Lysine – Arginine Antagonism in the Chick [1]

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ABSTRACT When a casein-gelatin diet supplemented with excess L-lysine was fed to young chicks, it produced symptoms that included abnormalities in bone composition and reduced growth rate. Free amino acids were compared in tissues from chicks receiving the diet with added lysine (2%) and from controls. The most marked effects of the lysine were a reduction in arginine and an increase in lysine in tissues. Supplementary arginine in the diet eliminated the symptoms, increased tissue arginine concentration, and returned growth rate and bone composition to normal. A decrease in cellular K concentration, calculated either from chloride or from sodium space, was related only to dietary supplementation with lysine and not to growth rate. Chicks receiving 2 and 4% lysine added to a soy α-protein ration did not exhibit symptoms; plasma and muscle lysine concentrations increased, and arginine concentration decreased slightly in muscle only. Since these animals and those receiving the casein-gelatin diet supplemented with lysine and arginine had the highest tissue lysine concentrations, lysine per se is not toxic. A difference in amino acid composition of the basal diets does not appear to be the explanation for these observations. It is suggested that excess dietary lysine induces an arginine deficiency by decreasing the availability of arginine from casein or gelatin or from a combination of them.

Addition of excess L-lysine, either as the free base or as the monohydrochloride, to casein-gelatin diets depressed growth rate and caused toxicity symptoms when fed to one-day-old chicks for 14 to 21 days. Electrolyte distribution in muscle was altered and high levels of lysine were observed in the tissues of the animals receiving the supplemental lysine (1). A later report [2] demonstrated that the toxicity was due to a lysine-arginine antagonism that occurred in chicks receiving the casein-gelatin diet, but not in chicks receiving a crude stock or soy α-protein diet. The evidence for this antagonism between lysine and arginine is presented and discussed with reference to other reports in which high levels of lysine or of lysine-containing mixtures were fed to chicks.

EXPERIMENTAL

Cornish × White Rock chicks were distributed into uniform groups of 10 to 12 chicks each on the basis of body weight and housed in electrically heated batteries with feed and water supplied ad libitum. Single groups of chicks were used for the experiments shown in figures 1 and 2. Numbers of chicks used for determinations for the other experiments are noted in the appropriate tables. Except when indicated otherwise, all chicks were one day old when fed the test diets. The basal diet described by Jones (1) was used in most of the experiments. It contained: (in per cent) casein, 18; gelatin, 10; corn oil, 4; DL-methionine, 0.3; and minerals, vitamins, and sucrose to 100. In some experiments, this diet was modified to contain 26.9% soy α-protein with an added 0.15% glycine and 0.4% DL-methionine as the protein source. The amino acid composition of these diets is shown in table 1. Amino acids [3] replaced sucrose when added to the diets. When the complete commercial mash was used, added lysine replaced an equal amount of mash. L-Lysine at a supplemental level of 2% was used most frequently because it consistently caused predictable growth depression and symptoms in chicks without significantly reducing food consumption for the first 6 days.[4]

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[1] This investigation was supported in part by Research Grant no. A-6336 from the Public Health Service.

[2] Jones, J. D. 1962 Observations on the toxicity of lysine. Federation Proc., *21*: 1 (abstract).

[3] L-Lysine and L-arginine were added as monohydrochloride. L-Lysine hydrochloride and crystalline B vitamins were kindly furnished by Merck and Company, Rahway, New Jersey; L-arginine hydrochloride was kindly furnished by General Mills, Minneapolis, Minnesota.

[4] From an experiment in which graded levels of L-lysine were added to the casein-gelatin diet, the following data were recorded for the first 6 days the diets were fed: lysine, 0, 1.5, 2.0%; mean daily food consumption, 8.7, 8.3, 8.1 g; mean daily weight gain, 7.3, 6.1, 5.5 g.

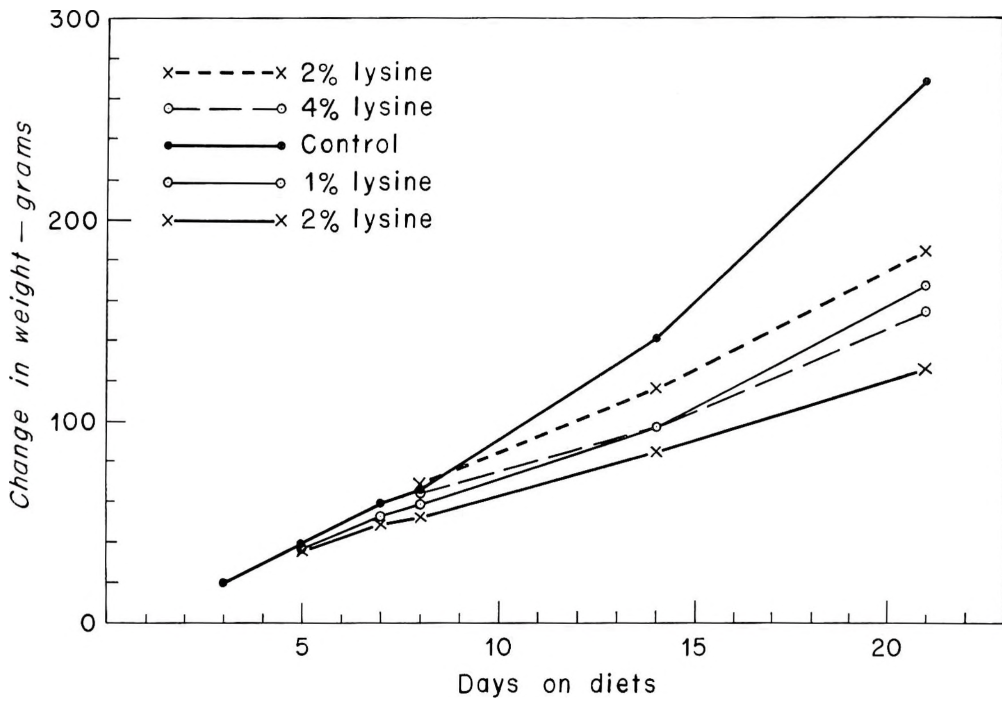

Fig. 1 Effect of added dietary lysine on growth of chicks as shown by growth curves of chicks fed at varying levels of lysine from zero to 21 days and from 8 to 21 days of age (broken lines).

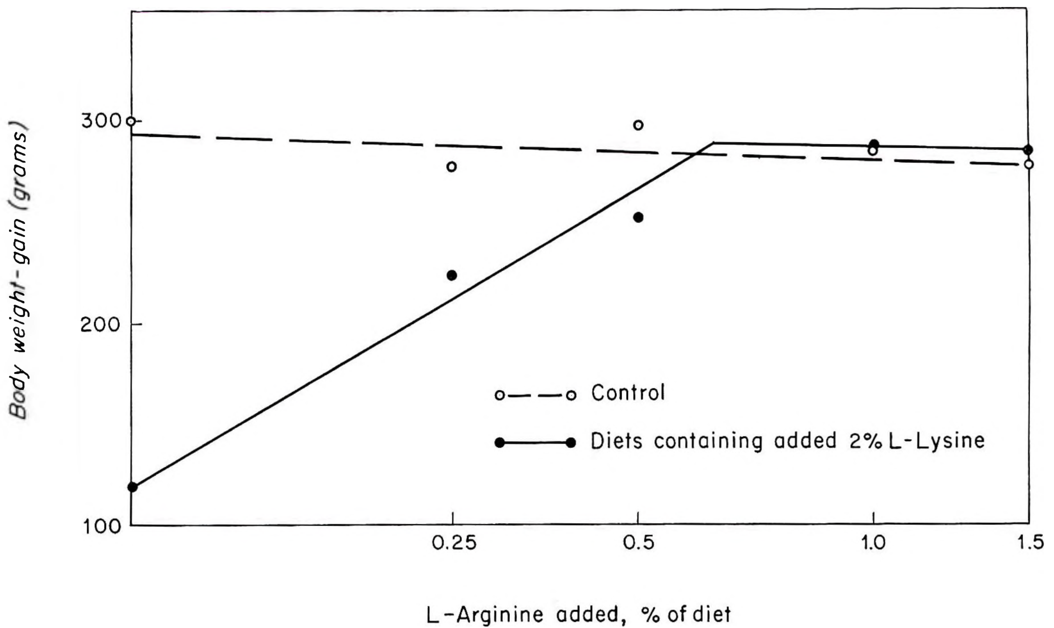

Fig. 2 Effect of graded levels of dietary L-arginine on growth of chicks fed casein-gelatin diets with and without 2% L-lysine. Regression equation for control curve is Y (body wt) = 294 — 11.0X (arginine as percentage of diet); slope of this line is not significantly different from 1.0.

TABLE 1

Amino acid composition of basal rations [1]

Amino acid	Ration [2]		
	Soy α-protein	Casein-gelatin	Required [3]
Lysine	1.06	1.65	1.0
Histidine	0.49	0.51	0.30
Arginine	1.44	1.30	1.2
Hydroxylysine	—	0.10	—
Aspartic acid	2.96	1.79	—
Threonine	0.82	0.90	0.6
Serine	1.24	1.28	—
Glutamic acid	4.57	4.60	—
Proline	1.21	3.11	—
Glycine	1.12	2.65	1.0
Alanine	0.97	1.56	—
Cystine	—	—	0.35 of methionine
Valine	1.12	1.28	0.8
Methionine	0.69	1.00	0.8
Isoleucine	1.17	1.03	0.6
Leucine	1.99	1.79	1.4
Tyrosine	0.99	1.05	0.7 of phenylalanine
Phenylalanine	1.32	1.03	1.4
Hydroxyproline	—	0.96	—

[1] Proteins were hydrolyzed with 6 N HCl in sealed evacuated tubes at 110° for 24 hours.

[2] Expressed as percentages of ration. Values include 0.15% glycine and 0.4% DL-methionine added to soy diet and 0.3% DL-methionine added to casein-gelatin diet.

[3] NRC requirement (2).

The data for controls obtained at the intervals noted were compared with those from similar groups of birds receiving the same diet supplemented with amino acid(s). Methods of sampling and analysis were those reported previously (1) with the following additional techniques: ion exchange chromatography of picric acid extracts of plasma and muscle and of protein hydrolysates was used for amino acid determinations (3); chloride in plasma and muscle was determined by the method of Keys (4); lipid was determined by loss in weight after exhaustive extraction of samples with $CHCl_3$:MeOH (3:1); and calcium and magnesium in tibias ashed at less than 480° were determined by chelometric methods. The gastrocnemius muscle was used in tissue analyses and is referred to simply as muscle in the text.

RESULTS AND DISCUSSION

To test the effect of age on susceptibility to excess dietary lysine, diets containing 1 and 2% L-lysine added to the basal casein-gelatin mixture were fed to one-day-old chicks and diets containing 2 and 4% lysine were fed to chicks which had received the basal ration alone for 8 days (fig. 1). The 8-day-old chick was less susceptible to excess lysine than the one-day-old bird. This was more evident in a similar experiment in which the birds receiving 2 and 4% lysine were fed the diets at 12 days of age. The total changes in body weight for 12 and 21 days were: control, 136 and 315 g; 2% L-lysine from day 1, 92 and 145 g; 2% L-lysine from day 12 to day 21, 138 and 274 g; and 4% L-lysine from day 12 to day 21, 132 and 232 g. This difference was also evident in that the ataxia and tremors that occur by 5 to 7 days, and the leg deformities that occur by 18 to 21 days in chicks receiving 2% L-lysine from day 1, were infrequent in any of the groups first fed the lysine-supplemented diet at 8 or 12 days of age. These intervals were chosen because it has been shown that the rapid changes in tissue electrolytes toward the mature pattern have occurred by this time (1).

Preliminary estimations of free amino acids in plasma and muscle of chicks fed the casein-gelatin basal and 2% lysine diets for 14 days were made by a manual ion exchange method (5). Addition of lysine to the diet increased the plasma and muscle lysine from 60 to 173 μmoles/100 ml of plasma and from 346 to 684 μmoles/100 g of muscle and decreased the plasma and muscle arginine from 20 to 7 μmoles/100 ml and from 101 to 32 μmoles/100 g, respectively. With the exception of a possible increase in plasma histidine and decrease in alanine and glycine, the concentrations of other amino acids were relatively unaffected. This observation plus the similarity of the symptoms described by Newberne et al. (6) in the arginine-deficient chick to those observed in the chick fed excess lysine and the dependence of the arginine requirement on protein source (7) suggested the following experiments: 1) supplementation of the diet containing added lysine with graded levels of arginine, and 2) determination of the effect of lysine added to different basal rations.

Casein-gelatin diet. Graded levels of L-arginine had no effect on growth when

added to the control diets, but eliminated visible symptoms and stimulated growth when added to diets containing added L-lysine (2%). A plot of weight gain verses the logarithm of percentage of added arginine yielded an extrapolated additional arginine requirement of 0.65% (fig. 2). A comparable value (slightly more than 0.5%) was obtained in a similar plot from data from another experiment (table 2, exp. B, lines 5 to 11).

The concentrations of free amino acids in the plasma and muscle of chicks fed diets supplemented with L-lysine or L-arginine, or both, for 24 days confirmed preliminary results that supplemental lysine, but not arginine, alters the plasma and muscle lysine concentration, whereas both alter the plasma and muscle arginine concentration (table 3). There are indications that supplemental lysine at a level of 2% may increase the concentration in plasma and muscle of other amino acids in addition to lysine — for example, proline and valine. An interpretation of these data and those obtained from chicks receiving both lysine and arginine (group 7) is not readily apparent. The variations observed between groups probably can be attributed to differences in food consumption or in growth rates, or in both. It is likely that the amino acids that occur in the diet in high concentrations and that are not the most active metabolically — such as proline and hydroxyproline — tend to accumulate in the tissues when diet consumption is increased, and that the increase in ornithine in those animals receiving supplemental arginine is a reflection of arginine degradation.

Two significant observations in this experiment are: 1) the highest plasma and muscle lysine concentrations were reached in group 7 in which arginine concentrations were returned to above normal by supplementation and in which there was normal growth; and 2) free amino acid concentrations in muscle are a direct reflection of plasma concentrations.

Analysis of bones demonstrated a significant difference in the lipid and ash content between controls and lysine-fed chicks (table 2, exp. A, lines 1 and 2). Although the weight gain was not affected by lysine added to the crude diet, the bone lipid content was significantly increased (table 2, exp. A, lines 3 and 4). The experiment, extended to include arginine as an additional variable, was repeated (table 2, exp. B, lines 5 to 11). Lysine supplementation again significantly increased the bone lipid content, decreased the bone

TABLE 2

Growth and bone composition of chicks fed diets supplemented with lysine, arginine or both

No.	Diet		Weight gain	Bones [1]					
	Lysine	Arginine		Dry weight	Lipid	Ash	Ash		
							Ca	Mg	P
	%	%	g	g	% dry wt	% dry wt	%	%	%
				Experiment A, 18 days					
1	0	0	170 ± 11 [2]	1.357 ± 0.077 [2]	17.8 ± 1.1 [2]	34.7 ± 5 [2]	—	—	—
2	2	0	84 ± 14	0.857 ± 0.062	30.1 ± 2.1	28.4 ± 0.8	—	—	—
3 [3]	0	0	145	1.114	16.0 ± 0.8 [4]	33.4	—	—	—
4 [3]	2	0	142	1.068	23.3 ± 0.2	31.9	—	—	—
				Experiment B, 24 days					
5	0	0	295 [5]	1.809 ± 0.086 [5]	20.3 ± 0.9 [5]	34.4 ± 0.4 [5]	35.7	0.68	16.9
6	2	0	108 [5]	0.635 ± 0.032 [5]	37.1 ± 1.6 [5]	26.4 ± 0.8 [5]	35.7	1.41	16.4
7	2	0.5	266	1.826 ± 0.136	17.0 ± 1.6	33.7 ± 0.5	34.6	0.74	17.3
8	2	1.0	275 [5]	—	—	—	—	—	—
9	0	0.5	304	1.633	17.3	35.8	34.8	0.68	16.9
10	0	1.0	296	1.657	15.8	35.1	35.2	0.76	16.3
11	2	1.5	297 [5]	1.576 ± 0.133 [5]	16.4 ± 1.3 [5]	34.6 ± 0.4 [5]	34.4	0.73	17.2

[1] All values are mean ± SD of at least 5 samples.
[2] Significant difference between diets 1 and 2.
[3] Crude commercial diet.
[4] Significant difference between diets 3 and 4.
[5] Significant difference between diets 5 and 6, 6 and 7, 6 and 8, and 6 and 11.

TABLE 3

Free amino acids in chicks fed casein-gelatin diets supplemented with lysine, arginine, or both

	Plasma						Muscle					
Treatment no.	1	2	3	5	6	7	1	2	3	5	6	7
L-Lysine added, %	0	2	2	0	0	2	0	2	2	0	0	2
L-Arginine added, %	0	0	0.5	0.5	1.0	1.5	0	0	0.5	0.5	1.0	1.5
	μmoles/100 ml		*μmoles/100 ml*		*μmoles/100 ml*		*μmoles/100 ml*		*μmoles/100 ml*		*μmoles/100 ml*	
Amino acid:												
Taurine	22 ± 3 [1]	33 ± 1.4	—	—	—	21 ± 6	451 ± 69	469 ± 38	—	—	—	1145 ± 45
Hydroxyproline	15 ± 1	13 ± 1	—	—	—	46 ± 3	71 ± 11	56 ± 6	—	—	—	169 ± 18
Aspartic acid	2 ± 0.3	5 ± 0.8	—	—	—	3 ± 0.7	128 ± 20	220 ± 24	—	—	—	80 ± 3
Threonine	47 ± 10	74 ± 15	—	—	—	66 ± 8	150 ± 22	217 ± 10	—	—	—	172 ± 15
Serine	51 ± 10	84 ± 4	—	—	—	90 ± 4	322 ± 51	428 ± 17	—	—	—	395 ± 27
Asparagine, Glutamine	35 ± 0	52 ± 11	—	—	—	63 ± 7	660 ± 136	834 ± 42	—	—	—	630 ± 45
Proline	28 ± 3	47 ± 9	—	—	—	185 ± 21	93 ± 11	126 ± 15	—	—	—	505 ± 65
Glutamic acid	9 ± 0.1	14 ± 1.4	—	—	—	19 ± 1	272 ± 30	379 ± 14	—	—	—	292 ± 28
Glycine	64 ± 3	77 ± 5	—	—	—	140 ± 22	546 ± 75	394 ± 34	—	—	—	744 ± 44
Alanine	33 ± 5	46 ± 2	—	—	—	61 ± 5	277 ± 30	288 ± 34	—	—	—	285 ± 12
α-Amino-*n*-butyric acid	4 ± 0.4	7 ± 0.7	—	—	—	4 ± 0.6	16 ± 0	77 ± 36	—	—	—	27 ± 1
Valine	17 ± 1	33 ± 4	—	—	—	31 ± 2	29 ± 2.6	39 ± 5	—	—	—	39 ± 2
Half cystine	2 ± 0.6	11 ± 2	—	—	—	3 ± 1.7	—	—	—	—	—	—
Methionine	5 ± 0.5	5 ± 0.6	—	—	—	9 ± 2	8 ± 1.7	8 ± 2.8	—	—	—	14 ± 2
Isoleucine	9 ± 2.2	15 ± 1.9	—	—	—	10 ± 0.8	14 ± 2.5	18 ± 0.9	—	—	—	11 ± 1
Leucine	13 ± 1.9	21 ± 1.7	—	—	—	20 ± 1	20 ± 2	26 ± 1.7	—	—	—	25 ± 3
Tyrosine	13 ± 2	19 ± 1.7	—	—	—	12 ± 1	24 ± 4	29 ± 2	—	—	—	20 ± 0
Phenylalanine	7 ± 1.1	10 ± 1	—	—	—	9 ± 0.9	12 ± 4	13 ± 1	—	—	—	14 ± 1
β-Alanine	—	—	—	—	—	—	27 ± 3	14 ± 2	—	—	—	27 ± 2
Ornithine	2 ± 0.6	2 ± 0.4	6 ± 0.3	4 ± 1.6	6 ± 3	14 ± 4	9 ± 0.6	7 ± 1.4	24 ± 1.9	23 ± 7	24 ± 13	61 ± 7
Lysine	54 ± 11	150 ± 8	211 ± 2	57 ± 2	74 ± 9	193 ± 40	427 ± 68	643 ± 84	1340 ± 14	351 ± 57	446 ± 87	1430 ± 20
Histidine	4 ± 0.7	6 ± 2.4	9 ± 0.6	6 ± 0.4	7 ± 1.7	8 ± 0.7	22 ± 3	30 ± 1	28 ± 4	27 ± 6	22 ± 5	25 ± 3
Anserine	—	—	—	—	—	—	450 ± 76	324 ± 18	328 ± 13	485 ± 75	441 ± 102	259 ± 2
Carnosine	—	—	—	—	—	—	138 ± 10	116 ± 58	68 ± 10	143 ± 18	101 ± 31	57 ± 14
Arginine	16 ± 1.7	5 ± 1.4	16 ± 3.5	26 ± 3.5	28 ± 8	26 ± 5	126 ± 8	21 ± 1	90 ± 28	184 ± 38	180 ± 31	170 ± 7
Body wt, mean increase, g [2]	295	108	266	304	296	297	—	—	—	—	—	—

[1] Values are means ± SD of 3 pooled samples of 5 chicks each, taken after feeding the diets for 24 days.

[2] For treatment 4 (2% lysine + 1.0% arginine), mean increase = 275 g.

weight and ash content, but did not alter the calcium, magnesium, phosphorus, sodium, or potassium content of the ash. Arginine at supplemental levels of 0.5 and 1.5% reversed the effects of lysine.

Other diets. Addition of lysine to a commercial chick-starting mash at levels of 2 and 4% did not produce symptoms and had less effect on average total body weight gain at 28 days (weight gain: 359, 352, and 267 g for controls, 2, and 4% lysine, respectively) than it did with the casein-gelatin diet. (Weight gains of chicks fed mash at 24 days were 272, 270, and 229 g for controls, 2, and 4% lysine, respectively; see also table 2, lines 3 and 4.)

When lysine was added to a soy α-protein diet at levels of 2 and 4%, the growth rate was not affected (table 4). The free amino acid concentrations in plasma and muscle were relatively unchanged except for an increase in plasma and tissue lysine and an apparent decrease in tissue arginine with increasing dietary lysine. This alteration of tissue arginine may be an indication of competition between these 2 amino acids at the tissue level. The highest plasma and tissue lysine levels observed to date occurred in this experiment but had no obvious adverse effects on the chicks, indicating that lysine per se is not toxic. Also, lysine did not appear to interfere with arginine utilization from soy α-protein.

Tissue electrolytes were measured in 2 experiments to determine whether the reported alterations in the sodium and potassium distribution caused by excess dietary lysine were due to a disturbed cellular-extracellular distribution and whether a normal distribution existed in the presence of high lysine and normal arginine levels in tissue. The derived data were calculated as described by Barlow and Manery

TABLE 4

Free amino acids [1] in chicks fed soy α-protein

	Plasma			Muscle		
L-Lysine added, %	0	2	4	0	2	4
	μmoles/100 ml			*μmoles/100 g*		
Amino acid:						
Taurine	17	22	13	—	—	—
Hydroxyproline	—	—	—	45	29	36
Aspartic acid	2	5	4	113	95	90
Threonine	22	30	23	84	88	67
Serine	83	96	65	603	828	492
Asparagine, Glutamine	78	97	83	1178	1100	1068
Proline	19	36	33	91	148	136
Glutamic acid	15	24	17	364	369	359
Glycine	37	51	47	327	318	241
Alanine	44	64	56	412	389	391
α-Amino-*n*-butyric acid	3	3	3	9	23	39
Valine	25	33	29	35	39	30
Methionine	6	9	11	12	15	17
Isoleucine	9	19	19	18	22	16
Leucine	18	26	20	25	24	19
Tyrosine	17	21	19	40	28	27
Phenylalanine	11	15	14	19	17	17
β-Alanine	—	—	—	26	14	11
Ornithine	3	5	4	11	17	12
Lysine	39	268	351	250	1570	1770
Histidine	10	13	13	45	47	44
Anserine	—	—	—	586	309	239
Carnosine	—	—	—	280	93	58
Arginine	22	17	21	116 [2]	102	80 [3]
Body wt, mean increase, g	169	166	162	—	—	—

[1] Values are means of 2 pooled samples of 5 chicks each, taken after the diets had been fed for 17 days.
[2] Values were 110 and 122.
[3] Values were 71 and 89.

(8) using both chloride and sodium as indexes of extracellular space.

Cellular Na concentration in breast and leg muscle apparently was increased by supplemental lysine; when arginine was also supplemented, cellular Na apparently was decreased to a value lower than the control values (table 5). Although it was a consistent result and would lead one to correlate increased cellular Na concentration with symptoms in chicks fed lysine added to the casein-gelatin diets, the cellular Na concentration was increased by lysine in the soy diet, and there was no adverse effect other than a slight reduction of growth rate. (The growth data obtained in these experiments were representative of those obtained at this interval in the longer experiments reported here.) Excess dietary lysine decreased cellular K concentration which did not return to normal when supplemental arginine was added to the casein-gelatin diet. Lysine consistently decreased cellular K concentration regardless of the effect on growth rate or other symptoms; furthermore, when cellular K concentration is calculated on the basis of Na space, the groups which had the largest reduction in cellular K were those least affected by the dietary lysine. Therefore, it is concluded that the symptoms caused by excess dietary lysine are not directly related to the distribution of K in the tissues. The relationship of symptoms to Na distribution remains undetermined.

TABLE 5

Electrolyte distribution in the young chick fed lysine or arginine or both

	Experiment A [1]				Experiment B [1]					
	Casein-gelatin diet				Casein-gelatin diet				Soy protein diet	
Supplemental L-lysine, %	0		2		0	2	0	2	0	4
Supplemental L-arginine, %	0		0		0	0	1.5	1.5	0	0
Gain in body wt, g	145		81		132	92	129	124	121	109
Composition of plasma:										
H_2O, g/liter	—		—		953	952	954	956	954	948
Na, mEq/liter	139.1		141.7		143.5	141.0	143.0	140.2	140.8	139.1
K, mEq/liter	4.4		4.6		4.3	4.4	3.9	4.4	4.7	4.7
Cl, mEq/liter	114.8		119.0		120.9	124.5	121.8	122.5	119.3	122.8
Composition of muscle:										
H_2O, g/kg wet wt	774		769		766	760	763	765	760	750
Na, mEq/kg wet wt	32.0		37.3		29.7	33.8	27.8	26.0	25.6	27.2
K, mEq/kg wet wt	92.2		84.7		107.1	101.7	108.3	100.7	110.3	94.5
Cl, mEq/kg wet wt	23.0		26.0		23.4	25.6	21.7	24.6	22.1	21.3
Data derived from average results given above: [2]										
$(H_2O)_E^{Cl}$, g/kg	187	115 [3]	203	147 [3]	180	192	165	188	172	161
$(H_2O)_c$, g/kg	588	634 [3]	566	622 [3]	586	568	599	578	588	589
$[Na]_c$, mEq/kg $(H_2O)_c$	10.7	7.4 [3]	15.7	10.4 [3]	7.0	12.5	7.8	0.2	2.9	8.5
$[K]_c$, mEq/kg $(H_2O)_c$	156	160 [3]	148	150 [3]	181	178	180	173	186	159
$[K]_c^{Na}$, mEq/kg $(H_2O)_c^{Na}$	148	159 [3]	145	148 [3]	176	176	178	160	177	154

[1] Values are means from determinations on 8 to 16 chicks after 12 to 13 days of receiving the diets.
[2] Distribution of electrolytes calculated from Cl space except $[K]_c^{Na}$ which was calculated from Na space.
[3] Values obtained from breast muscle in the same birds.

That a lysine-arginine relationship can be demonstrated in the chick appears to be a generally accepted premise.[5,6] However, a common interpretation of the cause and specificity of the relationship is not readily accepted. This is due in part to the differences in experimental approach. Dietary proteins and levels of protein and of supplemental amino acids have not always been comparable. Amino acid requirements, particularly of arginine, appear to be genetically determined (9) but breeds of chicks have not been comparable (the large variability noted in the experiments reported here can be attributed in part to this). Also, age of the chicks at the start of the experiments has varied from 1 to 7 days.

The dependence of arginine requirement on protein source in the absence of added lysine has been observed frequently. The relatively high concentration of lysine per se with respect to arginine in casein has been suggested as the reason for an increased requirement for arginine in chicks fed casein or casein supplemented with lysine or mixtures of amino acids containing lysine (10, 11). The suggestion that the levels of essential amino acids, specifically arginine and lysine, are more favorable in soy or plant proteins becomes less tenable because, when more than 2% L-lysine was added to a soy ration (which has an amino acid composition quite similar to that of the casein-gelatin diet), an arginine deficiency still was not induced as measured under the conditions of the present experiments. A low or suboptimal protein ration or one to which crystalline amino acids have been added to obtain desired levels may produce somewhat different conclusions. It appears that the casein-gelatin diet used here does furnish adequate arginine to the chick and that additional lysine acts in a specific manner that decreases the availability of the arginine: a portion of the analytically determined amino acid is not used by the animal. This is in contrast with amino acid imbalance which is caused by addition of a quantity of an unbalanced protein or amino acid mixture to a diet which is usually low in protein (12), whereas these diets contain adequate levels of protein.

The mechanism of this antagonism remains to be determined, but some of the possibilities include interference with digestion, absorption, degradation (or use of arginine for synthesis of another amino acid), or excretion. Lysine does not interfere significantly with the absorption of arginine because 1) low levels of supplemental arginine produce increased tissue levels of arginine and reversal of the adverse effects of lysine; and 2) little effect of lysine is noted in animals fed the soy protein ration. In contrast with the rat,[7] the chick apparently cannot synthesize arginine; however, the chick does continue to consume the ration that contains added lysine and, as a result, it is possible to show a depression of arginine in the tissues. The utilization of amino acids may be affected by the presence of a high concentration of lysine since the pattern of free amino acids in the tissue is altered somewhat even when the tissue levels of arginine are returned to normal by supplementation. However, a competition at the tissue level is not likely since the tissue levels directly reflect plasma levels.

A competition for reabsorption at the kidney is also an unlikely mechanism of the antagonism since the concentration of plasma lysine is greater in the chicks fed lysine-supplemented soy ration than in the chicks fed lysine-supplemented casein-gelatin rations, but plasma arginine remains normal in chicks fed the soy diets. Similar arguments may be advanced against an altered degradation of arginine that has been absorbed.

It was postulated, from experiments in which supplementation with glycine and arginine alleviated the growth depression caused by the addition of 4% L-lysine to diets containing plant proteins, that glycine and arginine function by enhancing the excretion of excess nitrogen (13). The relationship between arginine and glycine has not been investigated in this laboratory; however, glycine had no effect when fed to chicks receiving the casein-gelatin

[5] O'Dell, B. L., C. L. Limbaugh and J. E. Savage 1962 Arginine-lysine antagonism and deficiencies of casein for the chick. Federation Proc., *21:* 8 (abstract).

[6] Lewis, D. 1962 Arginine in metabolism and nutrition. Federation Proc., *21:* 7 (abstract).

[7] Unpublished data.

diet plus 2% lysine, an observation similar to that of O'Dell et al.[8] who also reported a response to added K that we have not been able to obtain (their diet contained 0.4% K and ours, 0.7%). Tissue levels of glycine were relatively unaffected by the addition of high levels of lysine to either soy or casein diets. Amino acids other than arginine and glycine have not been tested for their ability to reverse the effects of excess lysine.

It is suggested that excessive dietary supplementation with lysine induces an arginine deficiency by decreasing the availability of arginine from casein, from gelatin, or from a combination of these proteins.

LITERATURE CITED

1. Jones, J. D. 1961 Lysine toxicity in the chick. J. Nutrition, *73:* 107.
2. National Research Council, Committee on Animal Nutrition 1960 Nutrient requirements of domestic animals, no. 1. Nutrient requirements of poultry, pub. 827. National Academy of Sciences — National Research Council, Washington, D. C.
3. Spackman, D. H., W. H. Stein and S. Moore 1958 Automatic recording apparatus for use in chromatography of amino acids. Analyt. Chem., *30:* 1190.
4. Keys, A. 1937 Microdetermination of chlorides in biological materials: Presentation of method and analysis of its use. J. Biol. Chem., *119:* 389.
5. Moore, S., D. H. Spackman and W. H. Stein 1958 Chromatography of amino acids on sulfonated polystyrene resins: An improved system. Analyt. Chem., *30:* 1185.
6. Newberne, P. M., J. E. Savage and B. L. O'Dell 1960 Pathology of arginine deficiency in the chick. J. Nutrition, *72:* 347.
7. Krautmann, B. A., S. M. Hauge, E. T. Mertz and C. W. Carrick 1957 The ariginine level for chicks as influenced by ingredients. Poultry Sci., *36:* 935.
8. Barlow, J. S., and J. F. Manery 1954 The changes in electrolytes, particularly chloride, which accompany growth in chick muscle. J. Cell. Comp. Physiol., *43:* 165.
9. Nesheim, M. C., and F. B. Hutt 1962 Genetic differences among white leghorn chicks in requirements of arginine. Science, *137:* 691.
10. Fisher, H., R. Shapiro and P. Griminger 1960 Further aspects of amino acid imbalance, with special reference to the high arginine requirement of chicks fed casein diets. J. Nutrition, *72:* 16.
11. Dobson, D. C., J. O. Anderson and R. E. Warnick 1964 A determination of the essential amino acid proportions needed to allow rapid growth in chicks. J. Nutrition, *82:* 67.
12. Harper, A. E., and U. S. Kumta 1959 Amino acid balance and protein requirement. Federation Proc., *18* (no. 4): 1136.
13. Snetsinger, D. C., and H. M. Scott 1961 Efficacy of glycine and arginine in alleviating the stress induced by dietary excesses of single amino acids. Poultry Sci., *40:* 1675.

[8] See footnote 5.

Factors Affecting the Utilization of "Carbohydrate-free" Diets by the Chick

I. LEVEL OF PROTEIN [1]

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ABSTRACT An experiment was conducted to study the role which protein plays in enabling chicks to utilize "carbohydrate-free" diets in which non-protein calories are supplied by fat. Results showed that neither growth nor nitrogen retention was decreased when fat was substituted isocalorically for glucose in diets containing 13.2, 15.4, 17.6, 19.8 and 22.0 kcal/g of protein. These results show that the requirement of the chick for carbohydrate can be met without diverting amino acids from protein to carbohydrate synthesis, at least when protein is supplied by a mixture of soybean protein, methionine and glycine. Studies to determine the efficiency with which chicks utilized energy when fed high carbohydrate and "carbohydrate-free" diets showed that neither level of protein nor source of non-protein calories affected caloric efficiency or caloric consumption. These results show that lack of carbohydrate in the diet does not affect the ability of the chick to utilize triglyceride and shows that the chick has the ability to utilize large quantities of fat. The results also confirm the concept that chicks eat to meet their energy requirement.

Donaldson et al. (1) and Rand and associates (2) have shown that the growing chick can tolerate high levels of dietary fat if nutrient balance is maintained. More recently Hill and Renner [2] observed that fat can completely replace carbohydrate in the diet of the chick without affecting rate of growth. Since carbohydrate is required for maintaining level of blood glucose, for fatty acid oxidation and in the formation of various body constituents, the question arose as to whether chicks fed diets free of preformed carbohydrate were deriving carbohydrate from excess protein in the diet, from glycerol in triglycerides or whether the chick, like certain plants (3, 4) and microorganisms (5, 6), was capable of converting fatty acids to carbohydrate.

The present study was undertaken to determine the role which protein plays in enabling chicks to utilize "carbohydrate-free" diets. Comparisons were made of the rate of growth and efficiency of utilization of protein and energy when chicks were fed high carbohydrate and "carbohydrate-free" diets containing from 13.2 to 22.0 kcal of metabolizable energy per gram of protein.

EXPERIMENTAL

Male White Plymouth Rock chicks were used in the experiment and were maintained in electrically heated, thermostatically controlled battery brooders with raised wire-screen floors in a temperature-controlled laboratory. Ten chicks were assigned at random at one day of age to each experimental group, and duplicate groups were fed each of the experimental diets. Feed and water were supplied ad libitum.

The composition of the experimental diets calculated to contain 13.2 kcal of metabolizable energy per gram of protein is shown in table 1. The high carbohydrate diet contained glucose as the major source of non-protein energy. Its "carbohydrate-free" counterpart was formulated by replacing glucose isocalorically by lard, using the values 3.64 and 8.75 kcal/g for the metabolizable energy content of glucose and lard, respectively. Cellulose was added to improve the texture of the "carbohydrate-free" diet in an amount to maintain

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[2] Unpublished data.

TABLE 1
Composition of diets [1]

	High carbohydrate	"Carbohydrate-free"
	g	*g*
Variables		
Glucose [2]	62.62	—
Lard	—	27.97
Cellulose [3]	—	13.33
Constants		
Soybean protein [4]	26.79	26.79
Methionine	0.94	0.94
Glycine	0.74	0.74
Soybean oil	2.00	2.00
Limestone	1.49	1.49
Dicalcium phosphate	1.70	1.70
Sodium chloride	0.60	0.60
Mineral mixture [5]	1.51	1.51
Vitamin mixture [5]	0.60	0.60
Chromic oxide mix [6]	1.00	1.00
Antioxidant [7]	0.025	0.025

[1] Diets contained 13.2 kcal/g protein.
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[4] Promine R, Central Soya, Chemurgy Division, Chicago.
[5] Mineral and vitamin mixtures supplied: (in mg/100 g diet) KH_2PO_4, 930; $MgSO_4 \cdot 7H_2O$, 495; KI, 0.29; $FeSO_4 \cdot 7H_2O$, 28; $CuSO_4 \cdot 5H_2O$, 0.78; $ZnCl_2$, 12.5; $CoCl_2 \cdot 6H_2O$, 0.17; $Na_2MoO_4 \cdot 2H_2O$, 0.83; Na_2SeO_3, 0.022; $MnSO_4 \cdot H_2O$, 37; thiamine, 1.0; riboflavin, 1.0; Ca pantothenate, 4.0; biotin, 0.04; pyridoxine, 2.0; niacin, 8.0; folacin, 0.3; menadione, 0.3; vitamin B_{12}, 0.005; choline chloride, 210; vitamin A, 1000 USP units; vitamin D_3, 150 IC units; vitamin E, 3.31 IU; and chlortetracycline, 1.
[6] Contained 30% Cr_2O_3 in soybean protein.
[7] Santoquin, Monsanto Chemical Company, St. Louis.

the energy in a given volume equal to that of its high carbohydrate counterpart. The diets contained approximately 2.35 kcal/cm^3.

The other high carbohydrate diets calculated to contain 15.4, 17.6, 19.8 and 22.0 kcal/g of protein were formulated by replacing part of the protein mixture (soybean protein, glycine and methionine) in the high carbohydrate diet (table 1) isocalorically, by a mixture of glucose and lard. It was assumed that the protein mixture contained 3.83 kcal of metabolizable energy per gram. The corresponding "carbohydrate-free" diets were formulated by substituting lard isocalorically for glucose, and adding cellulose to maintain the energy in a given volume equal to that of its high carbohydrate counterpart.

Data on growth and feed consumption were obtained weekly and feed wastage was determined daily. In addition, during the fourth week of the experiment, excreta were collected from each experimental group at 24-hour intervals on 3 successive days for the determination of metabolizable energy. Chromic oxide was incorporated in each of the diets at a level of approximately 0.3% as an index substance to eliminate the need for quantitative collection of excreta and quantitative measurement of feed intake. The methods for processing excreta, conducting chemical analyses for moisture, nitrogen, combustible energy and chromic oxide. and computing metabolizable energy from these data have been described previously (7, 8). The differences between determined and calculated metabolizable energy values ranged from −1.5 to 2.6%.

At 28 days of age the chicks were starved for 16 hours to permit their gastrointestinal tracts to empty, and were killed without loss of blood. The entire carcasses from each experimental group were frozen, ground, mixed and an aliquot was dried by lyophilization. In order that tissue gains could be determined, a representative lot of chicks was killed at the beginning of the experiment and prepared for analysis in a similar fashion. Carcass samples were analyzed for protein, fat and moisture using the methods as described by Hill and Anderson (7).

RESULTS AND DISCUSSION

The effect of source of non-protein calories on the growth of chicks fed diets of different protein contents is shown by the data summarized in table 2. Analysis of variance (9) of factorial arrangement of treatments indicated the response to level of protein was significant ($P < 0.01$); however, the response to source of energy was not significant ($P > 0.05$). Analysis also showed the interaction between protein

TABLE 2
Growth of chicks fed diets with and without carbohydrate

Calories/g protein	Average weight, 4 weeks	
	High carbohydrate	"Carbohydrate-free"
kcal	*g*	*g*
13.2	468 [1]	476
15.4	477	432
17.6	416	413
19.8	384	415
22.0	349	364

[1] Values are averages of duplicate groups.

level and source of energy to be significant ($P < 0.01$). The data indicate that the isocaloric substitution of fat for carbohydrate decreased growth when the diet contained 15.4 kcal/g protein and increased growth when the diet contained 19.8 kcal/g protein. Subsequent studies (10) have shown that chicks fed "carbohydrate-free" diets containing 15.4 kcal/g protein in which non-protein energy is supplied by soybean oil grow at least as rapidly as chicks fed similar diets containing glucose. These results indicate that the protein requirement of chicks fed "carbohydrate-free" diets is no greater than chicks fed diets containing carbohydrate when rate of growth is used as the criterion. The results also show that the chick has the ability to utilize large quantities of fat. Calculations show that chicks fed "carbohydrate-free" diets consumed 69 to 82% of their total dietary calories as fat.

Data showing the average amounts of protein retained by chicks fed high carbohydrate and "carbohydrate-free" diets of different protein content are presented in table 3. The values shown are based on data obtained from carcass analysis. Analysis of variance (9) of the data on protein retention, taking into account the factorial arrangement of treatments, indicated that the response to level of protein was significant ($P < 0.01$), but that the response to source of energy was not significant ($P > 0.05$).

Since chicks fed "carbohydrate-free" diets utilize protein just as efficiently as chicks fed carbohydrate-containing diets, it can be concluded that the requirement of the chick for carbohydrate can be met without diverting amino acids from protein to carbohydrate synthesis at least when protein is supplied by a mixture of soybean protein, methionine and glycine.

TABLE 3

Protein retained by chicks fed diets with and without carbohydrate

Calories/g protein	Protein retained [1]	
	High carbohydrate	"Carbohydrate-free"
kcal	%	%
13.2	55 [2]	53
15.4	57	57
17.6	59	60
19.8	62	65
22.0	64	66

[1] (Gain in carcass protein, g/protein consumed, g) × 100.
[2] Values are averages of duplicate groups.

Munro (11) observed in reviewing the literature, that the isodynamic exchange of fat for carbohydrate in the diet of both adult and growing animals was followed by an increase in nitrogen excretion, which in some cases returned in a short time to levels obtained with the diet preceding the change. Failure to observe a period of decreased growth or nitrogen retention in chicks fed "carbohydrate-free" diets may be due to the fact that the chick embryo develops on a medium containing only 1% carbohydrate (12) and, therefore, does not have to adapt to a high fat, low carbohydrate diet.

Summarized in table 4 are data showing the energy consumption of chicks fed high carbohydrate and "carbohydrate-free" diets of different protein content. Energy consumption was calculated using metabolizable energy values determined for each diet. Analysis of variance (9) showed that neither level of protein nor source of energy affected energy consumption. Since the diets were of equal caloric density (2.35 kcal/cm^3) these data also indicate that equal volumes of feed were consumed.

The observation that chicks did not compensate for the reduced protein level of the diet by increased feed consumption is in agreement with results reported previously by Hill and Dansky (13) and lend support to the concept that chicks eat to meet their energy requirement. Results of this experiment are not in complete agreement with the concept proposed by Donaldson et al. (14) and Combs (15) that chicks increase their energy intake in an effort to obtain sufficient protein. Interpretation of the data depends upon whether energy consumption is viewed as an absolute quantity per chick or in relation to body size. In the present experiment, even though energy intake was relatively constant per chick for the period, it was considerably higher in the protein-deficient groups relative to body size, which apparently accounts for the increased fat deposition.

The failure of chicks to increase energy consumption when calories from glucose were replaced by calories from lard is in

TABLE 4

Utilization of energy by chicks fed diets with and without carbohydrate

Diet		Calories consumed [1]	Energy gained		Caloric efficiency [2]
Source of energy	Calories/g protein		Fat	Protein	
	kcal	*kcal*	*kcal*	*kcal*	
Glucose	13.2	2105 [3]	337	507	0.40
	15.4	2332	431	490	0.40
	17.6	2156	449	417	0.40
	19.8	2088	469	370	0.40
	22.0	1966	482	328	0.41
Lard	13.2	2294	408	509	0.40
	15.4	2084	353	436	0.38
	17.6	2054	442	393	0.41
	19.8	2183	482	393	0.40
	22.0	2078	469	344	0.39

[1] Metabolizable energy content of each diet was determined.
[2] Calories gained/calories of metabolizable energy consumed.
[3] Values are averages of duplicate groups.

contrast with results reported by Carew and associates (16) and may be due to the high level of fat in the diet or to the fact that the glucose-containing diet was already adequately supplemented with essential fatty acids. Evidence that the lack of response was not due to the type of fat fed has been furnished in subsequent studies [3] which showed that chicks fed diets in which non-protein calories were supplied entirely by soybean oil consumed the same number of calories as chicks fed diets containing 2% soybean oil in which the remaining non-protein calories were supplied by glucose.

Data on caloric efficiency (table 4), calculated using determined metabolizable energy values, show that chicks gained on the average 0.40 kcal for each kilocalorie of metabolizable energy consumed, irrespective of level of protein or source of non-protein energy. In agreement with Hill and Dansky (13) and Donaldson and associates (14), it was observed that chicks fed protein-deficient diets deposited more calories as fat. Since caloric efficiency was unaffected, it can be concluded that chicks utilized calories just as efficiently for fat synthesis as for growth. Whether these calories were provided by fat or carbohydrate was of no consequence in this study. In contrast, Forbes et al. (17) and Carew and Hill (18) observed that rats and chicks, respectively, utilized calories from fat more efficiently than calories from carbohydrate when fat was incorporated in the diet at lower levels. Whether the equality of calories from lard and glucose in the present study is the result of the high level of fat fed, awaits further study.

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LITERATURE CITED

1. Donaldson, W. E., G. F. Combs, G. L. Romoser and W. C. Supplee 1957 Studies on energy levels in poultry rations. 2. Tolerance of growing chicks to dietary fat. Poultry Sci., 36: 807.
2. Rand, N. T., H. M. Scott and F. A. Kummerow 1958 Dietary fat in the nutrition of the growing chick. Poultry Sci., 37: 1075.
3. Kornberg, H. L., and H. Beevers 1957 The glyoxylate cycle as a stage in the conversion of fat to carbohydrate in castor beans. Biochem. Biophys. Acta, 26: 531.
4. Marcus, A., and J. Velasco 1960 Malate synthetase in germinating seeds. Biochem. Biophys. Acta, 38: 365.
5. Wong, D. T. O., and S. J. Ajl 1956 Conversion of acetate and glyoxylate to malate. J. Am. Chem. Soc., 78: 3230.
6. Kornberg, H. L., and N. B. Madsen 1957 Synthesis of C_4-dicarboxylic acids from acetate by a "glyoxylate bypass" of the tricarbooxylic acid cycle. Biochem. Biophys. Acta, 24: 651.
7. Hill, F. W., and D. L. Anderson 1958 Comparison of metabolizable energy and productive energy determinations with growing chicks. J. Nutrition, 64: 587.

[3] Unpublished data.

8. Hill, F. W., D. L. Anderson, R. Renner and L. B. Carew, Jr. 1960 Studies of the metabolizable energy of grain and grain products for chickens. Poultry Sci., *39:* 573.
9. Snedecor, G. W. 1956 Statistical Methods. The Iowa State College Press, Ames.
10. Renner, R., and A. M. Elcombe 1964 Factors affecting the utilization of "carbohydrate-free" diets by the chick. II. Level of glycerol. J. Nutrition, *84:* 327.
11. Munro, H. N. 1951 Carbohydrate and fat as factors in protein utilization and metabolism. Physiol. Rev., *31:* 449.
12. Romanoff, A. L., and A. J. 1949 The Avian Egg. J. Wiley and Sons, Inc., New York.
13. Hill, F. W., and L. M. Dansky 1954 Studies of the energy requirements of chickens. 1. The effect of dietary energy level on growth and feed consumption. Poultry Sci., *33:* 112.
14. Donaldson, W. E., G. F. Combs and G. L. Romoser 1956 Studies on energy levels in poultry rations. 1. The effect of calorie-protein ratio of the ration on growth, nutrient utilization and body composition of chicks. Poultry Sci., *35:* 1100.
15. Combs, G. F. 1962 The interrelationships of dietary energy and protein in poultry nutrition. Nutrition of Pigs and Poultry. Butterworths, London, England.
16. Carew, L. B., Jr., M. C. Nesheim and F. W. Hill 1963 The relationship of dietary energy level and density to the growth response of chicks to fat. Poultry Sci., *42:* 710.
17. Forbes, E. B., R. W. Swift, R. F. Elliot and W. H. James 1946 Relation of fat to economy of food utilization. J. Nutrition, *31:* 203.
18. Carew, L. B., Jr., and F. W. Hill 1958 Studies of the effect of fat on metabolic efficiency of energy utilization. Poultry Sci., *37:* 1191.

Factors Affecting the Utilization of "Carbohydrate-free" Diets by the Chick

II. LEVEL OF GLYCEROL [1]

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ABSTRACT Experiments were conducted to study the role which glycerol plays in enabling chicks to utilize fatty acids in "carbohydrate-free" diets. Studies have shown that removal of glycerol from a "carbohydrate-free" diet, by substituting soybean fatty acids for soybean oil, results in a marked depression in growth rate. The amount of glycerol required for the maximal growth response of chicks fed diets containing 15.4 kcal/g protein in which non-protein energy is supplied by soybean fatty acids has been shown to be the amount required for theoretical conversion of fatty acids to triglyceride, i.e., 0.108 g glycerol/g fatty acids. Other studies have shown that glucose is at least as effective as glycerol in enabling chicks to utilize fatty acids. The amount of glucose required for the maximal growth response varied from one-third to the same amount required for theoretical conversion of fatty acids to triglyceride, i.e., 0.035 to 0.105 g glucose/g fatty acids.

Results of a previous study (1) have shown that calories from fat can completely replace calories from carbohydrate in the diet of the chick without altering growth rate or nitrogen retention, even when sub-optimal amounts of protein are fed. The present experiments were undertaken to determine the role which glycerol plays in enabling chicks to utilize "carbohydrate-free" diets. In addition, studies were conducted to determine the requirement for glycerol and to ascertain the effectiveness of glucose in meeting this requirement.

EXPERIMENTAL

Diets devoid of carbohydrate and of carbohydrate and glycerol were formulated from the high carbohydrate diet (table 1) by substituting either soybean oil or soybean fatty acids, isocalorically, for glucose, using the values 3.64, 9.21 and 8.65 kcal/g for the metabolizable energy content of glucose, soybean oil and soybean fatty acids, respectively.

Diets containing graded levels of glycerol or glucose were formulated from the high carbohydrate diet (table 1) by the isocaloric substitution of mixtures of glycerol and soybean fatty acids or glucose and soybean fatty acids for glucose. Glycerol was assumed to be completely absorbed and to have a metabolizable energy value of 4.32 kcal/g.

The diets were formulated to contain 15.4 kcal/g protein since previous work (1) had shown that at this ratio protein is present in sufficient quantities to promote rapid growth, but is not in excess. The caloric density of the experimental diets was maintained approximately equal to that of the high carbohydrate diet by the addition of cellulose.[2]

The soybean fatty acids were prepared by alkaline hydrolysis of crude degummed soybean oil. After acidification the fatty acids were separated from the acid seat by decantation and water-washed to remove acid and glycerol. Traces of water were removed by heating under reduced pressure in an atmosphere of nitrogen.

White Plymouth Rock or Dominant White × White Plymouth Rock male chicks were used. They were maintained in electrically heated, thermostatically controlled battery brooders with raised wire-screen floors in a temperature-controlled laboratory. Feed and water were supplied ad libitum. Data on growth and feed con-

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[2] Solka Floc S.W.-40-A, Brown Forest Products Limited, Montreal, Quebec.

TABLE 1

Composition of high carbohydrate diet

	%
Soybean protein [1]	23.59
Glycine	0.63
Methionine	0.81
Soybean oil	2.00
Limestone	1.49
Dicalcium phosphate	1.70
Sodium chloride	0.60
Mineral mixture [2]	1.50
Vitamin mixture [2]	0.52
Antioxidant [3]	0.025
Glucose [4]	67.14

[1] Promine R, Central Soya, Chemurgy Division, Chicago.

[2] Mineral and vitamin mixtures supplied: (in mg/100 g diet) KH_2PO_4, 930; $MgSO_4 \cdot 7H_2O$, 495; KI, 0.29; $FeSO_4 \cdot 7H_2O$, 28; $CuSO_4 \cdot 5H_2O$, 0.78; $ZnCl_2$, 12.5; $CoCl_2 \cdot 6H_2O$, 0.17; $Na_2MoO_4 \cdot 2H_2O$, 0.83; Na_2SeO_3, 0.022; $MnSO_4 \cdot H_2O$, 37; thiamine, 1.0; riboflavin, 1.0; Ca pantothenate, 4.0; biotin, 0.04; pyridoxine, 2.0; niacin, 8.0; folacin, 0.3; menadione, 0.3; vitamin B_{12}, 0.005; choline chloride, 210; vitamin A, 1000 USP units; vitamin D_3, 150 ICU; vitamin E, 3.31 IU; and chlortetracycline, 1.

[3] Santoquin, Monsanto Chemical Company, St. Louis.

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

sumption were obtained weekly and feed wastage was determined daily.

Experiments 1, 2, 3 and 4 were conducted using duplicate groups of 10 chicks/treatment. The chicks were reared to 4 days of age with a "carbohydrate-free" diet in which non-protein energy was supplied by soybean oil. They were then allotted on the basis of body weight to the experimental groups and fed the experimental diets to 28 days of age. A similar procedure was followed in experiment 5, except that chicks were reared to 7 days of age with the "carbohydrate-free" diet. In experiment 5, 3 replicate groups of 9 chicks were used in each treatment.

RESULTS

Summarized in table 2 are data showing the average weights, energy consumption and caloric efficiencies of chicks fed diets in which non-protein energy was supplied by glucose, soybean oil, soybean fatty acids and soybean fatty acids plus glycerol (exp. 1). The results show that growth of chicks was markedly depressed when glycerol was deleted from the "carbohydrate-free" diet by substituting soybean fatty acids for soybean oil. The addition of 0.108 g glycerol/g fatty acids, i.e., the amount required for theoretical conversion of fatty acids to triglyceride significantly increased growth ($P < 0.05$). The addition of twice this amount of glycerol had no further effect.

Chicks fed the diet in which non-protein energy was supplied by soybean fatty acids consumed fewer calories and utilized them less efficiently than when non-protein energy was supplied by soybean oil or glucose. The addition of 0.108 or 0.216 g glycerol/g fatty acids significantly increased energy consumption and caloric efficiency ($P < 0.05$).

Since the possibility existed that the reduced growth of chicks fed soybean fatty acids was due to failure to absorb this high level of fatty acids, fat absorbability was determined during the fourth week of life. Results showed the absorbability of soybean oil and soybean fatty acids to be 98 and 92%, respectively. Thus, inability to absorb free fatty acids was not the cause of the growth depression.

To determine the effect on growth of feeding glycerol at lower levels and to determine the effectiveness of glucose in enabling chicks to utilize fatty acids in "carbohydrate-free" diets, chicks were fed diets containing one-third, two-thirds, and the same amount of glycerol or glucose required for theoretical conversion of fatty

TABLE 2

Effect on growth of deleting glycerol from a "carbohydrate-free" diet

Source of energy	Avg wt, 4-week	Calories consumed	Calories consumed /g gain
	g	*kcal*	*kcal*
Glucose	464 [1]	2179	5.40
Soybean oil	490	2266	5.30
SFA [2]	298	1496	6.30
SFA + 0.108 g glycerol/g fatty acids	445	2064	5.38
SFA + 0.216 g glycerol/g fatty acids	453	2066	5.26

[1] Values are averages of duplicate groups.

[2] Soybean fatty acids.

acids to triglycerides (table 3). Analysis of variance and application of Duncan's multiple range test (2) to the data showed that growth was increased significantly ($P < 0.05$) by the addition of either 0.072 g glycerol/g fatty acids or 0.035 g glucose/g fatty acids. The addition of higher levels of glycerol or glucose did not cause any further significant increase in growth rate, although the difference in growth of chicks fed 0.108 g instead of 0.072 g glycerol/g fatty acids approaches significance. In agreement with results of the first experiment, growth of chicks fed diets containing 0.108 g glycerol/g fatty acids did not differ significantly ($P > 0.05$) from growth of chicks fed the diet containing soybean oil.

In contrast with the results of experiment 1, the utilization of energy by chicks in this experiment did not decrease significantly ($P > 0.05$) when soybean fatty acids replaced soybean oil as the source of non-protein energy.

Subsequent studies (exps. 3, 4 and 5), conducted to determine the glucose requirement of chicks fed diets in which non-protein energy was supplied by soybean fatty acids, have shown that the response to glucose is variable (table 4). Results show that the addition of 0.018 g glucose /g fatty acids (exps. 3 and 4) was ineffective in stimulating growth. In all experiments (exps. 3,4,5) the addition of 0.035 g glucose/g fatty acids resulted in a significant increase in growth ($P < 0.05$). Only in experiment 3 was a further significant growth response ($P < 0.05$) obtained from the addition of higher levels of glucose, i.e., 0.105 g glucose/g fatty acids.

Analysis of variance and application of Duncan's multiple range test (2) to data on caloric efficiency (table 4) showed that

TABLE 3

Response of chicks to glycerol and glucose

Treatment			Avg wt, 4-week	Calories consumed /g gain
Source of energy	Glycerol	Glucose		
	g/g SFA	*g/g SFA*	*g*	*kcal*
SFA [1]	—	—	343 [2]	5.44
SFA	0.036	—	371	5.41
SFA	0.072	—	413	5.44
SFA	0.108	—	450	5.24
SFA	—	0.035	432	5.32
SFA	—	0.070	428	5.32
SFA	—	0.105	420	5.20
Soybean oil	—	—	486	5.00
Glucose	—	—	439	5.17

[1] Soybean fatty acids.
[2] Values are averages of duplicate groups.

TABLE 4

Growth and energy utilization of chicks fed diets containing graded levels of glucose

Treatment		Experiment 3		Experiment 4		Experiment 5	
Energy source	Glucose	Avg wt, 4-week	Calories/g gain	Avg wt, 4-week	Calories/g gain	Avg wt, 4-week	Calories/g gain
	g/g SFA	*g*	*kcal*	*g*	*kcal*	*g*	*kcal*
SFA [1]	—	304 [2]	6.51 [2]	335 [2]	5.60 [2]	337 [3]	5.59 [3]
SFA	0.0099	307	6.29	—	—	—	—
SFA	0.018	335	6.07	338	5.63	—	—
SFA	0.026	361	5.70	—	—	—	—
SFA	0.035	379	5.84	425	5.22	399	4.99
SFA	0.105	437	5.33	400	5.44	400	5.16
SFA	0.210	—	—	460	5.44	423	4.93
Soybean oil	—	492	5.15	490	5.09	515	4.92
Glucose	—	—	—	447	5.59	514	5.25

[1] Soybean fatty acids.
[2] Values are averages of duplicate groups.
[3] Values are averages of triplicate groups.

in experiments 3 and 5 the utilization of energy by chicks fed diets in which non-protein energy was supplied by soybean fatty acids was less efficient than when diets containing soybean oil were fed. The addition of as little as 0.026 g glucose/g fatty acids to the diet containing soybean fatty acids resulted in significant increases in energy utilization.

DISCUSSION

The ability of the chick to utilize fatty acids in the absence of dietary carbohydrate and glycerol is limited. Studies have shown that deletion of glycerol from a "carbohydrate-free" diet by substituting soybean fatty acids for soybean oil decreased growth by approximately 35%. The addition of 0.108 g glycerol or 0.035 to 0.105 g glucose/g fatty acids permitted growth to increase to approximately 88% of that of chicks fed comparable diets containing soybean oil. The role which glycerol and glucose play in enabling chicks to grow when fed diets containing 15.4 kcal/g protein and in which non-protein energy is supplied by soybean fatty acids is unknown.

The efficiency of utilization of energy by chicks fed diets in which non-protein energy is supplied by soybean fatty acids is variable. Statistical treatment of the data on energy utilization in the 5 experiments has shown, however, that chicks fed diets in which non-protein energy is supplied by soybean fatty acids utilize energy less efficiently than when energy was supplied by soybean oil or soybean fatty acids plus the amount of carbohydrate required for theoretical conversion of fatty acids to triglycerides. Whether the energy is lost as heat or as excretory products requires further study.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Renner, R. 1964 Factors affecting the utilization of "carbohydrate-free" diets by the chick. I. Level of protein. J. Nutrition, *84:* 322.
2. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics with Special Reference to the Biological Sciences. McGraw-Hill Book Company, Inc., New York.

Effects of Dietary Moisture on the Determination of the Nutritional Value of Foods

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ABSTRACT A series of breakfast cereal, whole milk solids and sugar mixtures, and a dry dog food were fed, with and without the addition of water, to weanling rats for 28 days. The cereal mixtures, containing 11.3, 12.6 and 16.8% protein from corn-, wheat- and oat-based products, respectively, were rehydrated to a 68% moisture level which represents the moisture level consumed in a cereal and milk breakfast. The dog food (25.8% protein) was fed at 6, 24 and 54% moisture levels. The addition of water to the cereal and milk mixtures resulted in an increase in the amount of water consumption from the water bottles. No increase in the volume of drinking water was noted when water was added to the dog food. The increase in moisture of the cereal diet containing the lowest protein content resulted in significant increases in body weight gain and protein efficiency utilization. The addition of water to the other 2 cereal diets did not result in significant increases in body weight gain or protein efficiency utilization. The dog food with the highest moisture level gave significantly poorer body weight gains and protein efficiency utilization when compared with the dry product.

Keane et al. (1) in 1962 demonstrated that the addition of 20% of water to a purified diet of low moisture content resulted in increases in the body weight gains and protein efficiency ratios when rats were fed at dietary protein levels of 6 to 12%. This effect was not noted at the 18% protein level. In a second paper (2) the same group reported that this effect of moisture occurred when different sources of protein were used. However, when cornstarch was used in place of sucrose as the source of carbohydrates no significant increase in the protein efficiency was noted with the addition of 20% water.

Lepkovsky et al. (3) studied the effects of water intake and showed that the withholding of water during meals resulted in a reduction of food intake. Experiments by Harper and Spivey (4) suggested that the capacity of different carbohydrates to exert osmotic pressure can influence the growth rate of rats fed low protein diets.

The data presented in these publications indicate that the water content of the diet and the type of carbohydrate ingested, as well as other probable unknown factors, may have important effects on the growth rate and protein efficiency utilization of rats. The present study was carried out to determine the effects of the moisture content of the diet on the body weight gains and protein efficiency utilization with rats fed a series of diets containing natural foods rather than semi-purified rations. Breakfast cereal and milk combinations and a dry dog food were selected since these mixtures vary in protein content and usually promote good body weight gains in the growing rat.

EXPERIMENTAL

Groups of 10 rats each were fed diets containing breakfast cereal, milk and sugar combinations in the ratio of 28.4 g of cereal, 118 ml of whole milk and 8 g of sugar. For the "dry" diets whole milk solids[2] were substituted for fresh whole milk. These diets contained 55.8% cereal, 28.4% whole milk solids and 15.8% sugar.[3] The whole milk solids were rehydrated with distilled water to the moisture level in whole milk when the "wet" diets were fed. The dry diets contained 2 to 3% moisture and the wet diets, 68% moisture. Corn flakes, wheat flakes and oat flakes were the type of cereals selected for this study since they resulted in diets which differed considerably in protein con-

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[2] Parlac, The Borden Company, New York 10017.
[3] Domino 10X Sugar, American Sugar Refining Company, New York.

tent as shown in table 1. A dry dog food was fed at 3 moisture levels. This product was fed wet by adding one part of water per one part of food. This is the customary procedure used in feeding this product to dogs. In addition a lower level of water was added to the dog meal to result in a calculated level of 28% moisture.

The corn flakes, oat flakes, and wheat flakes products were commercial breakfast cereals. The protein in the corn cereal was derived from corn grits, that in the wheat flakes cereal from wheat, wheat flour and malted barley flour, and the oat product was made from oat flour with a small supplement of rice flour, soy flour and milk protein concentrate. Meat and bone meal, hominy feed, wheat flakes, soybean oil meal, and wheat flour were the major constituents of the dog food included in this study.

The animals were male weanling rats of the Wistar strain[4] weighing between 48 and 59 g at the beginning of the study. They were housed in individual raised-bottom wire cages in an air conditioned room kept at 22 to 25°. Water and food were given ad libitum throughout the 28-day test period. Fresh food was prepared each day for the animals fed the wet diets. The cereals were ground to a fine-particle size with a Fitzpatrick Mill before they were blended into the basal diets. When the water was added to the cereal diets the mixtures were homogenized in a Waring Blendor. The water consumption from the water bottle on each cage was recorded during the last 2 weeks of the test.

RESULTS AND DISCUSSION

The body weight gain and food utilization data are summarized in tables 2 and 3. It is shown that the addition of water to the cereal diets resulted in increases in body weight. The weight gain and protein efficiency data were subjected to analysis by the variance technique, and it was considered that a significant difference occurred when P was 0.05 or less. The corn flakes diet is the only one in which the increase in body weight gain was different at the 5% significance level.

[4] Charles River Breeding Laboratories, North Wilmington, Massachusetts.

TABLE 1
Analyses of test diets

Diets	Protein level[1]	Moisture level	
	"Dry" diets	"Dry" diets	Wet diets
	%	%	%
Corn flakes, whole milk solids, sugar	11.3	3.2	67.8
Wheat flakes, whole milk solids, sugar	12.6	2.3	68.1
Oat flakes, whole milk solids, sugar	16.8	2.0	67.5
Dog meal	25.3	6.8	54.1 (23.5)[2]

[1] Protein based on Kjeldahl nitrogen × 6.25 factor.
[2] Two levels of water added to dog food diet.

TABLE 2
Summary of body weight gain and food utilization data — cereal diets

	Body weight gains		Protein efficiency values[1]		Food efficiency values[2]	
	"Dry" diets	Wet diets	"Dry" diets	Wet diets	"Dry" diets	Wet diets
	g	g				
Corn flakes, milk, sugar	80.3 ± 3.5[3]	113.9 ± 6.1	2.42 ± 0.06	2.88 ± 0.06	0.283	0.336
Wheat flakes, milk, sugar	106.0 ± 4.5	113.4 ± 7.6	2.47 ± 0.07	2.63 ± 0.07	0.318	0.341
Oat flakes, milk, sugar	126.0 ± 9.7	142.9 ± 8.5	2.25 ± 0.11	2.34 ± 0.08	0.391	0.399

[1] Protein efficiency values calculated as grams of body weight gain per gram of protein consumed.
[2] Food efficiency values calculated as grams of body weight gain per gram of dry food consumed.
[3] Mean values ± SE for body weight gains and protein efficiency values.

When the protein efficiency values were compared there was a similar situation in that all the cereal diets showed an increase in protein efficiency utilization when water was added to the diets. Again the corn flakes diet is the only one showing a difference at the 5% significance level.

The dog meal gave a statistically significant poorer body weight gain value when the high moisture product was compared with the dry meal. Both of the dog foods with the higher moisture levels gave significantly lower protein efficiency values when compared with the dry product.

The food intake data presented in table 4 show that there was considerable increase in food intake when water was added to either the corn flakes or the dog meal. In the cereal diet series, the only significant increase in food consumption as a result of the water addition was noted with the corn flakes diet. When the dog food was fed, both levels of water resulted in significantly higher food consumption.

It is shown also that there was very little variation in water consumption on the basis of a gram of dry food intake when the dry diets were fed. Unexpectedly the "wet" cereal diets resulted in an increase in water consumption on the basis of a gram of dry food consumed. On the other hand, the addition of the water to the dog meal-type ration was not accompanied by an increase in fluid intake. The water intakes in this table include the moisture obtained from the food as well as the fluid from the water bottles on the cages.

Radford (5) compared the water balance in rats fed either commercial laboratory chow [5] or a semi-purified diet and his observations suggested that water intake was determined by the minimal urine water for solute excretion or by changes in fecal volume. The relatively high sucrose content of the cereal diets may have been a factor in the regulation of water intake due to its greater osmotic effect. However, Harper and Spivey (4) noted that rats consuming sucrose drank less water than those consuming dextrin as the source of carbohydrate. Cizek (6) reported that the total water intake increased in dogs when greater than 2 parts of water were added to one part of a dry dog food ration. In our study the cereal-milk mixtures were made by adding slightly more

[5] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

TABLE 3

Summary of body weight gain and food efficiency utilization data — dog food diets

Diet	Body wt gain	Protein efficiency value [1]	Food efficiency value [2]
	g		
"Dry" dog food	150.7 ± 6.5 [3]	1.36 ± 0.04	0.396
Dog food, 23.5% moisture	152.4 ± 7.5	1.02 ± 0.04	0.299
Dog food, 54.1% moisture	132.6 ± 5.7	1.04 ± 0.04	0.275

[1] Protein efficiency values calculated as grams of body weight gain per gram of protein consumed.
[2] Food efficiency values calculated as grams of body weight gain per gram of dry food consumed.
[3] Mean values ± SE for body weight gains and protein efficiency values.

TABLE 4

Food and water consumption data

Diet	Dry food consumed [1]		Water consumed/g solid intake [2]	
	"Dry" diets	Wet diets	"Dry" diets	Wet diets
	g	*g*	*ml*	*ml*
Corn flakes	284	339	1.4	2.4
Wheat flakes	333	333	1.5	2.4
Oat flakes	322	356	1.6	2.4
Dog meal	403	483 (509) [3]	1.5	1.4 (1.2) [3]

[1] Average values after 4-week feeding period.
[2] Water and food consumption measured during last 2 weeks of test.
[3] Values in parentheses obtained from diet adjusted to 23.5% moisture.

than 2 parts of water to one part of the dry cereal, with solids and sugar mixtures.

The data obtained in this experiment correlate rather well with the observations made by Keane et al. (1) in that the addition of moisture showed no beneficial effects on protein utilization at the higher dietary protein levels. There does not appear to be an obvious explanation for the poor performance of the animals fed the dog foods with the addition of water. None of the animals in these groups showed any evidence of abnormalities such as respiratory disease. A previous experiment had shown that a similar sample of dog food resulted in a considerable increase in food intake without significant effects on body weight gain or protein efficiency utilization when the moisture level was increased to 50%. The earlier study had been made with a different source of rats which did not grow as rapidly as the animals used in this study. Other unpublished experiments have indicated that, with either casein or a dry dog food as the source of dietary protein, there were poorer body weight gains at high dietary protein levels. It may be that the considerable increases in food intake with the wet dog food diets gave an increase in protein consumption which in turn could cause a retardation of body weight gain and protein efficiency utilization. Additional investigation would be necessary to determine this.

It appears, therefore, that, when the rat is used in nutritional value studies, the moisture level of the diet may have an important effect on nutritive value when body weight gain and protein efficiency utilization are used as a criterion of nutritional adequacy of the diet. This effect appears to be present when either a mixture of natural foods or a semi-purified diet is fed.

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LITERATURE CITED

1. Keane, K. W., C. J. Smutko, C. H. Krieger and A. E. Denton 1962 The addition of water to purified diets and its effect upon growth and protein efficiency ratio in the rat. J. Nutrition, *77:* 18.
2. Keane, K. W., C. J. Smutko, C. H. Krieger and A. E. Denton 1963 Water addition to purified diets. II. Effects of water and carbohydrate on protein efficiency ratio in rats. J. Nutrition, *81:* 87.
3. Lepkovsky, S., R. Lyman, D. Fleming, M. Nagumo and M. M. Dimick 1957 Gastrointestinal regulation of water and its effect on food intake and rate of digestion. Am. J. Physiol., *188:* 327.
4. Harper, A. E., and H. E. Spivey 1958 Relationship between food intake and osmotic effect of dietary carbohydrate. Am. J. Physiol., *193:* 483.
5. Radford, E. P., Jr. 1959 Factors modifying water metabolism in rats fed dry diets. Am. J. Physiol., *196:* 1098.
6. Cizek, L. J. 1959 Long term observations on relationship between food and water ingestion in the dog. Am. J. Physiol., *197:* 342.

Electrophoretic Study of the Effects of Changes in Dietary Protein Concentration and of DL-Ethionine on the Soluble Proteins of Rat Liver

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ABSTRACT Measurements, by means of electrophoresis, of the concentrations of soluble proteins in rat liver indicate that the concentrations of these materials vary with the protein concentration of the diet, but the change in concentration is not the same for all liver proteins. One group of proteins of relatively low mobility was observed in the same concentration in the livers of rats receiving either a high protein diet or a low protein diet. These proteins occurred in the mitochondria-plus-microsomes fraction and in the cell sap. The proteins which did change in concentration had greater electrophoretic mobility than the above group. They were derived from the mitochondria-plus-microsomes and from the nuclei. The cell sap contained proteins of similar mobilities but these proteins did not change in concentration as the dietary protein concentration changed. The effect of feeding DL-ethionine on the concentrations of soluble liver proteins was qualitatively very similar to the effect produced by feeding a low protein diet.

Kosterlitz (1) showed that the concentration of total liver protein in rats varies with the level of dietary protein and that the change results from gains or losses of whole cytoplasm, both particulate and interparticulate matter being affected; the number of liver cells does not change. Essentially in agreement with these observations are the results of Luck (2) who reported the albumin, pseudoglobulin, euglobulin and nucleoprotein fractions of rat liver to vary in concentration with the dietary protein concentration; the change in concentration was nearly the same for all fractions.

The rather uniform changes in the concentrations of the various liver protein fractions, as implied by the above results, do not apply to the liver enzymes (3). The concentrations of some liver enzymes, namely, apyrase, cytochrome c, cytochrome oxidase, glycylglycine peptidase and glutamic-oxaloacetic transaminase, do not change when the level of dietary protein is decreased. However, the concentration of some liver enzymes decreases under the same conditions, namely, ATPase, catalase, choline oxidase, cytochrome c reductase, succinoxidase, glutamic-pyruvic transaminase, uricase and xanthine oxidase.

It seemed worthwhile, therefore, to reinvestigate this problem with the use of electrophoresis. By this means it was possible to demonstrate a selective retention and a selective loss of liver proteins when rats were fed a low protein diet.

Studies of the electrophoretic properties of rabbit and rat liver proteins have been reported by Sorof and Cohen (4, 5) and by Anderson (6).

EXPERIMENTAL

Two groups of 8 female, albino rats[3] having body weights of about 100 g were fed complete, purified diets (7)[4] with casein concentrations of 8 and 30% for 18 to 34 days. After decapitation of the

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[3] Obtained from The Charles River Breeding Laboratories, North Wilmington, Massachusetts.

[4] The diet contained per 100 g diet: (in grams) casein (General Biochemicals Inc., Chagrin Falls, Ohio), 30 (high protein diet) and 8 (low protein diet); hydrogenated cottonseed oil (Crisco, Procter and Gamble, Cincinnati), 4; cottonseed oil (Wesson Oil, The Wesson Oil Co., New Orleans, La.), 2; cod liver oil, 1; salt mixture (Phillips, P. H., and E. B. Hart, J. Biol. Chem., *109:* 657, 1935), 4; glucose, 59 (high protein diet) and 81 (low protein diet); and (in milligrams) choline chloride, 100.0; niacin, 2.5; Ca pantothenate, 1.0; thiamine·HCl, 0.4; riboflavin, 0.4; pyridoxine·HCl, 0.4; biotin, 0.1; folic acid, 0.2; and (in micrograms) vitamin B_{12}, 0.1.

animal, the liver was perfused *in situ* with cold 0.9% saline (8) to remove residual blood. It was then excised, blotted free of excess saline, and after weighing, was minced and homogenized in a chilled, glass Potter-Elvehjem homogenizer with 3.0 ml of cold veronal buffer (pH 8.6, ionic strength 0.1) (9) per g of liver. The homogenate was dialyzed in a nitrogen atmosphere at 5° against 1,200 ml of the buffer containing 500 mg of cysteine·HCl neutralized to pH 8.6 with 1 M KOH. After 10 to 12 hours, a fresh supply of buffer mixture was substituted and the dialysis continued for an additional 12 hours (4).

The dialyzed homogenate was centrifuged at 0° for 30 minutes at 80,000 × *g*. The clear, amber supernatant liquid was collected and used for the electrophoresis run which was made in an electrophoresis-diffusion apparatus [5] using a micro cell of 2-ml capacity. A current of 4.0 ma was applied for 180.0 minutes.

A second series of determinations was made as above except that the perfused livers were divided; one part was treated as before and the remainder was homogenized with 9 parts of cold, isotonic sucrose to isolate the cell fractions. The nuclei and debris were removed by centrifuging at 0° for 10 minutes at 500 × *g*. This precipitate was washed twice in 2.5 volumes of the sucrose solution and the sucrose supernatant solutions were combined and centrifuged at 0° for one hour at 105,000 × *g*.

The sediment, consisting of mitochondria plus microsomes, was homogenized in 3.0 ml of veronal buffer per gram based on the original weight of liver. The final sucrose supernatant solution, representing the soluble fraction of the liver cells (cell sap), was lyophilized and the residue dissolved in the same volume of buffer as used for the mitochondria-plus-microsomes fraction. Dialysis and electrophoresis of both cell fractions were carried out as above.

A third series of determinations was made using 2 groups of rats as above; one group was fed the 30% casein diet and the other was fed the same diet to which DL-ethionine was added (0.33 g/100 g of diet). These animals were fed the diets

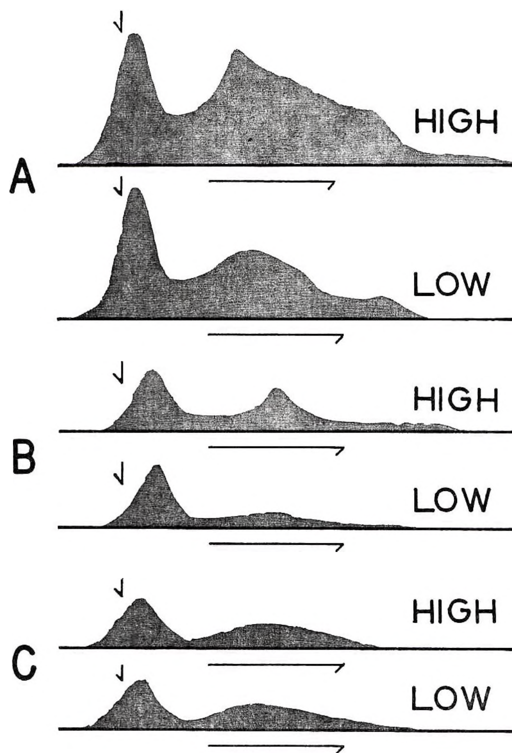

Fig. 1 Representative tracings of descending boundaries (Schlieren patterns) obtained by electrophoresis of extracts of soluble liver proteins from rats fed a high protein (30% casein) diet and a low protein (8% casein) diet: A, extracts from whole homogenate; B, extracts of mitochondria-plus-microsomes; C, cell sap. The vertical arrows indicate the position of the boundary at the start of the experiment; the horizontal arrows indicate the direction of migration.

for 24 days and were then treated as in the first series.

Representative tracings of the Schlieren patterns of the descending boundaries from typical electrophoresis runs are shown in figure 1 for the animals fed high and low protein and in figure 2 for the ethionine-fed rats and their controls.

The photographs of such boundaries were enlarged 2.75 times by projecting the image onto a paper and tracing around it. The tracings were divided into 2 distinct areas by drawing a perpendicular line to the base from the minimum between the 2 major peaks. The area of faster migrating components was obviously inhomo-

[5] Model H. Spinco Division, Beckman Instruments, Inc., Palo Alto, California.

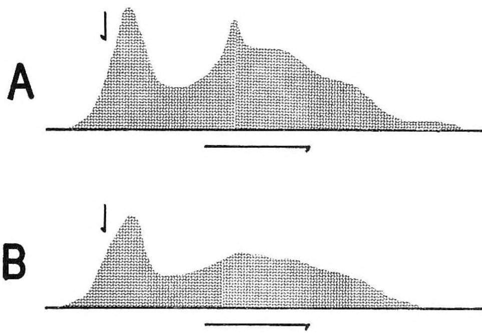

Fig. 2 Representative tracings of descending boundaries (Schlieren patterns) obtained by electrophoresis of extracts of soluble proteins prepared from whole homogenates of liver from: A, control rats fed a 30% casein diet; B, rats fed the same diet plus 0.33 g DL-ethionine/100 g of diet. The vertical arrows indicate the position of the boundary at the start of the experiment; the horizontal arrows indicate the direction of migration.

geneous; the area of slower migrating components consisted of a single peak which undoubtedly represented a mixture of proteins with similar mobilities. The total area of each tracing and the area under the peak representing the slower moving components were measured with a planimeter; the area of the faster moving components was obtained by difference.

RESULTS

The results of these measurements and other pertinent data are shown in table 1 for the animals fed high and low protein, and in table 2 for the animals fed ethionine, and their controls.

The liver weights of the rats fed the 30% casein diet were greater than the liver weights of those animals fed the 8% casein diet. However, the ratios of liver weight to body weight were the same for both groups.

From the total areas of the electrophoresis patterns it may be concluded that the extractable proteins are in a higher concentration in the livers of the rats fed the 30% casein diet. These observations are consistent with those of Kosterlitz (1).

The difference in concentrations of extractable protein in the livers of the animals fed high and low protein occurred only among the faster migrating components; the concentrations of slower migrat-

TABLE 1

Soluble protein concentrations in livers of rats fed high and low protein diets

Diet [1]	Wt gain	Relative liver wt	Electrophoretic analysis: Whole homogenate		Mitochondria + microsomes		Cell sap	
			Slow area [2]	Fast area	Slow area	Fast area	Slow area	Fast area
	g/rat/day	*g/g body wt*						
High protein	2.7 ± 0.2 [3] (12) [4]	0.050 ± 0.003(11)	133 ± 3.9(10)	396 ± 16 (10)	62 ± 3.9(5)	126 ± 10 (5)	71 ± 12(4)	161 ± 29(4)
Low protein	1.5 ± 0.1 *(11)	0.048 ± 0.002(12)	128 ± 4.0(10)	290 ± 14 *(10)	70 ± 3.6(6)	65 ± 4 * (6)	77 ± 10(5)	142 ± 19(5)

[1] Diet consumptions for the 2 groups of animals: high protein, 13.1 ± 0.8(8) g/rat/day; low protein, 13.3 ± 0.8(7) g/rat/day.
[2] Areas are expressed in units read from the planimeter.
[3] Mean ± SE.
[4] Number of determinations is shown in parentheses.
* Denotes significance at $P \leq 0.01$; Fisher's test.

TABLE 2

Soluble protein concentrations in livers of rats treated with DL-ethionine

Rats [1]	Wt gain	Relative liver wt	Electrophoretic analysis, Whole homogenate	
			Slow area [2]	Fast area
	g/rat/day	*g/g body wt*		
Control	3.52 ± 0.2 [3] (8) [4]	0.045 ± 0.001(8)	132 ± 9(8)	356 ± 22(8)
Ethionine-treated	2.41 ± 0.1 (8) *	0.055 ± 0.003(8) *	108 ± 6(7) **	208 ± 23(7)*

[1] Diet consumptions for the 2 groups of animals: control, 8.9 ± 0.5(4) g/rat/day; ethionine treated, 7.4 ± 0.7(4) g/rat/day.
[2] Areas are expressed in units read from the planimeter.
[3] Mean ± SE.
[4] Number of determinations is shown in parentheses.
* Denotes significance at $P \leqq 0.01$; Fisher's test.
** Denotes significance at $P = 0.05$; Fisher's test.

ing components were not influenced by the concentration of dietary protein. Similarly, only the faster migrating components of the mitochondria-plus-microsomes fractions were affected by the change in dietary protein concentration. This difference in extractable protein concentrations in the mitochondria-plus-microsomes fractions accounts for 58% of the total difference of protein concentrations in the liver as observed in the extracts from whole homogenates.

Comparisons of electrophoretic mobilities indicated that probably the slow moving components of the mitochondria-plus-microsomes fractions and of the soluble supernatant fractions contributed to the slow moving components observed in the patterns of extracts from the whole homogenates. Furthermore, the sum of the concentrations of these components in the extracts of the cell fractions equaled the concentration of slow moving components in the extracts from the whole homogenates for animals fed both the high and low protein diets. It may be concluded therefore that the slow moving components in the extracts of the whole homogenates are largely derived from the mitochondria, microsomes and cell sap.

About 70% of the fast-moving proteins of extracts of whole homogenates are derived from the mitochondria, microsomes and cell sap. The remaining 30% are presumably derived form the nuclear fraction. Since 58% of the difference in concentrations of extractable proteins, observed between extracts from whole homogenates of the livers of animals fed the high and low protein, was accounted for by the difference in concentrations of fast-moving components derived from the mitochondria-plus-microsomes fractions, it may again be presumed that the remaining 42% of the difference is due to a difference in concentrations of proteins which are extractable from the nuclei.

Ethionine feeding resulted in a depression of growth rate and in a significant increase in relative liver weight (table 2). Since female rats were used in these experiments, this increase in relative liver weight is probably due to fat accumulation in the liver (10).

Electrophoresis of extracts from whole liver homogenates prepared from ethionine-fed rats revealed a decrease in the concentration of both the slow-moving and fast-moving components. The 18% decrease in the former can be accounted for on the basis of the 18% increase in relative liver weight attributable to fat accumulation; the decrease in the concentration of the fast-moving components is too large to be explained in this way and must be due to an effect of the ethionine on liver protein metabolism.

In pilot studies, the soluble liver proteins, extracted from the whole liver homogenate as above, were separated into albumin, globulin and nucleoprotein fractions. Electrophoresis of these materials indicated that no specific area of the electrophoresis patterns was associated with any one of these classes of proteins.

DISCUSSION

The concentration of soluble protein in liver decreased when the rat was fed a low protein diet and the decrease was due primarily to a loss of the fast-moving proteins from the mitochondria, microsomes and presumably the nuclei. The concentrations of extractable proteins were approximately the same in the cell sap derived from the livers of animals fed the high and low protein.

It is concluded that the difference in protein concentrations in the livers of the 2 groups of rats cannot be due to differences in whole liver cytoplasm. The observation that there is a group of soluble proteins of relatively low electrophoretic mobility, which is in the same concentration in the livers of animals fed high and low protein, is further evidence that the difference in liver protein concentrations in the 2 groups of animals is not due to a difference of whole cytoplasm. This group of proteins is derived from the cell sap and the mitochondria- plus-microsomes but apparently not from the nuclei.

Since the effect of feeding ethionine was qualitatively similar to the effect produced by feeding the low protein diet, it is reasonable to conclude that the administered ethionine blocked the synthesis of liver proteins. This conclusion is consistent with the observation (11) that ethionine is a metabolic antagonist of methionine.

LITERATURE CITED

1. Kosterlitz, H. W. 1947 The effects of changes in dietary protein on the composition and structure of the liver cell. J. Physiol. (London), *106:* 194.
2. Luck, J. M. 1949 The liver proteins. Cold Spring Harbor Symposia on Quant. Biol., *14:* 127.
3. Knox, W. E., V. H. Auerbach and E. C. C. Lin 1956 Enzymatic and metabolic adaptations in animals. Physiol. Rev., *36:* 164.
4. Sorof, S., and P. P. Cohen 1951 Electrophoretic studies of the soluble proteins of rabbit liver. J. Biol. Chem., *190:* 303.
5. Sorof, S., and P. P. Cohen 1951 Electrophoretic and ultracentrifugal studies of soluble proteins of rat livers. J. Biol. Chem., *190:* 311.
6. Anderson, N. G. 1957 Labile colloidal complexes of the cytoplasm. J. Cell. Comp. Physiol., *49:* 221.
7. Higgins, E. S., D. A. Richert and W. W. Westerfeld 1956 Molybdenum deficiency and tungstate inhibition studies. J. Nutrition, *59:* 539.
8. Price, J. M., E. C. Miller and J. A. Miller 1948 The intracellular distribuiton of protein, nucleic acids, riboflavin, and protein-bound aminoazo dye in the livers of rats fed *p*-dimethyaminoazobenzene. J. Biol. Chem., *173:* 345.
9. Longsworth, L. G. 1941 The influence of pH on the mobility and diffusion of ovalbumin. Ann. N. Y. Acad. Sci., *41:* 267.
10. Farber, E., M. V. Simpson and H. Tarver 1950 Studies on ethionine. II. The interference with lipid metabolism. J. Biol. Chem., *182:* 91.
11. Stekol, J. A., and K. Weiss 1949 A study on growth inhibition by D-, L-, and DL-ethionine in the rat and its alleviation by the sulfur-containing amino acids and choline. J. Biol. Chem., *179:* 1049.

Passage of Selenium across the Placenta and also into the Milk of the Dog [1]

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ABSTRACT After a single, subtoxic, subcutaneous injection of radioactive selenium, as selenious acid, selenium passed through the mammary glands into the milk of the dog. The administered inorganic selenium was converted into organoselenium in the milk, because nearly all the Se^{75} was present in the proteins of the milk. It was observed that selenium was distributed equally between casein and milk serum proteins. At the time of the second pregnancy, the radioactive isotope was still detectable in the bitch and appeared in the milk when lactation began after the second pregnancy. Selenium-75 was transported across the placental membrane, since it was observed in the pups delivered by Caesarean section.

Selenium may be eliminated from the animal body through the kidney, gastrointestinal tract, lung and mammary glands into the milk of lactating animals. Several investigators have reported that selenium was present in the milk of domestic animals (1–3), and in the rat (4). Since it is known that selenium readily incorporates into tissue proteins (5–8), the question arises whether selenium is incorporated also into the milk proteins of the dog. Our results indicate that selenium was present in the various protein fractions of dog milk.

Placental transfer of selenium has been demonstrated (9, 4). In a concurrent experiment designed to obtain quantitative information on the transport of Se^{75} across the placental membrane, a pregnant dog previously injected with $Se^{75}O_3^{=}$ was delivered by Caesarean section, and the pups were assayed for Se^{75} activity. It was found that Se^{75} passes the placental barrier and is incorporated into various tissues of the pups. A preliminary report of these experiments was made in a recent review (10).

EXPERIMENTAL PROCEDURE

Lactating dogs were injected subcutaneously with $H_2Se^{75}O_3$ in amounts shown in table 1. Milk samples were collected at various intervals from 2 hours throughout 2 successive lactation periods. Radioactive determinations were made on whole milk, whole blood, plasma and red blood cells. The milk was centrifuged for 30 minutes at 40°, to separate the fat and the skim milk (11). The skim milk was fractionated into casein and milk serum by isoelectric preciptation of the casein at pH 4.6. The milk fractions were then assayed for Se^{75} radioactivity (7). A counting efficiency of 43% was obtained. Aliquots of whole milk, skim milk and the milk serum were treated with 10% trichloroacetic acid and the protein precipitate was washed twice to give the protein-bound selenium values. The 5 pups from dog no. 744 (first litter) were nourished with the mother's milk and were killed at various intervals over a 2-month period, following which the tissues were assayed for Se^{75}. The dogs treated with Se^{75} were bred again without further injection of the isotope (table 1). The second litter produced 2 pups each from dogs no. 744 and no. 863 which were delivered by Caesarean section. Milk and blood samples of the bitch and the tissues of the pups were assayed for Se^{75}. Tissue Se^{75} analysis of the pups from dog no. 744 only was made.

RESULTS AND DISCUSSION

Figures 1 and 2 show the amounts of Se^{75} in the whole milk throughout the 2 lactating periods of dogs no. 863 and no.

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TABLE 1

Experimental details

	Subcutaneous injection $H_2Se^{75}O_3$		Mode of birth and days after injection	No. pups	Days pups killed after birth	Final milk Se^{75} assay time
	mc	*μg Se*				*days*
			Dog no. 863			
1st Pregnancy [1]	0.50	35	natural	3 [2]	at birth	
2nd Pregnancy	nil	nil	Caesarean, 193 days	2	at birth	220
			Dog no. 744			
1st Pregnancy [1]	0.64	7	natural	5	12 days(pup 1) 12 days(pup 2) 23 days(pup 3) 46 days(pup 4) 52 days(pup 5)	
2nd Pregnancy	nil	nil	Caesarean, 235 days	2	0 days(pup 1) 22 days(pup 2)	278

[1] Injected one day after parturition.
[2] Three pups from a 9-pup litter allowed to suckle.

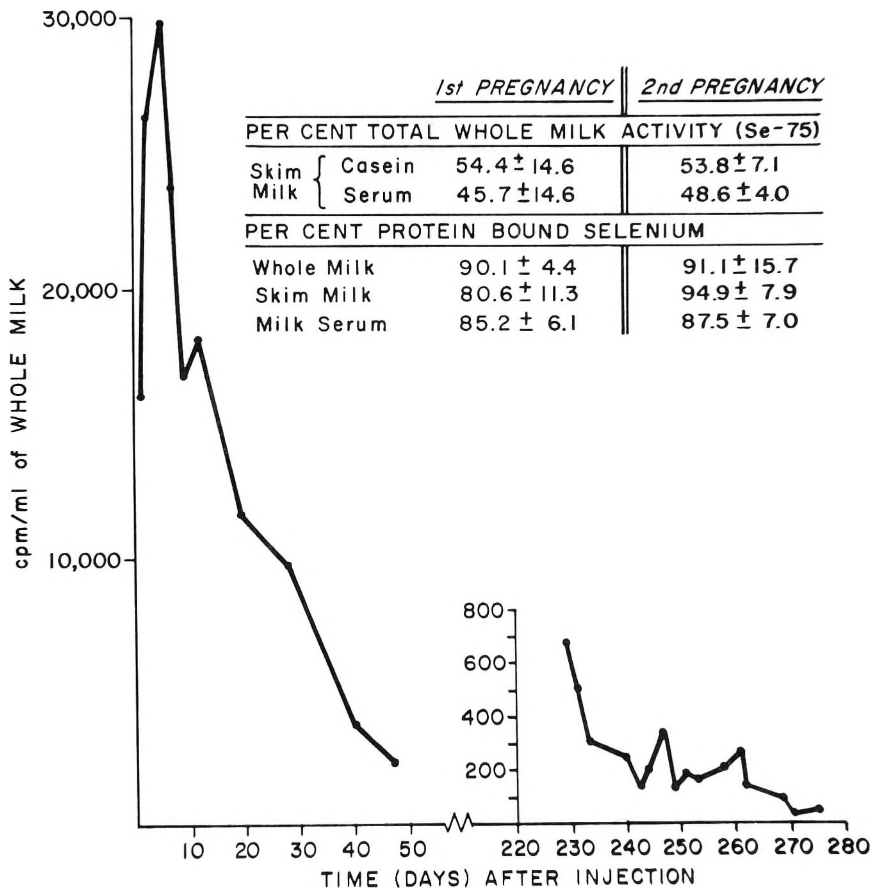

Fig. 1 Time distribution of Se^{75} in milk of dog no. 744 injected with $Na_2Se^{75}O_3$.

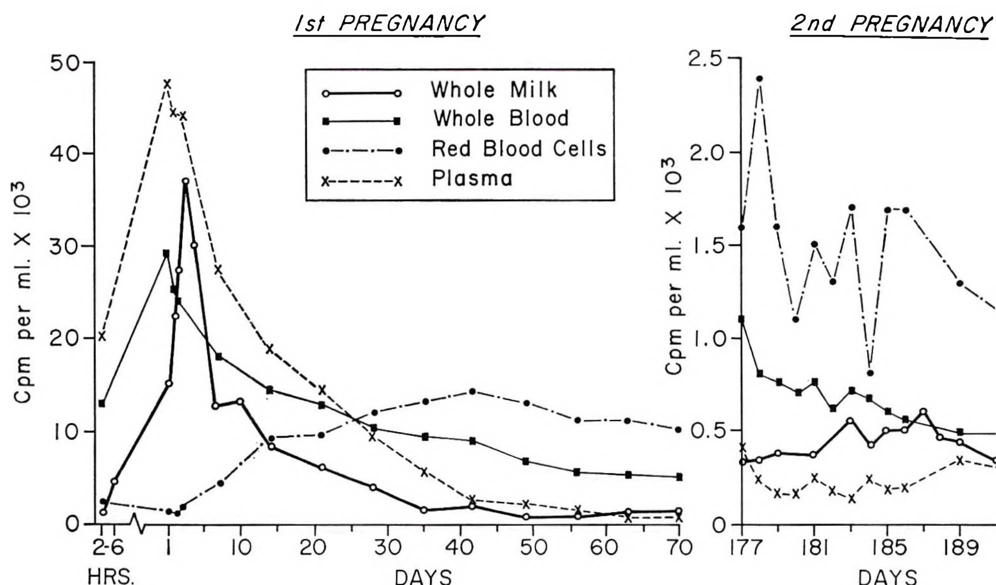

Fig. 2 Time distribution of Se^{75} in milk and blood of dog no. 863 injected with $Na_2Se^{75}O_3$. pregnancy: percentage of skim milk Se^{75} in milk serum, 36.8 ± 11.3 (15 samples); percentage o milk Se^{75} in casein, 63.2 ± 9.9 (15 samples); percentage of protein-bound selenium, 88.5 ± 7. samples). Second pregnancy: percentage of skim milk Se^{75} in milk serum, 52.1 ± 14.2 (14 sar percentage of skim milk Se^{75} in casein, 47.9 ± 14.2 (14 samples).

744. As early as 2 hours after injection (dog no. 863), Se^{75} was detected in the milk. Maximal concentration of the isotope in milk was reached in 4 days by dog no. 863, and in 5 days by dog no. 744. At the time of Caesarean section after the second pregnancies (235 days in dog no. 744, 193 days in dog no. 863 after injection), selenium increased in the milk as shown in figures 1 and 2. The final Se^{75} determinations on the milk were made 275 and 220 days after injection of $Se^{75}O_3$ = in dogs no. 744 and no. 863, respectively.

Figure 1 shows that inorganic selenium administered to the lactating dog was converted into milk organoselenium, since nearly all the selenium was present in the proteins of the milk. About 90% of the whole milk and skim milk selenium was protein-bound, and more than 85% of milk serum was protein bound. Isolated and washed casein contained Se^{75}. The Se^{75} was present in the milk proteins until lactation ceased after the second pregnancy. The distribution of the selenium in the various protein fractions of the milk (fig. 1) showed that Se^{75} was equally distributed between the casein and milk serum. Dog no. 863 (fig. 2) showed essentiall same distribution pattern of seleniu tween casein and milk serum as do 744. A preliminary report on the i poration of selenium in whole b plasma and red blood cells has been m A detailed report on the incorporati selenium in whole blood, plasma an blood cells on a different series of an will appear elsewhere. In the pr study, the observed increase in the centration of selenium (cpm/ml) i whole blood of lactating dog no. 863 2) was accompanied by a similar inc in whole milk selenium content (cpm Whole milk and whole blood seleniu ues decreased at about the same rate. plasma fraction reached a maximal nium value (24 hours) before the fractions. In this particular experi the red blood cells, which presumabl little-known relationship to the milk tions, reached a maximal value at 6 w

The 5 pups of dog no. 744, which nourished with the mother's milk,

[2] McConnell, K. P., and D. M. Roth 1957 D tion of selenium-75 disappearance from dog Federation Proc., 16: 219 (abstract).

TABLE 2

Distribution of Se^{75} in pups from dog no. 744 injected with $H_2Se^{75}O_3$

Organ	1st Pregnancy					2nd Pregnancy	
	Male no. 1	Female no. 2	Female no. 3	Male no. 4	Female no. 5	Female no. 1	Male no. 2
			% total activity			% total activity	
Heart	0.93	0.78	1.39	2.08	1.65	1.88	0.53
Lungs	3.28	2.52	2.96	3.31	2.81	7.02	1.61
Liver	17.83	20.96	23.91	16.87	14.09	23.64	10.15
Spleen	0.92	1.35	1.56	1.21	0.60	1.19	0.83
Kidney	8.39	7.71	7.45	6.71	4.76	9.24	2.82
Diaphragm	0.64	0.51	0.72	0.98	0.65	2.24	0.32
Small intestine	16.53	16.55	10.54	8.79	9.04	6.14	7.44
Large intestine	1.96	2.36	2.35	1.89	1.49	1.68	0.83
Adrenals	0.05	0.05	0.06	0.08	0.05	0.02	0.03
Muscle [1]	38.59	32.00	45.50	53.78	46.02	44.31	71.67
Pancreas	0.72	0.64	0.64	0.86	0.76	0.32	0.54
Stomach	2.05	3.11	2.99	3.35	2.37	2.31	1.48
Stomach content	0.63	4.40	0.12	—	—	—	1.25
Bile	0.05	0.05	0.01	0.07	0.07	—	0.04
Blood	7.44	7.03	—	—	15.63	—	—
Total cpm × 10^6	4.52	4.77	5.55	1.36	1.44	0.0053	0.051
Age at killing, days	13	13	34	57	63	born dead	22
Days after injection of mother	12	12	33	56	62	236	258

[1] Total musculature based on 40% of body wt.

killed at different time intervals and the tissues assayed for Se^{75}. The radioactivity of the tissues expressed as counts per minute per organ remained high for more than 2 weeks and then decreased (table 2). It was at this time that the pups' food intake was supplemented with commercial dog chow.[3] The percentage of the total body counts observed at any one time in the various tissues remained fairly constant throughout the experimental period, with the highest concentration of Se^{75} in the total muscle, liver, small intestine and kidney. Despite the metabolic demands of 2 lactations and a pregnancy period, dogs injected with a single dose of microgram amounts of inorganic selenium maintained or conserved selenium in such a manner that it was available in the maternal organism and transmitted to the pups by placental and mammary passage. Should future studies prove that selenium plays an essential role in growth and development, the manner in which the lactating and pregnant dogs maintain or conserve selenium may have some bearing on its role in nutrition.

LITERATURE CITED

1. Dudley, H. C., and H. C. Byer 1935 Determination of selenium. Determination on animal matter and clinical test in urine, Ind. Eng. Chem., (Anal. ed), 7: 3.
2. Smith, M. I., B. B. Westfall and E. F. Stohlman 1937 The elimination of selenium and its distribution in the tissues. Pub. Health Rept., 52: 1171.
3. Moxon, A. L. 1937 Alkali Disease or Selenium Poisoning. South Dakota Agricultural Experiment Station Bull. 311, Brookings, p. 3.
4. McConnell, K. P. 1948 Passage of selenium through the mammary glands of the white rat and the distribution of selenium in the milk proteins after subcutaneous injection of sodium selenate. J. Biol. Chem., *173*: 653.
5. Westfall, B. B., and M. I. Smith 1940 The distribution of selenium in plasma and liver proteins and its fractionation in tryptic liver digests. Pub. Health Rept., 55: 1575.
6. McConnell, K. P., and B. J. Cooper 1950 Distribution of selenium in serum proteins and red blood cells after subcutaneous injection of sodium selenate containing radioselenium. J. Biol. Chem., *183*: 459.
7. McConnell, K. P., and C. H. Wabnitz 1957 Studies on the fixation of radioselenium in proteins. J. Biol. Chem., 226: 765.

[3] Purina Dog Chow, Ralston Purina Company, St. Louis.

8. McConnell, K. P., and D. M. Roth 1962 Se^{75} in rat intracellular liver fractions. Biochim. Biophys. Acta, *62:* 503.
9. Westfall, B. B., E. F. Stohlman and M. I. Smith 1938 Placental transmission of selenium. J. Pharmacol. Exp. Therap., *64:* 55.
10. McConnell, K. P. 1963 Metabolism of lenium in the mammalian organism. J. Food Chem., *11:* 385.
11. Nilsson, T. 1958 Blood and milk prot and protein-bound carbohydrates in bc chronic mastitis. Acta Path. Micro Scand., *125* (supp. 2): 32.

Factors Affecting the Activity of Pentose Phosphate-metabolizing Enzymes in Rat Liver [1]

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ABSTRACT A direct spectrophotometric method for the assay of pentose phosphate-metabolizing enzyme activity, also referred to as transketolase activity, is described. This method was used to study the effect of diets and hormones on the liver activity of this enzyme system. High protein, high fructose, and a starvation-refed regimen all produced significant increases in pentose phosphate-metabolizing enzyme activity. Administration of hydrocortisone or cortisone also caused increases and feeding iodinated casein or thyroxine administration had a negligible effect on the activity of this system. A 4-day fast, diabetes, adrenalectomy, and hypophysectomy all caused significant decreases in liver pentose phosphate-metabolizing enzyme activity. Both vitamin-free and thiamine-free diets produced large decreases in the activity of this system, whereas a pyridoxine-free diet causd only a slight decrease in activity. The addition of oxythiamine to the thiamine-free diet caused a further decrease in activity of the pentose phosphate-metabolizing enzymes.

Recently there has been considerable interest in the measurement of pentose phosphate-metabolizing enzymes, which have also been referred to as transketolase. However, transketolase is only one of 3 enzymes required for the usual measurement of "transketolase," the other two are phosphoribose isomerase and phosphoribulose epimerase. Since the substrates for all three of these enzymes are pentose phosphate, a better term for the system than transketolase would be pentose phosphate metabolizing enzymes (PPME) which will be used hereafter in this report.

Much of the interest in this system has been due to the work of Brin (1) who has shown a decreased activity of the PPME as a result of thiamine deficiency. This decreased activity has been observed in red blood cells (1, 2) and other tissues (2), the former being used for diagnostic purposes. Other groups have also found decreases in activity of PPME during a thiamine deficiency (3, 4).

The method used for these assays depends upon the disappearance of ribose or appearance of hexose or heptulose after incubation with ribose-5-phosphate (1, 3). However, due to the very large blanks or endogenous values compared with the actual changes observed in the complete reaction, very small errors in sampling will be compounded into much larger errors in the final calculation. The assays by this method are also long and tedious; therefore, a more sensitive and rapid method to assay this system was studied. The present report will present this method, as well as results on the effect of various diets and hormones on the activity of the PPME, using the new assay system.

METHODS

Male Sprague-Dawley rats were used throughout. The enzyme preparations were obtained by killing the rat with a sharp blow on the head, exsanguating, rapidly removing and chilling the tissues to be studied, homogenizing in a Potter-Elvejhem homogenizer with 4 or 9 volumes of ice-cold 0.14 M KCl, and centrifuging at zero to 4° at 20,000 × g for 30 minutes. The resultant supernatant solution was used as the enzyme source. The kinetic studies were performed using liver as the enzyme source. The basic design of the assay was as follows: 0.3 ml of 0.17 M arsenate + 0.27 M glycine at pH 9.0, 0.6 ml of 0.1 M cysteine at pH 9.0, 0.1 ml of 0.1 M $MgCl_2$, 0.25 ml of DPN^+ (40 mg/ml),

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glyceraldehyde-3-phosphate dehydrogenase sufficient to oxidize at least 2 μmoles of glyceraldehyde-3-phosphate/minute under the prevailing assay condition at 27°, enzyme, water to 2.8 ml and the reaction was started with 0.2 ml of ribose-5-phosphate (50 μmoles/ml). The absorption at 340 mμ was followed using a Gilford model 2000 multiple sample absorbance recorder. The reactions were carried out at 27°.

The basis of this method is the formation of xylulose-5-phosphate from ribose-5-phosphate via ribulose-5-phosphate followed by the transketolase reaction between ribose-5-phosphate and xylulose-5-phosphate forming sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate. The latter product is converted to 3-phosphoglyceric acid by the glyceraldehyde-3-phosphate dehydrogenase in the presence of arsenate with the concomitant formation of DPNH, which is measured by its absorption at 340 mμ. One unit of activity is defined as the amount of activity required to form 1 μmole DPNH/minute at 27° under the specified assay conditions.

In the original studies on the method, certain changes were made in the conditions such as in pH, DPN^+ concentration, ribose-5-phosphate concentration, and glyceraldehyde-3-phosphate dehydrogenase concentration. Table 1 shows the effect of pH on the reaction. The middle of the broad pH optimal range was near the pH of 9.0 and therefore this pH was used for all further studies because of the greater stability of cysteine at this pH. Studies on the variation of reaction rates with time revealed a time lag varying from less than one to several minutes. However, after the lag period the reaction rate was constant for periods up to 15 minutes in most cases.

TABLE 1

Effect of pH on the activity of the pentose phosphate-metabolizing enzymes

pH [1]	Relative activity [2]
7.7	94–97
8.0	100
9.0	93–97
9.5	93–97

[1] A glycine-arsenate buffer was used at all pH values (see Methods section for further description).
[2] The activity is reported as percentage of activity at pH 8.0.

Only the rates obtained after the lag period were used in the calculations of activity. It was assumed that the lag period represented the time required to convert sufficient ribose-5-phosphate to xylulose-5-phosphate to permit the transketolase reaction to proceed at an optimal rate. To test this theory, xylulose-5-phosphate[4] (5 μmoles) was added to the reaction at the start; this completely eliminated the lag.

The reaction rate during the linear phase was considerably greater (50%) for the mixture of pentose phosphates than for the ribose-5-phosphate alone; thus there is some limitation in the activity of the isomerase or epimerase, or both (table 2).

TABLE 2

Effect of pentose phosphate concentrations on the activity of pentose phosphate-metabolizing enzymes

Substrate	No. of assays	Relative rate [1]
10 μmoles ribose-5-phosphate	—	100
5 μmoles ribose-5-phosphate	6	73
20 μmoles ribose-5-phosphate	7	113
5 μmoles ribose-5-phosphate + 5 μmoles xylulose-5-phosphate	13	150

[1] Relative rate is reported as percentage of the activity obtained using 10 μmoles of ribose-5-phosphate.

Doubling the concentration of ribose-5-phosphate or DPN^+ had only a slight effect on the reaction rate, although reducing either of these to one-half the suggested concentration caused a decrease in rate. Doubling or halving the glyceraldehyde-3-phosphate dehydrogenase had no observable effect upon the reaction rate.

The reaction rate was linear with enzyme concentration (table 3); thus the assay can be used in its present form as a quantitative estimation of PPME activity. This assay was then used in a number of tissues to evaluate the activity of PPME and these activities were compared with those obtained using Brin's method (2). Attempts to obtain ribose disappearance data using Brin's method were unsuccessful. The very high initial levels of ribose in the tissue homogenates compared with the very small decreases during incuba-

[4] This was a gift from Dr. W. A. Wood, Michigan State University.

TABLE 3

Effect of added liver supernatant solution on the activity of the pentose phosphate-metabolizing enzymes

	Activity [1]			
	Animal no. 1		Animal no. 2	
ml of supernate [2]	*mμmoles* DPNH/*min*	*mμmoles* DPNH/*min g liver*	*mμmoles* DPNH/*min*	*mμmoles* DPNH/*min g liver*
0.15	3.9	0.517	3.5	1.17
0.30	8.2	0.547	7.7	1.29
0.45	12.4	0.550	10.6	1.18

[1] All rates are corrected for endogenous activity.
[2] A 5% supernate was used for animal no. 1, and a 2% supernate was used for animal no. 2.

tion were the primary causes for the failure to obtain ribose disappearance data. Hexose accumulation data were obtained. A preliminary survey with three 400-g animals indicated that the activities in the various tissues relative to those in the liver were similar to those observed by Brin, but that the overall activity was about 15% of the rates reported by Brin et al. Part of the discrepancy in absolute rates between the 2 methods can be accounted for by the differences in the temperatures of the reaction mixtures, 38° for the Brin method and 27° for the direct spectrophotometric assay. The direct spectrophotometric assay could not be used on blood because of the high initial optical density of the sample due to hemoglobin. The reproducibility of the spectrophotometric assay was examined in one rat using 6 replicates in 3 tissues: liver, kidney, spleen, with the following activities, 37.4 ± 1.1; 15.6 ± 0.7; and 23.4 ± 0.8 μmoles DPNH produced/hour/g tissue ± SE of the mean. In an earlier study 11 replicates run on one liver sample throughout the complete day yielded a rate of 49.4 ± 1.6 with a coefficient of variation of 10.7%.

Animals. For the hormonal and dietary studies animals were housed in individual screen-bottom cages. The control diet consisted of 65% glucose, 25% casein, 5% corn oil, 4% salts mix,[5] and 1% vitamins.[6] The fructose and protein diets contained fructose and casein in place of the glucose, respectively. The iodinated casein diet contained 4% iodinated casein in place of 4% of the glucose. Vitamin-free casein was used for the vitamin-free diets, thiamine-deficient diets, and pyridoxine-deficient diets (6). The vitamin mixes were made up from purified vitamins (6). Food was given ad libitum to the rats, with the exception of the fasted animals; all animals were permitted water ad libitum. Hormone treatments were as follows: cortisone and hydrocortisone (ip), 5 mg/day/rat; thyroxine (ip), 1 mg/day/rat; diabetes was produced by alloxan, 200 mg/kg (ip), and only rats with blood sugar values over 300 mg/100 ml were considered diabetic. All animals were maintained with the diet or kept on treatment for 5 days prior to killing, except the fasted-refed rats which were fasted 48 hours and refed 24 hours prior to killing. Food was withheld from the fasted animals for 96 hours. The adrenalectomized rats received 1.5% NaCl in their drinking water. The hypophysectomized rats were kept one month after surgery before use.[7] Proteins were measured by the biuret method (7). All values are reported as activity per 100 g of body weight and/g of liver, with standard errors presented for the former values.

RESULTS

A tissue distribution study for PPME was undertaken. The results showed the

[5] Phillips, P. H., and E. B. Hart 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657, plus added cobalt. The salt mixture contained: (in per cent) dipotassium phosphate, 32.2; Ca carbonate, 30.0; NaCl, 16.7; magnesium sulfate (hydrate), 10.2; Ca phosphate ($CaHPO_4 \cdot 2H_2O$), 7.5; ferric citrate, 2.75; manganese sulfate, 0.51; KI, 0.08; copper sulfate, 0.03; Zn chloride, 0.025; cobalt chloride, 0.005. (Salt Mixture P–H, obtained from Nutritional Biochemicals Corporation, Cleveland.)

[6] Vitamin mixture contained: (mg/kg of diet) vitamin A concentrate (200,000 units/g), 45.0; vitamin D concentrate (400,000 units/g), 2.5; α-tocopherol, 50.0; ascorbic acid, 450.0; inositol, 50.0; choline chloride, 750.0; menadione, 22.5; *p*-aminobenzoic acid, 50.0; niacin, 45.0; riboflavin, 10.0; pyridoxine·HCl, 10.0; thiamine·HCl, 10.0; Ca pantothenate, 30.0; biotin, 0.2; folic acid, 0.9; and vitamin B_{12}, 0.0135 (Vitamin Diet Fortification Mixture, obtained from Nutritional Biochemicals Corporation, Cleveland).

[7] The surgically altered rats were obtained from Diablo Laboratories, Berkeley, California.

highest activity in the liver (37.4 units/g) followed by spleen (23.4 units/g) and kidney (15.6 units/g), then brain (1.1 units/g) with much lower activity. The standard errors and coefficients of variation indicate that the results are very reproducible with the same preparation and the coefficient of variation which averages about 9.4% can be considered as an indication of the reproducibility of the method. Since liver contained the highest PPME activity and has been shown to be adaptable in enzyme content under various physiological conditions, only the liver activity of this system was examined during subsequent dietary and hormonal manipulations.

Various feeding regimens were examined for their effects on the liver PPME activity (table 4). A 4-day fast caused a decrease in activity per unit of body weight, but due to the decreased liver weight-to-body weight ratio, an increase in activity per gram of liver was observed. The same phenomena was observed with the group fed a 4% iodinated casein diet for 5 days; however, in this case the decrease in activity per unit of body weight was not significant. A description of metabolic rate and liver glycogen level for these animals has been described elsewhere (8, 9).

A high protein or high fructose regimen caused a significant increase in liver PPME activity both on a fresh liver weight and body weight basis after 5 days of feeding. A 48-hour fast followed by feeding a high glucose diet (the control diet) caused a marked increase in activity both on body weight and liver weight basis.

Various hormone treatments were examined for their effect on the liver PPME activity (table 5). Both cortisone and hydrocortisone caused a marked increase in activity. Thyroxine had approximately the same results as observed with 4% iodinated casein diets, including the absence of liver glycogen. Alloxan diabetes caused a significant decrease in activity. The removal of the adrenal or pituitary glands also caused very marked decreases in activity per gram of liver and per unit of body weight.

TABLE 4

Effects of dietary treatment on the activity of the liver pentose phosphate-metabolizing enzymes

Diet	No. of animals	Activity (as % of control value)	
		Per gram liver	Per 100 g body wt
Control	44	100.0 ± 3.25 [1]	100.0 ± 3.51
4-Day fast	10	140.2 ± 10.7 **	81.8 ± 3.7 **
High protein	10	184.0 ± 20.4 **	155.0 ± 20.0 *
Fasted-refed	8	130.0 ± 5.3 **	155.0 ± 8.0 **
Fructose	4	172.0 ± 8.6 **	142.5 ± 11.6 **
Iodinated casein, 4%	11	134.3 ± 7.0 **	91.1 ± 4.3

[1] SE of mean, control activity units/g liver, 1.19; units/100 g body wt, 5.69.
* Differs from the control value at $P = 0.05$ or less.
** Differs from the control value at $P = 0.01$ or less.

TABLE 5

Effects of hormonal treatment on the activity of the liver pentose phosphate metabolizing enzymes

Treatment	No. of animals	Activity (as % of control value)	
		Per gram liver	Per 100 g body wt
Control	44	100.0 ± 3.26 [1]	100.0 ± 3.51
Hydrocortisone	14	143.9 ± 9.3 **	155.9 ± 8.6 **
Cortisone	12	171.6 ± 18.3 **	166.0 ± 12.4 **
Thyroxine	6	122.8 ± 5.9 **	103.0 ± 5.1
Diabetes	6	69.8 ± 7.4 **	59.8 ± 7.7 **
Adrenalectomized	4	62.0 ± 7.0 **	65.0 ± 9.1 **
Hypophysectomized	4	55.0 ± 5.0 **	46.7 ± 6.4 **

[1] SE of mean, control activity units/g liver, 1.19, units/100 g body wt, 5.69.
** Differs from the control value at $P = 0.01$ or less.

TABLE 6

Effect of vitamin-deficient diets on the activity of liver pentose phosphate-metabolizing enzymes

Diet	Time fed diet	Normal wt [2]	Vitamin-deficient wt	Activity [1]	
				Per gram liver	Per 100 g body wt
	days	*g*	*g*	%	%
Vitamin-free	14	124	52	24	29
Vitamin-free	14	92	40	31	32
Vitamin-free	21	164	48	21	21
Vitamin-free	21	111	41	19	26
Pyridoxine-free	14	170	75	101	84
Thiamine-free	14	158	76	46	36
Thiamine-free + oxythiamine	14	158	53	10	12

[1] All activities are reported as a percentage of the corresponding normal or control value.
[2] Weight of the corresponding control or normal animal.

The relationship of PPME activity and thiamine deficiency has been important in experimental and clinical nutrition; therefore it was important to examine the effect of thiamine deficiency on the PPME activity using the new assay method (table 6). Vitamin-free diets caused a decrease in weight gain and also a decrease in PPME activity; this decrease was somewhat greater after feeding the vitamin-free diet for 21 days than after 14 days. To test further the assumption that the observed decrease was due to a thiamine deficiency and not that of another vitamin, a pyridoxine-free diet, thiamine free-diet and thiamine-free diet plus oxythiamine were tested for their effect on the PPME activity. Oxythiamine has been shown to be effective in decreasing "transketolase" activity (2). The pyridoxine-free diet had no appreciable effect on the PPME activity per gram of liver and the slight decrease per unit of body weight may be due to some overlapping of fasting effects. The thiamine-deficient animals had a marked decrease in PPME activity, although the weight gains were similar for the pyridoxine- and thiamine-free groups. Oxythiamine administration (100 μg/day) further accentuated the decrease in PPME activity. Thus, it appears that there is a direct relationship between thiamine deficiency and low liver PPME activity.

DISCUSSION

The direct spectrophotometric assay for pentose phosphate metabolizing enzymes (PPME) has the advantages of being rapid, shorter in duration, and allowing a direct observation of the reaction rate with time The addition of xylulose-5-phosphate to the reaction mixture causes a 50% greater rate than the addition of equal amounts of ribose-5-phosphate. This indicates that the transketolase reaction is more rapid than the over-all PPME reaction, even after the lag period. Therefore, the proposed name of pentose phosphate-metabolizing enzymes is probably more accurate than transketolase for this and similar non-direct assays. When xylulose-5-phosphate becomes commercially available it will be feasible to add this compound and ribose-5-phosphate, which should permit the direct assay of transketolase.

Certain dietary treatments have been shown to have a considerable effect on the activity of liver glucose-6-phosphate dehydrogenase (G-6-PD). Since this enzyme catalyzes the first step of the pentose phosphate pathway and the PPME represents an important segment of this pathway, it seemed worthwhile to examine the activity of the PPME under these various conditions. Fasting, which decreases G-6-PD per unit body weight (9,10), also has the same effect on PPME activity. A high protein diet (11), a high fructose diet (9, 12, 13), and a fasted-refed regimen (10), which cause increases in G-6-PD activity, also produced significant increases in PPME activity. The iodinated casein diet which had no significant effect on G-6-PD activity (9) also had no effect on PPME activity. Thus it appears that these conditions not only increase or decrease the activity of the initiating step of the pentose phosphate pathway, but have a pronounced

effect upon the activity of the entire pathway.

A similar pattern was observed with various hormonal treatments. In this case both cortisone and hydrocortisone produced increased PPME activity; these experimental groups also had elevated G-6-PD activities (14). Thyroxine, as in the case of iodinated casein, had no observable effect on PPME activity. Conditions which decrease G-6-PD activity appreciably, namely, diabetes (9, 15), adrenalectomy (9, 14, 16), and hypophysectomy (17), also produced marked decreases in PPME activity. With the hormonal treatments, as in the dietary study, there was a correlation between G-6-PD and the PPME activities.

Since one of the PPME's, transketolase, requires thiamine pyrophosphate as a coenzyme it would be expected and has been shown that a thiamine deficiency decreases the PPME activity (2, 3). Therefore, as a further test of the direct spectrophotometric assay, the PPME activity of animals with various vitamin deficiencies was examined (table 6). A vitamin-free diet caused a very significant decrease in PPME activity after both 14 and 21 days on the regimen. The use of a pyridoxine-deficient or a thiamine-deficient diet shows that the decrease in PPME activity is correlated with the thiamine deficiency and is not due to a general or nonspecific deficiency. The slight decrease caused by the pyridoxine-free diet may be the result of decreased food intake as fasting also causes a marked decrease in PPME activity. The use of oxythiamine, which further aggrevates the thiamine deficiency, also caused a further decrease in the PPME activity. When xylulose-5-phosphate becomes available and a more specific assay for transketolase becomes practicable, these systems can be examined more closely. It will be particularly important to ascertain whether a thiamine deficiency causes a decrease only in transketolase or whether the entire PPME system is affected.

LITERATURE CITED

1. Brin, M., M. Tai, A. S. Ostashever and H. Kalinsky 1960 The effect of thiamine deficiency on the activity of erythrocyte hemolysate transketolase. J. Nutrition, *71:* 273.
2. Brin, M. 1962 Effects of thiamine deficiency and of oxythiamine on rat tissue transketolase. J. Nutrition, *78:* 179.
3. Sie, H. G., V. N. Nigam and W. H. Fishman 1961 Capacity of rat tissues to form heptulose phosphates in thiamine deficiency. Biochem. Biophys. Acta, *50:* 277.
4. Dreyfus, P. M. 1962 Clinical application of blood transketolase determinations. New England J. Med., *267:* 596.
5. Brin, M., and R. H. Yonemoto 1958 Stimulation of the glucose oxidative pathway in human erythrocytes by methylene blue. J. Biol. Chem., *230:* 307.
6. Stielau, W. J., and J. H. Meyer 1963 Periodic B-vitamin supplementation of growing rats fed a vitamin-free diet. J. Nutrition, *81:* 330.
7. Mokrasch, L. G., and R. W. McGilvery 1956 Purification and properties of fructose-1,6--diphosphatase. J. Biol. Chem., *221:* 909.
8. Freedland, R. A., and C. H. Sodikoff 1961 Effect of diets and hormones on two urea cycle enzymes. Proc. Soc. Exp. Biol. Med., *109:* 394.
9. Freedland, R. A., and J. K. Barnes 1963 The effect of adrenalectomy on the adaptation of glucose-6-phosphate metabolizing enzymes in the liver. J. Biol. Chem., *238:* 1915.
10. Tepperman, J., and H. M. Tepperman 1958 Effects of antecedent food intake pattern on hepatic lypogenesis. Am. J. Physiol., *193:* 55.
11. Schimke, R. T. 1962 Adaptive characteristics of urea cycle enzymes in the rat. J. Biol. Chem., *237:* 459.
12. Fitch, W. M., and I. L. Chaikoff 1960 Extent and patterns of adaptation of enzyme activities in livers of normal rats fed diets high in glucose and fructose. J. Biol. Chem., *235:* 554.
13. Fitch, W. M., R. Hill and I. L. Chaikoff 1959 The effect of fructose feeding on glycolytic enzyme activities of the normal rat. J. Biol. Chem., *234:* 1048.
14. Willmer, J. S. 1960 Changes in hepatic enzyme levels after adrenalectomy. II. Glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. Canad. J. Biochem. Physiol., *38:* 1449.
15. Fitch, W. M., R. Hill and I. L. Chaikoff 1959 Hepatic glycolytic enzyme activities in the alloxan-diabetic rat: Response to glucose and fructose feeding. J. Biol. Chem., *234:* 2811.
16. Weber, G., G. Banerjee and S. B. Bronstein 1961 Role of enzymes in homeostasis. III. Selective induction of increases of liver enzymes involved in carbohydrate metabolism. J. Biol. Chem., *236:* 3106.
17. Tepperman, H. M., and J. Tepperman 1962 Role of hormones in glucose-6-phosphate dehydrogenase adaptation of rat liver. Am. J. Physiol., *202:* 401.

Effects of Dietary Selenium, Methionine, Fat Level and Tocopherol on Rat Growth [1]

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ABSTRACT It is possible to relate the tocopherol requirement of the rat to the dietary or tissue lipid fatty acids by means of growth curves when the methionine levels and other components of the diet are controlled and the environment is optimal. One of the dependent variables is the amount of selenium available to the animal. Conversely, if the α-tocopherol content of the diet is controlled at a low level, the growth curves can be related to the levels of biologically available selenium. Such growth curves show that the selenium requirement of the tocopherol-deficient rat has a very narrow optimal range and that the toxicity of selenium may be decreased by adding methionine. The selenium content of casein is variable and, to only a moderate degree, biologically available. When rats were fed low levels of dietary fat (1 to 7.5%) in a diet essentially devoid of tocopherol, the time required for the production of creatinuria, a sign of nutritional muscular dystrophy, was inversely proportional to the dietary fat level. The tocopherol requirement also decreased markedly as the dietary fat level was lowered. The time in weeks required to produce creatinuria was correlated with the fatty acid composition of the muscle phospholipid and neutral lipids.

Since the major, if not sole, function of *d*-α-tocopherol is as a biological antioxidant, it is logical that dietary polyunsaturated fatty acids (PUFA) are prime factors in the production of tocopherol deficiency (1, 2). It is well known, however, that a complex interrelationship also exists between biologically available selenium, sulfur amino acids, and tocopherol (3, 4). In the chick, by careful manipulation of the diet, it is possible to produce any one of the following: encephalomalacia, exudative diathesis, or nutritional muscular dystrophy as the distinctive deficiency state (5–7).

The tocopherol requirements of the rat (2, 8) and the chick (9), using various specific criteria of deficiency, have been shown to vary with the amount of fat fed. At moderate-to-high levels (7.5 to 25%) of a given dietary fat, however, the muscle lipids of the rapidly growing rat reach equivalent equilibrium fatty acid compositions and the tocopherol requirement by the criterion of creatinuria is independent of the amount of fat fed (10).

In a previous paper (10) the potential in vivo peroxidizability of the PUFA was explored in detail and an attempt was made to define the tocopherol requirement of the rat as a function of n in $CH_3(CH_2)_x(CH=CH\,CH_2)_n(CH_2)_yCO_2H$ under a specific set of experimental conditions assuming that creatinuria is evidence of the onset of lipid peroxidation in muscle. The present report [2] deals mainly with the growth of rats and demonstrates that classical growth curves may be useful in evaluating tocopherol needs, if all variables are properly controlled. An investigation was conducted of the role of biologically available selenium, methionine, and amount of fed fat on the tocopherol requirement of the rat.

MATERIALS AND METHODS

The experimental diets and procedures have been described in detail in a previous paper (10). Briefly, the diet consisted of casein,[3] glucose monohydrate,[4] fat, vita-

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[2] A preliminary report of this work was given at the 1964 meeting of the Federation of American Societies for Experimental Biology, Chicago. L. A. Witting and M. K. Horwitt 1964 Effect of dietary selenium, methionine, and fat level on the requirement of the rat. Federation Proc., 23: 395 (abstract).
[3] Casein obtained from Nutritional Biochemicals Corporation, Cleveland, or General Biochemicals Inc., Chagrin Falls, Ohio.
[4] Cerelose, Corn Products Company, Argo, Illinois.

mins (2) and salt mix 446 (11). Vitamins, salts, and protein were added at a constant ratio to calories. Casein, therefore, always supplied 21.8% of calories. The weight percentage of casein was 20.8, 21.5, 22.7, 24.1, 25.9 and 27.5% when the diet contained 1, 3, 7.5, 12.5, 19 and 25% fat, respectively.

Dietary fats were prepared by methylation of tocopherol-free fatty acids (10), and sodium methoxide catalyzed ester interchange of the freshly molecularly distilled methyl esters with glyceryl triacetate under vacuum. The fatty acid composition of the 2 fats used was balanced to achieve an iodine value of 82.0 (12). The so-called "dienoic fat" therefore contained 42.5% saturated; 21.2% monoenoic; and 36.0% dienoic acids; and the so-called "trienoic fat" contained 58.5, 10.0, 8.4, and 23.1%, respectively, of saturated, monoenoic, dienoic and trienoic acids. The dietary fats were stored in filled glass containers at − 5° prior to use. Diets were mixed every other day and kept refrigerated when not in use. Fresh feed was supplied to the animals daily and particular care was taken to remove any portion of the previous ration not consumed. In a prior experiment (10), no advantages were noted when butylated hydroxyanisole was added to the fat to stabilize it prior to ingestion.

Tocopherol supplements, dissolved in coconut oil freed of tocopherol as previously described (10), were administered by dropper 3 times a week. Dosage of α-tocopheryl acetate in milligrams per kilogram of rat body weight per week, was adjusted according to 25-g animal weight brackets (50 to 75 g; 75 to 100 g; 100 to 125 g, etc.). Rats, Sprague-Dawley strain, were weighed weekly. Each dietary group consisted of 5 rats. Methionine, when used as a supplement, was fed at only one level (0.4% DL-methionine) but *d*-α-tocopheryl acetate and sodium selenite were fed at various levels as indicated. Some of the experimental periods were shorter than others because of the need to kill animals for tissue analysis (10).

Selenium was determined by the method of Watkinson (13). Creatine and creatinine were determined by the method of Bonsnes and Taussky on 24-hour urine samples (14).

RESULTS

Effect of supplemental selenium and methionine on growth. Eight groups of 5 male weanling white rats each, were fed the synthetic "trienoic fat" described in the experimental section at the 12.5% level in a tocopherol-deficient ration. Three groups received oral supplements of 0.2, 0.4, or 0.6 mg *d*-α-tocopheryl acetate/kg body weight/week. This ration contained approximately 0.25 ppm of selenium largely derived from the casein. Growth curves, broken lines, are shown in figure 1. Methionine (0.4% DL-methionine) and selenium (0.10 ppm of selenium as sodium selenite) were added to the ra-

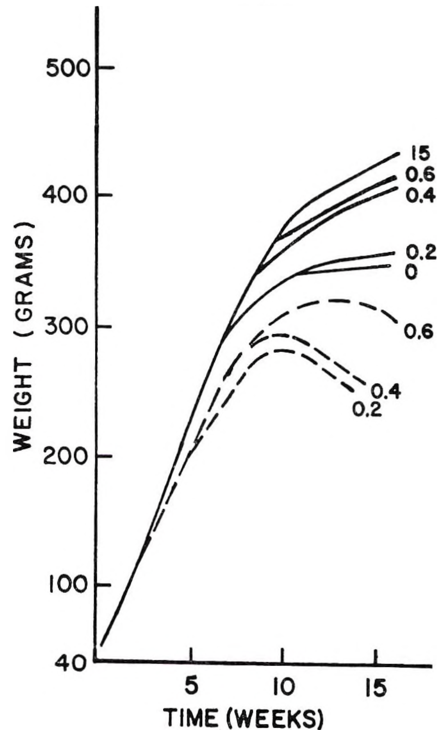

Fig. 1 Effect of added selenium and methionine on the growth response of the tocopherol-deficient rat to low levels of tocopherol supplementation. Broken lines indicate basal ration, average weight of 5 rats per group. Solid lines indicate basal ration plus 0.4% DL-methionine and 0.10 ppm of selenium as sodium selenite, average weight of 5 animals per group. Tocopherol (mg *d*-α-tocopheryl acetate/kg rat/week indicated by numbers at end of each line.

tion fed to the remaining 5 groups and α-tocopheryl acetate was administered orally to four of the groups at dose levels of 0.2, 0.4, 0.6, or 15 mg/kg rat/week. Growth curves, solid lines, are shown in figure 1.

Mason (15) noted that tocopherol-deficient rats grew as well as animals given the tocopherol supplement, for several months. Thereafter, however, the weight of the deficient animals stayed at a plateau for 3 to 4 months, and then decreased. In the present experiment, differences in average weight and standard error of the mean between groups at early periods were sufficiently small so that individual group average lines were not drawn until a clearly divergent trend was evident. Addition of selenium and methionine to the basal ration significantly (table 1) increased the average growth response (fig. 1) of tocopherol-deficient rats at each of 3 levels of tocopherol supplementation.

Effect of selenium supplementation on growth response. Five groups of 5 male weanling rats each were fed the synthetic "trienoic fat" (10) at the 12.5% level in the tocopherol-deficient ration. Selenium was added to the ration at the levels of zero, 0.03, 0.10, 0.30, or 1.00 ppm. In the control group in this and each succeeding experiment, 0.10 ppm of selenium and 0.4% DL-methionine were added to the ration, and oral supplements of 15 mg *d*-α-tocopheryl acetate/kg body weight/week were given. Growth curves are shown in figure 2(A). Best growth was noted in the group receiving 0.10 ppm of added selenium. Addition of 0.30 ppm of selenium produced better growth than addition of 0.03 ppm of selenium, but was inferior to the addition of 0.10 ppm of selenium. Four of 5 rats died during the eleventh week in the group fed 1.00 ppm of added selenium. The levels of significance of the observed differences in group average weights are noted in table 1 in which the effects of the deviations in weight of the individual rats from the group average are taken into consideration.

Effect of selenium supplementation in absence and presence of added methionine. Five groups of 5 male weanling rats each were fed the synthetic "trienoic fat" at the 12.5% level in the tocopherol-

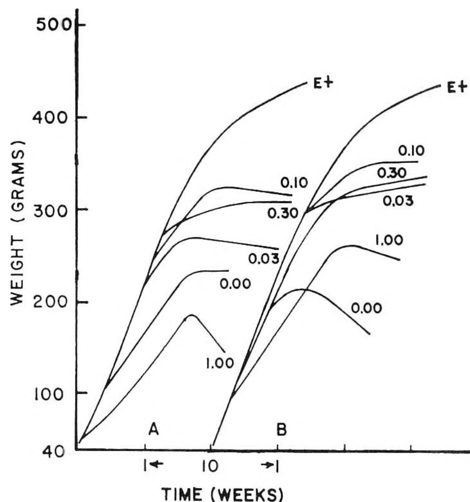

Fig. 2(A) Effect of added selenium on the growth of the tocopherol-deficient rat. Average weight of 5 rats/group. Selenium additions in parts per million indicated at end of each line. E+ denotes the control group fed the basal ration containing 0.10 ppm of added selenium and 0.4% of added DL-methionine and supplemented with 15 mg *d*-α-tocopheryl acetate/kg rat/week; (B) Effect of added selenium in the presence of added methionine (0.4% DL-) on the growth of the tocopherol-deficient rat, average weight of 5 animals per group. Selenium additions in parts per million indicated by numbers at end of each line.

deficient ration to which 0.4% DL-methionine had been added. Selenium was added to the rations at levels of zero, 0.03, 0.10, 0.30, or 1.00 ppm. Growth curves are shown in figure 2(B). Adjacent growth curves were compared statistically to determine the significance of observed weight differences (table 1). Then the growth of the groups at each level of selenium addition (fig. 2(A) and (B) was compared to evaluate the effect of methionine supplementation (table 1). Added methionine enhanced the growth-promoting properties of added selenium and decreased the toxic effects of adding 1.0 ppm of selenium. Although four of five rats died during the eleventh week of receiving the 1.0-ppm supplement, all survived for the duration of the experiment when 0.4% DL-methionine was added.

Effect of level of tocopherol supplementation on the growth responses to added selenium and methionine. The effect of 4 levels, zero, 0.03, 0.10, and 0.30 ppm of

TABLE 1

Selected data representing levels of significance between growth curves shown in figures 1 and 2 at pertinent times[1]

	Week of growth compared	α-Tocopheryl acetate	Se	0.4% Methionine	α-Tocopheryl acetate	Se	0.4% Methionine	Significance
		mg/kg/rat/week	*ppm*		*mg/kg/rat/week*	*ppm*		
Figure 1	10	0.2	0.00	−	0.2	0.10	+	$P < 0.005$
	10	0.4	0.00	−	0.4	0.10	+	$P < 0.05$
	12	0.4	0.00	−	0.4	0.10	+	$P < 0.005$
	10	0.6	0.00	−	0.6	0.10	+	$P < 0.05$
	14	0.6	0.00	−	0.6	0.10	+	$P < 0.005$
	13	0.2	0.00	−	0.4	0.00	−	ns
	13	0.4	0.00	−	0.6	0.00	−	$P < 0.01$
	14	0	0.10	+	0.2	0.10	+	ns
	14	0.2	0.10	+	0.4	0.10	+	$P < 0.05$
	14	0.4	0.10	+	0.6	0.10	+	ns
Figure 2(A)	9	0	1.00	−	0	0.00	−	$P < 0.01$
	5	0	0.00	−	0	0.03	−	$P < 0.005$
	8	0	0.00	−	0	0.03	−	$P < 0.01$
	9	0	0.03	−	0	0.30	−	$P < 0.01$
	7	0	0.30	−	0	0.10	−	ns
	8	0	0.30	−	0	0.10	−	$P < 0.01$
	9	0	0.30	−	0	0.10	−	ns
Figure 2(B)	9	0	0.03	+	0	0.30	+	ns
	9	0	0.30	+	0	0.10	+	$0.10 > P > 0.05$
	9	0	0.00	+	0	1.00	+	$P < 0.001$
	9	0	1.00	+	0	0.03	+	$P < 0.02$
Figures 2(A) and 2(B)	9	0	0.00	−	0	0.00	+	$P < 0.005$
	9	0	0.03	−	0	0.03	+	$P < 0.05$
	9	0	0.10	−	0	0.10	−	ns
	9	0	0.30	−	0	0.30	−	ns
	9	0	1.00	−	0	1.00	+	$P < 0.01$

[1] For example, line 1 above: comparison, after 10 weeks, of the weights of 5 rats receiving 0.2 mg/kg/week of α-tocopheryl acetate and no added selenium or methionine, with 5 rats receiving the same level of α-tocopheryl acetate plus 0.1 ppm selenium and 0.4% DL-methionine shows a significant difference of $P < 0.005$.

selenium addition alone and in combination with 0.4% DL-methionine was evaluated at 5 levels of tocopherol supplementation, 0.2, 0.4, 0.6, 1.2, and 2.4 mg/kg rat/week, respectively. Only 30 of the required 40 groups necessary for the complete experiment were actually used (table 1). In each case, each group contained 5 male weanling rats and the dietary fat was the synthetic "trienoic fat" fed at the 12.5% level. Growth at 0.2 mg/kg rat/week was similar to that noted in figure 2(A) and (B) for zero tocopherol supplementation. Growth curves obtained at 0.4 mg *d*-α-tocopheryl acetate/kg rat/week at various levels of selenium addition are shown for the basal diet (fig. 3(A)) and for the basal diet with added methionine (fig. 3(B)). Growth at 0.6 mg/kg rat/week was similar to that noted in figure 3(A) and (B) for 0.4 mg/kg rat/week. Similar

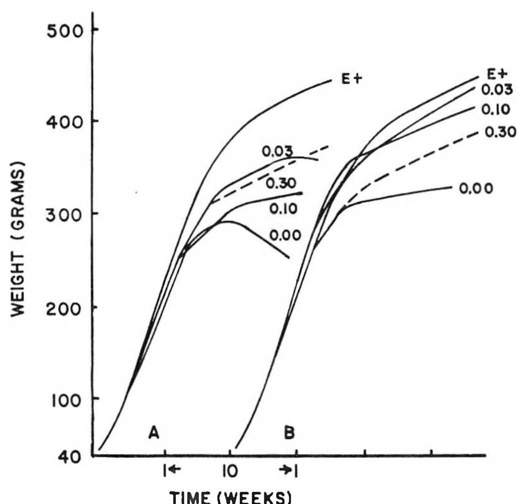

Fig. 3(A) Effect of added selenium on the growth response of tocopherol-deficient rats to supplementation with 0.4 mg *d*-α-tocopheryl acetate/kg rat/week, except broken line, 0.6 mg/kg rat/week. Average weight of 5 animals/group. Selenium additions in parts per million indicated at end of each line. E+ denotes the control group fed the basal ration containing 0.10 ppm of added selenium and 0.4% of added DL-methionine and supplemented with 15 mg *d*-α-tocopheryl acetate/kg rat/week; (B) effect of added methionine (0.4% DL-) on the growth response of tocopherol-deficient rats to supplementation with 0.4 mg *d*-α-tocopheryl acetate/kg rat/week, except broken line, 0.6 mg/kg rat/week. Average weight of 5 animals/group. Selenium additions in parts per million indicated by numbers at end of lines.

data subsequently obtained at a later date at a higher level, 1.2 mg/kg rat/week, of tocopherol supplementation are shown in figure 4. Growth curves at 2.4 mg/kg rat/week were similar to those noted in figure 4.

Creatinuria. The following summarizes observations with respect to the onset of creatinuria as affected by different levels of selenium, methionine and tocopherol. When the ration did not contain added selenium and methionine, creatinuria occurred in approximately 7 weeks, and tocopherol supplements (0.2 to 0.6 mg/kg rat/week) were ineffective in delaying the development of creatinuria (10). However, when larger tocopherol supplements, 1.2 and 2.4 mg/kg rat/week were administered, creatinuria developed in 9 to 10 and 29 weeks, respectively. If only selenium, (0.03 to 0.30 ppm) was added, the onset of creatinuria was not delayed by low levels of tocopherol supplementation (0.2 to 0.6 mg/kg rat/week). At a higher dose level of tocopherol (1.2 mg/kg rat/week, the times of onset of creatinuria were 24, 24, and 30 weeks, respectively, when 0.03, 0.10, or 0.30 ppm of selenium was added to the basal ration. Adding

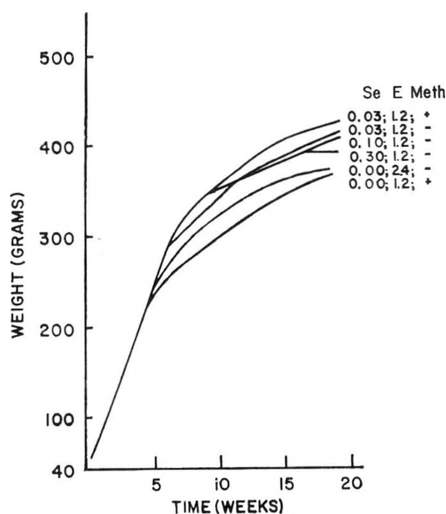

Fig. 4 Effect of added selenium and methionine (0.4% DL-) on the growth response of tocopherol-deficient rats to supplementation with a relatively high dose level of α-tocopherol, average weight of 5 rats/group, selenium (ppm), tocopheryl acetate (mg/kg rat/week), and methionine (0.4% DL-) as indicated.

methionine and 1.2 mg *d*-α-tocopheryl acetate/kg rat/week delayed the onset of creatinuria until 12 weeks if no selenium was added and until 30 weeks when 0.03, 0.10, or 0.30 ppm of selenium was added. In the most extensively studied series, 0.10 to 0.13 ppm added selenium plus 0.4% DL-methionine, a linear dose response was obtained to between 0.2 to 1.75 mg/kg rat/week. Without tocopherol supplementation, creatinuria occurred in 9 weeks and the onset was delayed 17 to 18 weeks/mg *dl*-α-tocopheryl acetate/kg rat/week.

Effect of low fat diets on growth and onset of creatinuria. When the "dienoic" synthetic fat was fed at the 1 and 3% levels in a diet essentially devoid of tocopherol, growth (fig. 5) was initially inferior to that noted when this fat was fed at the 7.5 and 12.5% levels (10). Sustained growth, however, was markedly superior at the lower fat levels.

As the level of synthetic "dienoic" dietary fat was lowered from 7.5 to 3% and then to 1%, the period of feeding required to produce creatinuria (fig. 6) increased from 11 to 14 weeks and then to 33 weeks, respectively, when the diet was essentially devoid of tocopherol (fig. 5).[5] The delay in onset of creatinuria per 0.4 mg of *d*-α-tocopheryl acetate/kg rat body weight fed per week similarly increased from 7 weeks to 13 weeks and then to 15

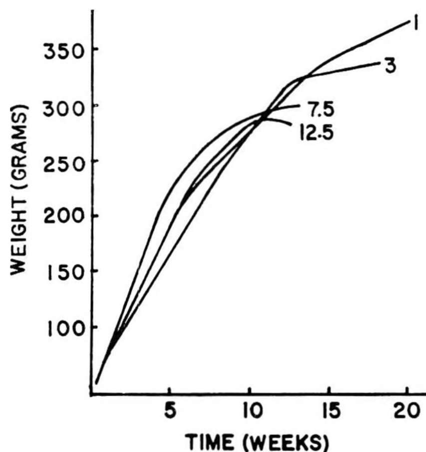

Fig. 5 Effect of dietary fat level on the growth of rats fed a tocopherol-free diet. Growth curves (average of 5 rats/group) are labeled as to the percentage of fat fed.

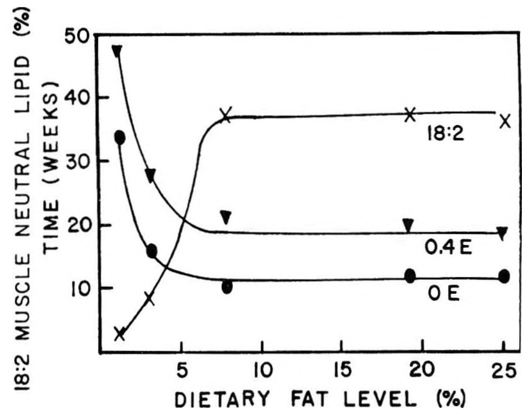

Fig. 6 Effect of dietary fat level on the time of onset of creatinuria in tocopherol-deficient rats and rats given a supplement of 0.4 mg *d*-α-tocopheryl acetate/kg rat/week, and effect of dietary fat level on the linoleic acid (18:2) content of the muscle neutral lipids. Circles indicate data from groups fed tocopherol-free diets (5 animals/group), triangles indicate data from groups given the tocopherol supplement (0.4 mg/kg rat/week), and crosses indicate the percentage of linoleic acid in the gastrocnemius and quadriceps muscle neutral lipid fatty acids from control rats given a supplement of 15 mg *d*-α-tocopheryl acetate/kg rat/body weight/week.

weeks. At the low fat levels (1 and 3%), only a small change in muscle phospholipid linoleic and arachidonic acid composition was noted (table 2), whereas the muscle neutral lipid linoleic acid content was drastically altered. Muscle neutral lipid linoleic acid (average of 5 rats/point) observed in control animals given the tocopherol supplement (15 mg/kg rat/ week) is plotted in figure 6 for comparison with the rate of production of creatinuria.

DISCUSSION

Several factors must be considered in discussing the present data. Considerable care was taken to reduce the tocopherol content of the ration, and the levels of tocopherol supplementation were rather low. For example, using round numbers, for a 300-g rat consuming 150 g of feed/week, given a supplement orally by dropper of

[5] It was reported previously (10) that feeding the "dienoic" fat at the 25% level resulted in a more rapid production of creatinuria. Subsequent experiments have shown that these results were attributable to the use of a small batch of casein unusually low in biologically available selenium. Only the points for data derived from experiments at the 19% fat level are taken from reference 10.

TABLE 2

Effect of level of synthetic dietary fat on the fatty acid composition of muscle[1] *neutral lipids and phospholipids*

Phospholipids					
Dietary fat [2] level, %	1	3	7.5	19	25
No. of samples	5	4	3	9	5
	%	%	%	%	%
Muscle fatty acid composition:					
Monoenoic	17.3 ± 2.2 [3]	12.0 ± 1.5	9.9 ± 0.8	8.7 ± 2.3	6.9 ± 2.0
Dienoic	15.2 ± 1.7	20.0 ± 0.7	21.0 ± 0.2	21.2 ± 2.5	22.1 ± 2.1
Trienoic	2.3 ± 0.2	0.9 ± 0.1	0.6 ± 0.1	0.4 ± 0.2	0.4 ± 0.1
Tetraenoic	16.7 ± 0.9	18.8 ± 2.0	18.3 ± 0.4	21.4 ± 1.0	20.5 ± 1.7
Pentaenoic	2.6 ± 0.5	3.0 ± 0.5	4.5 ± 0.7	4.7 ± 1.6	3.4 ± 1.2
Hexaenoic	2.6 ± 0.3	2.6 ± 0.6	5.1 ± 0.5	4.9 ± 1.1	4.8 ± 0.2
Total PUFA's	39.4 ± 2.3	45.3 ± 1.5	49.6 ± 0.6	52.8 ± 1.3	51.1 ± 1.1
Neutral lipids					
Dietary fat level, %	1	3	7.5	19	25
No. of samples	5	3	1	9	5
	%	%	%	%	%
Muscle fatty acid composition:					
Monoenoic	56.2 ± 3.4	53.7 ± 3.2	33.2	31.3 ± 2.1	31.3 ± 0.2
Dienoic	3.3 ± 0.5	7.9 ± 0.9	36.8	36.8 ± 1.7	35.7 ± 0.9
Trienoic	0.4	0.2	0.2	0.5	0.3
Tetraenoic	0.5	0.5	1.7	1.8	0.5
Pentaenoic	0.3	0.4	0.9	0.9	0.3
Hexanenoic	0.2	0.1	0.4	0.1	0.1
Total PUFA's	4.6 ± 0.8	9.3 ± 1.0	40.0	40.1 ± 1.1	37.4 ± 0.4

[1] Gastrocnemius and quadriceps.
[2] Fatty acid composition: (in per cent) saturated, 42.5; monoenoic, 21.2; dienoic, 36.0; trienoic, 0.3.
[3] Average ± SD.

0.4 mg or 1.2 mg *d*-α-tocopheryl acetate/kg body weight/week, the tocopherol levels would be equivalent to 70 and 220 μg/100 g diet, respectively. Tocopherol-deficient rations containing more tocopherol than the first amount have been used frequently in experiments reported from this and other laboratories (16).

Certain foreign caseins (17, 18) have been reported to be low in biologically available selenium. Domestic casein, however, prevents liver necrosis when fed at the 15 to 20% level (18). The present basal ration contains approximately 0.25 ppm of selenium. Similarly, although casein is known (19) to be low in sulfur amino acids, when fed at a high level (21.8% of calories) as in the present experiment, evidence of such an amino acid deficiency would not be anticipated. Whether the high level of casein in the diet caused an amino acid imbalance that could be partially ameliorated by supplementing with methionine, could not be evaluated in the present experiment.

An analogy to the work of Scott (20) with chicks appears evident. With the tocopherol-deficient diet used in his laboratory low levels of selenium supplementation (0.1 ppm) prevented exudative diathesis. Higher levels of selenium (1 to 5 ppm) " . . . reduced the incidence of muscular dystrophy but failed to prevent this disease in young chicks when *severely* vitamin E-deficient diets were used." (Italics ours). In these same experiments low levels of added methionine (0.15%) stimulated growth but had no effect on the dystrophy, whereas higher levels (0.38% DL-methionine) prevented the nutritional muscular dystrophy. The diet used by Scott contained small amounts (0.0125%) of synthetic antioxidant (ethoxyquin, 6-ethoxy-2,2,4-trimethyl-1:2 dihydroquinoline or DPPD, N, N′ diphenyl-*p*-phenylenediamine) to prevent encephalomalacia.

Also, Schwarz (21) observed that supplemental sulfur amino acids reduced tenfold the tocopherol requirement of rats fed the torula yeast diet. Growth with this ration, however, is relatively poor.

Effect of selenium and methionine on growth and tocopherol requirement. In the present experiments, the addition of selenium (0.10 ppm selenium as sodium selenite) and methionine (0.4% DL-methionine) significantly improved (table 1) the growth response (fig. 1) of tocopherol-deficient rats obtained by giving supplements of 0.2, 0.4, and 0.6 mg *d*-α-tocopheryl acetate/kg body weight/week. When these factors were considered separately (fig. 2(A) and (B)) 0.4% DL-methionine alone was not found to improve the growth of tocopherol-deficient rats. However, when the animals were fed diets containing between 0.03 and 0.30 ppm of added selenium alone, significant improvements (table 1) in growth were noted (fig. 2(A)). Significant (table 1) improvements in growth responses attributed to the added methionine were observed when the diet contained 0.03 and 1.00 ppm of added selenium (fig. 2(A) and (B)). Similar growth stimulation attributed to the added methionine was observed at the intermediate levels of added selenium (0.10 and 0.30 ppm), but these differences were not significant with the small groups (5 rats per group) used. Results (figs. 3(A) and (B), and 4) at 2 levels of tocopherol supplementation (0.4 and 1.2 mg/kg rat/week) and at other levels (0.2, 0.6, and 2.4 mg/kg rat/week) for which the data were not presented, were similar. Selenium addition stimulated growth, and methionine addition effected an additional stimulation of the growth response. However, with increasing levels of tocopherol supplementation, the spread of the groups decreased and the growth stimulation, therefore, became less significant.

With the present severely tocopherol-deficient diet, selenium toxicity was noted at an unusually low dietary level of this trace element (0.25 ppm in basal + 1.00 ppm added). Under these stressful conditions, lower levels of added selenium (0.03 to 0.30 ppm) stimulated growth but did not delay the onset of creatinuria. Under less severe conditions, supplementation with 1.2 mg *d*-α-tocopheryl acetate/kg rat/week, it was evident that the selenium supplementation lowered the apparent tocopherol requirement of the rat (i.e., delayed onset of creatinuria). Methionine supplementation decreased the lethal effect of the previously toxic level of selenium and also decreased the apparent tocopherol requirement of the rat by the same criterion of delay in onset of creatinuria.

Selenomethionine has been shown to be a more potent antioxidant than α-tocopherol (22) and it is likely that the selenium added to the diet is incorporated into the tissue proteins to function in a manner which decreases the requirement for tocopherol as a tissue antioxidant. In view of the recent paper of Shimazu and Tappel (23), on the protective action of selenoamino acids against free radical damage to protein, a possible mechanism for the lowered tocopherol requirement may be formulated. The formation of polar fatty acid peroxide groups within the hydrophobic membrane, a bimolecular-oriented phospholipid layer, results in an "unfolding" of the membrane, bringing peroxidized groups into the aqueous phase in contact with the surrounding protein. Damage to the protein is then minimized when the protein contains selenium. An analogous relationship between aqueous and lipid phases in the cell may prove useful in understanding the function of ascorbic acid as an antioxidant.

Effect of dietary fat level on tocopherol requirement. Except insofar as growth and the tocopherol requirement of the rat were affected, the dietary fat level had very little connection with the rest of the present investigation. In gathering data on factors known to affect the tocopherol requirement, however, it was necessary to include this variable due to the contradictory statements occurring in the literature (2, 8–10). The present data (table 2) taken in conjunction with those presented previously (10) suggest that muscle phospholipid fatty acid composition is dependent qualitatively, but not quantitatively, on the nature of the dietary polyunsaturated fatty acids available. Polyunsaturated fatty acids "overflow" into the neutral lipids only to a very slight extent

until surplus quantities are ingested. Even then, only linoleic and linolenic acids appear in any appreciable quantity in the neutral lipids.

At relatively low levels of dietary fat (7.5%) both the neutral lipid and phospholipid fatty acid achieved relatively "fixed" compositions in equilibrium with the fatty acid composition of the fat fed (table 2). Still lower levels of dietary fat represent an unusual nutritional situation encountered only in some experimental animal diets or in certain human disease states. In these special cases, the tocopherol requirement is markedly reduced, not because of any great change in the potential estimated in vivo peroxidizability of the phospholipid fatty acids, but rather by the greatly reduced estimated peroxidizability (10) of the much greater quantity of neutral lipid in the tissue (fig. 5).

Although the data presented are consistent with the concept that tocopherol functions largely, if not solely, as a fat-soluble, biological antioxidant, no evidence of lipid peroxidation has been presented. The decreased concentration of some polyunsaturated fatty acids during tocopherol deficiency, the presence of lipofuscin or ceroid pigments, and studies of the liver by means of electron microscopy will be covered in future papers.[6] The present data apply only to the young, growing rat in equilibrium with his dietary fat.

Adipose tissue from adult humans fed normal diets contains approximately 8 to 10% of linoleic acid (24), but this level may increase to 40% or more when subjects are fed diets containing large amounts of corn oil or safflower oil (25). The data illustrated in figure 6, support the concept that the tocopherol requirement of man can be related to the linoleic acid content of his tissue as influenced by dietary fat (26). Fortunately, increased concentration of polyunsaturated fats in human diets is usually paralleled by the presence of increased levels of tocopherol. The problems which may arise when this ratio is disturbed remain to be studied.

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LITERATURE CITED

1. Horwitt, M. K. 1961 Vitamin E in human nutrition — an interpretative review. Borden's Rev. Nutrition Res., 22: 1.
2. Century, B., and M. K. Horwitt 1960 Role of diet lipids in the appearance of dystrophy and creatinuria in the vitamin E-deficient rat. J. Nutrition, 72: 357.
3. Schwarz, K. 1954 Factors protecting against dietary necrotic liver degeneration. Ann. N. Y. Acad. Sci., 57: 878.
4. Schwarz, K., and C. M. Foltz 1957 Selenium as an integral part of factor 3 against dietary liver degeneration. J. Am. Chem. Soc., 79: 3292.
5. Bunnell, R. H., L. D. Matterson, E. P. Singsen, L. M. Potter, A. Kozeff and E. L. Jungherr 1955 Studies on encephalomalacia in the chick. III. The influence of feeding or injection of various tocopherols and other antioxidants on the incidence of encephalomalacia. Poultry Sci., 34: 1068.
6. Nesheim, M. C., and M. L. Scott 1958 Studies on the nutritive effects of selenium for chicks. J. Nutrition, 65: 601.
7. Calvert, C. C., M. C. Nesheim and M. L. Scott 1962 Effectiveness of selenium in prevention of nutritional muscular dystrophy in the chick. Proc. Soc. Exp. Biol. Med., *109:* 16.
8. Gottlieb, H., F. W. Quackenbush and H. Steenbock 1943 The biological determination of vitamin E. J. Nutrition, 25: 433.
9. Century, B., M. K. Horwitt and P. Bailey 1959 Lipid factors in the production of encephalomalacia in the chick. Arch. Gen. Psychiat., *1:* 420.
10. Witting, L. A., and M. K. Horwitt 1964 Effect of degree of fatty acid unsaturation in tocopherol-deficiency induced creatinuria. J. Nutrition, 82: 19.
11. Mameesh, M. S., and B. C. Johnson 1958 The effect of penicillin on the intestinal synthesis of thiamine in the rat. J. Nutrition, 65: 161.
12. Woodman, A. G. 1941 Food Analysis, ed. 4. McGraw-Hill Book Company, Inc., New York, p. 185.
13. Watkinson, J. H. 1960 Fluorometric determination of traces of selenium. Analyt. Chem., 32: 981.
14. Bonsnes, R. W., and H. H. Taussky 1945 On the colorimetric determination of creatinine by the Jaffe reaction. J. Biol. Chem., *158:* 581.
15. Mason, K. E. 1944 Physiological action of vitamin E and its homologues. Vitamins and Hormones, 2: 141.
16. Green, J., A. T. Diplock, J. Bunyan, E. E. Edwin and D. McHale 1961 Ubiquinone

[6] J. J. Theron, L. A. Witting and M. K. Horwitt, unpublished data.

(coenzyme Q) and the function of vitamin E. Nature, *190:* 318.

17. Bieri, J. G., H. Dam, I. Prange and E. Søndergaard 1961 Effect of dietary selenium dioxide, cystine, ethoxyquin and vitamin E on lipid autoxidation in chick tissues. Acta Physiol. Scand., *52:* 36.
18. Bonetti, E., and F. Stirpe 1962 Conditions affecting dietary liver necrosis and liver regeneration in rats. J. Nutrition, *77:* 179.
19. Vars, H. M., and F. N. Gurd 1947 Effect of dietary protein upon the regeneration of liver protein in the rat. Am. J. Physiol., *151:* 399.
20. Scott, M. L. 1962 Vitamin E in health and disease of poultry. Vitamins and Hormones, *20:* 621.
21. Schwarz, K. 1962 Lipids and antioxidants in dietary necrotic liver degeneration. In Symposium on Foods: Lipids and Their Oxidation, eds., H. W. Schultz, E. A. Day and R. O. Sinnhuber. Avi Publishing Company, Inc. Westport, Connecticut, p. 387.
22. Hamilton, J. W., and A. L. Tappel 1963 Lipid antioxidant activity in tissues and proteins of selenium-fed animals. J. Nutrition, *79:* 493.
23. Shimazu, F., and A. L. Tappel 1964 Selenoamino acids: Decrease of radiation damage to amino acids and proteins. Science, *143:* 369.
24. Hegsted, D. M., C. W. Jack and F. J. Stare 1962 The composition of human adipose tissue from several parts of the world. Am. J. Clin. Nutrition, *10:* 11.
25. Horwitt, M. K. 1962 Interrelations between vitamin E and polyunsaturated fatty acids in adult men. Vitamins and Hormones, *20:* 541.
26. Horwitt, M. K. 1960 Vitamin E and lipid metabolism in man. Am. J. Clin. Nutrition, *8:* 451.

Interrelationships between Dietary Levels of Sodium, Chlorine and Potassium

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ABSTRACT A series of experiments was conducted on the dietary relationship of the cations, sodium and potassium, and the anions, chloride and sulfate. The data showed that excesses of dietary chloride or sulfate ions supplied as glutamic acid hydrochloride, calcium chloride or calcium sulfate markedly depressed growth rate of chicks unless these anions were balanced with equimolar levels of sodium or potassium supplied in the diet with a metabolizable anion such as glutamate or carbonate. Excess sodium supplied as sodium glutamate was also detrimental unless a high level of chloride in one of the above forms was also present. Chicks tolerated excesses of potassium better than excesses of sodium. These experiments provide a probable explanation of many discrepant results reported in the literature on the dietary requirements of chicks and rats for sodium and potassium.

Although the physiological role of sodium, potassium and chloride in the regulation of osmotic pressure and acid-base balance in the animal has been well established (1), the application of these principles to nutritional investigations has received little study. This could be particularly important in nutritional investigations requiring the use of high purified diets, since large quantities of amino acid hydrochlorides or other acid-forming salts are sometimes used.

In this laboratory, certain differences, depending upon the mineral mixture used in a purified diet, were noted in the growth rate of chicks. The tested mineral mixtures differed only in form and level of chloride. These observations led to a series of studies of the effect of feeding amino acid hydrochlorides or other "acid" chlorides upon the sodium and potassium needs of the young chick.

MATERIALS AND METHODS

The composition of the 2 purified diets used in these studies is shown in table 1. One diet contained casein (diet A) as a source of protein and the other, isolated soybean protein (diet B). Alterations in the diet were made at the expense of glucose, except where indicated with the basal diet used in each individual experiment. Mineral mixture no. 1 was used in all basal diets except in experiment 1 (table 2) in which mineral mixture no. 2 was used. The basal diets were analyzed as described by Leach and Nesheim (2). The total amount of dietary chloride is shown in all of the tables.

White Plymouth Rock male chicks were fed the experimental diets from 2 to 29 days of age in experiment 1, and from 2 to 15 days of age in the remaining experiments. Chicks were housed in electrically heated cages with raised wire floors, and feed and demineralized water were supplied ad libitum.

Statistical analyses were conducted by procedures described by Federer (3).

EXPERIMENTS AND RESULTS

In a preliminary experiment, the growth rate of chicks fed a diet similar to diet A (table 1) varied, depending upon which of 2 mineral mixtures was used. The major differences in the 2 mineral mixtures were the levels of chloride and carbonate they contained.

To determine more precisely the effects of changes in the chloride level of the mineral mixture, an experiment was conducted in which the chloride, sodium and potassium levels were altered in several ways. The dietary variables were studied

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TABLE 1
Composition of diets

	Diet A	Diet B
	%	%
Glucose	56.67	58.47
Isolated soybean protein	—	27.00
Casein	27.00	—
Arginine·HCl	1.50	—
DL-Methionine	0.30	0.70
Glycine	1.00	0.30
Corn oil	3.00	3.00
Cellulose	3.00	3.00
Vitamin mixture [1]	1.23	1.23
Mineral mixture no. 1 [2,3]	6.30	6.30
Protein (N × 6.25), %	25.86	24.82
Metabolizable energy, kcal/g	3.18	3.35

[1] Supplies the following per 100 g of diet: inositol, 25.0 mg; niacin, 5.0 mg; Ca pantothenate, 2.0 mg; pyridoxine·HCl, 0.45 mg; folic acid, 0.40 mg; menadione sodium bisulfite, 0.125 mg; biotin, 0.02 mg; vitamin B_{12}, 2.0 μg; α-tocopheryl acetate, 6.6 IU; vitamin A, 540 IU; vitamin D_3, 98 ICU; thiamine·HCl, 1.1 mg; riboflavin, 1.1 mg; ethoxyquin, 12.5 mg; and choline chloride, 220 mg.

[2] Mineral mixture no. 1 supplied the following per 100 g of diet: $CaCO_3$, 2.80; $CaHPO_4 \cdot 2H_2O$, 0.34 g; KH_2PO_4, 1.38 g; $NaH_2PO_4 \cdot H_2O$, 1.44 g; $MgSO_4$, 0.25 g; $MnSO_4 \cdot H_2O$, 33.3 mg; $FeSO_4 \cdot 7H_2O$, 33.3 mg; KI, 0.26 mg; $CuSO_4 \cdot 5H_2O$, 1.67 mg; $CoSO_4 \cdot 7H_2O$, 0.20 mg; $NaMoO_4 \cdot 2H_2O$, 0.83 mg; and $ZnCO_3$, 11.5 mg.

[3] Mineral mixture no. 2 used in experiment 1 (table 3), 6.10 g/100 g of diet supplied the following per 100 g of diet: $CaCO_3$, 1.83 g; $CaHPO_4$, 1.70 g; KH_2PO_4, 1.38 g; $NaHCO_3$, 0.87 g; $MgSO_4$, 0.25 g; $MnSO_4 \cdot H_2O$, 33.3 mg; $FeSO_4 \cdot 7H_2O$, 33.3 mg; KI, 0.26 mg; $CuSO_4 \cdot 5H_2O$, 1.67 mg; $CoCl_2 \cdot 6H_2O$, 0.17 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.83 mg; and ZnO, 7.46 mg.

and the results obtained are presented in table 2.

In this experiment, increasing the chloride content of the diet from 0.45 to 0.81% depressed growth rate. This effect was reversed by the addition of sodium and potassium bicarbonate to the diet. The level of 0.67% chloride, which coincides with that in the casein diets supplemented with arginine hydrochloride and mineral mixtures of the type commonly used in purified chick diets, was achieved by substituting sodium chloride for the sodium bicarbonate in the basal mineral mixture. The basal levels of sodium (0.24%) and potassium (0.40%) exceeded the known requirements for the elements (4, 5).

A further increase in chloride content to 1.50% severely reduced growth rate. This effect was minimized when the chloride was supplied in the form of sodium and potassium salts, resulting in concomitant increases in sodium and potassium which allowed the chicks to tolerate better the effects of excess chloride. The results of this experiment suggest that the differences in growth rate with different chloride levels and salts are primarily due to effects on acid-base balance of the various salts used in the diets. Thus, the chloride supplied as arginine hydrochloride, glutamic acid hydrochloride, or $CaCl_2$ may have produced a metabolic acidosis which was neutralized by the addition of extra sodium and potassium in conjunction with metabolizable anions such as bicarbonate and acetate.

To determine the amount of excess chloride that would be detrimental, calcium chloride was included at graded levels in a diet for chicks (table 3). The amount of calcium in all diets was kept constant by substituting $CaCl_2$ for $CaCO_3$ in the mineral mixture. The amounts of sodium and potassium were also maintained constant at 0.24 and 0.40%, respectively.

The data in table 3 show that the basal diet (0.02% Cl) was deficient in chloride since growth of chicks was considerably improved when 0.30% chloride was added to the diet. This observation led to a further study of chloride deficiency in chicks (2). All levels of chloride above 0.3% depressed growth of chicks fed these diets as compared with diet 2. Because of the

TABLE 2
Effect of excess chloride, sodium and potassium on the growth rate of chicks fed a purified diet (diet A)

Source of chloride	Level of chloride	Level of Na	Level of K	Avg wt, 4 weeks [1]
	%	%	%	g
Arginine·HCl	0.45	0.24	0.40	567
Arginine·HCl, NaCl	0.81	0.24	0.40	508
Arginine·HCl, NaCl	0.81	0.45	0.76	572
Arginine·HCl, NaCl, $CaCl_2$	1.50	0.24	0.40	391
Arginine·HCl, NaCl, glutamic acid·HCl	1.50	0.24	0.40	415
Arginine·HCl, NaCl, KCl	1.50	0.45	0.76	507

[1] Values represent averages of 2 groups of 15 chicks/treatment.

TABLE 3

Effect of feeding calcium chloride at various levels to chicks (diet B)

Treatment[1] Level of chloride	Avg wt,[2,3] 2 weeks	Mortality
%	g	%
0.02	148	21
0.32	209	4
0.62	183	4
0.92	173	0
1.22	144	0
1.52	122	8

[1] $CaCl_2 \cdot 2H_2O$ added to the diet at the expense of $CaCO_3$ to maintain constant the calcium level in all diets.

[2] Represents the average weight of 3 groups of 8 male White Plymouth Rock chicks/treatment.

[3] Statistical analysis of these data indicated that the weight of chicks receiving the 0.02% chloride level was significantly different ($P < 0.01$) than weights of chicks receiving higher levels of chloride. The chloride levels fed, depressed growth rate in a linear fashion with the regression of chloride level on body weight being highly significant ($P < 0.01$).

results obtained in this study, in later experiments a level of 1% chloride added as glutamic acid hydrochloride was used for a "high-chloride" diet.

The next experiment was conducted to determine the effect of feeding diets high in an amino acid hydrochloride along with variable levels of sodium and potassium. To keep the alterations in other anions or cations in the diet from confounding the results, chloride was added as glutamic acid hydrochloride, and sodium and potassium as sodium or potassium glutamate. Thus the total amount of glutamate or glutamate salt was kept constant in all diets. The design and results of this experiment are shown in table 4.

A series of sodium and potassium levels was studied at 2 levels (0.45 and 1.50%) of dietary chloride. The high level of chloride used represented the addition of 1% chloride to the basal diet as glutamic acid hydrochloride. Additional amounts of sodium and potassium were added to the basal diet (0.24 Na and 0.40 K) at levels which were 0.75 M (treatments 2 and 5), equimolar (treatments 3 and 6), and 1.25 M (treatments 4 and 7) with the 1% added chloride. In treatment 8, a mixture of sodium and potassium glutamate was used to make the total added sodium and potassium levels equimolar to the added chloride.

Levels of 0.89 and 1.05% added sodium were detrimental in the low chloride series as evidenced by the high mortality of chicks and the low body weight of survivors in treatments 3 and 4. Most of the mortality in chicks fed the high levels of sodium occurred in the first 3 to 5 days of feeding. In groups of chicks fed at the high levels of potassium, little mortality occurred although the growth rate of chicks fed the highest level was slightly lower than that of chicks fed at the 2 lower levels. In the high chloride series of treatments there was little mortality from feeding high levels of sodium. The chloride appeared to protect the chick from sodium "toxicity."

TABLE 4

Relationship of various levels of sodium and potassium to the chloride content of a diet for chicks (diet A)

Treatment no.	Level of Na	Level of K	Level of chloride[1] 0.45% Avg wt, 2 weeks[2]	0.45% Mortality	1.50% Avg wt, 2 weeks	1.50% Mortality
	%	%	g	%	g	%
1	0.24	0.40	180	8	143	8
2	0.73	0.40	191	0	165	4
3	0.89	0.40	182	67	184	4
4	1.05	0.40	122	92	174	0
5	0.24	1.22	186	0	165	4
6	0.24	1.50	190	12	185	0
7	0.24	1.78	176	0	192	8
8	0.56	0.95	197	8	186	12

[1] Statistical analysis of these data was carried out by an analysis of variance, and the difference in mean treatment weights required for significance (at $P < 0.05$) according to Tukey's HSD test (3) is 25.5 g.

[2] Each value represents the average of 3 groups of 8 chicks/treatment.

Increasing the chloride content of the basal diet (treatment 1) from 0.45 to 1.50% markedly depressed growth rate of chicks. Increasing the level of sodium or potassium alleviated the growth depression observed in chicks fed at the high chloride levels. In each case it appeared that the equimolar level, or above, of the cation was needed to completely reverse the growth depression caused by feeding excess chloride as glutamic acid hydrochloride. When a combination of sodium and potassium was used, equimolar to the added chloride, the growth rate of chicks was not depressed.

An additional experiment was conducted to compare glutamic acid hydrochloride and calcium chloride as sources of excess chloride in the diets used. In addition, the influence of extra nitrogen as glutamic acid in a diet high in calcium chloride was studied along with the effectiveness of potassium carbonate in reversing the effects on chicks of consuming diets with high chloride levels. The data from this experiment are shown in table 5. Chicks fed at high levels of chloride, either as the glutamic acid hydrochloride or as calcium chloride, grew at a considerably slower rate than chicks fed the basal diet. The calcium chloride depressed growth significantly more ($P < 0.05$) than the glutamic acid hydrochloride. The difference between the 2 sources of excess chloride may be at least partially due to the ammonia supplied with the glutamic acid, because chicks receiving the calcium chloride treatment supplemented with glutamic acid did not grow at rates significantly different from those receiving the glutamic acid hydrochloride. The potassium carbonate was partially effective in reversing the chloride toxicity.

In view of the results obtained in previous experiments with excess chloride and sodium and potassium levels, it was considered that similar results would also be obtained with excess sulfate supplied as calcium sulfate. Sulfate at graded levels was fed to chicks in the presence or absence of a combination of 0.64% sodium and 1.08% potassium. The glutamate content of all diets was kept constant and sodium or potassium glutamate was used to bring the sodium and potassium content to the above levels. The calcium level in all diets was kept constant by adjustments in the level of calcium carbonate used. The design and results of this experiment are shown in table 6.

As the sulfate level in the diet was increased, the growth rate of chicks was depressed. Except for the highest level of sulfate, extra sodium and potassium as the glutamic acid salts in the diet completely reversed the growth depression. Possibly a higher level of sodium or potassium than used may be necessary. The sulfate was not as growth-depressing as the chloride, probably because of poorer absorption of the sulfate as compared with chloride.

An attempt was made to determine whether the other alkali elements, lithium or rubidium, could function to counteract excess chloride levels in a diet, but these elements proved to be highly toxic and chicks died within a week after being fed these materials. In addition, the alkaline earth elements, calcium and magnesium, were studied but these were also ineffective in counteracting chloride toxicity.

TABLE 5

Influence of glutamic acid and potassium carbonate on excess dietary chloride (diet A)

	Treatment	Avg wt,[1,2] 2 weeks
		g
1	Basal diet (0.45% Cl)	192 d
2	1% Chloride (as glutamic HCl)[3]	153 b
3	1% Chloride (as glutamic·HCl) + 1.95% K_2CO_3	179 c
4	1% Chloride (as $CaCl_2 \cdot 2H_2O$)	129 a
5	1% Chloride (as $CaCl_2 \cdot 2H_2O$) + 10% glutamic acid	141 ab

[1] Each value represents the average of 3 groups of 8 chicks each/treatment.
[2] Means not followed by the same letter are significantly different ($P < 0.05$) by Duncan's multiple range test (3).
[3] In treatments 2 and 3 the glutamic acid·HCl replaced glucose in the basal diet, in treatments 2 and 5 the $CaCl_2$ replaced $CaCO_3$ and the dietary calcium level remained constant.

TABLE 6

Effect of sodium and potassium on reversing sulfate toxicity (diet B)

Treatment [1,2]	0.24% Na, 0.40% K	0.64% Na, 1.08% K
SO_4 added	Avg wt, 2 weeks [3]	Avg wt, 2 weeks [3]
%	g	g
0	235	242
0.5	233	240
1.0	213	246
1.5	213	240
2.0	206	233
2.5	193	221

[1] The SO_4 was added as $CaSO_4 \cdot 2H_2O$ replacing $CaCO_3$ to maintain a constant percentage of Ca in all diets. The sodium and potassium were added as sodium and potassium glutamate. All diets had the same glutamate level by adjusting glutamic acid or appropriate sodium or potassium salt. Mineral mixture no. 1 (table 1) was altered for use in the basal diet to supply 0.416 g $MgCl_2 \cdot 6H_2O$/100 g of diet instead of the $MgSO_4$; 0.11 g NaCl/100 g diet; and the $NaH_2PO_4 \cdot H_2O$ was reduced to 1.18 g/100 g diet (diet contains 0.23% Cl).

[2] Statistical analyses of these data by analysis of variance showed that there was a significant effect ($P < 0.01$) from the sulfate levels fed and the different sodium and potassium levels fed. There was also a significant interaction ($P < 0.05$) between sulfate levels and sodium and potassium levels fed.

[3] Each value represents the average of 3 groups of 8 chicks each/treatment.

DISCUSSION

These studies show that a rather close balance of sodium and potassium to chloride and sulfate must be maintained in diets in which the forms of chloride and sulfate may increase dietary acidity. Thus, levels of amino acid hydrochloride in purified diets for chicks must be balanced with nearly equimolar amounts of sodium or potassium supplied with a metabolizable anion such as carbonate. Conversely, excessive sodium supplied with a metabolizable anion can also be detrimental to chicks unless it is balanced with sufficient chloride or other anion. Excesses of potassium appear to be less detrimental than sodium.

The results of these experiments with chicks are similar to those reported by Thacker (6). Growth rates of rabbits were improved by supplementing the basal diet with salts of calcium, magnesium, sodium and potassium carrying an anion metabolizable to CO_2 and H_2O. The chloride and sulfate salts of the above cations were ineffective. The mineral mixture used in the basal diet was high in calcium and potassium sulfates.

Presumably the results obtained are those of the maintenance of body acid-base balance. Excess acidity neutralized by the bicarbonate buffer system of the body results in chloride excretion in the kidney. Since pH 4 is usually the lowest value for urine in most animals, very little HCl can be excreted and the chloride must be excreted primarily as an ammonium salt or neutral salt such as sodium or potassium chloride. Thus with high amounts of dietary acidity a sodium and potassium deficiency may be induced. No measurements of urinary excretion of sodium, potassium and chloride were made in these studies. However, recently Koike et al.[2] reported that considerable sodium excretion may occur under certain conditions through the large intestine as well as in the urine.

These results are of particular importance in the formulation of purified diets for chicks. Thus in diets using casein as the source of protein, and supplemented with arginine hydrochloride, the sodium and potassium in the mineral mixture should be supplied as carbonate or bicarbonate salts. Similarly, mineral mixtures in diets with crystalline amino acids as the source of protein should be formulated to take into account the high levels of amino acid hydrochloride often used in such diets.

Several observations in the literature can probably be explained because of problems encountered in acid-base balance of dietary components. A purified diet for chicks containing high levels of arginine and lysine hydrochlorides was improved by the addition of potassium acetate, citrate or bicarbonate.[3] Leach et al. (7) presented data on the toxicity of dietary sulfate which was greater than that observed in experiments reported in this paper, probably because the basal diet used already contained a substantial amount of chloride as arginine hydrochloride.

The relationships observed in these experiments also provide a likely explanation for variations in sodium and potassium requirement reported in the literature.

[2] Koike, T., D. H. Lumijarvi and F. W. Hill 1964 Studies of sodium movement and excretion in chickens. Federation Proc., 23: 185 (abstract).

[3] O'Dell, B. L., C. L. Limbaugh and J. E. Savage 1962 Arginine-lysine antagonism and deficiencies of casein for the chick. Federation Proc., 21: 8 (abstract).

Burns et al. (8) reported a sparing action of sodium level in the diet on the amount of potassium required for maximal growth rate in chicks. At 0.1% dietary sodium, 1% potassium was required for maximal growth rate, whereas when 0.18% sodium was included in the diet, only 0.33% potassium was needed. The mineral mixture used in the experiments contained calcium chloride to supply 0.44% chloride. The sodium and potassium was supplied as the bicarbonate salts. As shown by the data in this paper, the variation in potassium requirement at different sodium levels was probably due to the need for sodium or potassium for excretion of the excess chloride supplied by the calcium chloride in the mineral mixture. Similarly, McWard and Scott (5) reported that the dietary sodium requirement of the chick varied from 0.11% in a diet containing isolated soybean protein as the source of protein to 0.2% in a diet containing casein. The casein diet contained the same mineral mixture as in the studies of Burns et al. (8) with 0.44% chloride supplied as calcium chloride, whereas the diet containing soybean protein had 0.18% chloride supplied as KCl. Thus the higher sodium requirement with the casein diet was probably due to the increased need for sodium caused by the excess chloride supplied by the calcium chloride.

In further experiments, using the diet containing isolated soybean protein, McWard and Scott [4] were unable to confirm the dietary studies of Burns et al. (8) who reported that increasing the level of sodium or potassium would decrease the requirement for the other element. The sodium and potassium requirement studies with rats have also been complicated by the use of diets containing calcium chloride in the mineral mixture while using bicarbonates of sodium and potassium to supply the levels of these elements studied. Thus Grunert et al. (9) observed that there was a slight sparing effect of the sodium requirement by high dietary potassium levels and a larger effect on the potassium requirement by increased sodium levels. The mineral mixture used supplied calcium in the form of calcium chloride to the diet. In the studies of St. John (10) the sodium requirement of the rat was found to be 0.3% of the diet. These studies were also complicated by the use of 1.7% HCl in the mineral mixture.

LITERATURE CITED

1. Best, C. H., and N. B. Taylor 1961 The Physiological Basis of Medical Practice. The Williams and Wilkins Company, Baltimore.
2. Leach, R. M., Jr., and M. C. Nesheim 1963 Studies on chloride deficiency in chicks. J. Nutrition, *81:* 193.
3. Federer, W. T. 1955 Experimental Design. The Macmillan Company, New York.
4. Leach, R. M., Jr., R. Dam, T. R. Zeigler and L. C. Norris 1959 The effect of protein and energy on the potassium requirement of the chick. J. Nutrition, *68:* 89.
5. McWard, G. W., and H. M. Scott 1961 Sodium requirement of the young chick fed purified diets. Poultry Sci., *40:* 1026.
6. Thacker, E. J. 1959 Effect of a physiological cation-anion imbalance on the growth and mineral nutrition of rabbits. J. Nutrition, *69:* 28.
7. Leach, R. M., Jr., T. R. Zeigler and L. C. Norris 1960 The effect of dietary sulfate on the growth rate of chicks fed a purified diet. Poultry Sci., *39:* 1577.
8. Burns, C. H., W. W. Cravens and P. H. Phillips 1953 The sodium and potassium requirements of the chick and their interrelationship. J. Nutrition, *50:* 317.
9. Grunert, R. R., J. H. Meyer and P. H. Phillips 1950 The sodium and potassium requirements of the rat for growth. J. Nutrition, *42:* 609.
10. St. John, J. L. 1928 Growth on a synthetic ration containing small amounts of sodium. J. Biol. Chem., *77:* 27.

[4] McWard, G. W., and H. M. Scott 1960 The potassium and sodium requirement of the young chick fed a purified diet. Poultry Sci., *39:* 1274 (abstract).

Comparison of Mo^{99} Metabolism in Swine and Cattle as Affected by Stable Molybdenum [1]

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ABSTRACT Data from yearling cattle and growing swine having similar molybdenum, copper and sulfate intakes demonstrated marked species differences in absorption and excretion of Mo^{99}. Mo^{99} from oral doses reached a peak in blood of swine at 2 to 4 hours, whereas the average time in cattle was 96 hours. Over 75% of both oral and intravenously dosed Mo^{99} was excreted in urine of swine in 120 hours. In contrast, fecal excretion was the main route of excretion in both oral and intravenous doses in cattle with only 15% excreted in urine in 168 hours by intravenously dosed cattle. Added stable molybdenum increased urinary Mo^{99} excretion in swine but had little influence on Mo^{99} excretion in cattle. These data demonstrate a definite species difference and support the differences in toxicity of molybdenum in swine and cattle.

Molybdenum as a component of xanthine oxidase is widely distributed in animal and plant tissue (1). The normal concentration of molybdenum in mammalian muscle is usually 0.05 to 0.10 ppm on a dry matter basis (2). It has been suggested that molybdenum be considered an essential dietary trace element as a part of xanthine oxidase and other enzyme systems (3, 4). More recently, Handler et al. (5) reported that 2 atoms of molybdenum were required as an essential molecular part of both xanthine oxidase and aldehyde oxidase. However, molybdenum has been considered primarily as a toxic mineral for ruminants and laboratory animals, with no toxicity reported in swine fed 1000 ppm for 3 months (2). The convenience and reliability of radioactive molybdenum as a tool for research on this mineral element have been demonstrated (6–9), but most of these data were obtained with dosing solutions of low specific activity which may have influenced the data. Buescher[5] suggested that specific activity of the dosing solution affected excretion of Mo^{99} of swine dosed orally.

The purpose of these investigations was to compare absorption and excretion of Mo^{99} of the porcine and bovine species and to investigate the effects of stable carrier molybdenum.

EXPERIMENTAL PROCEDURE

Twenty barrows of mixed breeding, whose body weights averaged 39 kg, were fed ad libitum a practical growing ration (10) which contained 1.2 ppm molybdenum and 9.3 ppm copper. During a 14-day pre-experimental period the barrows were kept in individual pens. This was followed by a 4-day adjustment period in a stall designed for complete separation and collection of urine and feces (11). Blood samples were collected at 1, 2, 4, 8 and 16 hours following the oral or intravenous administration of Mo^{99}. Blood, urine and fecal samples were collected thereafter at 24-hour intervals for 5 consecutive days.

Six Hereford steers averaging 418 kg in body weight were used in a reversal-type experiment involving 2 routes of administration and 2 levels of stable carrier molybdenum so that each of the steers was used in the 4 treatments. The steers were fed a

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[4] Operated by the Tennessee Agricultural Experiment Station for the U. S. Atomic Energy Commission under Contract no. AT-40-1-GEN-242.

[5] Buescher, R. G. 1961 The effect of excessive calcium upon the absorption and utilization of Se^{75}, Mo^{99}, Cu^{64} and Fe^{59} in swine. M. S. Thesis, University of Tennessee.

ration (table 1) containing 0.9 ppm molybdenum and 7.5 ppm copper during the 21-day adjustment periods and throughout the experiment. An interval of at least 7 days between the end of one collection period and the succeeding dosing was maintained. The steers were placed into individual stalls for separation and collection of urine and feces for a preliminary period of 7 days prior to dosing. Blood, urine and fecal samples were collected for 7 or 12 days folowing the oral or intravenous administration of Mo^{99}.

Mo^{99} as ammonium molybdate in approximately 1 N ammonium hydroxide solution was used. Specific activity averaged 0.2 mc/mg for the control dosing solution and 0.025 mc/mg when stable molybdenum, as ammonium molybdate, was added. Swine were orally dosed with 0.5 mc through a stomach tube or intravenously into the jugular vein with needle and syringe. Cattle were orally dosed with 4 mc adsorbed on cellulose in a gelatin capsule or intravenously into the jugular vein. Three-gram aliquots of feces and 3-ml aliquots of urine and blood were assayed for Mo^{99} using a well-type scintillation counter. Appropriately diluted standards of the dosing solution were also counted for dose calculations. Blood volume was assumed as 7% of body weight for swine and 7.7% of body weight for cattle (12).

TABLE 1
Composition of cattle ration [1]

Complete ration	
	%
Cottonseed hulls	40.0
Crushed corn	37.3
Alfalfa meal	10.0
Soybean meal	10.0
Urea	1.0
Dicalcium phosphate	1.0
Salt	0.5
Trace mineral mix	0.2
Total	100.0

Trace mineral mix	
	%
$MgSO_4 \cdot 7H_2O$	86.000
$FeSO_4 \cdot 7H_2O$	10.000
$MnSO_4 \cdot H_2O$	1.000
$Na_2B_4O_4 \cdot 10H_2O$	1.000
$CuSO_4 \cdot 5H_2O$	0.300
$ZnSO_4 \cdot 7H_2O$	1.400
KI	0.291
$CoCl_2 \cdot 6H_2O$	0.003
$NaSeO_3$	0.003
MoO_3	0.003
Total	100.000

[1] 500 IU vitamin A and 200 IU vitamin D added/kg of ration.

Stable molybdenum was determined by neutron activation analysis at the Oak Ridge National Laboratory and copper was extracted by the procedure of Kalthoff and Sandell (13) and was then determined on a flame photometer at 325.1 and 324.7 mμ.

RESULTS

Blood. Mo^{99} in the whole blood of swine dosed orally averaged 6% of the dose at 2 and 4 hours after an oral dose. This declined rapidly to 0.07% at 48 hours (fig. 1). In contrast, the highest blood Mo^{99} from an oral dose to cattle was observed as 2.6% of the dose at 96 hours. Blood Mo^{99} in these cattle declined slowly to 0.9% at 168 hours. Four hours after dosing, blood Mo^{99} from an intravenous dose was almost identical with that from an oral

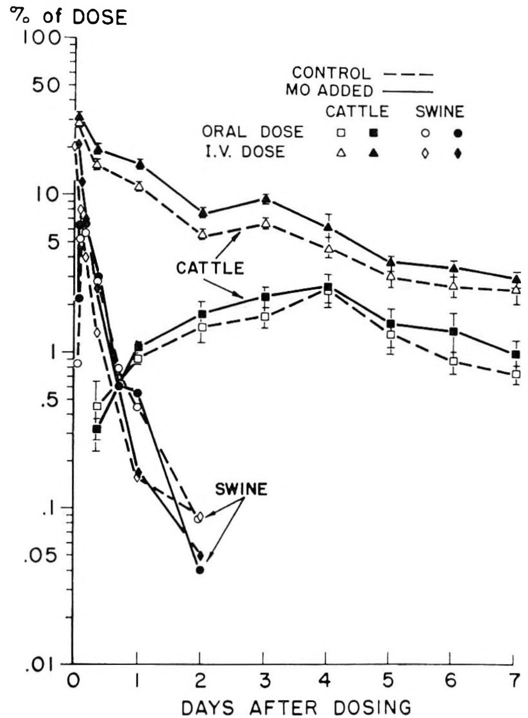

Fig. 1 Comparison of blood Mo^{99} in swine and cattle. The vertical bars (I) represent SE of means.

dose in swine. At one hour after an intravenous dose, blood Mo^{99} was 21.5% of the dose in swine compared with 29.7% in cattle. By 48 hours, blood Mo^{99} averaged 0.07% in both orally and intravenously dosed swine. Mo^{99} in blood of cattle dosed intravenously declined very slowly and averaged 2.8% of dose in 168 hours. Added carrier molbydenum did not consistently influence blood Mo^{99} in swine; however, the blood levels of cattle were consistently higher when stable molybdenum was added to the dosing solution. Intravenous Mo^{99} disappeared from blood of steers at an uneven rate and data in figure 1 indicate recycling of Mo^{99} with either some body pool or with the gastrointestinal contents. Blood Mo^{99} content averaged 6.7% at 48 hours then increased to 8.1% at 72 hours. This was followed by a slow but steady decline in blood Mo^{99}.

Urine. Both oral and intravenously administered doses of Mo^{99} were rapidly excreted in the urine of swine. These values, for the first 24 hours, ranged from 53% for the intravenously dosed control swine to 86% for both the oral and intravenously dosed swine given carrier molybdenum. Urinary excretion during the 5-day collection period cleared 79% of the dose in the intravenously dosed control swine and 97% in the intravenously dosed swine receiving added carrier molybdenum.

In contrast the cattle excreted less than 0.5% of the oral dose and less than 3% of the intravenous dose during the first 24 hours. At the end of 7 days, urinary excretion averaged 4.5% of the orally administered dose and 9.5% of the intravenous dose. Urinary excretion of intravenously administered Mo^{99} reached a plateau at 9 days and slowly increased to 15% of the dose by the end of 12 days. Added stable molybdenum did not affect urinary or fecal Mo^{99} excretion by cattle (fig. 2).

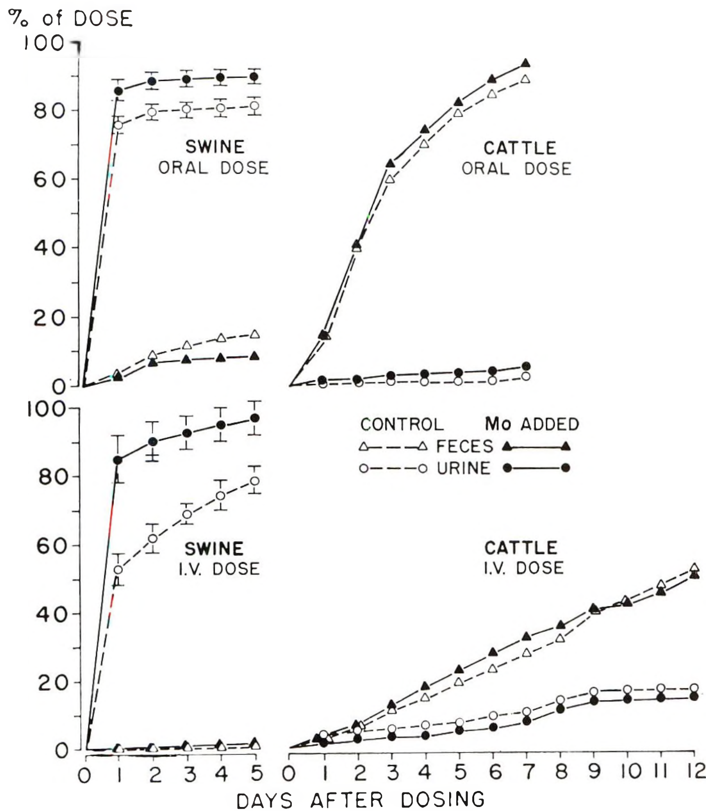

Fig. 2 Accumulative Mo^{99} excretion from oral and intravenous (I.V.) dose to swine and cattle. The vertical bars (I) represent SE of means.

Feces. The proportion of an oral dose of Mo^{99} excreted in the feces of swine was reduced by addition of stable molybdenum with the total 5-day excretion averaging 14.6% for the control swine and 8.6% for the swine receiving added molybdenum (fig. 2). Only 0.6% of intravenously administered Mo^{99} was excreted in the feces by swine in 5 days. Fecal excretion of Mo^{99} in 7 days by steers averaged 92.4% of the oral dose and 30.2% of the intravenous dose. The intravenously dosed cattle showed a linear excretion pattern, with a total of 51.4% excreted in the feces in 12 days.

Retention. Excretion data in table 2 show that Mo^{99} is retained for a much longer period by cattle than by swine. Most of the Mo^{99} was excreted during the first 24 hours by swine. Intravenously dosed cattle excreted only 66.4% in 12 days, whereas intravenously dosed swine excreted 69.4% during the first 24 hours.

DISCUSSION

A nearly exponential rate of clearance of Mo^{99} from the blood of pigs occurred regardless of route of administration or specific activity of dosing solution. Shirley et al. (14) reported that 0.2% of an oral dose remained in the blood of swine after 30 hours. Teekell and Lyke (9) reported that a maximum of 1.3% of oral Mo^{99} could be recovered in the blood of chicks 5 hours after the administration of a tracer dose. Disappearance of blood molybdenum was much slower in the chick than in swine, since 48-hour blood levels were 0.20% in the chick and 0.07% in swine. The clearance of Mo^{99} from the blood of human subjects (8, 15) was similar to that of swine. In contrast, the concentration of Mo^{99} in blood of cattle followed a markedly different pattern. Mo^{99} from an oral dose was below measurable quantities at 2 and 4 hours after dosing, coincident to the highest blood levels (6.1%) of orally dosed swine. The highest average blood level of 2.6% observed at 96 hours suggests a slow absorption and slow excretion rate. Comar et al. (6) reported that 2.9% of an oral dose of Mo^{99} was present in the blood of cattle 41 hours following an oral dose. The slow excretion rate was confirmed by the data obtained in the present study following the intravenous administration of Mo^{99} to cattle and by others using cattle and sheep (2). Data in figure 1 are in agreement with studies reviewed by Underwood (2) showing that higher molybdenum intakes result in increased blood molybdenum levels in ruminants. Blood Mo^{99} following an intravenous dose decreased slowly for 48 hours, then increased, being significantly higher at 72 hours than at 48 hours regardless of level of stable molybdenum administered. This was probably due to 1) recycling of the Mo^{99} excreted into the upper portion of the gastrointestinal tract; 2) uptake in tissue for temporary storage followed by release; 3) biological synthesis into usable compounds which were gradually released; or 4) the complexing of excess molybdenum by detoxification processes with subsequent release as excretory mechanisms were brought into action.

TABLE 2

Total excretion of Mo^{99} by swine and cattle

Animals	Treatment group	Day 1	Day 5	Day 7	Day 12
		%	%	%	%
Swine	Oral control	79.8 ± 2.4 [1]	97.0 ± 2.0		
	Oral + Mo	89.3 ± 3.4	99.1 ± 2.9		
	Intravenous control	53.1 ± 4.6	79.5 ± 3.7		
	Intravenous + Mo	85.6 ± 6.6	98.0 ± 4.8		
Cattle	Oral control	15.4 ± 1.7	81.4 ± 4.1	93.3 ± 4.2	
	Oral + Mo	16.0 ± 2.0	87.6 ± 1.8	101.0 ± 2.7	
	Intravenous control	4.6 ± 1.3	25.5 ± 2.0	37.7 ± 3.0	67.7 ± 4.2
	Intravenous + Mo	2.8 ± 0.3	28.2 ± 1.8	41.9 ± 2.2	65.0 ± 6.1

[1] Mean ± SE.

Added stable molybdenum increased urinary excretion of Mo^{99} more in intravenously dosed swine than in orally dosed swine suggesting the existence of renal processes in swine which rapidly eliminate excess molybdenum. Added stable molybdenum slightly increased Mo^{99} urinary excretion in cattle; nevertheless very little Mo^{99} was excreted in urine by cattle in comparison with swine. Data reviewed by Underwood (2) show that dietary molybdenum and dietary sulfate influence both blood and urine molybdenum concentrations. Kirschgessner et al. (16) showed that excess copper increased urinary and decreased fecal molybdenum excretion. The small differences in dietary molybdenum and copper could not account for the large differences observed in urinary excretion between swine and cattle. Neither total sulfur nor sulfate was measured, but the calculated levels were similar for the swine and cattle rations. Gross kidney functions between cattle and swine are assumed to be the same; however, these data show a real difference in the renal clearance of Mo^{99}. Perhaps there are differences in the binding of Mo^{99} in blood of swine compared with the bovine. Underwood (2) cited work of Scaife who has shown that molybdenum is readily dialyzable from both plasma and red blood cells from sheep.

In contrast with the data from 24 collection periods of the present study, the data of Comar et al. (6) showed that most of the Mo^{99} was excreted in urine from both an orally dosed steer and an intravenously dosed steer. These animals, however, received 4.67 and 1.27 g of molybdenum as MoO_3 in the dosing solutions in comparison with the tracer levels of 0.02 and 0.16 g of molybdenum as ammonium molybdate in the doses given to the steers in the present experiment. Data reported by Shirley et al. (14) show an average of 55% of oral Mo^{99} excreted in 30 hours in urine from pregnant sows. However, stable molybdenum as Na_2MoO_4 ranged from 0.7 to 1.5 g/animal.

Fecal excretion of Mo^{99} by swine was a minor pathway of elimination of both oral and intravenous doses. Stable molybdenum added to oral doses reduced Mo^{99} in feces from 15 to 9% showing that absorption was actually increased. This could not be explained as less re-excretion because less than 1% of an intravenous dose was excreted in the feces by either the controls or those given additional molybdenum. Net retention of Mo^{99} was reduced slightly when stable molybdenum was added because urinary Mo^{99} excretion was increased. These values are similar to data reported by Shirley et al. (14) which showed that an average of 8% of an oral dose of low specific activity Mo^{99} was excreted in 30 hours in feces of pregnant sows. Similar data have been reported for man by Rosoff and Spencer (15). In contrast with the swine and human data, apparent absorption of Mo^{99} by cattle was low and apparent re-excretion into the gastrointestinal tract was high (fig. 2). These data are also in contrast with data presented by Comar et al. (6) showing a 12-day fecal excretion of 34% from oral Mo^{99} and 11% from intravenous Mo^{99}. Again these differences may in part be due to differences in quantities and chemical form of stable molybdenum administered.

In a review, Underwood (2) stated that all cattle are susceptible to molybdenum toxicity from teart pastures. Sheep were less susceptible and horses were not affected. Davis (17) reported no toxicity from feeding 1000 ppm molybdenum to pigs for 3 months. Underwood (2) attributed this to low absorption of molybdenum, but the present data demonstrate rapid absorption and rapid excretion.

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LITERATURE CITED

1. Westerfield, W. W., and D. A. Richert 1953 Distribution of the xanthine oxidase factor (molybdenum) in foods. J. Nutrition, *51:* 85.
2. Underwood, E. J. 1962 Trace Elements in Human and Animal Nutrition, ed. 2. Academic Press, Inc., New York.
3. Totter, J. R., W. T. Burnett, Jr., R. A. Monroe, I. B. Whitney and C. L. Comar 1953 Evidence that molybdenum is a nondialyzable component of xanthine oxidase. Science, *118:* 555.
4. De Rengo, E. C. 1962 Molybdenum. In Mineral Metabolism, vol. 2, part B, eds. C. L.

Comar and F. Bronner. Academic Press, Inc., New York, p. 483–498.

5. Handler, P., K. V. Rajagopalan and V. Aleman 1964 Structure and function of iron-flavoproteins. Federation Proc., *23:* 30.
6. Comar, C. L., L. Singer and G. K. Davis 1949 Molybdenum metabolism and interrelationships with copper and phosphorus. J. Biol. Chem., *180:* 913.
7. Bruner, H. D. 1955 Distribution of intravenously injected radiomolybdate ion in the rat. Am. J. Physiol., *183:* 600.
8. Sorensen, L. B., and M. Archambault 1963 Visualization of the liver by scanning with Mo^{99} (molybdate) as tracer. J. Lab. Clin. Med., *62:* 330.
9. Teekell, R. A., and W. A. Lyke 1961 Protein binding of molybdenum-99 in plasma, liver, and kidney of chicks. Poultry Sci., *40:* 1277.
10. Buescher, R. G., M. C. Bell and R. K. Berry 1961 The effect of excessive calcium on selenium-75 in swine. J. Animal Sci., *20:* 368.
11. Mayo, R. H. 1961 Swine metabolism unit. J. Animal Sci., *20:* 71.
12. Schalm, O. W. 1961 Veterinary Hematology. Lea and Febiger, Philadelphia, p. 16–17.
13. Kalthoff, I. M., and E. B. Sandell 1962 Textbook of Quantitative Inorganic Analysis, ed. 3. The Macmillan Company, New York, p. 639.
14. Shirley, R. L., M. A. Jeter, J. P. Feaster, J. T. McCall, J. C. Outler and G. K. Davis 1954 Placental transfer of Mo^{99} and Ca^{45} in swine. J. Nutrition, *54:* 59.
15. Rosoff, B., and H. Spencer 1964 Fate of molybdenum-99 in man. Nature, *202:* 410.
16. Kirschgessner, M., V. Wiser and H. Friescke 1963 Cu and Mo metabolism in growing pigs. Ztschr. Tierphysiol., Tierernaehrung Futtermittelk., *18:* 239. (cited in Biol. Abstr., *45:* 24975, 1964).
17. Davis, G. K. 1950 The influence of copper on the metabolism of phosphorus and molybdenum. In Copper Metabolism, eds., W. C. McElroy and B. Glass. Johns Hopkins Press, Baltimore, p. 216–229.

Bone Density Measurements throughout the Life Cycle of the Rat [1,2]

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ABSTRACT The effect of calcium intake, growth, reproduction and senescence on the bone density of rats was studied through 2 generations. A low bone density index was accompanied by retarded growth and poor lactation. A high bone density is indicative of an adequate calcium status, but if it is achieved at the expense of normal growth and reproduction, then the intake producing this density cannot be considered adequate. The mean range of bone densities of rats 74 days old to death fed 0.1% dietary calcium was 0.39 to 0.66 x-ray equivalent grams of ivory/cm^3 of bone, whereas that of rats fed 0.5% dietary calcium ad libitum was 0.73 to 0.91. With restricted consumption, diets containing 0.3 and 0.5% calcium produced variable physiological performance as a resultant of concomitant effects of growth, lactation and calcium intake.

Uncertainty with respect to human calcium requirements still exists (1) as well as a concern for causes and early detection of bone rarefaction. Leitch and Aitken (2) in a re-examination of calcium requirements note the need for methods of determining calcium status in addition to balance techniques. Keane et al. (3) suggest the desirability for a quantitative estimate of the calcification of bones in pathological studies. Walker (1) notes the paucity of knowledge concerning mineral matter per unit volume of bone, "... which measurement reflects incidence and severity of osteoporosis." Calcium status of the rat as affected by calcium intake has been determined by using balance experiments and by measuring the processes of growth, adult maintenance, and reproduction. The present study uses bone density measurements to determine calcium status during these processes. It extends the work of previous studies in this laboratory by recording the effects of a continuous difference in calcium intake throughout the life cycle of 2 generations of rats. The effects of various biological phenomena on the bone density of rats fed at different calcium levels have implications for the corresponding processes in man. These animal studies are being used as guide lines for concomitant studies on bone density measurements as a criterion for calcium status with human subjects.

LITERATURE REVIEW

Previous work by Williams et al. (4) showed that in the *growing* rat the density of the ninth caudal vertebra increased with increased calcium intake. At all levels of calcium intake, the animals showed some increase of bone density with age, but those receiving 0.1% dietary calcium never achieved as high bone densities as those with higher calcium intakes. In the case of adult rats, when stock diet had been fed until maturity, there was no regression of bone density on level of calcium intake after being fed the experimental diet for 9 months. However, a significant difference was found in the bone density of rats fed at the lowest calcium level (0.1%) and those fed at the higher levels. With reduced requirements for adults, bone densities were about the same for animals fed at 0.3 and 0.5% calcium levels whether the food was restricted or fed ad libitum. However, differences in growth in favor of those fed ad libitum were noted. This study also noted that tall, heavy women who had achieved a greater than usual growth had lower than average bone den-

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sities suggesting an increased need for calcium. In a study by Williams and Samson (5), out of a total of 32 subjects, 4 unusually tall, heavy ones similarly showed an increased calcium need by lower than average bone densities. Conversely, 5 short, light (e.g., 40-kg) subjects had higher than average densities, suggesting a reduced calcium requirement due to retarded growth. For the remaining 22 subjects of "normal" variation in size, bone density increased with body weight. These observations suggested the importance on adult mineral status of the influence of previous history, genetic or nutritional.

The validity and application of bone density measurements for quantitative determination of mineralization has been studied by Schraer and associates (6, 7) and by Melton.[5] Vose (8) has enumerated in vivo techniques other than x-ray methods for the determination of mineral status. Microradiography has been used by Vose (9), Rowland et al. (10) and Jowsey (11) to investigate bone changes in aging and in osteoporosis. The hypothesis advanced in 1948 by Albright and Reifenstein (12) that osteoporosis is caused by a disturbance of the protein matrix of bone, with calcium intake having little or no importance, has discouraged research on dietary causes. Their hypothesis has been challenged by the recent radiocalcium studies of Heaney (13), Heaney and Whedon (14), Harrison and Fraser (15), Harrison et al. (16) and by studies of human osteoporotics by Nordin (17, 18) who reports improvement of 7 out of 9 patients with increased calcium intake. Practical difficulties of quantitative radiological assessment of bone density on pathological material have been pointed out by Mayo (19) and by Doyle (20).

PROCEDURE

A preliminary experiment was conducted. Rats of the Wistar strain were fed diets of 0.1, 0.3 and 0.5% calcium content when 29 to 31 days old. The diet groups were designated as A(0.1%), B(0.3%), C(0.5%) and D(0.5% ad libitum). Four animals from the same litter were distributed among the groups so that average weights were equal. Group A was fed ad libitum. The food intakes of groups B and C were restricted to the intake of group A. Group D animals were fed ad libitum.

The percentage composition of the diet was: cornstarch, 60.5; casein, 18.0; vegetable shortening, 8.0; brewer's yeast, 7.5; cod liver oil, 2.0; and salt mixture, 4.0. The salt mixture was that of Osborne and Mendel (21) modified by the addition of copper as $CuSO_4$ and by varying the $CaCO_3$ so that the diets would contain 0.1, 0.3 or 0.5% calcium when 4% of the mixture was used. This mixture contributed 0.3% phosphorus to the diet. All calcium analyses were carried out by a modification of the McCrudden method (22) and phosphorus by the AOAC (23) micro-method modified for a photocolorimeter. A choice had to be made between keeping the Ca-to-P ratio the same and varying only the calcium content with phosphorus at the minimal adequate level. The latter was chosen. The total phosphorus of the diet by analysis was 0.39%. Distilled water was given ad libitum.

Each dietary group contained 2 breeding lots made up of 2 males and 3 females each and one non-breeding lot made up of 4 males and 2 females each so that there were 8 males and 8 females in each group. Animals were housed individually. At breeding time, the breeding lot was housed in one cage. Females were removed from the breeding cages when pregnancy was evident by increased body weight. They were remated 2 weeks after the death or weaning of young. Records were kept of weight, food consumption, age of mother at birth of first litter, number of young born, weight of young at birth and at weaning, number of young raised to weaning, reproductive span, and pathological manifestations. Autopsies were performed at termination. The bodies were analyzed for calcium.

Young of normal weight were born after 8 weeks on experiment but soon died. The diet was not adequate for lactation. In an attempt to improve lactation, fresh pork liver was added to the diet at the rate of 10 g/female/feeding, fed 3 times

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[5] Melton, A. W. M. 1957 A nutrition education study with preschool children of low socio-economic status families, involving medical nutrition observations and tests. Ph.D. Thesis, Texas Woman's University, University Hill, Denton, Texas.

weekly. This was shared with the males when in the breeding cages. Young were raised to weaning after the liver was added to the diet. X-ray pictures of the ninth caudal vertebra for bone density determinations were made at the beginning, at 72, 114 and 213 days on experiment. These determinations are referred to as series 1, 2, 3 and 4. The theory and method used for determining the bone density index as x-ray equivalent grams of ivory per cubic centimeter of bone is as given by Williams and Mason (24), with an ivory wedge[6] being used instead of the alloy. Exposure conditions used were 50 kv, 10 to 12 ma, 0.75 seconds with a 91-cm focal distance.

This experiment was terminated after 213 days with plans to redesign the basal diet and to include pork liver from the beginning. This preliminary study is designated experiment 1.

When the experiment was redesigned, each dietary group contained 2 breeding lots and one non-breeding lot, as previously. The non-breeding lots were made up of 6 females so that their bone density might be compared with that of the 6 breeding females to study the effect of lactation. An extra breeding lot was added and designated group E. This group was fed the 0.5% calcium diet ad libitum but was not x-rayed in order to check the effect on reproduction of the amount of x-ray exposure used in the technique. Breeding lots of the second generation were made up from the first 2 litters, from different mothers within the group, which contained 2 males and 3 females each. They continued to be fed the diets of their parents.

The diet of experiment 1 was modified by reducing the brewer's yeast to 3.75%, and by omitting the cod liver oil because of possible destruction of vitamin E by the unsaturated fish oil. Vitamins and minerals were used in the amounts suggested by Cuthbertson (25). Fifty per cent of the vegetable shortening was replaced with cottonseed oil which carried *d*-α-tocopheryl acetate and menadione. Fresh pork liver was fed 3 times weekly from the beginning at the rate of 6 g/male and 10 g/female/feeding. Males and females had equal access to all the liver while in the breeding cages. The liver increased the protein of the diet to approximately 25%. The calcium content of the liver was negligible but it contributed phosphorus at approximately the same level as the basal diet.

X-ray pictures were made at the beginning, at 72, 114 and 213 days on experiment and every 6 months thereafter until death.

The redesigned experiment was designated 2P for parent animals and 2Y for offspring.

In addition to the statistical analyses for variability and significance of differences between means, the stepwise regression analysis program (STRAP) described by Efroymson (26) was used so that the order of importance and the degree of significance of the independent variables on the dependent variable in the regression equation were printed out. In this way the relative effects of such factors as the level of dietary calcium and lactation on bone density as well as on other parameters were assessed during different stages of the life cycle. STRAP also prints out all possible correlations between the variables in the regression equations.

RESULTS AND DISCUSSION

The measurement of bone density as a criterion of the effect of calcium intake on various physiological functions was studied throughout the life cycle of 2 generations of rats. The bone density index, serving as an additional quantitative measurement, was compared with the usual ones for growth, reproduction including lactation, longevity, and chemical analysis. Data are presented in table 1 for bone density measurements and body weights at stated intervals for experiment 1 and experiment 2 for parents and young.

Results obtained during the period of growth confirmed those previously reported from this laboratory. The extension of the study through a second generation indicates the usefulness of bone density measurements in determining calcium status in adult rats when other factors are duly considered.

Growth. By the time of x-ray series 5, 6, and 7 of the first generation in experi-

[6] A gift from P. B. Mack.

TABLE 1

Bone density[1] *and body weights of rats fed at various levels of calcium*

X-ray series	Exp. no.	0.1% Ca in diet (A)			0.3% Ca in diet (B)			0.5% Ca in diet (C)			0.5% Ca in diet ad lib. (D)		
		No. rats	Bone density	Body wt	No. rats	Bone density	Body wt	No. rats	Bone density	Body wt	No. rats	Bone density	Body wt
				g			*g*			*g*			*g*
1	1	16	0.31 ± 0.10 [2]	57 ± 8	16	0.35 ± 0.02	56 ± 8	16	0.35 ± 0.09	56 ± 8	16	0.38 ± 0.12	56 ± 9
1	2P [3]	16	0.38 ± 0.12	55 ± 6	16	0.34 ± 0.12	55 ± 5	16	0.30 ± 0.07	54 ± 5	16	0.37 ± 0.10	55 ± 5
1	2Y [4]	10	0.40 ± 0.15 *	42 ± 4	10	0.47 ± 0.13	44 ± 5	0			10	0.55 ± 0.08 *	43 ± 7
2	1	16	0.41 ± 0.11 **	163 ± 38 **	16	0.62 ± 0.10	176 ± 34	16	0.70 ± 0.11	176 ± 34	16	0.73 ± 0.12 **	204 ± 38 **
2	2P	16	0.41 ± 0.07 **	161 ± 33 *	16	0.66 ± 0.13	175 ± 34	16	0.97 ± 0.18	175 ± 35	16	0.87 ± 0.13 **	186 ± 43 *
2	2Y	5	0.42 ± 0.20 **	88 ± 14 **	10	0.54 ± 0.18	176 ± 36	0			10	0.73 ± 0.16 **	196 ± 37 **
3	1	16	0.39 ± 0.06 **	213 ± 38 **	16	0.68 ± 0.18	240 ± 47	16	0.76 ± 0.12	242 ± 42	16	0.78 ± 0.13 **	268 ± 58 **
3	2P	16	0.44 ± 0.12 **	204 ± 46 *	16	0.77 ± 0.17	219 ± 50	16	0.91 ± 0.11	221 ± 52	16	0.91 ± 0.16 **	245 ± 60 *
3	2Y	3	0.54 ± 0.15 *	78 ± 15 **	10	0.58 ± 0.11	237 ± 54	0			9	0.80 ± 0.14 *	254 ± 65 **
4	1	16	0.52 ± 0.10 **	264 ± 47 *	16	0.75 ± 0.08	284 ± 62	16	0.78 ± 0.11	282 ± 55	16	0.75 ± 0.10 **	310 ± 68 *
4	2P	16	0.47 ± 0.08 **	254 ± 68	16	0.75 ± 0.13	264 ± 62	15	0.76 ± 0.09	272 ± 77	16	0.86 ± 0.16 **	285 ± 82
4	2Y	1	0.90	102	10	0.73 ± 0.12	310 ± 90	0			8	0.80 ± 0.13	329 ± 101
5	2P	11	0.55 ± 0.11 **	265 ± 51	13	0.73 ± 0.12	286 ± 51	12	0.87 ± 0.10	279 ± 84	11	0.83 ± 0.13 **	303 ± 89
5	2Y	1	0.98	118	8	0.65 ± 0.15	372 ± 111	0			7	0.86 ± 0.17	362 ± 116
6	2P	7	0.66 ± 0.18 *	284 ± 27 **	7	0.92 ± 0.20	310 ± 50	8	0.95 ± 0.18	327 ± 92	10	0.87 ± 0.27 *	336 ± 39 **
6	2Y	0			6	0.61 ± 0.09	322 ± 100	0			6	0.74 ± 0.12	355 ± 92
7	2P	5	0.56 ± 0.08 **	260 ± 45 **	5	0.73 ± 0.19	274 ± 32	6	0.76 ± 0.15	311 ± 50	7	0.75 ± 0.08 **	329 ± 41 **
7	2Y	0			4	0.83 ± 0.17	368 ± 41	0			5	0.78 ± 0.17	279 ± 40

[1] Bone density is expressed as x-ray equivalent grams of ivory per cubic centimeter of bone.
[2] Mean ± SD.
[3] 2P = Experiment 2 (parent groups).
[4] 2Y = Experiment 2 (offspring).
* Difference between means for groups A and D is significant ($P < 0.05$).
** Difference between means for groups A and D is highly significant ($P < 0.01$).

ment 2 some animals had died, approximately the same number in each group but not corresponding to their distribution from the same litters. Although the average values are, thus, not strictly comparable, they indicate clearly that there is a calcium intake below which rats (group A) consuming it continue to grow but not as well as those fed the same amounts of food containing more calcium (table 1 and fig. 1). Furthermore, their average bone densities (table 1 and fig. 2) are consistently lower throughout maturity and at death than those receiving larger amounts of dietary calcium. Fisher's *t* test indicates a highly significant difference ($P < 0.01$) in bone density between means for groups A and D animals throughout life. The average bone densities of group C animals are equal to or greater than the corresponding ones for group D with comparable standard deviations, therefore it should follow that there is a significant difference for average bone densities between groups A and C. Schraer et al. (27) report differences of bone density at different levels of calcium intake using the seventh caudal vertebra and femur of the rat for measurement.

Breeding results. The second generation showed the most marked effects of calcium deficiency by any test. Average weights at weaning were lower for group A young than for all other groups (table 2 and fig. 1). Group A rats were older than those of the other groups when the first litter was born, thus shortening their reproductive span in contrast with the others.

Group A offspring were stunted in growth throughout life (table 1 and fig. 1). They never reached their parents' weight at the same age as did the young of all other groups. In fact, they weighed less than one-half as much as their parents at corresponding ages. Neither did they live as long. Mortality was 50% by the time of x-ray series 2. Only one rat in this group lived to maturity (series 4 and 5). This stunted mature rat achieved a high bone density (fig. 2). This rat is a striking example of the adaptation of a young animal to a deficiency of a nutrient (calcium in this case) by failure to grow, thereby reducing the requirement for the nutrient. Thus, for this mineral, a deficiency for the function of skeletal growth became a great sufficiency for the function of rigidity of support as evidenced by a high bone density. Possible similar human examples have been referred to earlier in this paper and also in a recent publication by Williams et al. (28) on 9 underprivileged children.

Data in table 2 present the comparative breeding performance of the different groups in experiment 2P. The records show that fertility could not be associated with level of calcium intake of the animals x-rayed. Group C rats produced very few offspring. Group B rats produced the most. In experiment 1 group C rats produced about the same number as did groups B and D. The number of young born to each group was 21(A), 46(B), 41(C), and 44(D) by time of termination of the experiment. Termination of this experiment was necessary because of failure of lactation, not of fertility. The reason for the poor breeding performance of the group C females in experiment 2P is not evident but the possible effects on bone density may be noted.

The influence on bone density of several concomitant physiological phenomena is reflected when group C animals (0.5% calcium diet restricted in intake) are compared with those in group D (0.5% calcium diet fed ad libitum). Group C animals exhibited retarded growth, produced very few young, but maintained equal or higher densities than rats in group D. Thus, in using bone density as a criterion for calcium status, other biological phenomena must be considered if this measurement is to be clinically useful.

Group E rats (based on results from one breeding litter) produced a larger number of young over a longer reproductive span than group D animals. The percentage raised and average weight at weaning were less than for group D animals. Group B rats with the same amount of x-radiation but fed a restricted diet produced more young than group D but less than group E. Group C rats of experiment 2 were fed the same diet as groups E and D but restricted, and produced very few young. The reason for the differences in fertility of the various

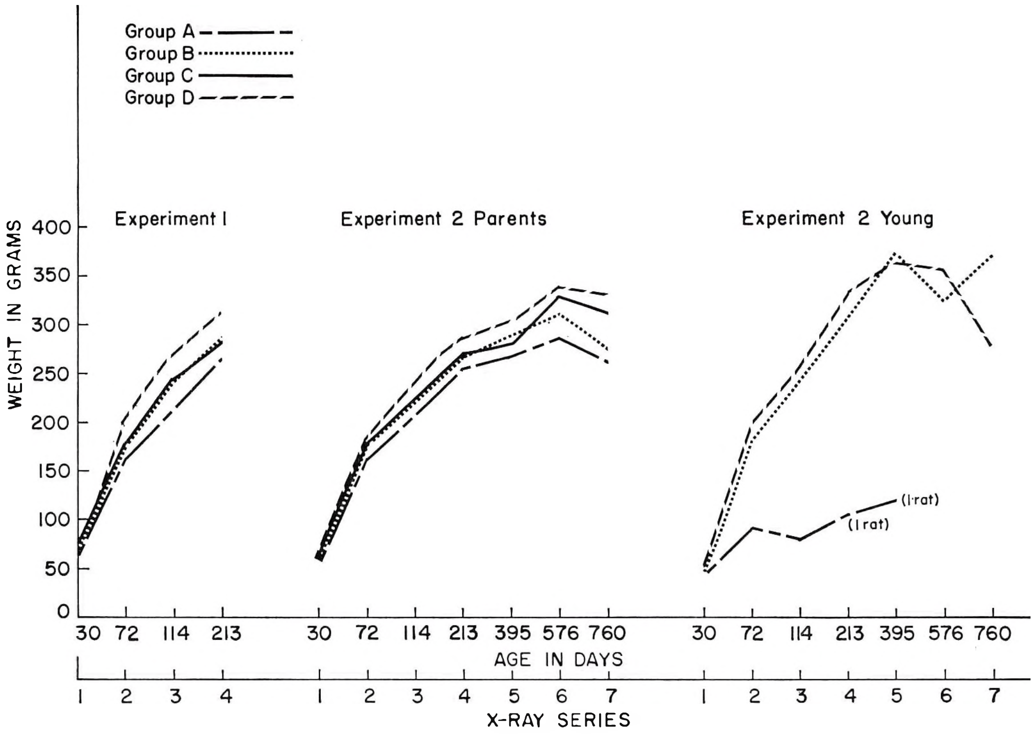

Fig. 1 Mean body weight versus age.

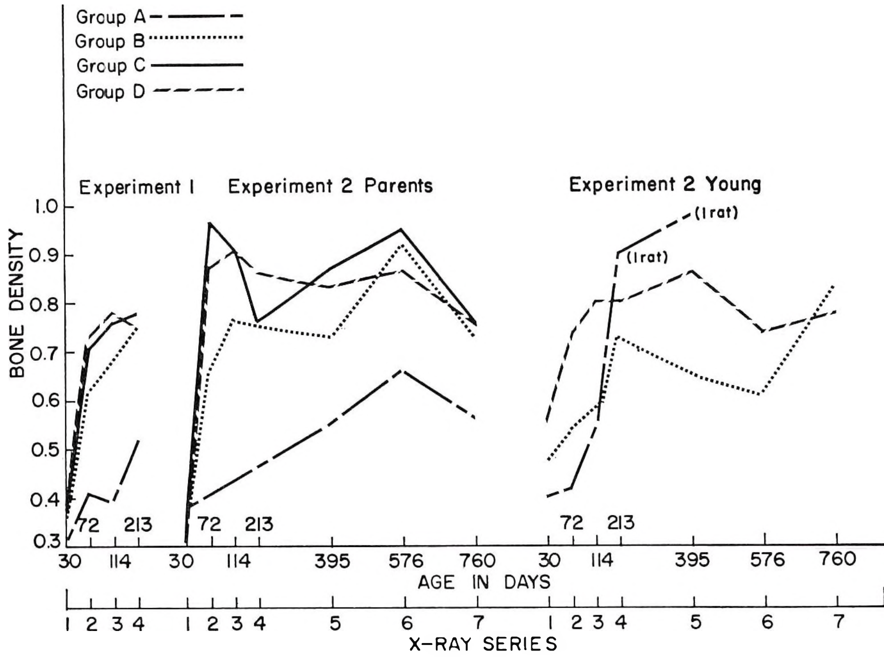

Fig. 2 Mean bone density versus age.

TABLE 2

Breeding and longevity data for rats in experiment 2P

Group	Ca in diet	Avg age at birth of 1st litter	Litters	Total young			Avg wt		Repro-ductive span	Life span
				Born	Raised		At birth	Weaning		
	%	*days*	*no.*	*no.*	*no.*	%	*g*	*g*	*days*	*days*
A	0.1	173	13	90	36	40	5.0	23.2	93	571
B	0.3	129	32	204	119	58	4.9	31.7	239	561
C	0.5	100 [1]	2	14	7	50	5.0 [1]	33.4	— [2]	653
		184 [3]	1	8	5	62	3.6 [3]	25.8	— [2]	
D	0.5 ad lib.	107	21	153	78	57	5.0	32.3	162	661
E	0.5, ad lib., non-x-ray	100	34 [4]	238 [4]	116 [4]	49	5.0	28.2	261	613

[1] Average for 2 healthy litters.
[2] One litter per female.
[3] For 1 sickly litter.
[4] Data for 1 breeding lot multiplied by 2 for comparison.

groups of rats, as previously stated, is not clear, but the comparison of groups B and E appears to indicate that the amount of x-radiation given was not the predominating factor.

Longevity. The group A parents had about the same average life span as animals in group B (table 2). Neither group lived as long as those in groups C and D. There was a marked difference in average length of life between the offspring (exp. 2Y) of group A and those of groups B and D (105, 645 and 585 days, respectively) and between them and their parents. Pathological findings at autopsy could not be associated with calcium intake or with x-ray exposure. Respiratory, kidney and liver involvement occurred about equally in all groups.

Total body calcium at termination. Table 3 shows results of calcium analyses at death both as total calcium and as percentage of body weight. For animals in experiments 1 and 2P, as calcium intake increased the total amount of body calcium increased. In terms of percentage of body weight, the figures for all animals in the A groups are lower than for those in the same experiment at higher intake levels. In groups B, C and D there is no percentage difference in calcium content at death since the marked loss of weight which often occurs before death masks the actual difference in total calcium reserves.

TABLE 3

Mean calcium content of body at death of rats

Group	No. of rats	Ca in diet	Total Ca in body	
		%	*g*	*% of body wt*
		Experiment 1		
A	16	0.1	1.80	0.72
B	16	0.3	2.66	0.97
C	16	0.5	2.76	1.02
D	16	0.5 ad lib.	3.07	1.04
		Experiment 2P		
A	16	0.1	2.35	1.08
B	16	0.3	3.05	1.36
C	16	0.5	3.14 [1]	1.40
D	16	0.5 ad lib.	3.37	1.38
		Experiment 2Y		
A	10	0.1	0.39	0.55
B	10	0.3	3.39	1.22
D	10	0.5 ad lib.	3.35	1.44

[1] Total Ca becomes 3.36 when bone tumor Ca is added; percentage of Ca becomes 1.44 when bone tumor Ca is added.

The results described regarding the effects of dietary calcium level on growth, reproduction, calcium content of body and longevity in rats are in agreement with earlier works by Sherman (29) and coworkers (30, 31). The data presented indicate that the level of calcium intake also has a significant effect on the density of bone. A quantitative measurement of bone density has been achieved in contrast with the qualitative visual comparison of relative depth of x-ray shadows such as observed by Moore et al. (32).

TABLE 4

Stepwise regression analysis with bone density the dependent variable

Experiment 1					Experiment 2P				
X-ray series	Independent variable	F [1]	No. of cases	R_2^2	X-ray series	Independent variable	F	No. of cases	R^2
				First entering variable [3]					
2	Ca intake	79.36 **	64	0.56	2	Ca intake	138.80 **	64	0.69
3	Ca intake	78.04 **	64	0.56	3	Ca intake	105.30 **	64	0.63
4	Ca intake	47.89 **	64	0.44	4	Ca intake	56.99 **	63	0.48
					5	Ca intake	49.16 **	47	0.52
					6	Y raised	8.93 **	32	0.23
					7	sex [4]	16.46	23	0.44
				Second entering variable [3]					
2	Sex	1.29	64	0.57	2	mated vs. unmated	1.10	64	0.72
3	Weight	7.67 **	64	0.61	3	$(\text{Ca intake})^2$	5.96 *	64	0.66
					4	$(\text{Ca intake})^2$	7.63 **	63	0.54
					5	Y raised	7.07 *	47	0.59
					6	weight	5.50 *	32	0.35
					7	Ca intake	13.19 **	23	0.66

[1] *F* is the Fisher *F* statistic as tabled in Snedecor.
[2] R^2 is the correlation squared.
[3] Indicates order of importance.
[4] Since no young were raised between series 6 and 7, the factor "sex" was entered in place of "Y raised" in the regression equations.
* $P < 0.05$.
** $P < 0.01$.

Statistical interpretations. The stepwise regression analysis program (table 4) for experiments 1 and 2P, series 2 and 3, shows calcium intake had the greatest effect on bone density of any of the factors analyzed. Similarly, highly significant ($P < 0.01$) correlation coefficients of bone density with calcium intake range from 0.60 to 0.83 for the period through the first 114 days of life. Although throughout life, sex and body weight are highly correlated ($P < 0.01$) with each other, sex and bone density show no correlation. In other words, males with larger skeletons normally have a larger amount of calcium at their disposal than females yet do not have more dense bones. Moore et al. (32) reported in rats fed adequate calcium throughout their rapid growing period (125 days) no differences in mean percentage ash between males and females. When the diet was fed for shorter periods, the percentage femur ash content was higher in females than in males, explained by the bone growth rate of females. Thus, according to the authors, in a competition between growth and bone mineralization, in females growth would exert a lesser force than in males. The correlation values (r) for calcium intake on body weight (0.14 to 0.54 for exps. 1 and 2P, series 2 through 7) are smaller and less frequently significant than those for calcium intake on bone density (0.36 to 0.83). Apparently bone density is more drastically affected by the level of calcium intake than is growth. Thus in a competition for calcium during development, the tendency for the skeleton to grow is greater than the tendency to acquire greater density or strength. The bone density index becomes a more sensitive criterion for calcium status than does growth during this period.

During maturity (x-ray series 4 to 7) the need for calcium was reduced mainly to that required for maintenance and reproduction. STRAP for parent rats, experiment 2P, indicates that in early maturity (series 4) the level of calcium intake still exerted the most and highly significant influence on bone density which is also true for experiment 1. In series 5, calcium intake still had the most important influence on bone density but the next entering variable, and a significant one, is the effect of raising young (or lactation). That young had been raised appears as the most important factor in series 6. There was a significant

($P < 0.01$) negative correlation between the number of young raised and bone density, and between the amount of calcium in the body at death and young raised ($P < 0.05$). Thus, the effect of lactation on bone density was evident until death, not just through the lactation period. Statistical analysis thus gives an objective indication that bone density measuresment could serve effectively as a criterion for the determination of calcium status supplemental to the usual methods.

These rat data indicate that a diet inadequate for all physiological functions for calcium, including maintenance of normal bone density, such as the 0.1% calcium diet, is deficient. However, it remains to demonstrate a similar quantitative level for a deficient diet for man. Bone density values for rats give evidence that animals have remarkable powers to adapt to adverse conditions. This is undoubtedly true for human beings as well. Nordin (18) suggests that human adults adapt to low calcium intake either by increased absorption or by decreased elimination of the mineral. Those who fail to adapt eventually become osteoporotic. Malm (33) has also produced evidence that senile osteoporosis may be due to a calcium deficiency. Detection of calcium deficiency in rats by means of bone density measurement suggests its usefulness for detection of a deficiency in humans who fail to adapt to a low intake.

Bone density can be used to detect bone rarefaction. Bone rarefaction is presumed to be the result of disturbances in calcium metabolism. These disturbances may be a reflection of limited dietary intake, hormonal imbalance or immobilization. Using this tool, it is conceivable that experiments could be designed to study the effect of these different variables. As improved methods of determining bone density become available, selected segments of the human population can be measured and the results might indicate a revision of the recommended calcium requirement. A low bone density means bone rarefaction. A high bone density would be positive evidence for lack of bone rarefaction representing an adequate intake for the *individual*. However, a high bone density, achieved at the expense of normal growth, lactation or other collateral functions, cannot serve as a basis for setting up calcium requirements for normal human beings. Bone density measurements, properly interpreted in relation to other manifestations, should be a useful clinical and research tool for nutritional and medical studies.

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LITERATURE CITED

1. A.M.A. Council on Foods and Nutrition 1963 Symposium on human calcium requirements. J. A. M. A., *185:* 588.
2. Leitch, I., and F. C. Aitken 1959 The estimation of calcium requirement: A re-examination. Nutrition Abstr. Rev., *29:* 393.
3. Keane, B. E., G. Spiegler and R. Davis 1959 Quantitative evaluation of bone mineral by a radiographic method. Brit. J. Radiol., *32:* 162.
4. Williams, D. E., B. B. McDonald, E. Morrell, F. A. Schofield and F. L. MacLeod 1957 Influence of mineral intake on bone density in humans and in rats. J. Nutrition, *61:* 489.
5. Williams, D. E., and A. Samson 1960 Bone density of East Indian and American students. J. Am. Dietet. A., *36:* 462.
6. Schraer, H. 1958 Variation in the roentgenographic density of the os calcis and phalanx with sex and age. J. Pediat., *52:* 416.
7. Schraer, H., R. Schraer, H. G. Trostle and A. D'Alfonso 1959 The validity of measuring bone density from roentgenograms by means of a bone density computing apparatus. Arch. Biochem. Biophys., *83:* 486.
8. Vose, G. P. 1961 An investigation of intrinsic sites of demineralization in bone: A microradiographic study. Texas Rep. Biol. Med., *19:* 676.
9. Vose, G. P. 1962 Quantitative microradiography of osteoporotic compact bone. Clin. Orthopaed., *24:* 206.
10. Rowland, R. E., J. Jowsey and J. H. Marshall 1959 Microscopic metabolism of calcium in bone. III. Microradiographic measurements of mineral density. Radiation Res., *10:* 234.
11. Jowsey, J. 1960 Age changes in human bone. Clin. Orthopaed., *17:* 210.

12. Albright, F., and E. C. Reifenstein 1948 The Parathyroid Glands and Metabolic Bone Disease. Williams and Wilkins, Baltimore.
13. Heaney, R. P. 1962 Radiocalcium metabolism in disuse osteoporosis in man. Am. J. Med., *33:* 188.
14. Heaney, R. P., and G. D. Whedon 1958 Radiocalcium studies of bone formation rate in human metabolic bone disease. J. Clin. Endocrinol., *18:* 1246.
15. Harrison, M., and R. Fraser 1960 Bone structure and metabolism in calcium deficient rats. J. Endocrinol., *21:* 197.
16. Harrison, M., R. Fraser and B. Mullan 1961 Calcium metabolism in osteoporosis. Lancet, *1:* 1015.
17. Nordin, B. E. C. 1961 The pathogenesis of osteoporosis. Lancet, *1:* 1011.
18. Nordin, B. E. C. 1962 Calcium balance and calcium requirement in spinal osteoporosis. Am. J. Clin. Nutrition, *10:* 384.
19. Mayo, K. M. 1961 Radiological assessment of bone density. II. Quantitative measurement of bone mineral content in normal adult bone. Brit. J. Radiol., *34:* 693.
20. Doyle, F. H. 1961 Radiological assessment of bone density. III. Ulnar bone mineral concentration in metabolic bone diseases. Brit. J. Radiol., *34:* 698.
21. Osborne, T. B., and L. B. Mendel 1919 The nutritive value of the wheat kernel and its milling products. J. Biol. Chem., *37:* 557.
22. Association of Official Agricultural Chemists 1950 Official Methods of Analysis, ed. 7. Washington, D. C., p. 97.
23. Association of Official Agricultural Chemists 1950 Official Methods of Analysis, ed. 7. Washington, D. C., p. 104.
24. Williams, D. E., and R. L. Mason 1962 Bone density measurements in vivo. Science, *138:* 39.
25. Cuthbertson, W. F. J. 1957 Nutrient requirements of rats and mice. Proc. Nutrition Soc., *16:* 70.
26. Efroymson, M. A. 1960 Multiple regression analysis. In Mathematical Methods for Digital Computers, eds., A. Ralston and H. S. Wilf. John Wiley and Sons, Inc., New York, p. 191.
27. Schraer, H., W. J. Siar and R. Schraer 1963 Changes in bone mass and density in living rats during the manipulation of calcium intake. Arch. Biochem. Biophys., *100:* 393.
28. Williams, D. E., B. B. McDonald and S. I. Pyle 1964 Bone density and skeletal maturation as indexes of mineral status in children. Am. J. Clin. Nutrition, *14:* 91.
29. Sherman, H. C. 1956 Chemistry of Food and Nutrition, ed. 8. The Macmillan Company, New York, p. 280.
30. Sherman, H. C., and F. L. MacLeod 1925 The calcium content of the body in relation to age, growth and food. J. Biol. Chem., *64:* 429.
31. Campbell, H. L., O. A. Bessey and H. C. Sherman 1935 Adult rats of low calcium content. J. Biol. Chem., *110:* 703.
32. Moore, T., S. G. Impey, P. E. N. Martin and K. R. Symonds 1963 Meat diets. II. Effect of the age of rats on their ability to withstand the low calcium intake induced by a diet of minced beef. J. Nutrition, *80:* 162.
33. Malm, O. J. 1958 Calcium requirement and adaptation in adult men. Oslo University Press, Oslo, p. 77.

The Magnitude and Significance of the Protein Reserves in Rats Fed at Various Levels of Nitrogen [1]

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ABSTRACT Rats were fed at various levels of casein or wheat gluten protein in a semi-purified diet from the time of weaning, over a period of 28 days. Gain in body weight and protein-to-DNA and RNA-to-DNA ratios in liver and muscle were determined at the end of the experimental period. Maximal body weight was obtained in rats fed 15% of casein protein, whereas the highest intakes resulted in a slight decrease in growth rate. Liver size, protein-to-DNA and RNA-to-DNA ratios increased with the quantity of casein consumed. The protein-to-DNA ratio in the muscles, however, increased to a maximum in animals fed 15% of casein protein in the diet and decreased when greater amounts of protein were consumed. When animals were pre-fed 9 or 70% of casein, the average survival time during starvation and protein deprivation was significantly less than that for animals fed 18% of casein. The animals pre-fed 40% of wheat gluten survived for a shorter time than the animals fed 9% of casein even though the 2 groups had gained an equal weight over the pre-feeding time. The ability of the animal to resist the stress of food deprivation appeared to be correlated with the magnitude of the protein-to-DNA ratio in the muscles.

Variations of the protein-to-DNA and RNA-to-DNA ratios in several tissues as a result of alteration of both the type and quantity of dietary protein have been investigated by Allison et al. (1). These ratios were raised in the liver and muscle of young rats by either increased nitrogen intake or nutritive value of the dietary protein, or both. In the kidney, these parameters increased with nitrogen intake, but were essentially unaffected by the quality of the dietary protein. In the brain, however, both ratios were independent of type and quantity of protein consumed over the experimental period used. When rats were fed sufficient casein or wheat gluten to produce a gain of 50 g in body weight over a 28-day experimental period, the cellular proteins in both the liver and muscle were greater in animals fed casein than in those fed wheat gluten (3). The magnitude of the cellular protein of liver and muscle has been associated with the "protein reserves" of the animal (2). The reserves were defined as the body proteins which can be reversibly depleted and repleted by variation in nitrogen intake.

Banks et al. (3) observed that feeding 5 to 6 g of casein nitrogen (approximately 15% protein in the diet) over a period of 28 days to young rats produced the maximal increase in the liver and muscle protein-to-DNA ratios. When the casein intake was increased to 12.0 g of nitrogen (approximately 30% protein in the diet), there was little change in the protein-to-DNA ratio of either liver or muscle. It was suggested that this plateau might be related to maximal filling of the protein reserves (7). Henry et al. (5), however, reported that the liver-protein nitrogen increased as rats were fed casein intakes which varied from 2.0 to 8.0 g of nitrogen/kg of body weight/day (4 to 35 g of nitrogen/28 days). These results suggest that liver protein may continue to increase when diets contain a large excess of nitrogen. There is a need to study the effect of excess dietary nitrogen upon various tissue proteins.

Samuels et al. (6) observed that rats pre-fed 92% lactalbumin did not survive starvation as well as animals fed 20% of

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this protein. Halac (7) reported that 20% casein in the diet gave maximal growth and survival to nitrogen deprivation and that feeding a diet containing 45% did not improve either measurement. In another experiment, Halac (8) observed that rats fed a diet containing 70% casein were not able to withstand stress as well as those fed 20% of this protein in the diet.

In the present study, the response of the protein and RNA in liver and skeletal muscle was investigated when rats were fed within a wide range of levels of casein or wheat gluten in the diet. The rats which had been pre-fed the various protein diets were fed a starvation or protein-free diet and mean survival rates were estimated.

METHODS

Experiment A. Groups of 10 male weanling Wistar rats were fed diets containing various concentrations of casein or wheat gluten protein. These diets were prepared in an agar gel base (table 1) and fed ad libitum. Water was also supplied ad libitum. Daily food consumption and weekly body weight changes were recorded.

After 28 days, the animals were killed and samples were taken of the gastrocnemius muscle and liver. A 2.0-g sample of the liver was homogenized in 10.0 ml of a 0.35 M sucrose solution buffered with bicarbonate to a pH of 7.4. Weighed samples of the muscle were homogenized in 5.0 ml of the sucrose buffer. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were determined using a modification of the ultra-violet absorption techniques suggested by Hutchison and Munro (9). Total protein was estimated by the buiret method of Layne (10) on the alkaline hydrolysate of an acid-insoluble precipitate of the homogenate from which the lipid had been extracted.

Experiment B. Groups of male weanling Wistar rats were fed diets containing 40% of wheat gluten, 9, 18, or 70% of casein. These diets were prepared in an agar gel base (table 1) and fed ad libitum for 28 days. Daily food consumption and weekly body weight changes were recorded.

After 28 days, animals fed at various protein levels were divided into 2 groups of 10 rats each. For each protein dietary level one group was deprived of food, and the other group was fed a protein-free diet. The time of death was recorded for each animal, and the mean survival time was calculated for each dietary group.

TABLE 1
Basal agar gel diet [1]

	g		*mg/1000 g dry diet*
Protein [2]	180	thiamine	10
Sucrose	134	riboflavin	20
Dextrose	212	pyridoxine	10
Dextrin	140	menadione	15
Lard	231	niacin	80
Salt mixture [3]	40	pantothenic acid	80
Agar	33	inositol	200
Cod liver oil	20	folic acid	0.5
α-Tocopherol [4]	10	biotin	0.5
	1000	*p*-aminobenzoic acid	80.0
Water	1400	choline	2000.0
	2400	ascorbic acid	2.0
			units/1000 g dry diet
		vitamin A	40,000
		vitamin D	4,000

[1] The water, lard and agar were heated until the solid material had melted. This solution was added to dry ingredients and mixed. The vitamins were added when this mixture had cooled but was still fluid.
[2] The protein content of the diet can be varied by isocaloric replacement of the protein with sucrose and dextrose (3).
[3] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.
[4] 100 mg in 10 g hydrogenated cottonseed oil (Crisco, Procter and Gamble, Cincinnati).

RESULTS AND DISCUSSION

Feeding the diet containing 15% casein protein (N × 6.25) to young rats produced maximal gain in body weight (fig. 1). There was an equal or slightly lower gain in body weight in rats fed higher percentages of casein. This optimal casein diet (15%) supplied 13% of the calories in the form of protein calories. As the dietary wheat gluten protein (N × 5.7) was increased from zero to 38% of the diet, there was a linear increase in body weight gain over 28 days. At the high wheat gluten intake, the rats did not reach the maximal growth observed in animals fed casein. In these experiments, the commercial source of wheat gluten contained only 54.8% protein (N × 5.7). To keep the diet isocaloric and at a 4% salt level, it was technically impossible to make a diet which contained more than 38% wheat gluten protein. It might have been possible to produce maximal growth by feeding a wheat gluten diet if the intake had been increased.

As the percentage of dietary casein or wheat gluten was increased (fig. 1), there was a linear elevation in nitrogen intake (g/kg/day). Although the animals on the low protein intakes ate less food, the caloric intake per kilogram of body weight per day was slightly higher in these animals than in those fed the diets which contained high percentages of protein. In these experiments, the over-all caloric intake of the weanling rats was approximately 400 kcal/kg/day.

The data in figure 2 demonstrate that 2.5 to 2.8 g of casein nitrogen/kg of body weight/day were necessary to result in maximal growth of these rats. As the casein intake was increased from 3.0 to 7.5 g of nitrogen/kg/day, there was a definite decrease in body weight. When Holt et al. (11) fed a diet containing 70% casein to

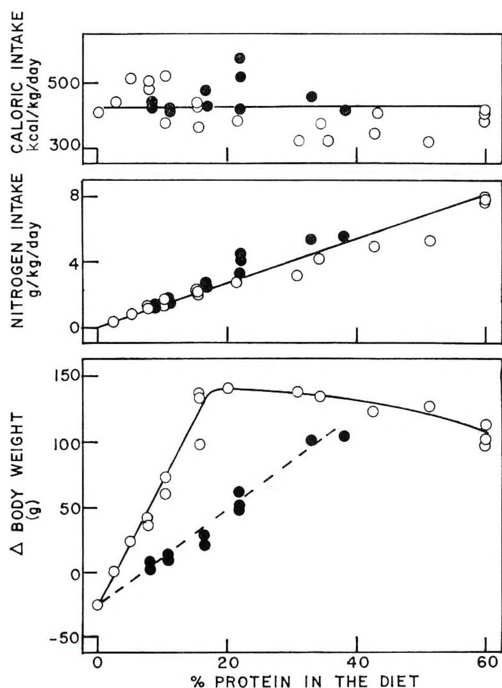

Fig. 1 Gain in body weight over 28 days, nitrogen intake in grams of nitrogen per kilogram of body weight per day and caloric intake in kilocalories per kilogram of body weight per day plotted against the percentage of protein in the diet. Open circles indicate casein-fed rats and solid circles, wheat gluten-fed animals. Each point is the mean of 10 animals.

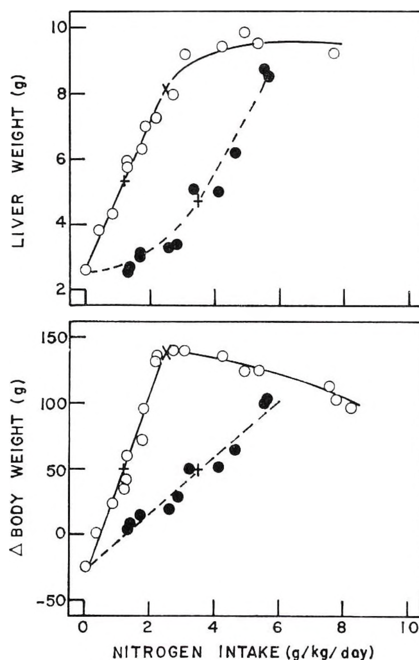

Fig. 2 Gain in body weight over 28 days and liver weight in grams plotted against nitrogen intakes in grams of nitrogen per kilogram per day. Open circles indicate casein-fed rats and solid circles, wheat gluten-fed animals. Each point is the mean of 10 animals. (+) Represents a gain of 50 g in body weight. (×) Represents the nitrogen intake which produced maximal gain in body weight.

rats, they observed a marked decrease in growth rate when compared with rats fed a diet containing 20% casein (approximately 3.0 g N/kg/day).

At a casein intake of 0.5 g of nitrogen/kg/day, the weanling rats were just able to maintain body weight. Thus, this amount of nitrogen may be required for maintenance, and an additional 0.7 g of casein nitrogen/kg/day to gain 50 g in body weight ((+), fig. 2) over the 28-day experimental period. When wheat gluten was utilized as a source of dietary protein, 1.1 g of nitrogen/kg/day was needed for maintenance, and an additional 2.4 g of nitrogen/kg/day had to be supplied to produce a 50-g gain in body weight over the 28-day period. Thus, approximately twice as much wheat gluten nitrogen as casein was needed to maintain the body weight in weanling rats, whereas nearly 3 times as much wheat gluten was utilized to produce a 50-g gain in body weight.

With increased intakes of casein or wheat gluten nitrogen, there was a curvilinear increase in liver weight (fig. 2). This increase in liver weight was correlated with elevated cellular RNA-to-DNA and protein-to-DNA ratios (fig. 3). Similarly, Halac (8) noted a larger liver in rats fed 70% casein when compared with those fed 20% casein in the diet. Henry et al. (5) also reported that increasing casein nitrogen from 2.0 to 8.0 g/kg/day, resulted in a corresponding increase in liver nitrogen content. From these data, it appears that the liver continues to store protein even at high dietary intakes, and which is in excess of that needed for maximal growth. It is possible that this storage of nitrogen at high levels of protein intake might be due in part to the higher levels of enzymes required to metabolize large amounts of amino acids.

The protein-to-DNA and RNA-to-DNA ratios of the muscle (fig. 3) rose sharply with increased casein intake and reached a maximum at a protein intake of 2.5 to 3.0 g of nitrogen/kg/day, whereupon a sharp decrease in this value was observed when higher intakes of casein were consumed. This optimal muscle cellular protein occurred at a casein intake which corresponded to maximal body weight gain. In the rats fed wheat gluten, the protein-to-DNA and RNA-to-DNA ratios continued to slowly increase at all nitrogen intakes studied but never reached a maximal level observed in the animals fed casein.

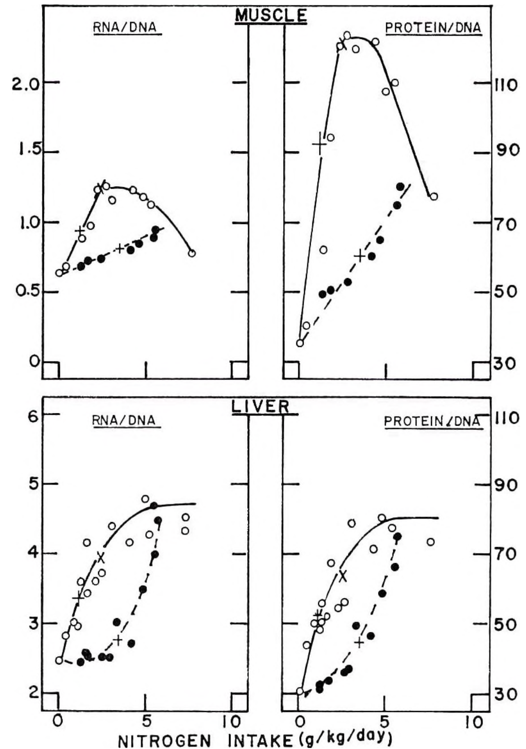

Fig. 3 Liver and muscle RNA-to-DNA and protein-to-DNA ratios plotted against nitrogen intake in grams of nitrogen per kilogram per day. Open circles indicate casein-fed rats and solid circles, wheat gluten-fed animals. Each point is the mean of 10 animals. (+) Represents a gain of 50 g in body weight. (×) Represents the nitrogen intake which produced maximal gain in body weight.

When rats maintained with the 2 protein sources were compared on the basis of equal body weight gain of 50 g/28 days ((+) fig. 3), the liver and muscle cellular protein and RNA content were higher in the animals fed casein when compared with the groups fed wheat gluten. This higher cellular protein in the liver and muscles of the rats fed casin suggests a larger protein reserve which could be drawn upon during starvation by these animals. To test this hypothesis, rats, which had been pre-fed the diets for 28 days, were placed on a starvation or protein-deprivation regimen. Under these

stresses, the animals fed wheat gluten had a significantly lower ($P > 0.01$) mean survival time than did the equal weight rats fed casein (table 2). Although both protein sources promoted equal gain in weight, the animals fed wheat gluten, with lower tissue proteins, had decreased ability to withstand the nutritional stresses of starvation or protein deprivation.

If rats were fed 1.3 g of casein nitrogen/kg/day (50-g gain in body weight), they had a lower muscle and liver cellular protein and RNA content than those which ingested 2.24 g casein nitrogen/kg/day ((x) fig. 3). In this latter group, the higher tissue proteins could be correlated with an increased ability to withstand the stresses of starvation or protein deprivation (table 2). These results are in argeement with those of Halac (7), who observed that increasing casein from 5 to 20% of the diet, resulted in a corresponding increase in resistance of the rats to the stress of nitrogen deprivation.

When rats were fed very high intakes of casein (7.90 g N/kg/day), there was a decrease in growth rate, in muscle protein and an increase in liver size and cellular protein content when compared with the animals fed 2.24 g nitrogen/kg/day. Similarly, rats injected with adrenal corticoids demonstrated an increased liver protein-to-DNA ratio and a decreased muscle protein content and growth rate (12). Thus, the high intakes of casein protein may have stimulated an adrenal stress response in these animals. Rats fed the high intake of casein did not survive the stresses of starvation or nitrogen deprivation as well as the animals fed the lower intake which was optimal for growth (table 2). Samuels et al. (6) and Halac (8) also reported that rats fed high intakes of casein or lactalbumin did not survive stress as well as those fed a 20% protein diet.

These data demonstrate a correlation between the cellular protein content of the muscle tissue and the ability of the animal to survive the stresses of starvation or protein deprivation. It is possible that the muscle, because of its large mass, is the major source of protein reserve in the body. There is an optimal filling of the muscle protein, and feeding a large excess of nitrogen could result in a stress response in the animals. Feeding excess nitrogen above the amount necessary to produce maximal formation of tissue proteins could be wasteful. Some of these tissue proteins, called reserves, can be reversibly depleted and repleted, thereby contributing to the amino acid pool of the body during nutritional and other stresses. These reserves may be represented by as much as 20% of body nitrogen (2). They are components of the tissues such as enzyme systems and need not be considered excess or stored proteins for the sole purpose of supplying amino acids. The data presented in this paper, however, suggest that filling the cellular proteins maximally in the liver may not represent optimal nutrition for muscle tissues. More information is needed concerning the relationship between maximal cellular proteins and optimal nutrition.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Edith Rosenblum for her technical assistance in this work.

TABLE 2

Mean survival rate of weanling rats fed either casein or wheat gluten protein for 28 days and then starved or fed a protein-free diet (PFD)

Dietary protein	Protein in diet	Gain in body wt	Nitrogen intake	Mean survival rate	
				Starved	Fed protein-free diet
	%	g	g/kg/day		
Wheat gluten	40	49	3.70	5.9 ± 0.8 [1]	43 ± 6
Casein	9	51	1.30	8.9 ± 1.2	65 ± 6
Casein	18	137	2.24	13.6 ± 0.9	90 ± 4
Casein	70	105	7.90	8.9 ± 1.2	75 ± 10

[1] SE of mean.

LITERATURE CITED

1. Allison, J. B., R. W. Wannemacher, Jr., W. L. Banks, Jr., W. H. Wunner and R. A. Gomez-Brenes 1962 Dietary proteins correlated with ribonuclease, ribonucleic acid and tissue proteins. J. Nutrition, *78:* 333.
2. Allison, J. B., R. W. Wannemacher, Jr. and W. L. Banks, Jr. 1963 Influence of dietary proteins upon protein biosynthesis in various tissues. Federation Proc., *22:* 1126.
3. Banks, W. L., Jr., J. B. Allison and R. W. Wannemacher, Jr 1964 Supplementation of wheat gluten protein. J. Nutrition, *82:* 61.
4. Allison, J. B., and W. H. Fitzpatrick 1960 Dietary Proteins in Health and Disease. Charles C Thomas Company, Springfield, Illinois.
5. Henry, K. M., H. W. Kosterlitz and M. H. Guenouille 1953 A method for determining the nutritive value of a protein by its effects on liver protein. Brit. J. Nutrition, *7:* 51.
6. Samuels, L. T., R. C. Gilmore and R. M. Reinecke 1948 The effects of previous diet on the ability of animals to do work during subsequent fasting. J. Nutrition., *36:* 639.
7. Halac, E. Jr. 1962 Studies of protein reserves. The relation between protein intake and resistance to protein deprivation. Am. J. Clin. Nutrition, *11:* 574.
8. Halac, E., Jr. 1961 Effects of stress on animals fed high protein diets. Am. J. Clin. Nutrition, *9:* 557.
9. Hutchison, W. C., and H. N. Munro 1961 The determination of nucleic acid in biological materials. Analyst, *86:* 786.
10. Layne, E. 1957 Spectrophoretic and turbidimetric methods of measuring proteins. III. Biuret method. In Methods in Enzymology, vol. 3. Academic Press, Inc., New York, p. 450.
11. Holt, L. E., Jr., E. Halac, Jr. and C. N. Kajdi 1962 The concept of protein stores and its implication in diet. J. A. M. A., *181:* 699.
12. Yatvin, M. B., and R. W. Wannemacher, Jr 1964 The effect of thiouracil and adrenal corticoids upon protein metabolism in liver and muscle tissue. Endocrinology, in press

Metabolic Patterns in Preadolescent Children

XI. RESPONSE OF VITAMIN A AND CAROTENE SERUM LEVELS TO DIETARY PROTEIN AND VITAMIN A [1]

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ABSTRACT Thirty-six preadolescent girls were studied under controlled experimental conditions in which amount and source of dietary protein varied. The protein intake ranged from 0.6 to 3 g/kg and the protein from plant sources varied from 25 to 100% of the total protein intake. The effect of age, weight, and amount and source of dietary protein and vitamin A on the vitamin A and carotene serum levels was evaluated. All of the factors studied accounted for 61% of the variation in serum vitamin A and 78% of the variation in serum carotene. Although there was no conclusive evidence that the vitamin A and carotene serum levels were influenced by the levels of protein fed in these diets, each serum level was related to a particular source of protein. Specifically, serum vitamin A was associated with plant protein, whereas serum carotene was associated with animal protein. The single factor accounting for the greatest amount of variation in serum vitamin A was serum carotene. There was no significant difference in either serum level between groups receiving 22 and 40 g of plant protein/day.

The primary dietary factors influencing vitamin A and carotene serum concentrations in humans are the amount and source of vitamin A and protein. The 2 serum levels do not exhibit the same response to dietary vitamin A intake. Carotene serum levels reflect the daily intake of carotene, whereas vitamin A serum levels normally exhibit random variation and remain relatively stable regardless of daily dietary fluctuations (1–6). Serum levels of both constituents have been observed to rise as the quantity and quality of dietary protein increased (7–9).

Information concerning vitamin A and carotene serum levels of children have been obtained primarily from dietary surveys made under normal and deficient conditions. The present study evaluates vitamin A and carotene serum levels of preadolescent girls under controlled experimental conditions. Results of the influence of certain physical and dietary factors on the vitamin A and carotene serum levels of the subjects will be reported.[2]

EXPERIMENTAL PROCEDURES

A total of 37 normal girls, ranging in age from seven to ten, participated in metabolic studies conducted in the summers of 1956, 1958 and 1962 at Virginia Polytechnic Institute. They received controlled diets in which the principle variables were level or source of dietary protein, or both. Dietary vitamin A was supplied from the normal food intake so that no supplementation was necessary. The intakes of protein and vitamin A from various sources are shown in table 1. The other nutrients were relatively constant within each year and were based on the recommended levels for this age group (10, 11). The experimental designs and chemical and statistical procedures are summarized below; a detailed description of the experiments is given elsewhere (12, 13).

1. *Experimental designs and dietary descriptions (table 1). 1956:* The 12 subjects were divided into 2 groups on the basis of body weight; the mean weight of the lighter group was 25.5 kg, and that of

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[1] This study was a phase of the Southern Regional Research Project, S-28, Requirements and Utilization of Selected Nutrients by Preadolescent Children, supported in part by funds appropriated to the U. S. Department of Agriculture under the Research and Marketing Act of 1946 and the Hatch Act, as amended. The Human Nutrition Division of the Agricultural Research Service was a cooperator in this project.

[2] Some of the data presented herein are taken from theses submitted by Hei Soo Rhee and Sarah T. Ehrlich to the Graduate School of Louisiana State University for the degree of Master of Science.

the heavier group was 31.6 kg. Three of the subjects in each weight group received 2 g of protein/kg of body weight (diets 2 and 4) and the other 3 subjects in each group received 3 g of protein/kg of weight (diets 3 and 5). Each subject received an average of 76 kcal/kg of weight during the sixteen 4-day experimental periods.

1958. For five 6-day periods the subjects received 22 g of protein and 2178 kcal (diet 8). During the last periods the subjects received 18 g of protein and 2235 kcal (diet 8′).

1962. Two levels of plant protein were fed to the subjects for six 6-day periods; one group of six received 22 g of plant protein (diet 9) and the other group of six received approximately 40 g of plant protein (diet 11). The levels of riboflavin and niacin for the 2 groups were not constant and evaluation of the vitamin A and carotene serum levels was made considering these differences.

2. *Chemical.* Blood samples were obtained primarily by finger puncture from the subjects at the beginning of every period (except periods 4 and 6 in 1962). The serum samples were frozen and later analyzed by the micromethod of Bessey et al. (14), to determine their vitamin A and carotene content.

3. *Statistical.* Data from all the experimental periods in 1956 and 1962 were evaluated statistically using analysis of variance to test for significant differences between experimental treatments (15). Data from the final periods of 1956, 1958 and 1962 were compiled and analyzed using multiple regression to evaluate the influence of certain physical and dietary factors on the vitamin A and carotene serum levels. Means, standard deviations and correlation coefficients for vitamin A and carotene serum levels from all experimental periods are shown in table 2.

RESULTS AND DISCUSSION

1956 Analysis of variance. Vitamin A. The serum vitamin A levels of the girls consuming the high and low protein diets did not differ significantly. This indicates that either: 1) the vitamin A serum levels were not influenced by the protein intake, or that 2) both diets supplied adequate amounts of protein for vitamin A metabolism. Differences in serum vitamin A between periods were significant ($P < 0.01$) due to lower vitamin A serum levels in several periods followed by increasing levels. Since the vitamin A serum levels between groups receiving different levels of protein were not significantly different, the period effect may have been due to a general improvement in dietary or physical conditions, or both, of all of the subjects due to the experimental regimen.

Although neither weight group exhibited any definite trend in serum vitamin A levels, the interaction between body weight and period was significant ($P < 0.01$). This was due to inconsistent differences in serum vitamin A levels between periods for the 2 weight groups.

Carotene. The group receiving 3 g of protein/kg of weight had increasingly higher serum carotene levels throughout

TABLE 1

Descriptions and mean protein and vitamin A intakes for the various experimental groups

Year	Description	Diet [1] no.	No. of subjects	Mean wt	Protein intake			Vitamin A intake		
					Total	Plant	Animal	Total	Plant	Animal
				kg	*gN*	*gN*	*gN*	*IU*	*IU*	*IU*
1956	Light wt, 2 g protein/kg	2	3	24	7.7	2.3	5.4	3594	2039	1555
	Light wt, 3 g protein/kg	3	3	27	11.6	2.6	9.0	3441	1603	1838
	Heavy wt, 2 g protein/kg	4	3	31	9.4	2.6	6.7	3557	1538	2019
	Heavy wt, 3 g protein/kg	5	3	33	14.1	2.7	11.4	3613	1451	2150
1958	Periods 1–5, 0.8 g protein/kg	8	13	28	3.5	1.7	1.8	3687	2795	892
	Periods 6–8, 0.6 g protein/kg	8′	13 [2]	29	2.9	1.8	1.2	3505	2795	710
1962	0.8 g Protein/kg	9	6	27	3.5	3.5	0	3926	3926	0
	1.4 g Protein/kg	11	6	29	6.3	6.3	0	3220	3220	0

[1] Diet numbers which are omitted were concerned with other aspects of Metabolic Studies in Preadolescent Children.
[2] These same subjects consumed diet 8.

the experiment than the subjects receiving 2 g of protein/kg of weight (fig. 1). However, the difference between these 2 groups was not statistically significant because of the large amount of variation between subjects. On the basis of weight groupings, the light subjects had significantly higher serum carotene levels ($P < 0.05$) than did the heavy subjects (fig. 2). The large amount of individual variation between subjects probably masked the treatment effects even though definite trends for weight and protein groups were apparent.

1962 Analysis of variance. The vitamin A serum levels of the subjects in the 2 protein groups were not significantly different. The vitamin A serum levels of each subject tended to remain the same throughout the experiment. These results are not unexpected since vitamin A serum levels fluctuate only slightly under normal

TABLE 2

Means, standard deviations, and correlation coefficients (r) for serum vitamin A and serum carotene for all experimental groups

Year	Experimental description	Diet no.	Serum vitamin A	Serum carotene	r value
			μg/100 ml	μg/100 ml	
1956			50.5 ± 9.9	180.0 ± 44.2	0.32**
	Light wt, 2 g protein/kg	2	45.8 ± 7.7	181.9 ± 54.9	0.42**
	Light wt, 3 g protein/kg	3	52.7 ± 7.3	213.8 ± 39.3	0.48**
	Heavy wt, 2 g protein/kg	4	53.9 ± 14.4	146.8 ± 26.4	0.70**
	Heavy wt, 3 g protein/kg	5	49.9 ± 6.2	179.3 ± 21.7	0.37*
1958			27.3 ± 7.6	107.6 ± 22.5	0.71**
	Periods 1–5, 0.8 g protein/kg	8	29.0 ± 7.8	112.3 ± 23.7	0.68**
	Periods 6–8, 0.6 g protein/kg	8′	24.0 ± 6.6	100.2 ± 18.4	0.71**
1962			36.2 ± 8.6	112.3 ± 24.6	0.21
	0.8 g Plant protein/kg	9	36.0 ± 6.6	101.4 ± 21.7	0.14
	1.4 g Plant protein/kg	11	36.3 ± 10.3	123.2 ± 22.7	0.28
All years			40.7 ± 13.8	146.1 ± 50.3	0.69**

* Significant at the 5% level of probability.
** Significant at the 1% level of probalility.

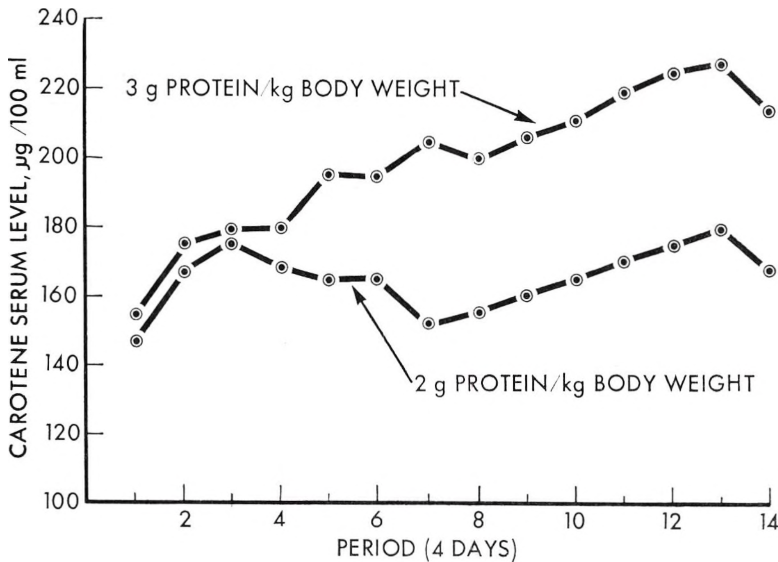

Fig. 1 Serum carotene concentrations of subjects receiving 2 levels of protein in 1956. Each point represents the mean serum level of 6 subjects.

conditions. Apparently preadolescent girls, as well as adults, maintain stable vitamin A serum levels except for random variation. There is no indication that the respective levels of protein, riboflavin, and niacin affected the vitamin A serum levels of the 2 groups.

Carotene serum levels between the 2 groups of subjects were not significantly different. However, there was a significant interaction between protein level and period. This was the result of the large initial differences between the serum levels of the 2 groups which decreased as the experiment progressed. The results indicate that the dietary conditions of each group influenced carotene serum levels so that the 2 groups became more similar as the experiment progressed (fig. 3). The carotene intake on the plant protein diets

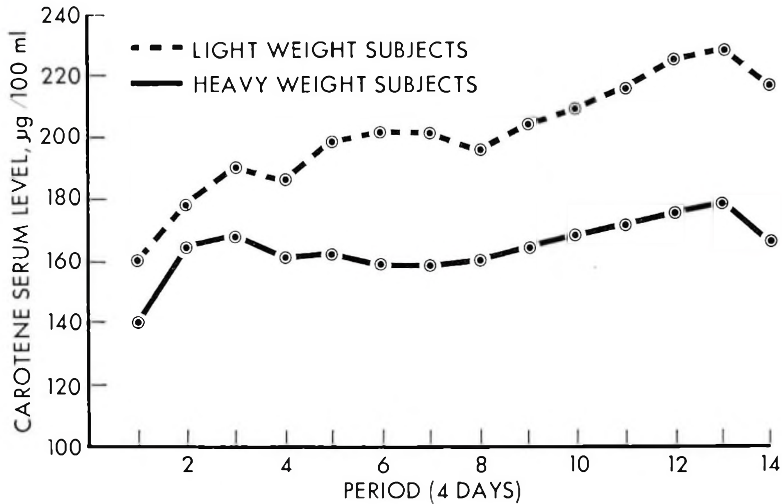

Fig. 2 Serum carotene levels of light- and heavy-weight subjects in 1956. Each point represents the mean serum level of 6 subjects.

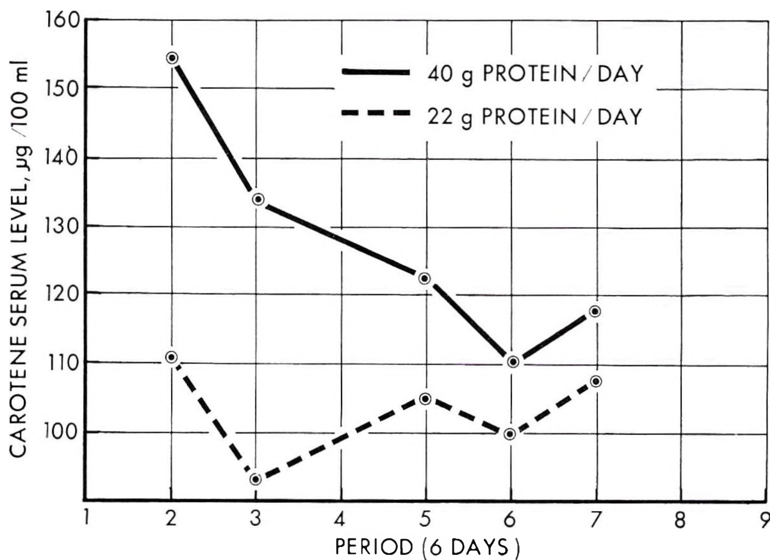

Fig. 3 Mean carotene serum levels of 6 subjects receiving 22 g of plant protein/day and of 6 subjects receiving 40 g of plant protein/day in 1962.

was essentially the same; therefore, the converging carotene serum levels reflect the similar dietary intake of carotene by the two groups.

REGRESSION ANALYSIS

Multiple regression was used to evaluate the influence of certain physical and dietary factors on vitamin A and carotene serum levels. These factors were age, weight, source and amount of dietary protein, and source and amount of dietary vitamin A. The analysis consisted of 2 phases: 1) investigation of all variables with successive deletion of the independent variable having the least effect on the dependent variable, and 2) regression of the dependent variable on independent variables of special nutritional interest. The R^2 values for all combinations tested were significant ($P < 0.01$). Table 3 summarizes the factors having the greatest influence on the 2 serum levels.

1) *Effect of all variables.* All of the factors considered accounted for 61% of the variation in serum vitamin A and 78% of the variation in serum carotene. These factors accounted for a greater amount of the variation in serum carotene than in serum vitamin A, thus indicating that serum carotene levels reflect immediate dietary intake to a greater extent than do serum vitamin A levels.

Results of this analysis indicate that the 2 factors which have the most influence on serum vitamin A are serum carotene and plant protein intake ($R^2 = 0.56$). Likewise, the 2 best predictors of serum carotene are serum vitamin A and animal protein intake ($R^2 = 0.72$). These results substantiate theories concerning the relationship between the 2 serum levels (1, 6) and between the serum levels and protein intake (8, 9). They also indicate that a specific source of protein is associated with each serum level.

The single factor which affected serum vitamin A levels most was serum carotene concentration ($r^2 = 0.47$). This supports the theory that an increase in serum carotene levels is accompanied by an increased serum vitamin A level. The one variable affecting serum carotene most was animal protein intake ($r^2 = 0.57$).

2) *Combinations of special nutritional interest.* Since the results of the above analysis did not provide a complete picture of some of the important nutritional interrelationships, selected combinations of dietary and physical factors were analyzed. These relationships are believed to be functional, especially those which include protein components. All combinations of independent variables tested produced highly significant R^2 values ($P < 0.01$) when either serum vitamin A or serum carotene was used as the dependent variable.

Serum vitamin A was found to be significantly influenced by total protein and total vitamin A intakes ($R^2 = 0.45$). This reinforces previous observations that vitamin A serum levels are proportionately related to dietary intakes of protein and vitamin A (1, 4, 8, 9). A second relationship indicated that protein given on the basis of body weight influenced vitamin A serum levels ($R^2 = 0.43$). None of the combinations tested accounted for more than 50% of the variation in serum vitamin A levels indicating that there are other factors which affect serum vitamin A.

Serum carotene levels reflected the influence of dietary protein intake more than did serum vitamin A levels. This is illustrated by the fact that R^2 values between the independent variables and serum carotene are much greater than the corresponding values for serum vitamin A levels (table 3). Previous observations have shown that serum carotene levels

TABLE 3

Factors accounting for the greatest amount of variation in vitamin A and carotene serum levels

	Independent variable	Dependent variable	R^2 **
1	Serum carotene Plant protein intake	serum vitamin A	0.56
2	Serum vitamin A Animal protein intake	serum carotene	0.72
3	Protein intake Vitamin A intake	serum vitamin A	0.45
4	Weight Protein intake	serum vitamin A	0.43
5	Weight Protein intake	serum carotene	0.70
6	Weight Animal protein intake	serum carotene	0.63

** All R^2 values are significant at the 1% level of probability.

were affected by protein intake (16) and possibly by protein intake per kilogram of body weight. Results from these experiments indicated that this relationship between protein intake based on body weight and serum carotene levels existed in these experiments ($R^2 = 0.70$). In addition, serum carotene levels were found to be specifically related to animal protein intake per kilogram of body weight ($R^2 = 0.63$).

The above data obtained from healthy children under controlled experimental conditions indicated that 1) serum carotene is influenced more by dietary factors than is serum vitamin A; 2) factors other than age, weight, protein intake and vitamin A intake affect serum vitamin A levels; 3) there is a high positive relationship between serum vitamin A and serum carotene; 4) serum vitamin A is more influenced by protein intake from plant sources, whereas serum carotene is influenced by protein intake from animal sources; and 5) the various amounts of protein fed did not influence serum vitamin A or serum carotene.

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LITERATURE CITED

1. Moore, T. 1957 Vitamin A. Elsevier Publishing Company, Princeton, New Jersey.
2. Hume, E. M., and H. A. Krebs 1949 Vitamin A requirement of human adults. An experimental study of vitamin A deprivation in man, Spec. Rept. Series no. 264. H. M. Stationery Office, London.
3. Szymanski, B. B., and B. B. Longwell 1951 Plasma vitamin A and carotene determinations in a group of normal children. J. Nutrition, *45:* 431.
4. Moschette, D. S. 1955 Metabolic studies with preadolescent girls. I. Utilization of carotene. J. Am. Dietet. A., *31:* 37.
5. Roderuck, C. E., V. D. Sidwell, E. H. Jebe and E. S. Eppright 1963 Studies of serum carotenoids and vitamin A in Iowa school children. Am. J. Clin. Nutrition, *13:* 186.
6. Moschette, D. S. 1962 Blood serum vitamin A and carotene studies of preadolescent children, bull. 552. Louisiana Agriculture Experiment Station, Baton Rouge, Louisiana.
7. Berger, S., M. Rechcigl, Jr., K. Loosli and H. H. Williams 1962 Protein quality and carotene utilization. J. Nutrition, *77:* 174.
8. Gopalan, C., P. S. Venkatachalam, and B. Bhavani 1960 Studies of vitamin A deficiency in children. Am. J. Clin. Nutrition, *8:* 833.
9. Arroyave, G., D. Wilson, J. Mendez, M. Behar and N. S. Scrimshaw 1961 Serum and liver vitamin A and lipids in children with severe protein malnutrition. Am. J. Clin. Nutrition, *9:* 180.
10. National Research Council, Food and Nutrition Board 1954 Recommended dietary allowances, pub. 302. National Academy of Sciences — National Research Council, Washington, D. C. 20025.
11. National Research Council, Food and Nutrition Board 1958 Recommended dietary allowances, pub. 589. National Academy of Sciences — National Research Council, Washington, D. C. 20025.
12. Technical Committee of the Southern Regional Nutrition Research Project (S-28) 1959 Metabolic patterns in preadolescent children. I. Description of Metabolic Studies. Southern Cooperative Series, bull. no. 64. Virginia Agriculture Experiment Station, Blacksburg, Virginia.
13. Technical Committee of the Southern Regional Nutrition Research Project (S-28) 1964 Metabolic patterns in preadolescent children. X. Description of 1962 study. Southern Cooperative Series, bull. no. 94. Virginia Agriculture Experiment Station, Blacksburg, Virginia.
14. Bessey, O. A., O. H. Lowry, M. J. Brock and J. A. Lopez 1946 The determination of vitamin A and carotene in small quantities of blood serum. J. Biol. Chem., *166:* 177.
15. Snedecor, G. W. 1956 Statistical Methods. Iowa State College Press, Ames.
16. Guzman, M. A., G. Arroyave and N. S. Scrimshaw 1961 Serum ascorbic acid, riboflavin, carotene, vitamin A, vitamin E and alkaline phosphatase values in Central American school children. Am. J. Clin. Nutrition, *9:* 164.

A Further Study of the Specificity of the Vitamin E Requirement for Reproduction

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ABSTRACT A study was carried out to determine whether the ability of certain synthetic antioxidants to substitute for vitamin E in the diet of the female rat is due to a conservation of tocopherols in the diet or tissues, or to a direct substitution for the vitamin in its metabolic role. A highly purified basal diet was devised which was demonstrably free of vitamin E. When this diet was fed to rats of proven sterility, supplementation with the antioxidant DPPD (but not ethoxyquin) was observed to restore reproductivity. Under the conditions used, 0.75 mg of DPPD daily was more effective in this respect than 5 mg of *dl-α*-tocopheryl acetate. The results support the conclusion that DPPD is capable of direct metabolic substitution for tocopherols in metabolism, and that the function of vitamin E is entirely associated with its antioxidant properties.

Numerous experiments, both in vivo and in vitro, have been conducted in recent years in attempts to elucidate the biological function of vitamin E. The observation of Dam and co-workers (1, 2) that part of the vitamin E required by the rat and chick can be replaced by methylene blue has stimulated many additional experiments on the ability of various antioxidants to substitute for tocopherols in the prevention of specific deficiency diseases. As a result, it is generally agreed that a prime function of vitamin E in vivo is to serve as a lipid antioxidant, the presence of which is necessary for stabilization of various easily oxidizable components of the cells, notably the lipoprotein moieties of the cell wall and particulate membranes. This role also can be assumed by certain structurally unrelated synthetic antioxidants, of which N,N′-diphenyl-*p*-phenylenediamine (DPPD) and 1,2-dihydro-6-ethoxy-2,2,4-trimethyl-quinoline (ethoxyquin) [1] appear to be the most effective.

Whether the biological role of vitamin E resides entirely in its antioxidant properties, or whether it also has some additional (presumably coenzymatic) function is a subject of current controversy. Nason and associates (3) have recently revived the proposal that vitamin E is a co-factor in the DPNH-cytochrome c reductase of bovine heart muscle, and Martius and Fürer (4) have reported the isolation of a proposed "active form" of the vitamin from rabbit liver. Conversely, by feeding vitamin E-deficient diets to various species of animals it has been shown that DPPD is capable of preventing all of the classical tocopherol deficiency diseases, including resorption gestation (5), muscular dystrophy (6) and encephalomalacia (7). In addition, the major biochemical lesions associated with vitamin E deficiency can be prevented or alleviated by administration of this antioxidant. It cannot be proved by preventive experiments, however, whether the ability of an antioxidant to substitute for vitamin E in the diet is due to direct functional displacement in metabolism or to conservation of the vitamin in the tissues. In support of the former concept, DPPD has been shown to possess curative as well as preventive activity with respect to sterility (5) and dystrophy (6). A further consideration is the difficult technical problem of devising experimental diets composed of natural materials which are completely devoid of vitamin E yet adequate in all other respects. It has been contended (8, 9) that the basal diets used in some of the aforementioned antioxidant experiments contained small amounts of tocopherols which may have been sufficient, in the presence of

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[1] Santoquin, Monsanto Chemical Company, St. Louis.

DPPD, to satisfy the requirement for some specific non-antioxidant function.

Two conditions are essential in an experimental approach to the question of the nutritional indispensability of vitamin E. First, it must be unequivocally established that the basal diet used is completely free of tocopherols. Second, a curative procedure must be used, since a response to antioxidant administration in grossly deficient animals fed a vitamin E-free diet cannot be attributed to a conservation of tocopherol in the tissues. In the experiments described here, when these conditions were observed, DPPD was at least as effective as α-tocopherol in reversing sterility in female rats.

EXPERIMENTAL

Animals. The experimental animals were 90 weanling female rats of the Sprague-Dawley strain. In a preliminary experiment they were depleted of tocopherols by feeding a vitamin E-deficient diet and shown to be sterile by mating with normal males. They were subsequently remated and maintained throughout a regeneration cycle with a highly purified diet which was shown to be devoid of tocopherols. The curative effect of α-tocopherol, DPPD or ethoxyquin was determined in the second cycle.

Diet A (depletion cycle). The composition of this diet is shown in table 1. Weanling rats were maintained on this regimen for 44 days, at which age they weighed 200 g, and then were caged with normal males for a 5-day period. In the course of the ensuing reproductive cycle the females were observed to be uniformly sterile. They were subsequently remated and shifted to diet B.

TABLE 1

Composition of diet A

	%
Glucose monohydrate [1]	65.4
Casein, vitamin-free [2]	20.0
Corn oil (molecular-distilled) [3]	10.0
Salts 4164 [4]	4.0
Vitamin premix in glucose monohydrate [4]	0.5
Choline chloride	0.1
	100.0

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] "Vitamin-free" Casein, General Biochemicals Corporation, Chagrin Falls, Ohio.
[3] Distillation Products Industries, Rochester, New York.
[4] See Experimental section for composition. Vitamins A and D were added at levels of 25 and 2 IU/g of diet, respectively.

TABLE 2

Composition of diet B [1]

	%
Sucrose, ether-extracted	71.8
Synthetic amino acids	18.6
Glycerol	5.0
Salts 4164 [2]	4.0
Vitamin premix (in extracted sucrose)	0.5
Choline chloride	0.1
	100.0

[1] Each rat received 60 mg ethyl linoleate *per os* daily containing 100 μg crystalline vitamin A acetate. Vitamin D was given orally in two 100 IU doses at the beginning of the regeneration cycle. For composition of amino acid and vitamin mixtures see Experimental section.
[2] See Experimental section for composition.

Diet B (curative cycle). The gross composition of this diet is outlined in table 2, and the details of its formulation are described below to illustrate the measures used to assure a complete absence of vitamin E. This diet was not used in the preliminary depletion cycle because of economic considerations.

Although the residue obtained by ether extraction of a 200-g sample of commercial sucrose gave a negative Emmerie-Engel reaction for reducing substances, this component was nevertheless subjected to a 12-hour continuous extraction with diethyl ether using a Soxhlet apparatus. Casein, which has been reported to contain significant amounts of vitamin E (8, 9) was replaced in the curative diet by a synthetic amino acid mixture. This mixture, which was similar to that devised by Rao et al. (10) for the growing rat, was composed of the following amino acids: (% of diet) L-arginine·HCl, 0.38; L-histidine·HCl·H_2O, 0.50; L-lysine·HCl, 1.69; L-tryptophan, 0.23; DL-isoleucine (50% D-allo), 1.66; DL-valine, 1.66; L-leucine, 1.05; DL-threonine (allo-free), 1.50; L-methionine, 0.24; L-cystine, 0.51; DL-phenylalanine, 1.26; L-tyrosine, 0.45; L-aspartic acid, 1.50; L-glutamic acid, 1.50; and glycine, 4.45.

Salt mixture 4164 was composed of the following ingredients: (g/kg) NaCl, 108.09; $K_3C_6H_5O_7 \cdot H_2O$, 236.53; K_2HPO_4, 77.33; $CaHPO_4 \cdot 2H_2O$, 355.56; $CaCO_3$, 163.56; $MgCO_3$, 40.89; $FeC_6H_5O_7 \cdot 3H_2O$, 16.00; $CuSO_4 \cdot 5H_2O$, 0.18; $MnSO_4 \cdot H_2O$, 1.38; KI, 0.04; and $ZnCO_3$, 0.44.

Crystalline vitamins were added as a premix in ether-extracted sucrose so as to provide the following concentrations in the diet: (μg/g) thiamine, 10; riboflavin, 5; nicotinic acid, 25; pyridoxine·HCl, 5; Ca pantothenate, 10; menadione, 1; folic acid, 1; biotin, 0.1; and vitamin B_{12}, 0.1. Crystalline vitamin A acetate was administered in the methyl linoleate supplement (see below) at a rate of 100 μg/day. Two oral doses of 100 IU of vitamin D each were given on consecutive days at the beginning of the regeneration cycle.

The most difficult component of a diet to obtain free from vitamin E is the source of essential fatty acids. To insure an uncontaminated product, linoleic acid was isolated from maize oil as the ethyl ester by a modification of the bromination-debromination method of Rollett (11) as revised by Frankel and Brown (12). In this procedure, 1 kg of maize oil was saponified for 1 hour with KOH and the resulting mixture, after acidification with concentrated HCl, was extracted with petroleum ether. The fatty acids which crystallized in the cold were removed by vacuum filtration and a small excess of bromine was added to the filtrate at − 20°. The tetrabromostearic acid which precipitated in the cold was recrystallized from petroleum ether, the final product exhibiting a melting point of 114°. This material was dissolved in ethanol and reduced by refluxing for 2 hours after the addition of granular zinc and HCl gas. The resulting ethyl linoleate was recovered into petroleum ether solution which was washed with weak alkali and water and then dried. Final purification of the ester was achieved by molecular distillation at 0.07-mm pressure. The main fraction, which distilled at 147°, was a clear product which gave a negative Emmerie-Engel reaction when duplicate 2-g samples were tested. This material was administered orally to all rats during the regeneration cycle at the rate of 60 mg/rat/day.

Regeneration procedure. The 90 sterile females were remated by caging with normal males for 5 days, during which time the males were rotated among the cages. Subsequently all females were housed individually and fed basal diet B plus a daily oral supplement of ethyl linoleate containing vitamin A. One group of 10 rats was maintained as a negative control; the remainder were divided into 4 groups of 20 each and given daily oral supplements of vitamin E or antioxidant, as indicated: group 1, no supplement; group 2, 0.75 mg DPPD; group 3, 3.75 mg ethoxyquin; group 4, 5 mg *dl*-α-tocopheryl acetate; and group 5, 10 mg *dl*-α-tocopheryl acetate. In each case the supplement was incorporated into the ethyl linoleate-vitamin A mixture prior to administration. Group 5 represented a second positive control which was introduced into the experiment at a later date when a poor response to vitamin E was obtained in group 4. Twenty-one days from the beginning of the mating period all the females were killed, the uteri were examined for live pups or evidence of resorbed fetuses, and the hearts and livers were collected for vitamin E analysis.

α-Tocopherol determination. The livers of 6 animals from each group, along with 3 pooled samples of 3 hearts each, were analyzed for their vitamin E content. The organs were minced and refluxed for 1 hour with 1 volume of 0.25% pyrogallol in methanol and 0.5 volume of 60% KOH. After cooling and adding 3 volumes of water, the mixture was extracted 4 times with peroxide-free diethyl ether. The ether phase was washed 5 times with H_2O and dried with Na_2SO_4, the solvent was evaporated, and the residue was applied in petroleum ether (40° to 60°) to a 0.75-cm × 8-cm column of silicic acid-Celite (2:1). The chromatogram was developed with 5% diethyl ether in petroleum ether until the yellow ubiquinone band approached the bottom of the column. By use of appropriate standards it was predetermined that at this point α-tocopherol and vitamin A were present in the eluate, whereas the sterols, in addition to ubiquinone remained on the column. A further purification was carried out by means of thin-layer chromatography on silica gel using 1% methanol in benzene as the mobile phase. In this system pure α-tocopherol exhibited an R_f value of 0.63, vitamin A, 0.22, and ubichromenol (which was not detected in any of the tissue extracts), 0.78. Duplicate eluates from the column, together

with a pure α-tocopherol standard, were chromatographed on the same plate. One of the duplicates, plus the standard, was sprayed with Emmerie-Engel reagent to locate vitamin E and a band in the analagous position representing the second duplicate was eluted with diethyl ether. Vitamin E present in this eluate was determined quantitatively by the Emmerie-Engel reaction in a Coleman spectrophotometer. The recovery of α-tocopherol from the silicic acid column, determined by use of *d*-α-tocopherol-5-methyl-^{14}C, averaged 77% and that for the thin-layer procedure was 86%, giving an over-all recovery of 66%.

RESULTS AND DISCUSSION

The results of the regeneration cycle are summarized in table 3. Although the general level of reproductivity was below that observed using stock diets, there was a clear response to DPPD administration which was, in fact, superior to that obtained to α-tocopherol. The conditions of the experiment dictate, moreover, that this response must have been the result of a functional replacement of vitamin E in metabolism.

The failure of vitamin E to bring about more complete regeneration, even at the higher level (10 mg/day) administered to group 5, was unexpected in view of the substantial levels of the vitamin observed in the liver and heart. Ten litters were observed to be in resorption in this group, including five which also contained live fetuses. Consumption of the amino acid diet, which was introduced immediately after the second mating period, was initially poor, and all groups lost weight during the ensuing 2 days (average loss, 6 g). Subsequently food intake improved and, except in the negative control and ethoxyquin groups which yielded no live young, the weight loss was more than recovered. It appears likely that the general level of fecundity was depressed by the necessary rigor of the experimental regimen, and that this consideration accounts, at least in part, for the subnormal apparent conception rate. Death and complete resorption of the embryo at an early stage of pregnancy may explain the absence of evidence of conception in some animals at term. Notwithstanding, the response to DPPD was clearly adequate to demonstrate the efficacy of this compound in reversing sterility in female rats under conditions in which the effect cannot be attributed to a "sparing" of vitamin E in the diet or tissues. This observation is analagous to that recorded earlier with respect to muscular dystrophy in rabbits, which was shown to

TABLE 3

Effect of vitamin E and antioxidants on remission of sterility in female rats

Group no.	1	2	3	4	5
Treatment	None	DPPD	Ethoxyquin	Vitamin E	Vitamin E
Dose, mg/day	—	0.75	3.75	5.0	10.0
Reproductive performance					
No. females	10	20	20	20	20
No. litters *in utero*	8	9	8	4	11
No. live litters [1]	0	9	0	4	6
Total no. live pups	0	63	0	21	29
Avg. no. live pups [2]	0	7.0	0	5.3	4.8
No. litters in resorption [3]	8	3	8	4	10
Vitamin E content of tissues					
No. liver samples analyzed	6	6	0	0	6
No. heart samples analyzed [4]	3	4	0	0	3
Conc in liver, μg/g	nd [5]	nd	—	—	30.2 ± 5.8 [6]
Conc in heart, μg/g	nd	nd	—	—	13.0 ± 3.5

[1] Includes all litters containing live pups.
[2] Based on number of litters containing live pups.
[3] Includes some litters containing live pups.
[4] Each sample contained 3 hearts.
[5] None detected.
[6] Mean ± SD.

yield readily to DPPD administration even when the affected animals were maintained with a lipid-free amino acid diet (6). These results provide strong support for the view that the function of vitamin E is entirely associated with its antioxidant properties.

The failure of ethoxyquin to restore fertility in this experiment illustrates the fact that the specifications required of a fully effective biological antioxidant are extremely stringent. Many synthetic compounds possessing a favorable redox potential, lipid solubility, toxicity level and antioxidant potency in vitro have low efficacy as vitamin E substitutes. For example, butylated hydroxytoluene (BHT), an antioxidant which is highly effective in the stabilization of unsaturated fats in foods and feeds, does not support reproduction in rats (5) or poultry (13), a fact which may be explained by its limited absorption from the lumen of the intestine or inefficient transport across biological membranes (14, 15). Apparently for the same reason, butylated hydroxyanisole (BHA), another compound having a strong inhibitory effect on fatty acid perodixation, has been found ineffective in the prevention of several vitamin E deficiency diseases. Although DPPD is the most active tocopherol substitute which has been extensively tested, it too has been observed to be unsatisfactory in certain biological systems. Its failure to maintain fertility in poultry is apparently attributable to a limited deposition in the egg (13). Whereas it has been found capable of preventing muscular dystrophy in lambs in the preruminal stage (16), DPPD is ineffective in animals in which this organ is functional (17), indicating that the compound is destroyed in vivo by bacterial action. DPPD also possesses a high order of chronic toxicity for pregnant rats (5) which restricts the range of effective concentrations in the diet. These various considerations illustrate the complexity of the properties necessary in a biological antioxidant and suggest that, although there may be no absolute requirement for vitamin E in the functional sense, from an over-all physiological standpoint the tocopherol molecule may be uniquely suited to this purpose.

The minimal concentration of tocopherol in the liver which could be detected by the method used was about 0.5 μg/g, and the results indicate that less than this amount was present in the liver of the animals that did not receive a vitamin E supplement. Green and co-workers (8) have reported values as low as 0.4 μg/g in the liver of vitamin E-deficient rats and Gloor et al. (18) reported a similar amount. Bieri et al. (19) were unable to detect as much as 0.25 μg of vitamin E/g in the liver of deficient chicks, and Csallany and Draper (6) concluded that less than 0.1 μg/g was present in the liver of dystrophic rabbits.

Even the higher estimates of the vitamin E present in the liver of deficient animals represent only about one-tenth of the concentration required for stoichiometry with cytochrome c, a consideration which strongly militates against a suggested role for the vitamin in electron transport (3). Further, Pollard and Bieri (20) noted no difference in DPNH-cytochrome c reductase activity in heart muscle preparations from vitamin E-deficient and control chicks, even though no tocopherol could be detected in the tissue taken from the depleted birds. Similarly, Corwin and Schwarz (21) observed that vitamin E deficiency had no influence on the DPNH-cytochrome c reductase of rat liver mitochondria, and Allen et al. (22) reported elevated cytochrome c reductase activity in the muscle of dystrophic rabbits. On the other hand, taken together with the fact that the vitamin E requirement can be predicted from the amounts and proportions of polyunsaturated fatty acids in the diet (23) and that chicks can be raised to maturity in the absence of the vitamin if the intake of these acids is minimized (19), the results of the present experiment constitute persuasive evidence for the conclusion that the antioxidant role of vitamin E represents its exclusive function in metabolism. In further support of this concept, it has been observed [2] that the incipient symptoms of muscular dystrophy (creatinuria, red blood cell hemolysis) can be induced within hours by intravenous infusion of milligram quantities of methyl linoleate hydroperoxide.

[2] Kokatnur, M. G., J. G. Bergan and H. H. Draper, unpublished results.

LITERATURE CITED

1. Dam, H., and H. Granados 1952 The effect of dietary methylene blue on the reproduction capacity of vitamin E deficient rats. Acta Pharmacol. Toxicol., *8:* 47.
2. Dam, H., I. Kruse, I. Prange and E. Søndergaard 1951 Substances affording a partial protection against vitamin E deficiency symptoms. Acta Physiol. Scand., *22:* 299.
3. Nason, A., R. H. Garrett, P. P. Nair, F. D. Vasington and T. Detwiler 1964 Inhibition by digitonin of bovine heart and its specific reversal by the tocopherols. Biochem. Biophys. Res. Comm., *14:* 220.
4. Martius, C., and E. Fürer 1963 Über die Umwandlung des Tokopherols im Tierkörper. Biochem. Ztschr., *336:* 474.
5. Draper, H. H., S. Goodyear, K. D. Barbee and B. C. Johnson 1958 A study of the nutritional role of anti-oxidants in the diet of the rat. J. Nutrition, *12:* 89.
6. Csallany, A. S., and H. H. Draper 1961 On the occurrence of vitamin E in the liver of dystrophic and anti-oxidant-fed rabbits. Arch. Biochem. Biophys., *92:* 462.
7. Singsen, E. P., R. H. Bunnell, L. D. Matterson, A. Kozeff and E. L. Jungherr 1955 Studies on encephalomalacia in the chick. 2. The protective action of diphenyl-*p*-phenylenediamine against encephalomalacia. Poultry Sci., *34:* 262.
8. Green, J., A. T. Diplock, J. Bunyan, E. E. Edwin and D. McHale 1961 Ubiquinone (coenzyme Q) and the function of vitamin E. Nature, *190:* 318.
9. Diplock, A. T., E. E. Edwin, J. Bunyan and J. Green 1961 The effect of tocopherol, vitamin A, selenium and anti-oxidants on ubiquinone in the rat. Brit. J. Nutrition, *15:* 425.
10. Rama Rao, P. B., H. W. Norton and B. C. Johnson 1961 The amino acid composition and nutritive value of proteins. IV. Phenylalanine, tyrosine, methionine and cystine requirements of the growing rat. J. Nutrition, *73:* 38.
11. Rollett, A. 1909 Zur Kenntnis der Linolsaure. Ztschr. Physiol. Chem., *62:* 410.
12. Frankel, J. S., and J. B. Brown 1943 Studies on the chemistry of the fatty acids. XII. The preparation of alpha- and beta-linoleic acids by debromination in various solvents and observations on the chemistry of these acids. J. Am. Chem. Soc., *65:* 415.
13. Jensen, L. S., and J. McGinnis 1960 Influence of selenium, antioxidants and type of yeast on vitamin E deficiency in the adult chicken. J. Nutrition, *72:* 23.
14. Krishnamurthy, S., and J. G. Bieri 1962 Dietary antioxidants as related to vitamin E function. J. Nutrition, *77:* 245.
15. Francois, C. A., and A. Pihet 1960 Influence de l'ingestion de'antioxygenes sur la composition des certain tissus et sur la stabilité des graisses de reserve du porc et du poulet. Ann. de L'Inst. Nat. de la Recherche Agron., *9:* 195.
16. Draper, H. H., and B. C. Johnson 1956 N,N'- diphenyl - *p* - phenylenediamine in the prevention of vitamin E deficiency in the lamb. J. Animal Sci., *15:* 1154.
17. Hogue, D. E., J. F. Proctor, R. G. Warner and J. K. Loosli 1962 Relation of selenium, vitamin E and an unidentified factor to muscular dystrophy (stiff-lamb or white-muscle disease) in the lamb. J. Animal Sci., *21:* 25.
18. Gloor, U., F. Weber, J. Wursch and O. Wiss 1963 Verteilung der radioaktivität in der ratte nach verabreichung von (^{14}C)-*d,l-α*-tocopheryl-acetat. Helv. Chim. Acta, *46:* 2457.
19. Bieri, J. G., G. M. Briggs, C. J. Pollard and M. R. Spivey Fox 1960 Normal growth and development of female chickens without dietary vitamin E or other antioxidants. J. Nutrition, *70:* 47.
20. Pollard, C. J., and J. G. Bieri 1959 Studies on the biological function of vitamin E. I. Tocopherol and reduced diphosphopyridine nucleotide-cytochrome c reductase. Biochim. Biophys. Acta, *34:* 420.
21. Corwin, L. M., and K. Schwarz 1959 An effect of vitamin E on the regulation of succinate oxidation in rat liver mitochondria. J. Biol. Chem., *234:* 191.
22. Allen, J. R., B. A. Sullivan and H. L. Dobson 1960 Cytochrome oxidase and reductase in muscle from vitamin E-deficient rabbits. Arch. Biochem. Biophys., *86:* 6.
23. Horwitt, M. K., C. C. Harvey, B. Century and L. A. Witting 1961 Polyunsaturated lipids and tocopherol requirements. J. Am. Dietet. A., *38:* 231.

Effect of Essential Fatty Acid Deficiency and Dietary Cholesterol on Liver Ubiquinone Levels in the Rat[1]

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ABSTRACT The influence of feeding a fat-free diet, and of the administration of cholesterol, on the metabolism of ubiquinone in rat liver was investigated. It was observed that in advanced essential fatty acid (EFA) deficiency the concentration of ubiquinone was increased about twofold. Mevalonate-2-C^{14} incorporation experiments showed that the specific activity of liver ubiquinone was significantly reduced in EFA deficiency, but that, because of the increased amounts present, the total incorporation was similar to that in normal control animals. The biological half-life of ubiquinone in the liver of the deficient animals was approximately doubled. Administration of cholesterol in the diet resulted in a marked depression in ubiquinone concentration.

The concentration of ubiquinone in the tissues has been shown to be increased by a number of factors, including vitamin A deficiency (1), dinitrophenol (2, 3) and thyroxine (2–4). These increases occur even though the normal concentration of this substance in mitochondria is in marked molar excess of the concentrations of other components of the electron transport chain (5). Other dietary factors, such as the type of carbohydrate (6), also exert an influence on ubiquinone levels, as do additional nutritional factors of undetermined identity.

In connection with a study of dietary factors that affect tissue levels of ubiquinone, it was observed that a sharp increase in the concentration of the quinone occurs in the liver of rats acutely deficient in essential fatty acids (EFA) and that a depression ensues following the oral administration of cholesterol. The results of these experiments are reported here.

EXPERIMENTAL

Four experiments were conducted using weanling rats of the Sprague-Dawley strain. Except where otherwise indicated, they were fed a fat-free diet of the following composition: (in per cent) glucose, 71.4; casein,[2] 20.0; mineral mix (7), 4.0; glycerol, 4.0; vitamin mix in glucose (8), 0.5; and choline chloride, 0.1. Glycerol was included to improve the texture of the fat-free diet. In the control diets one-half of the glycerol was replaced with an EFA supplement consisting of 75% methyl linoleate (the remainder of which was primarily methyl oleate).[3] The diets were also supplemented with 100 IU of vitamin A, 5 IU of vitamin D and 0.5 mg of *dl*-α-tocopheryl succinate/g. The body weights of the rats were recorded weekly, and in one experiment feed consumption and efficiency of utilization were calculated.

In experiment 1, two groups of weanling female rats were maintained with either the fat-free diet or the basal formula supplemented with 2% methyl linoleate. After an 8-week experimental period the deficient rats averaged 20 g less in body weight than the controls and showed the characteristic dermatitis associated with an EFA deficiency. Experiment 2 was identical with experiment 1 except that the animals continued to be fed the test diets for an additional 12 weeks, during which time a moderate progression of symptoms occurred.

In experiment 3 a comparison was made of the ubiquinone content of the liver of weanling males fed the EFA-deficient or control diets. In this experiment the basal diet was modified slightly by replacing 2% of the glucose with tristearin and reduc-

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[2] Labco, The Borden Company, New York.

[3] Nutritional Biochemicals Corporation, Cleveland.

ing the vitamin A content from 100 to 50 IU/g of diet. Tristearin was included so that both the EFA-deficient and control groups would receive comparable amounts of fat. The experiment was terminated after 16 weeks, at which time the rats exhibited severe symptoms of EFA deficiency.

As the results of the above experiments indicated that an increase in ubiquinone content of the liver occurred in acute EFA deficiency, the influence of a mild deficiency was investigated in experiment 4. Two groups of weanling male rats were maintained with the deficient or the EFA-supplemented diet for 8 weeks, at which time the deficient animals showed the early symptoms of dermatitis on the feet and tail, as well as a slight depression in body weight.

To obtain additional information on the influence of EFA deficiency on ubiquinone metabolism, the incorporation of C^{14}-labeled mevalonic acid into this compound, as well as into cholesterol, was compared in deficient and control animals. In the first comparison (exp. 5) 2 severely deficient male rats and 2 normal control animals were given an intraperitoneal injection of 5.76×10^4 dpm of DL-mevalonate-2-C^{14}/g of body weight (specific activity 2.25 mc/mmole).[4] The animals were killed one hour after injection, the livers were removed, and ubiquinone and cholesterol determinations were made immediately. In the second comparison (exp. 6), a similar dose was administered to 2 mildly deficient males, 2 normal controls and 2 animals which had received the basal diet supplemented with 3% methyl linoleate concentrate and 3% cholesterol.

An attempt was made to accentuate the symptoms of EFA deficiency by adding cholesterol to the diet (9). Four groups of weanling male rats (exp. 7) were maintained for 8 weeks with the basal diet plus 3% cholesterol; 2 groups received, in addition, 6% methyl linoleate concentrate to replace the glycerol and 2% of glucose. A further short-term test (exp. 8) was made of the effect of cholesterol on the ubiquinone content of liver of rats fed a complete diet. In this instance both groups were fed the basal diet supplemented with 6% methyl linoleate concentrate (to replace glucose) for 7 days, and one was also given 5% cholesterol. High levels of cholesterol were used because of its inefficient absorption from a fat-free diet, and in order to determine the effect of saturating the tissues with cholesterol on the level of ubiquinone in the liver.

At the termination of each experiment the animals were killed and, with the exception of the mevalonate incorporation studies, the livers were stored at − 15° until assayed for ubiquinone. The method for ubiquinone determination has been described in detail previously (10). It consisted, in brief, of saponifying the liver in methanolic KOH in the presence of pyrogallol, then extracting with ether and chromatographing the ether residue on a silicic acid-Celite (2:1) column. The column was developed with light petroleum ether containing increasing amounts of diethyl ether. The fraction containing ubiquinone was dried under nitrogen, taken up in ethanol, and assayed by determining the change in optical density at 275 mμ upon reduction with $NaBH_4$. Although rat tissues contain predominantly ubiquinone-45, for comparative purposes a $\Delta E^{1\%}_{1cm}$ value of 142 for ubiquinone-50 was used in the calculations. This figure gives a value that is approximately 110% of that obtained when the data are calculated on the basis of ubiquinone-45 ($\Delta E^{1\%}_{1cm} = 156$).

To obtain the specific activity of labeled ubiquinone, carrier ubiquinone-50 was added to the crude fraction from the silicic acid-Celite column after determining the amount of ubiquinone originally present. The residue plus the carrier ubiquinone then were chromatographed on a Bio-Rad[5] neutral alumina column (deactivated with 6% water) to remove labeled sterol contaminants. The partially purified mixture of ubiquinone-45 and ubiquinone-50 was crystallized from ethanol-acetone-water (50:50:1) three times and then from ethanol until the specific activity was constant. The ubiquinone was estimated by the $NaBH_4$ reduction assay, and the radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. Before counting, the samples were taken up in

[4] New England Nuclear Corporation, Boston.
[5] Bio-Rad Laboratories, Richmond, California.

toluene containing 0.3% 2,5-diphenyl oxazole (PPO).

The crude sterol fraction from the silicic acid-Celite column was quantitatively assayed for cholesterol using the method of Nieft and Deuel (11). Carrier cholesterol was added and the sample was purified by the bromination-debromination procedure described by Schwenk and Werthessen (12). The purified cholesterol then was recrystallized from methanol at — 15° until a constant specific activity was observed.

The fact that a vitamin A deficiency is known to produce large increases in rat liver ubiquinone levels (1) prompted a study of the vitamin A content of livers from EFA-deficient rats. The determinations were made on livers from the severely deficient and control animals of experiment 3 by a modification of the method described by Hawk et al. (13).

The statistical significance of differences found between treatments was determined by applying an analysis of variance.

RESULTS

As observed by other workers, the effect of feeding the EFA-deficient diet was less pronounced in the female rats. After they had received the test diets for 8 weeks the average body weight of the deficient females was 94% of that of the controls, whereas that of the males was only 80%. After 16 weeks the body weights of the deficient groups were 82 and 76% of those of the controls for the females and males, respectively. Dermatitis of the feet and tail appeared in both sexes at about the eighth week, which in the males (but not appreciably in the females) became more severe as the experiment progressed. The excretion of blood in the urine of three of the deficient males during the fifteenth and sixteenth weeks indicated severe kidney damage.

Despite the differences in weight gain between deficient and control animals, total food consumption was observed to be about the same. The deficient rats of experiment 3 consumed an average of 2.07 kg/rat and the controls, 1.98 kg/rat. The deficient animals required 4.6 g of diet /g gain during the first 4-week period and 34.9 g during the last 3 weeks for an over-all efficiency of 8.2, whereas the controls required 3.9 and 16.5 g of diet/g gain for the first 4 weeks and last 3 weeks, respectively, for an over-all feed efficiency of 6.4.

The results presented in table 1 show significantly higher liver ubiquinone levels in the essential fatty acid-deficient females and severely deficient males. Whereas the increase was readily discernible in the females after 8 weeks, it was not evident in

TABLE 1

Liver ubiquinone levels of normal and essential fatty acid-deficient rats

Group no.	Treatment	No. rats	Sex	Body wt	Liver wt	Liver ubiquinone	
						Avg	Range
				g	g	μg/g	μg/g
			Experiment 1				
1	Without EFA	5	F	195	6.30	122*	85–149
2	With EFA	5	F	203	5.46	74	55–106
			Experiment 2				
1	Without EFA	5	F	235	7.63	141**	108–177
2	With EFA	6	F	267	6.49	73	54–99
			Experiment 3				
1	Without EFA	7	M	315	10.76	89**	70–98
2	With EFA	6	M	420	12.16	45	29–68
			Experiment 4 [1]				
1	Without EFA	5	M	279	12.19	65	58–69
2	With EFA	4	M	351	13.31	60	57–68

* Significantly different from control values ($P < 0.05$).
** Significantly different from control values ($P < 0.02$).
[1] Rats exhibited only mild deficiency symptoms.

the males until the deficiency had progressed to a more acute stage (exp. 3). The apparent implication that the female is more susceptible to a derangement in ubiquinone metabolism contrasts with the fact that the skin lesions are generally less severe than in the male, and requires further investigation.

Table 2 shows that the concentration of ubiquinone in the liver and the extent of labeling from mevalonate-2-C^{14} were similar for mildly deficient males and control animals (exp. 6). The deficient animals used in this experiment had been maintained with the basal diet for 8 weeks and exhibited only mild symptoms of dermatitis. The absence of elevated levels of ubiquinone in these rats is in agreement with similar results in experiment 4 (table 1). Nevertheless, there was a somewhat lowered incorporation of C^{14} into cholesterol in the mildly deficient rats, an effect which was clearly evident in the severely deficient animals (exp. 5). In the latter the incorporation of mevalonate-2-C^{14} into cholesterol was less than one-half that in the controls. Although the ubiquinone concentration in the liver of the acutely deficient animals was increased about twofold, the specific activity was about one-half that of the control rats. Consequently the values for the total incorporation of mevalonate into ubiquinone were similar for the deficient and normal animals.

The effect of cholesterol feeding on the incorporation of mevalonate-2-C^{14} into ubiquinone and cholesterol is also shown in table 2. As might be expected, the rate of labeling of cholesterol was greatly reduced in the cholesterol-fed animals, whereas the data for ubiquinone show a fourfold increase. The suggested depression of ubiquinone concentrations in the liver of the cholesterol-fed rats was confirmed by the results of experiment 8 (table 3), in which the inclusion of cholesterol in a complete diet for 7 days brought about a significant decrease in ubiquinone content.

No difference was noted in the liver vitamin A content of the EFA-deficient and control rats. The average concentration in the livers of the deficient and control animals of experiment 3, respectively, was 645 and 606 μg/g of fresh tissue. The

TABLE 2

Incorporation of mevalonate-2-C^{14} into ubiquinone and cholesterol by normal, cholesterol-fed, and essential fatty acid-deficient rats

Diet	Rat wt	Liver wt	Ubiquinone			Cholesterol		
	g	*g*	*μg/g liver*	*dpm/mg*	*Total dpm*	*mg/g liver*	*dpm/mg*	*Total dpm* ($\times 10^{-3}$)
			Experiment 5					
Without EFA	310 [1]	10.12	128.9	4335	5660	1.43	17,750	257.4
Without EFA	322 [1]	12.73	112.2	4420	6320	1.19	17,700	269.0
With EFA	420	11.59	68.0	9770	7710	1.49	40,500	692.5
With EFA	411	11.93	56.7	8410	5215	1.43	41,600	728.9
			Experiment 6 [2]					
Without EFA	325 [3]	13.60	68.8	1500	1410	1.55	16,150	338.7
Without EFA	240 [3]	9.78	61.9	2890	1700	2.46	13,400	321.6
With EFA + cholesterol	390	16.96	44.4	9050	6800	4.30	735	54.0
With EFA + cholesterol	390	17.58	45.3	6250	4970	3.94	1,800	124.0
With EFA	415	15.55	60.6	1310	1230	1.80	21,000	588.0
Wtih EFA	320	11.05	58.6	1930	1250	2.71	18,600	558.0

[1] Severely deficient males.
[2] 5.76×10^4 dpm/g body weight administered in experiment 5; 7.58×10^4 dpm/g body weight in experiment 6.
[3] Mildly deficient males.

TABLE 3

Effect on ubiquinone levels in the liver of adding cholesterol to an essential fatty acid-deficient or complete diet

Group no.	Treatment	No. rats	Body wt	Liver wt	Liver ubiquinone	
					Avg	Range
			g	*g*	*µg/g*	*µg/g*
	Experiment 7 (8 weeks)					
1	Without EFA + 3% cholesterol	6	248	10.56	51	38–72
2	With EFA + 3% cholesterol	5	352	14.98	42	21–64
3	Without EFA + 3% cholesterol	4	301	11.62	69**	65–84
4	With EFA + 3% cholesterol	4	354	14.31	41	35–45
	Experiment 8 (7 days)					
1	With EFA + 5% cholesterol	5	91	3.70	57*	31–85
2	With EFA, without cholesterol	5	97	3.97	98	73–121

* Significantly different from control value ($P < 0.05$).
** Significantly different from control value ($P < 0.02$).

increase in ubiquinone content of the liver in EFA deficiency, therefore, is apparently unrelated to the similar effect of vitamin A deficiency.

During the chromatography of the nonsaponifiable fractions from rat liver, a yellow band was observed that moved off the silicic acid-Celite column in the first light petroleum ether fraction. An ultraviolet absorption spectrum of this material revealed a strong absorption band at approximately 370 mµ with 2 smaller peaks at 350 and 390 mµ. Under certain conditions vitamin A forms cyclization products having a similar absorption spectrum.[6] As the test rats were receiving relatively high levels of vitamin A in their diets, the possibility was explored that the yellow material was an artifact of vitamin A formed during the procedure used for ubiquinone determination. Nine grams of pig liver were saponified in the usual manner after the addition of 7 mg of vitamin A acetate. During chromatography a marked increase was observed in a yellow material which moved off the column in the same fraction as the band from rat liver. The ultraviolet absorption spectra of the cyclized product and the yellow material from rat liver are shown in figure 1. This material was detected in rat, rabbit and pig liver, and, although no quantitative evaluation was attempted, the amount present appeared to vary directly with the vitamin A intake of the animal. The compound was not observed when vitamin A alcohol or its acetate ester was subjected to chromatography without prior saponification. This observation indicates that during the saponification step in the procedure used for ubiquinone determination vitamin A is partially converted to a cyclic form. Thin-plate chromatography indicated that more than one compound was present in the yellow material derived from both vitamin A acetate and liver. This result with respect to vitamin A is similar to the observation that refluxing ubiquinone in ethanolic KOH results in cyclization of its polyisoprenoid side chain to form ubichromenol and other isomers (14).

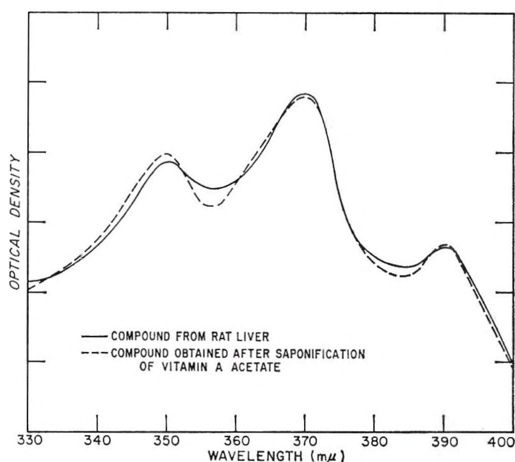

Fig. 1 Ultraviolet absorption spectra of a compound from rat liver and of vitamin A acetate following treatment with alkali.

[6] Rogers, W. E., personal communication.

DISCUSSION

The decrease in efficiency of utilization of dietary energy by the EFA-deficient rats, which occurred in the absence of a depression in food intake, is in accord with the observations of previous workers. Loeb and Burr (15) reported an increased metabolic rate and a decrease in efficiency of food utilization in rats receiving a fat-free diet. An indication of the changes in cell function which bring about these effects is given by the work of Klein and Johnson (16) and Levin et al. (17), who reported a decreased phosphorus uptake and an increased oxygen consumption by liver mitochondrial preparations from fatty acid-deficient rats. Smith and DeLuca (18) have reported an increased rate of oxidation of Krebs cycle intermediates by liver homogenates from deficient rats, and Hayashida and Portman (19) observed greater succinic dehydrogenase activity. In view of the apparent role of ubiquinone in electron transport, the increased levels of this compound noted in the tissues of EFA-deficient animals might be regarded as a reflection of the increased metabolic rate. However, the results indicate that the increase in liver ubiquinone levels does not occur until the more advanced stages of the deficiency, whereas the decreased efficiency of feed utilization and increased oxygen consumption are evident at a relatively early stage (20). The enhancement of oxygen consumption induced by thyroxine is also accompanied by an increase in ubiquinone levels (2–4).

The incorporation studies on mildly deficient male rats support the observation that ubiquinone metabolism is unaltered in the early stages of an EFA deficiency. However, a somewhat lowered incorporation of mevalonate-2-C^{14} into cholesterol was observed in these rats which was accentuated in the severely deficient animals. These observations agree with those of Avigan and Steinberg (21), who observed that the synthesis of cholesterol from acetate-1-C^{14} by rats was greatly increased when linoleic acid was fed. Mukherjee and Alfin-Slater (22) also reported that liver slices from EFA-deficient rats exhibited a depressed ability to synthesize cholesterol from acetate-1-C^{14}. It was postulated by these authors that the linoleic acid ester of cholesterol is important in the transportation and degradation of cholesterol in the body.

The incorporation of mevalonate-2-C^{14} into ubiquinone in the severely deficient rats indicates that there was no significant change in the amount of ubiquinone synthesized during the incorporation period. Although the specific activity of the ubiquinone obtained from the deficient animals was only one-half that of the controls, the total radioactivity incorporated was essentially the same. If it is assumed that all the ubiquinone present constituted one homogeneous pool, its lower specific activity in the deficient animals reflects a twofold increase in biological half-life. The accumulation of ubiquinone in EFA deficiency suggests some role of unsaturated fatty acids in the catabolism of this compound.

As anticipated, the incorporation of mevalonate-2-C^{14} into cholesterol was greatly decreased in the cholesterol-fed animals. The data shown in table 2 indicate that cholesterol feeding in experiment 6 resulted in a doubling of its concentration in the liver. The fourfold increase in incorporation of mevalonic acid into ubiquinone may be regarded as a reflection of the enhanced specific activity of the mevalonate pool in the cholesterol-fed animals. However, this does not explain the lowered ubiquinone levels. One feasible explanation is that in the presence of high concentrations of cholesterol a feed-back control mechanism is stimulated which results in either a reduced synthesis of mevalonate or in its decreased utilization for the synthesis of both ubiquinone and cholesterol. It is well known that the biosynthesis of cholesterol is suppressed when an exogenous source is supplied. A decreased rate of biosynthesis of ubiquinone also may have prevailed in the cholesterol-fed rats which was obscured by the greater incorporation of isotope that probably resulted from an increased specific activity of the mevalonate pool.

As in a previous study (10), a wide range of ubiquinone levels was observed in the livers of animals subjected to the same treatment. The results of this study further illustrate the multiplicity of factors which influence the concentration of ubi-

quinone in animal tissues, especially the liver, and emphasize the difficulty of establishing normal ranges for individual organs.

LITERATURE CITED

1. Lowe, J. S., R. A. Morton and R. G. Harrison 1953 Aspects of vitamin A deficiency in rats. Nature, *172:* 716.
2. Beyer, R. E., W. M. Noble and T. J. Hirschfeld 1962 Alterations of rat-tissue coenzyme Q (ubiquinone) levels by various treatments. Biochim. Biophys. Acta, *57:* 376.
3. Edwin, E. E., J. Green, A. T. Diplock and J. Bunyan 1960 Effect of thyroxine and 2,4-dinitrophenol on levels of ubiquinone and ubichromenol in the rat. Nature, *186:* 725.
4. Aiyar, A. S., and A. Sreenivasan 1962 Content and intracellular distribution of ubiquinone in the rat in experimental thyrotoxicosis. Biochem. J., *82:* 182.
5. Redfearn, E. R. 1960 The possible role of ubiquinone (coenzyme Q) in the respiratory chain. In Ciba Foundation Symposium on Quinones in Electron Transport, eds., G. E. W. Wolstenholme and C. M. O'Connor. Little, Brown and Company, Boston, p. 347.
6. Wiss, O., and G. Brubacher 1961 Zur Beeinflussing des Ubichinongehaltes der Leber waschsender Ratten durch verschiedene Diätformen. J. Biochem. (Tokyo), *49:* 609.
7. Mameesh, M. S., and B. C. Johnson 1958 The effect of penicillin on the intestinal synthesis of thiamine in the rat. J. Nutrition, *65:* 161.
8. Draper, H. H., S. Goodyear, K. D. Barbee and B. C. Johnson 1958 A study of the nutritional role of anti-oxidants in the diet of the rat. Brit. J. Nutrition, *12:* 89.
9. Peifer, J. J., and R. T. Holman 1955 Essential fatty acids, diabetes, and cholesterol. Arch. Biochem. Biophys., *57:* 520.
10. Lee, D. J., M. Chiu and H. H. Draper 1964 The effect of vitamin E deficiency on ubiquinone levels in rat and rabbit liver. Nature, in press.
11. Nieft, M. L., and H. J. Deuel, Jr. 1949 Studies on cholesterol esterases. I. Enzyme systems in rat tissues. J. Biol. Chem., *177:* 143.
12. Schwenk, E., and N. T. Werthessen 1952 Studies on the biosynthesis of cholesterol. III. Purification of C^{14}-cholesterol from perfusions of livers and other organs. Arch. Biochem. Biophys., *40:* 334.
13. Hawk, P. B., B. L. Oser and W. H. Summerson 1954 Practical Physiological Chemistry, ed. 13. The Blakiston Division, McGraw-Hill Book Company, Philadelphia, p. 1121.
14. Draper, H. H., and A. S. Csallany 1960 On the natural occurrence of ubichromenol. Biochem. Biophys. Res. Comm., *2:* 307.
15. Loeb, H. G., and G. O. Burr 1947 A study of sex differences in the composition of rats, with emphasis on the lipid component. Sex differences in susceptibility to essential fatty acid deficiency with high and low fat diets. J. Nutrition, *33:* 541.
16. Klein, P. D., and R. M. Johnson 1954 Phosphorus metabolism in unsaturated fatty acid-deficient rats. J. Biol. Chem., *211:* 103.
17. Levin, E., R. M. Johnson and S. Albert 1957 Mitochondrial changes associated with essential fatty acid deficiency in rats. J. Biol. Chem., *228:* 15.
18. Smith, J., and H. F. DeLuca 1963 Essential fatty acid deficiency and rat liver homogenate oxidations. J. Nutrition, *79:* 416.
19. Hayashida, T., and O. W. Portman 1963 Changes in succinic dehydrogenase activity and fatty acid composition of rat liver mitochondria in essential fatty acid deficiency. J. Nutrition, *81:* 103.
20. Panos, T. C., J. C. Finerty, G. F. Klein and R. L. Wall 1958 Metabolic studies in fat deficiency. In Essential Fatty Acids, ed., H. M. Sinclair. Academic Press, Inc., New York, p. 205.
21. Avigan, J., and D. Steinberg 1958 Effects of saturated and unsaturated fat on cholesterol metabolism in the rat. Proc. Soc. Exp. Biol. Med., *97:* 814.
22. Mukherjee, S., and R. B. Alfin-Slater 1958 The effect of the nature of dietary fat on synthesis of cholesterol from acetate-1-C^{14} in rat liver slices. Arch. Biochem. Biophys., *73:* 359.

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