 1500 ICU.
[5] Mineral mix supplied (in g/kg diet): $CaCO_3$, 20; dicalcium phosphate, 10; iodized salt, 6.4; K_2HPO_4, 2.2; $MgSO_4$, 1.21; $MnSO_4·H_2O$, 0.30; $FeSO_4·7H_2O$, 0.278; $CuSO_4·5H_2O$, 0.0078; and NaI, 0.0026.

10 chicks each by selection on the basis of 2 weeks' body weights and weight gains in a manner similar to that described by McKittrick (8). Duplicate lots of chicks were used per treatment, and the experiment terminated at four weeks of age.

Chicks were maintained in electrically heated, thermostatically controlled battery brooder units with raised wire floors. Water was given ad libitum. Chicks were fasted for an 18-hour period immediately before the start of the experimental period and again at the end of the experimental period to eliminate the influence of intestinal contents on body composition and weight gain values. Where restricted feeding was used, chicks were given their 24-hour feed allotment once daily at approximately the same time each day allowing sufficient feeder space to minimize competition. Daily corrections were made for feed wastage. Methods used in the determination of metabolizable energy values, and protein, fat and total energy gains of tissues have been described previously (4). Chicks receiving diethylstilbestrol were injected subcutaneously with a single 15-mg dose in a paste form [3] on the first day of experimentation. Reaction to diethylstilbestrol was checked 4 days later and was positive in all cases.

The experimental plan is shown in table 2. Three treatment series were established and within each series chicks were fed the same diet at 3 levels of energy intake. In the basal series (treatments 1, 2 and 3) control chicks were fed the basal diet ad libitum and at a restricted level equal to 60% of ad libitum intake. Also included in this series were chicks injected with diethylstilbestrol and fed the basal diet ad libitum. Hill et al. (3) reported that chicks treated in this manner greatly increase their energy intake but show no changes from the normal pattern in energy metabolism. Thus, advantage was taken of this knowledge to increase energy intake in this series without affecting efficiency of energy utilization.

In the corn oil series (treatments 4, 5 and 6) control chicks were fed the diet containing 20% added corn oil ad libitum and at a restricted level equal to 60% of the ad libitum intake of this diet. In the third treatment energy intake was restricted to that consumed by chicks fed the low fat diet ad libitum (treatment 2) to permit a direct comparison of the effects of corn oil on energy metabolism. This paired-feeding comparison was possible since increased levels of dietary corn oil will stimulate energy intake by the chick (9).

In series 3 (treatments 7, 8 and 9) chicks injected with diethylstilbestrol were fed the diet containing 20% added corn oil at 3 levels of energy intake: ad libitum; equal to untreated chicks fed the low fat diet ad libitum (treatment 2); and equal to untreated chicks fed the high fat diet ad libitum (treatment 4).

The data obtained for metabolizable energy values of the diets and 2-week weight gains and tissue protein gains of chicks were treated statistically by analysis of variance (10). Significance of differences between treatments was tested with Duncan's multiple range test (11).

RESULTS

The results of this study are presented in table 2. The metabolizable energy value of the basal diet was unaffected by restric-

[3] Mattox and Moore, Inc., Indianapolis, Indiana.

TABLE 2

Effect of dietary corn oil and diethylstilbestrol (DES) on metabolizable energy values of diets and weight gains, energy and protein intakes, and tissue gains of chicks

Treatment			Level of energy intake	ME [1] of diet [2]	2-week wt gains [2,3]	ME intake [1]	Protein intake	Tissue protein gain [2]	Tissue fat gain	Gain in carcass energy
Diet		DES								
				kcal/g	*g*	*kcal*	*g*	*g*	*g*	*kcal*
1	Basal	+	ad libitum	3.45 [a]	277 [a]	1834	154	50.9 [ab]	40.3	599
2	Basal	−	ad libitum	3.48 [a]	259 [bc]	1492	124	50.8 [ab]	24.5	453
3	Basal	−	60% of no. 2	3.46 [a]	137	940	79	30.5 [c]	8.9	215
4	+20% corn oil	−	ad libitum	4.32 [b]	276 [ab]	1579	140	53.2 [a]	30.8	519
5	+20% corn oil	−	equalized to no. 2	4.22 [c]	251 [c]	1392	126	48.8 [b]	29.5	488
6	+20% corn oil	−	60% of no. 4	4.23 [c]	161	977	88	33.0 [c]	16.1	294
7	+20% corn oil	+	ad libitum	4.25 [bc]	304	1924	173	52.0 [ab]	58.0	768
8	+20% corn oil	+	equalized to no. 2	4.13 [d]	233	1364	126	39.9	37.3	523
9	+20% corn oil	+	equalized to no. 4	4.18 [cd]	252 [c]	1542	141	44.3	43.0	594

[1] ME indicates metabolizable energy.
[2] Values not followed by same letter are significantly different ($P < 0.05$).
[3] Weight gains of treatments 5 and 8 adjusted to energy intake of treatment 2 are 269 and 258 g, respectively. Weight gain of treatment 9 adjusted to energy intake of treatment 4 is 258 g.

tion of energy intake or injection of the chicks with diethylstilbestrol (DES). However, when 20% corn oil was added to the diet, restriction of energy intake at the 2 levels studied with control chicks resulted in a significant decrease in dietary energy value. The depressive effect of restricted feeding on the energy value of the high fat test diet was enhanced by treatment of the chicks with diethylstilbestrol. DES alone tended to decrease the metabolizable energy value of the high fat diet when chicks were fed ad libitum, but the difference was not significant.

Energy intake of chicks fed ad libitum was increased markedly by treatment with DES and to a lesser degree by addition of corn oil to the diet. When both of these variables were included together in treatment 7, their effects on energy intake were additive. Because of the unexpected depression in metabolizable energy values due to restricted feeding and treatment with DES, equalization of energy intakes was not as accurate as the experimental plan anticipated.

Weight gains, in general, were correlated with the quantity of energy consumed. However, direct comparisons of these values may be influenced by the effect of treatment on both metabolic efficiency of energy utilization and body composition. Nevertheless, when ad libitum feeding was used, chicks treated with DES or fed the high level of corn oil had weight gains greater than chicks fed the basal diet, although the latter increase was not statistically significant. These effects appeared to be additive when studied simultaneously resulting in 17.4% faster weight gains. This marked increase in weight gain was associated with 29% greater intake of dietary energy.

Treatment with DES of chicks fed the basal diet ad libitum had no effect on the degree of protein synthesis measured as tissue protein gains. Furthermore, control or DES-treated chicks fed the high fat test diet ad libitum did not show significant increases in tissue protein gains although their protein intakes were greater than the control chicks fed the basal diet ad libitum.

When energy and protein intakes were equalized to the basal diet fed ad libitum in treatment 2, addition of 20% corn oil

to the diet produced a slight but nonsignificant decrease in tissue protein gain. Additional treatment with DES (treatment 8) resulted in a marked decrease in tissue protein gains when the high level of corn oil was fed. Although the depression in dietary energy value caused the energy intake of chicks in the latter treatment to be lower than expected, and thus prevented a direct comparison with treatment 2, the observed decrease in tissue protein gains cannot be attributed to the lower energy intake since chicks in treatment 9 with greater intakes of both energy and protein exhibited the same alteration in tissue protein gains.

Data are presented in table 2 showing the effect of the various treatments on total body fat content and average gain in total carcass energy. The latter value represents the sum of caloric gains in tissue fat and protein. Computation of this value has been described elsewhere (4). Both the addition of 20% corn oil to the basal diet, and the injection of chicks with DES, under conditions where ad libitum feeding was used, increased total body fat content as well as average gain in total carcass energy. Since differences in carcass energy content may simply be a reflection of differences in energy intake, the experimental data relating energy intake and tissue energy gains were compared graphically (fig. 1) to determine whether the treatments altered the energetic efficiency of growth. The data for control and DES-treated chicks fed the basal diet fit closely a single regression with a high degree of correlation ($R = 0.998$). These results confirm the report by Hill et al. (3) demonstrating that DES injection of chicks fed diets containing only small quantities of added fat causes no departure from the expected relationship between energy intake and the

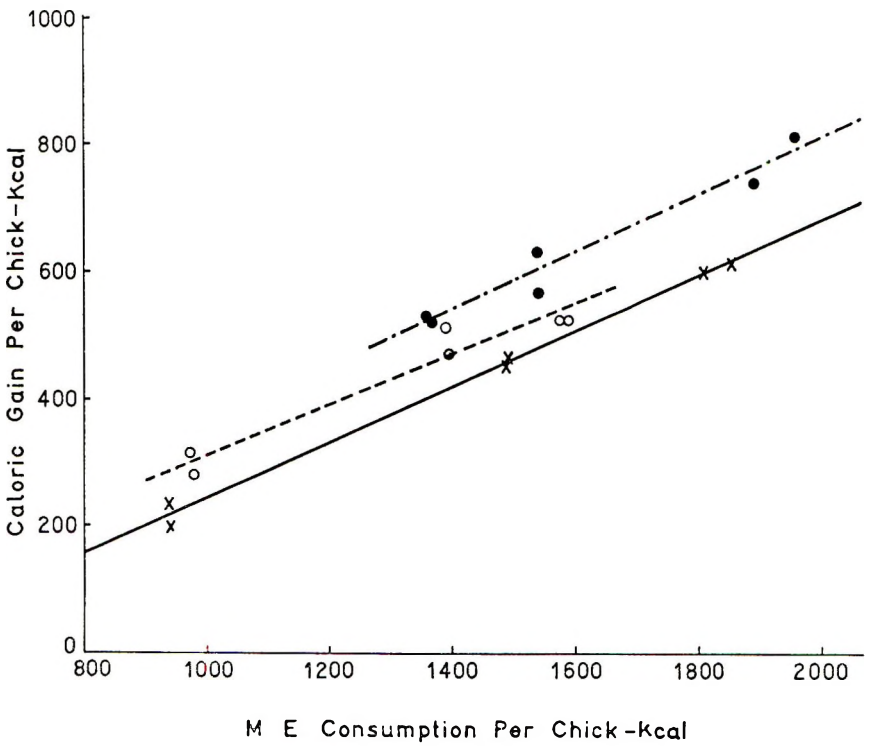

Fig. 1 Relationship between metabolizable energy consumption and tissue energy gain for chicks. Points represent average values/chick obtained with groups of 10 chicks each. X represents untreated and diethylstilbestrol-treated chicks fed the low fat basal diet; O represents untreated chicks fed the high fat diet; ● represents diethylstilbestrol-treated chicks fed the high fat diet.

efficiency of conversion to tissue energy. This regression, therefore, served as a base line with which could be compared the effect of other treatments on efficiency of energy utilization.

All average gains in total carcass energy obtained with untreated or DES-treated chicks fed the diet with 20% added corn oil were located above the basal regression. Separate regressions are shown for the untreated and DES-treated chicks. No significant differences were found between the slope values for the 3 lines ($P < 0.05$). Therefore, the carcass energy gain values for the three treatment series or regressions were subjected to an analysis of covariance (10) to remove the effects due to differing metabolizable energy intakes and to judge differences between treatment series. The analysis showed that after adjustment to a common energy intake, the average carcass energy gain values were significantly different for the three treatment series ($P < 0.05$). This meant that chicks located within each treatment series utilized dietary energy to significantly different degrees in the synthesis and maintenance of body tissues. Previous results (4, 5) have shown that certain dietary fats including corn oil increase the metabolic efficiency of energy utilization of chicks. The present investigation shows that further improvement in metabolic efficiency is possible if chicks fed diets high in fat content are physiologically altered by treatment with DES.

DISCUSSION

The results of this experiment demonstrate that the degree to which estrogen treatment of chicks influences certain physiological processes is dependent on the source of dietary energy. Previously Hill et al. (3) reported that both the expected metabolic efficiency of energy utilization as determined with control chicks and the metabolizable energy yield of the diet were unaffected by injection of chicks with diethylstilbestrol (DES) when glucose was the major energy component of the diet. The present investigation confirms these results, but further shows that both of these parameters of energy utilization may be altered by DES treatment when a large portion of dietary energy is supplied as a fat.

An explanation for the depression in metabolizable energy value of the high fat diet due to estrogen treatment of chicks is not available from these results. It appears most likely that this reflects a change in absorbability of a component of the dietary fat since estrogen treatment did not alter the metabolizable energy value of the low fat basal diet; unfortunately, this was not investigated in the course of the experiment.

Restricting the energy intake of control chicks fed the diet containing 20% corn oil also caused a reduction in the metabolizable energy value. The depressive actions of restricted feeding and estrogen treatment on the metabolizable energy value of the diet proved to be additive when studied together and resulted in a 4.4% decrease in this value. If this is due to a change in absorbability in the lipid components of the diet, it would amount to a decrease of more than 10% in the absorbability and metabolizable energy value of the corn oil.

The substitution of dietary glucose by corn oil has been reported to increase efficiency of energy utilization in the chick when measured as tissue energy gains per unit of energy consumed (4). The present work confirms these studies as demonstrated by the significant elevation above the base line in figure 1 of the points representing control chicks fed the high fat diet. This representation of data describes the partition of metabolizable energy intake into retention as tissue energy and loss as heat production. Therefore, for any given unit of energy intake, the more elevated a single point or locus on the plotted line, the greater the ability of the chick to retain energy in the form of tissue growth.

The data in figure 1 show a further increase in efficiency of energy utilization when chicks fed the high fat diet were treated with DES, as indicated by the uppermost line in the graph which occupied a position significantly different from the other 2 lines. Whether this represents a distinct effect of DES on the efficiency of metabolic processes of the chick or simply an augmentation of the increased metabolic efficiency observed in the chick due

to the ingestion of large quantities of corn oil is not known. It is clear, nevertheless, that a large quantity of corn oil was a predisposing nutritional condition necessary for the manifestation of the effect of DES on metabolic efficiency.

It might be suggested that the increased metabolic efficiency accompanying estrogen treatment is a result of the greater gain in body fat which may occur more efficiently than gain in tissue protein. This could occur even at restricted levels of energy intake since under these conditions there is a reduction in tissue protein gain accompanied by increased tissue fat gain. However, the observation that estrogen-treated chicks fed the low fat basal diet ad libitum showed a marked increase in body fat but no change from the expected pattern of energy utilization established by control chicks fed this diet does not support this thesis. Furthermore, Hill et al. (3) reported that DES treatment of chicks fed a diet high in carbohydrate resulted in a preferential synthesis of tissue fat at the expense of tissue protein but was unaccompanied by changes in metabolic efficiency of energy utilization.

DES treatment of chicks fed the high fat diet caused a marked reduction in tissue protein gains whenever energy and protein intakes were restricted. When DES-treated chicks fed the high fat diet were allowed ad libitum intake, which was accompanied by increased intakes of energy and protein, tissue protein gains were similar to those of control chicks fed ad libitum. Whether this effect was primarily due to the increased intake of energy or protein or required both of these changes cannot be determined from the data. Previous results (3) indicated a similar depressive action of DES on nitrogen retention in chicks with a high carbohydrate diet fed ad libitum, an effect which did not occur in the present study.

Hill et al. (3) reported that increased fat synthesis in DES-treated chicks fed a low fat diet is due primarily to increased energy consumption and to a lesser extent to preferential synthesis of fat at the expense of protein tissue. While these 2 factors have also been found responsible for the fattening effect in DES-treated chicks fed a diet high in corn oil, a third factor, increased metabolic efficiency of energy utilization, is also involved. In the present study the preferential synthesis of tissue fat at the expense of tissue protein was found to occur only at restricted levels of energy intake, and was not a contributing factor in the increased fattening of DES-treated chicks fed ad libitum.

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Changes in Aortic Extensibility Found in Sulfate-deprived Rats [1]

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ABSTRACT The influence of various levels of dietary sulfate on the extensibility and collagen content of rats' aortae was measured. It was found that the aortae of rats fed 0.0002% dietary sulfate would extend only 3.71 to 4.47 mm, whereas those from rats fed 0.02% or higher levels of dietary sulfate would extend 5.28 to 5.66 mm before breaking. In an effort to explain these results the aortae for several diet groups were fractionated in neutral-salt, acid-soluble and insoluble collagen. There were no differences between the groups in the percentage amounts of the various forms of collagen present. There was a significant decrease, however, in the total amount of hydroxyproline per milligram of fresh tissue present. The data were interpreted as suggesting a change in the overall structure of the connective tissue elements of the aortae which was related to the level of sulfate in the diet.

Dietary sulfate has long been regarded as being of little physiological consequence (1, 2). Michels and Smith (3) demonstrated that dietary sulfate contributes as significantly as organically bound sulfur to the body's metabolic pool of sulfur. Brown et al. (4) reported that sulfate-deprived rats showed rather profound changes in collagen metabolism, changes which were apparently related to defects in intramolecular crosslinkage. These workers (5) also noted that rats fed sulfate-poor diets had aortae which were weaker per milligram of collagen present than those of their controls. The change in the resistance of the aorta to breaking was interpreted as due to a change in the structure of the collagen present.

The present study reports the influence of sulfate-deprivation on other properties of the rat's aorta such as extensibility, percentage soluble collagen and the influence of added amounts of sulfate on extensibility.

MATERIALS AND METHODS

Littermate, weanling, male rats of the Wistar strain from the Department's colony were fed for 6 weeks the casein diets containing 0.02 and 0.0002% sulfate described in table 1. A second, identical experiment with male weanling rats, selected at random, from the same source was also conducted using the same procedure as in the first experiment. A third study was performed using 4 groups of weanling, male rats selected at random, in which the animals were fed peanut flour diets described in table 1 which provided 0.0002, 0.02, 0.10 and 0.45% sulfate. The third experiment was conducted to determine to what extent any changes in extensibility noted were related to the level of sulfate in the diet. The peanut flour diet was selected because it contained a minimal amount of the sulfur amino acids which the animal could oxidize to sulfate. Thus any changes in aortic properties which are due to physiologically available amounts of sulfate

TABLE 1

Composition of experimental diets

	Casein diet	Peanut diet
	g/100 g	*g/100 g*
Casein (vitamin-free)	18.0	—
Peanut flour (fat-free)	—	20.0
Cornstarch	35.0	68.0
Sucrose	35.0	—
Hydrogenated fat	6.0	6.0
Corn oil	1.0	1.0
Vitamin mixture [1]	2.0	2.0
Salt mixture [2]	3.0	3.0

[1] Purchased from General Biochemicals, Chagrin Falls, Ohio.

[2] The salt mixtures described by Brown et al. (4) were used in the 0.0002% and 0.02% sulfate diets. The salt mixtures of Button et al. (14) were used to provide 0.10% and 0.42% sulfate to the diets. These two salt mixtures are identical except for the amount of sulfate provided.

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from either internal or external sources would be more responsive to added dietary sulfate. The levels of dietary sulfate in this experiment were calculated using the levels of sulfate provided by the salt mixture.

After the animals reached the appropriate age they were decapitated and their thoracic aortae removed and placed in cold Ringer's solution. After measurement of length, the aorta segment was blotted, weighed and then promptly returned to cold Ringer's solution. The extension of a one-half inch of the aorta which was located in the same geometric location in all samples was accomplished with a modification of the method used by Brown et al. (5) in which a steel ruler calibrated in 0.01 inches was fixed to the moving member. The extension of the aorta to the breaking point was measured by counting the divisions of the ruler that passed a fixed point, which was measured through a magnifying glass as water was added at a constant rate. The measurements were converted into the metric system for purposes of reporting. The aortae from the second experiment were fractionated in neutral-salt-soluble, acid-soluble and insoluble hydroxyproline as described previously (4).

RESULTS

The aortae from the sulfate-deprived rats in the first experiment exhibited significantly less extensibility than those of their littermate controls (table 2). The standardized sections of the aortae from the sulfate-deprived group were significantly weaker than those from their controls. In both cases, the amounts of extension or breaking strength in the sulfate-deprived group were markedly lower than in the control group. The lower tensile strength of the standardized segments may have been due to a decreased collagen content although previous work (5) suggests that this may not be so.

The extension of a uniform 12.5-mm segment of the aorta appeared to be influenced by the level of dietary sulfate as shown by the results of the third experiment (table 3). In terms of aortic extensibility, the amount of sulfate provided in the 0.02% salt mixture is adequate since

TABLE 2

Extensibility and breaking strength of a 12.5-mm segment of aortae from control and sulfate-deprived rats

Measurement [1]	Sulfate in diet, %		$\bar{d}$	$S\bar{d}$	*P* value [2]
	0.0002	0.02			
Extension of aorta to breaking point, mm	4.470	5.664	1.194	0.173	0.025
Wt required/100 mm of extension, g	2,738.4	3,817.2	1,186.8	248.4	0.05
Breaking strength/mg/100 mm of aorta, g [3]	19.6	32.4	12.8	2.048	0.025

[1] Three pairs of littermates used. Measurements of length made in inches and converted to the metric system.
[2] All statistical comparisons by the paired-comparison technique. Values presented in table are averages. Data from first experiment.
[3] The term "mg/100 mm" refers to an idealized aortic segment which weighs one milligram and measures 100 mm in length. It is obtained by dividing the weight of the segment by the length.

TABLE 3

Extensibility of aortae of rats fed various levels of sulfate added to a low sulfur basal diet

	Sulfate in diet,[1] %			
	0.0002	0.02	0.10	0.42
Extension of aorta to breaking point, mm	3.71 ± 0.599 [2]	5.33 ± 0.368	5.28 ± 0.366	5.54 ± 0.645

[1] Five animals/group. All measurements were made in inches and converted to the metric system.
[2] Average ± SE of mean. The values found for the group fed 0.0002% of sulfate were significantly lower ($P < 0.05$) than those fed the other levels of sulfate as determined by Student's *t* test.

TABLE 4

Composition of aortae from male rats fed the 0.02 and 0.0002% of sulfate diets

Collagen fractions	Sulfate in diet, %		Difference	t	P
	0.02 [1]	0.0002 [1]			
	μg/100 μg hydroxyiroline				
Neutral-salt-soluble	8.02 ± 1.702 [2]	8.82 ± 0.817	0.80	—	ns
Acetic acid-soluble	16.12 ± 3.229	14.02 ± 1.031	2.10	—	ns
Insoluble	75.52 ± 5.870	77.52 ± 3.213	2.00	—	ns
	μg/mg fresh tissue				
Total hydroxyproline	14.06 ± 0.902	11.45 ± 0.330	2.61	3.030	< 0.05

[1] Four animals in the 0.0002% sulfate group and 3 animals in the 0.02% sulfate group.
[2] Figures shown are averages ± SE of mean. The letters ns indicate no statistical importance can be attached to these differences of the means as determined by Student's *t* test.

levels above that did not further improve aortic extensibility.

The data presented in table 4 indicate there were no significant differences in the amounts of soluble collagen present in the aortae, which is in direct contrast with findings in the skin (4). This may be indicative of no change in the rate of biosynthesis of collagen in the aorta. Most of the collagen present in the aortae was present as the more crosslinked, and mature, insoluble collagen. The collagen is generally credited with being the element responsible for the mechanical strength of the aorta (7, 8), and the least extensible of the several components of an aorta. Any defect present in the collagen system may be in the mature, insoluble form. The aortae from the sulfate-deprived animal contained less total hydroxyproline than their controls, which is in accord with previous observations.

DISCUSSION

The results of this study may be explained if the aorta is considered as a complex system of collagen and elastin, which are present in varying quantities, depending on the part of the aorta investigated (9).

Milch (10), in a recent review of the properties of the aging arterial wall, suggests that the loss of extensibility in aging can be attributed to failure in muscoloelastic system and fibrosis. Because of the young age of the animals in this study, it is doubtful that fibrosis accounted for any change in extensibility, which indicates that there may have been changes in the elastic system. Previous work (5) indicated that the aortae of rats fed these same low sulfate diets exhibited less tensile strength per milligram of collagen. This suggested that collagen in the aorta is structurally weak. In a longitudinal pull, the pulling is against a combination of collagen and elastin; collagen is the least extensible of the two (7, 8, 10).

If it is assumed that collagen furnishes the bulk tensile strength and elastin the larger part of extensibility in a manner much like that of the cord and rubber which make up the structure of the modern automobile tire, then the following hypothesis may be used to explain these data. If the collagen in the aorta is structurally weak, perhaps undergoing the similar changes in crosslinkage as shown in skins (4), it will not bear the added weight. This places the weight-bearing burden upon the structurally weaker elastin. The result is an early breaking of the aorta before it extends to the same length as the control. The hypothesis, however, does not rule out a change in the structure of the elastin which does not permit fullest extension before separation.

The results of these experiments may help in part to explain recent epidemiological findings.

In recent years there has been considerable interest in the influence of the hardness of water on the incidence of cardiovascular problems (11–13). It is difficult, however, to pinpoint any definitive cause and effect relationship between the absence of some minerals and the development of any one cardiovascular syndrome. It is of interest to speculate that as the water supply became softer the amount of

sulfate present declined; and to note that hypertension, which is due to inelasticity of the blood vessel, was found to be highly correlated with the degree of softness of the water supply (11). Possibly this may have been due in part to a decrease in sulfate supply. One cannot rule out possible interaction between minerals in the natural state which may contribute to the epidemiological findings. This controlled experiment did not attempt to find any synergistic effects of other ions with sulfate. In this connection, the findings of Morris et al. (12) must be noted. They reported that the incidence of cardiovascular problems associated with a soft water supply may be related to the level of carbonates, calcium and possibly sulfate. Schroeder (13) calls attention to the importance of the sulfate-to-carbonate ratio in Japanese rivers on the incidence of heart disease and cerebral hemorrhage. It would be of value to investigate the role of the various cations and anions on various parameters of cardiovascular performance under controlled laboratory conditions.

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Effect of Vitamin B_{12} in Choline Deficiency in the Rat [1]

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ABSTRACT Several diets varying in methionine content and low in choline and vitamin B_{12} were prepared using different amounts of extracted peanut meal supplemented with amino acids. Most of the diets contained added homocystine as well. Marked growth inhibition was observed within 2 to 3 weeks in weanling male rats and, provided the level of methionine in the diet was not too low, vitamin B_{12}, choline or methionine supplementation promoted rapid and approximately equal growth. The 3 supplements also prevented fatty liver, liver fibrosis, and kidney scarring although vitamin B_{12} was not always completely effective. Urinary formiminoglutamic acid (FIGLU) excretion was high with the deficient diet and was decreased to nearly zero by vitamin B_{12} or methionine supplements. The presence or absence of folic acid in these diets had little effect on the performance of the animals or the lesions seen although a moderate growth response occurred in the presence of vitamin B_{12}. When homocystine was not included in the diet (and methionine synthesis presumably blocked), supplements of vitamin B_{12} and folic acid did not completely prevent FIGLU excretion. The excretion of this metabolite would not appear to be due to a deficiency of vitamin B_{12} or folic acid per se.

It has long been recognized that it is relatively difficult to reproduce choline deficiency in all details in different laboratories. Whereas practically any diet moderately deficient in methyl groups will produce fatty liver, the amount of fat deposited, the extent and severity of kidney lesions, and the time required to produce fibrosis and cirrhosis may vary substantially and inspection of the diets may not reveal the cause of such differences. Certainly, a primary need for the production of severe deficiency is a diet restricted in methionine and other methyl donors but not so low that growth and food intake are markedly depressed, since food restriction partially prevents the development of the full-blown deficiency. Griffith and Nyc (1) reviewed the data thoroughly in 1954 and include the amount of protein, the level of cystine, and the kind and amount of dietary fat among the factors which influence the severity of the lesions produced.

At the time that review was written, the work of Schaefer et al. (2), Bennett (3) and others (4–6) was appearing which implicated vitamin B_{12} and folic acid in the transfer or synthesis of methyl groups. Vitamin B_{12} is now accepted as a lipotropic factor and many of the enzymatic functions involving vitamin B_{12} and folic acid in methyl synthesis and transport (7, 8) have been described. Many of the systems have been studied only in bacteria, however. The role of folic acid, if any, in the development of choline deficiency in the rat under ordinary conditions is unclear since it is known to be synthesized by the bacterial flora and this may meet requirements.

Despite these well-known observations, it does not appear that the nutritional or biochemical implications have been explored thoroughly. Some laboratories apparently find little effect on choline deficiency from the inclusion of vitamin B_{12} in diets (9) although most investigators omit vitamin B_{12} from diets designed to produce choline deficiency for obvious reasons. The extent to which vitamin B_{12} or folic acid is involved in the development of the lesions seen is usually unknown. The same kinds of questions may be asked relative to studies on vitamin B_{12} deficiency. The urinary excretion of formiminoglutamic acid (FIGLU) is elevated in the vitamin B_{12}-deficient rat and the ex-

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TABLE 1
Experimental diets

	1	2	3	4	5
	%	%	%	%	%
Extracted peanut meal [1]	32.00	25.50	19.00	12.50	6.00
Glucose	47.50	50.50	53.50	56.60	59.50
Salt mixture [2]	4.00	4.00	4.00	4.00	4.00
Coconut oil	15.00	15.00	15.00	15.00	15.00
Threonine	0.26	0.26	0.26	0.26	0.26
Lysine hydrochloride	0.73	0.73	0.73	0.73	0.73
Vitamin mix [3]	0.50	0.50	0.50	0.50	0.50
Amino acid mix [4]	0.00	3.50	7.00	10.50	14.00

[1] General Biochemicals, Chagrin Falls, Ohio.
[2] Jones, J. H., and C. Foster. J. Nutr., 24: 245, 1942.
[3] Vitamin A and D powder (325,000 IU/g and 32,500 IU/g), 5 g; thiamine·HCl, 200 mg; riboflavin, 400 mg; pyridoxine·HCl, 200 mg; Ca pantothenate, 1.25 g; niacinamide, 2.0 g; folic acid, 50 mg; biotin, 10 mg; menadione, 25 mg; α-tocopheryl succinate, 500 mg; and glucose to make 250 g.
[4] Contained: (in grams) DL-tryptophan, 3.0; DL-threonine, 5.0; DL-isoleucine, 14.0; L-leucine, 20; L-lysine·HCl, 9.0; L-cystine, 5.0; DL-phenylalanine, 16.0; L-tyrosine, 13.0; DL-valine, 13.0; L-arginine·HCl, 20; L-histidine·HCl, 6.0; DL-homocystine, 10.0; glycine, 108 and L-glutamic acid, 108.

cretion is markedly depressed by methionine administration (10, 11). Herbert and Sullivan (12) have also reported depression of FIGLU excretion in patients with megaloblastic anemias and pernicious anemia by the administration of methionine. Thus, the biochemical lesion in vitamin B_{12} deficiency may be largely dependent upon the kind of diet utilized in its production. It may be worthwhile to attempt to determine whether factors influencing choline deficiency do so by influencing the need for choline or methyl groups or by affecting methyl synthesis.

EXPERIMENTAL

The diets used in these studies are shown in table 1. The commercially available extracted peanut meal [2] apparently supplies insufficient amounts of lysine, threonine and methionine for the young rat (13). To produce a diet primarily lacking in the supply of available methyl groups and in vitamin B_{12}, this diet was supplemented with what appeared to be adequate amounts of lysine and threonine. This diet, supplying 15% of protein from the peanut meal, is designated diet 1 in table 1. To further reduce the methionine content, the peanut meal in other diets was decreased to supply 12, 9, 6, and 3% of protein in diets 2 to 5. The approximate amounts of the essential amino acids removed by this reduction were replaced by the amino acid mixture shown. The inclusion of glycine and glutamic acid at rather high levels was made so that the total nitrogen content of diets 1 to 5 was approximately the same. In addition, homocystine was included in the amino acid mixture at a fairly substantial level. It was thought that if the homocystine were methylated to form methionine, the methionine requirement would be adequately covered.

The animals were obtained from a commercial source [3] as weanling male rats and weighed between 45 and 55 g. They were housed in individual cages and food and water were supplied ad libitum. There were 6 animals per group. Although the cages contained screen bottoms, it is recognized that this does not prevent coprophagy.

The experiments were terminated after either 2 or 3 months. The data obtained varied somewhat in the different experiments reported. Liver fat was determined by chloroform-methanol extraction of fresh liver. The extracts were taken to dryness under reduced pressure, redissolved in petroleum ether, and the weight of lipid was determined after drying an aliquot in vacuo at 20° in tared aluminum dishes. Sections of liver and kidney were taken in 10% formalin or Zenker's solution for histologic examination. Formiminoglutamic acid (FIGLU) in urine was determined after dilution of 48-hour collections by the enzymologic method (14).

[2] General Biochemicals, Chagrin Falls, Ohio.
[3] Charles River Breeding Laboratories, North Wilmington, Massachusetts.

RESULTS

The results of the first experiment in which diets 1, 2 and 3 were fed are shown in table 2 and the growth curves obtained with diets 1 and 3 are shown in figure 1. When fed without vitamin B_{12} these diets allowed much less growth than the same diet supplemented with vitamin B_{12} and this was evident within 10 to 15 days. Diet 1 allowed substantially better growth than diet 3 when unsupplemented, but equivalent growth was obtained with either diet in the presence of vitamin B_{12}. As shown in table 2, the weight of the supplemented animals was similar to that noted in animals fed laboratory ration.

The vitamin B_{12}-deficient diets produced enlarged livers which contained approximately 30% of fat on a wet-weight basis. The vitamin B_{12} supplement reduced the weight and fat content to nearly normal levels. No substantial anemia was observed.

In the second experiment, the level of peanut meal was further reduced by feeding diets 3, 4, and 5. When the animals were killed after 60 days, the livers were graded for relative degrees of fatty infiltration and fibrosis on a zero-to-4 scale. The kidneys were graded for relative degrees of scarring. As shown in table 3, growth was severely inhibited with all diets deficient in vitamin B_{12}. Diets 4 and 5 did not support maximal growth even in the presence of vitamin B_{12}. Enlarged and severely fatty livers were noted in all deficient groups and this was not entirely prevented by vitamin B_{12}. The extent of kidney scarring was similar in diets 3 to 5 and the scarring was practically prevented in all groups by vitamin B_{12} supplementation.

In the next experiment, diet 3 was used but the folic acid was removed from the previously described vitamin mixture to determine whether folic acid was involved or necessary for the responses obtained with vitamin B_{12}. The supplements provided are shown in table 4. The addition of vitamin B_{12} and folic acid, together or singly, were compared with supplements of methionine and choline. A diet containing 5% tripropionin was also included since the addition of Ca propionate has

TABLE 2

Characteristics of animals fed diets with and without vitamin B_{12}

Diet		Time	Body wt	Liver			Blood values			
				Wt		Lipid	Hb	Hematocrit	RBC	WBC
		months	*g*	*g*	*% body wt*	*%*	*g %*	*%*	*10^6/mm³*	*10^3/mm³*
1	15% peanut protein — no vitamin B_{12}	3	259	14.9	5.8	27.1	12.8	43	8.4	8.1
1	15% peanut protein + vitamin B_{12}	3	364	10.4	2.8	7.6	13.6	46	8.1	7.3
3	9% peanut protein — no vitamin B_{12}	3	199	10.8	5.5	31.8	12.4	41	7.6	5.8
3	9% peanut protein + vitamin B_{12}	3	356	10.9	3.1	10.0	13.2	44	7.7	5.5
2	12% peanut protein — no vitamin B_{12}	1	169	12.7	7.7					
2	12% peanut protein + vitamin B_{12}	1	210	10.6	5.0					
	Laboratory ration	3	366	10.4	2.9	6.8	13.9	47	8.2	6.3

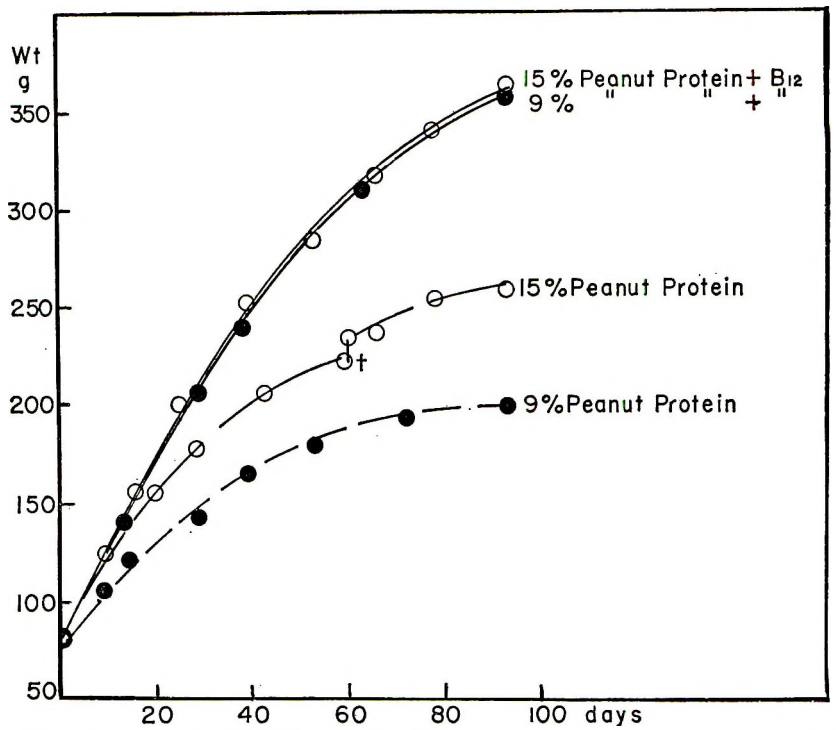

Fig. 1 Growth curves of animals with and without vitamin B_{12} fed diets 1 and 3 (table 1).

TABLE 3

Response of animals to vitamin B_{12} when fed diets with low levels of peanut protein (60-day experiment)

	Diet	Vitamin B_{12}	Gain (60 days)	Vitamin B_{12} response	Liver		Pathology score [1]	
					Wt		Liver fat	Kidney
			g	*g*	*g*	%		
3	9% peanut protein	—	62		9.0	8.6	4.0	2.3
4	6% peanut protein	—	32		6.3	8.6	4.0	2.3
5	3% peanut protein	—	14		5.4	9.7	4.0	3.0
3	9% peanut protein	+	191	129	8.8	3.7	2.5	0
4	6% peanut protein	+	174	142	9.2	4.3	2.0	0
5	3% peanut protein	+	122	108	9.3	5.6	2.8	0.5

[1] Degree of fatty liver was scored as follows:
0 Absent, no fat present.
1+ Mild, a small amount of fat seen here and there throughout the section.
2+ Moderate, fat seen throughout the section separating or at the margin of almost all liver liver lobules. Normal lobular architecture is preserved.
3+ Moderately severe, fat has replaced completely a large portion of liver lobules, at least 50%. However, a vestige of lobular architecture remains visible in some areas.
4+ Severe, fat has replaced almost completely the entire hepatic parenchyma. A few hepatic cells are seen here and there which are intact and have not been replaced by fat. Lobular architecture is not discernible.

Degree of fibrosis (cirrhosis) was scored as follows:
0 Absent, no fibrous tissue seen.
1+ Mild, an occasional strand of fibrous tissue proliferation seen extending from the portal areas.
2+ Moderate, fibrous tissue strands seen extending between portal areas and separating lobules in some areas.
3+ Moderately severe. (More extensive lesions than 2+ were not found in this study.)
4+ Severe. (More extensive lesions than 2+ were not found in this study.)

Kidneys were scored as follows:
0 Absent, normal kidney.
1+ Present, focal scars seen on occasion beneath the capsular surface of the outer cortex.
2+ Present, moderate number of scars seen in the same region of the cortex.
3+ Present, diffusely are many scars in the outer cortex resulting in U-shaped depressions of the surface. Glomeruli are intact but clumped in these areas along with golden brown, homogeneous, 10 to 15 μ in diameter hemosiderin pigment granules. Protein casts are present occasionally in dilated proximal convoluted tubules and at the cortico-medullary junction.
4+ Present, diffusely with cortico-medullary streaking are the same scars described above. Evidence of increased severity is noted by the pathologic process at the cortico-medullary junction.

TABLE 4

Effect of various supplements on diets lacking vitamin B_{12} and folic acid (90-day experiment)

Supplement	Body wt	Liver wt	Liver wt	Hemoglobin	Hematocrit	Pathology score [1]	
						Liver fat	Kidney
	g	*g*	*% body wt*	*g %*	*%*		
None [2]	202	14.0	7.4	11.9	39	3.5	1.8
5% tripropionin	200	12.8	6.8	11.5	39	3.7	1.3
Vitamin B_{12}	336	12.8	3.8	13.8	44	1.6	0.8
Folic acid	206	16.1	8.2	11.0	36	4.0	2.2
Vitamin B_{12} + folic acid	368	13.4	3.7	14.0	45	1.7	0
Methionine	374	13.8	3.7	14.6	47	0	0
Choline chloride	336	11.7	3.5	14.0	44	0	0

[1] See explanation of footnote to table 3.
[2] Diet 3 (table 1) with folic acid removed from the vitamin mixture.

been reported to increase the requirement of the rat for vitamin B_{12} (15).

As shown in table 4, the deficiency of vitamin B_{12} produced growth inhibition, severely fatty livers and moderate scarring of the kidneys. Mild anemia was also present. Folic acid additions had no effect in the absence of vitamin B_{12} but improved growth somewhat in the presence of vitamin B_{12}. Vitamin B_{12} alone greatly stimulated growth and reduced liver fat and kidney scarring. Methionine was somewhat more effective. This supplement produced maximal gain and eliminated liver and kidney pathology. Choline alone was less effective in supporting weight gain but also eliminated the accumulation of fat in the livers and kidney scarring. The addition of tripropionin had no apparent effect.

The data from the last experiment to be reported are summarized in table 5. These animals were killed after 90 days. In this study the effects of vitamin B_{12} supplementation with and without folic acid were compared with methionine using diet 3 as in the previous study. In addition, the same diet but with the homocystine removed from the amino acid mixture was fed, with and without vitamin B_{12} and folic acid. Thus, groups 6 to 9 were not only low in methionine but synthesis of additional methionine should be very limited since homocystine was not supplied. Urine was collected from each animal for 2 days after 3 weeks, 5 weeks and 3 months for FIGLU determinations (14).

The results in the first 5 groups are similar to those previously observed. Whereas either methionine or vitamin B_{12} and folic acid supplements gave equivalent growth at one month, the vitamin supplement was somewhat more effective after 3 months suggesting that functions of vitamin B_{12} other than methyl synthesis become more critical with time and the animals may become more depleted of the vitamin. The effects of vitamin B_{12} and methionine and lack of effect of folic acid upon the mild anemia and fatty liver is similar to that in previous experiments. FIGLU excretion was high in vitamin B_{12} deficiency and not appreciably affected by folic acid supplements. In contrast with earlier experiments, these animals showed a moderate amount of fibrosis of the liver which was prevented by methionine supplementation and nearly so by vitamin B_{12}. For some reason this group of animals was probably more susceptible than those used earlier and we assume that fibrosis would have been produced in the earlier experiments if the animals had been continued somewhat longer.

In the animals that received the diets lacking homocystine (groups 6 to 9) no growth response to vitamin B_{12} was observed during the first month but became clearly evident with time. As suggested above, it appears likely that either the requirement for methionine or methyl groups decreases with age and other functions of vitamin B_{12} become more important. Folic acid supplementation alone had little effect and may have been slightly detrimental as indicated by some increase in the degree of liver fibrosis and the lower hematocrit values. The addition of folic acid alone appears to have raised the level of FIGLU excretion (compare group 6 with 8) and did not decrease the excretion when added

TABLE 5

Responses to various supplements in diets with and without homocystine (90-day experiment)

Basal diet	Diet 3 [1] minus folic acid					Diet 3 [1] minus homocystine and folic acid			
Group	1	2	3	4	5	6	7	8	9
Supplement	None	Folic acid [2]	Vitamin B_{12} [3]	Folic acid + vitamin B_{12}	Methionine [4]	None	Vitamin B_{12}	Folic acid [2]	Folic acid + vitamin B_{12}
Wt at 1 month, g	110	113	174	202	198	93	102	98	101
Wt at 3 months, g	186	197	441	455	409	227	324	218	352
Liver wt, g	12.9	14.2	14.4	13.9	13.4	14.2	14.4	15.0	13.9
Liver wt, % body wt	6.9	7.3	3.2	3.0	3.3	6.4	4.4	7.1	4.0
Liver fat, % wet wt	36.5	35.4	13.4	8.6	7.8	34.0	25.6	35.9	29.2
Hemoglobin, g %	11.0	11.5	13.0	13.8	13.7	12.4	12.8	11.5	13.0
Hematocrit, %	35	37	42	43	44	45	42	37	42
FIGLU at 3 weeks, μg/day	3100	2300	175	42	0	640	1300	3000	720
FIGLU at 5 weeks, μg/day	> 8000	> 8000	46	37	370	2400	1100	> 8000	2300
FIGLU at 3 months, μg/day	6700	6600	330	120	290	4150	735	6000	1030
Liver score:									
Fatty change	4.0	4.0	1.8	1.1	0.1	3.8	3.0	4.0	2.6
Fibrosis	1.8	0.8	0	0	0	0.1	0	1.0	0
Kidney score	3.0	2.1	0.8	0.6	0	0	0	0	0

[1] See table 1.
[2] 1 mg folic acid/kg.
[3] 200 μg vitamin B_{12}/kg.
[4] 5 g methionine/kg.

with vitamin B_{12}. Group 9 cannot be assumed to be lacking in either vitamin B_{12} or folic acid but is lacking in either methionine or available methyl groups. Hence, the excretion of FIGLU is presumably more related to a deficiency of the latter than of either vitamin. Very large differences in FIGLU excretion are observed among animals receiving the same diet and only substantial differences can be considered to have any significance.

DISCUSSION

It does not appear possible to reconcile these results in all details with those in the literature. Bennett (3) showed that animals fed a "methyl-free" diet would grow when homocystine, vitamin B_{12} and folic acid were added but growth was poor. As du Vigneaud (16) has pointed out, the evidence suggests that some preformed methionine is probably required, if not for survival, at least for optimal performance. It is not strictly correct, therefore, to state, as have Silverman and Pitney (10), that Bennett showed that these supplements would replace the methionine requirement in rats. Evidence of severe choline deficiency, that is, fatty livers and kidney lesions, has been obtained with diets containing vitamin B_{12} and folic acid. For example, Young et al. (9) recorded a very high incidence of kidney lesions and fatty livers in young rats fed a diet said to contain 0.19% methionine and 3 mg of vitamin B_{12}/kg. Diet 1 (15% peanut protein) used here apparently contains between 0.13 and 0.19% methionine and diet 3 (9% peanut protein) between 0.08 and 0.12% methionine depending upon correct figures for the methionine content. The compilation by Orr and Watt (17) gives an average figure of 0.88% methionine in peanut protein and McOsker (13) gives figures of 1.3% for raw peanuts and 1.16% for toasted peanuts. In these studies, no animals died from acute kidney lesions although the scarring present at autopsy after 2 or 3 months on the experiment indicates that such lesions did develop. All evidence of the kidney lesions was prevented by the vitamin B_{12} supplementation. It appears likely, therefore, that consideration of factors other than methionine and vitamin B_{12} are required to reconcile results from different laboratories. It should be noted that the response to vitamin B_{12} was greater when the methionine intake was severely restricted and homocystine added to the diet (table 3) but the addition of homocystine is not required to obtain the marked growth response to vitamin B_{12}. We assume that methionine is spared by the presence of the vitamin.

It may be assumed that all of the animals fed the diets with vitamin B_{12} supplements developed fatty livers during the early part of the experiments. This was observed in a few animals fed diet 3 supplemented with vitamin B_{12} that were killed after 10 days and would be consistent with the results of Young et al. (9) and data published from this laboratory by Iwamoto et al. (18).[4] However, animals that received the vitamin-supplemented diets for 2 months had only moderately fatty livers (table 3) and those fed for 3 months only slightly fatty livers (tables 4 and 5). The ability of homocystine, folic acid, and vitamin B_{12} to replace methionine clearly depends upon the criteria used to evaluate this and the time the animals are fed the diet. Factors mentioned in the literature such as the cystine, protein, and fat content are presumably also involved. We do not find in the literature, either in studies designed to investigate vitamin B_{12} deficiency or those related to choline deficiency, studies on the qualitative relationships of methionine, choline, and vitamin B_{12}. The vitamin B_{12} requirement, based upon diets presumably but not certainly adequate in methionine or choline, are thought to be of the order of 10 to 20 μg/kg but such levels will not promote maximal growth with the diets used in the studies reported here.[5] Hence, it is not necessarily possible to evaluate the relative adequacy of diets reported in the literature.

Liver fibrosis was observed only in one experiment and this also was prevented by vitamin B_{12}. This group of animals was apparently more susceptible than those used in other experiments but, since fibrosis in many people's experience begins only after 3 or 4 months of choline-deficient diets, the animals in other experiments

[4] The vitamin mixture was not described in the paper but supplied 150 μg of vitamin B_{12}/kg.
[5] Unpublished data.
[6] Folic acid assay of the peanut meal using *L. casei* indicates approximately 0.38 μg/g.

might have developed similar lesions had they been continued for somewhat longer periods. We would like to point out, however, that in view of the discussion as to the cause of fibrosis and cirrhosis and whether this is the inevitable consequence of long continued fatty infiltration of the liver (19, 20, 21), a distinction between vitamin B_{12} deficiency and choline deficiency may be meaningful if it is possible.

The data on FIGLU excretion are generally similar to those reported by Silverman (10), Spivey Fox and Ludwig (22), and Stokstad et al. (11) in that either methionine or vitamin B_{12} is effective in lowering the urinary excretion. Again, the absolute levels of excretion are probably not interpretable because of differences in the diets including the histidine content. However, the fact that when homocystine was omitted from the diet, and methionine synthesis presumably blocked, vitamin B_{12} and folic acid were not capable of reducing FIGLU excretion may be important. It appears that the excretion of FIGLU is not due to a deficiency of vitamin B_{12} per se but rather to a deficiency of methionine which somehow influences the metabolism of FIGLU.

Although data were obtained with and without folic acid supplements in some experiments, it is recognized that these data indicate only that folic acid was apparently not a major factor in the results obtained. We assume that folic acid is either adequate in the diet or is synthesized in adequate or nearly adequate amounts by the intestinal flora.[6] It is of more interest that the addition of folic acid in the absence of homocystine and vitamin B_{12} actually increased FIGLU excretion.

In view of the general impression that vitamin B_{12} deficiency can be produced in rats only with difficulty and after prolonged depletion, it is worthwhile to emphasize the marked growth depression obtained with these diets which responds to vitamin B_{12}. Similar responses were obtained by Stokstad et al. (11) although they were somewhat smaller since the diet contained choline and a higher level of methionine. In the diets used by Silverman and Pitney (10), no growth response to vitamin B_{12} was noted although biochemical evidence of deficiency was apparent. The position that the deficiency being studied is either "choline or methyl" or "vitamin B_{12}" deficiency can easily be defended since the only criteria of a nutritional deficiency is the development of an abnormality which responds to a specific nutrient. However, it appears unlikely that the defects in enzyme systems which result would be identical when one or the other nutrient is primarily lacking or at various stages in the development of the deficiency. It should be of interest to determine to what extent evidence of vitamin B_{12} deficiency can be ameliorated under conditions in which methionine or choline deficiency have been assumed (but not proved) to be not involved.

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Invitation for Nominations for 1968 American Institute of Nutrition Awards

Nominations are requested for the 1968 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 and, where appropriate, a selected bibliography which supports the nomination. Seconding or 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, 1967*, to be considered for the 1968 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 during the banquet at the annual meeting.

1968 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.

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1955 – A. G. Hogan
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1957 – no award
1958 – L. D. Wright
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Corvallis, Oregon 97331

1968 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.

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University Park, Pennsylvania 16802

1968 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.

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The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

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Invitation for Nominations for 1968 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 may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

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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.

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Tulane University School of Medicine
New Orleans, Louisiana 70112

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
Dame Harriette Chick
F. W. A. Clements
Sir David P. Cuthbertson
Herbert M. Evans
Joachim Kühnau
Joseph Masek
Toshio Oiso
H. A. P. C. Oomen
Lord John Boyd Orr
Conrado R. Pascual
V. N. Patwardhan
Sir Rudolph A. Peters
B. S. Platt
Emile F. Terroine
Jean Tremolieres
Eric John Underwood
Artturi I. Virtanen

Invitation for Nominations for the 1968 Hoblitzelle National Award in the *Agricultural Sciences*

The Hoblitzelle Award Committee of the American Institute of Nutrition invites nominations for the 1968 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 1968 Award must fall within the period January 1, 1963 through December 31, 1966. Final date for submission of nominations is November 15, 1967.

AIN Hoblitzelle Award Committee:

Orville G. Bentley, *Chairman*
R. W. Engel
R. G. Hansen
H. O. Kunkel

Send nominations to:

Dr. Orville G. Bentley
Dean of Agriculture
University of Illinois
Urbana, Illinois 61803