Effect of Propionate on the Induction of Vitamin B_{12} Deficiency in Chicks and Rats [1]

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ABSTRACT A study was made to devise dietary conditions for the induction of simple vitamin B_{12} deficiency in experimental animals without the delay and complications involved in methods routinely used. Chicks maintained with a purified vitamin B_{12}-deficient diet showed a marked decrease in liver methylmalonyl-CoA mutase activity. Because of the role of vitamin B_{12} co-enzyme in the metabolism of propionate, branched-chain fatty acids, lysine and isoleucine, a study was carried out to examine the growth of chicks with diets containing these metabolites. The compounds tested included formate, lysine, propionate, butyrate and isoleucine. Vitamin B_{12} deficiency in terms of growth depression was markedly accentuated by the presence of sodium formate and sodium propionate at 5% levels. None of the other additions had a similar effect when incorporated in the vitamin B_{12}-deficient rations. In rats, vitamin B_{12} prevented growth depression caused by 2% sodium propionate. In addition, propionate markedly reduced liver methylmalonyl-CoA mutase activity both in the presence and absence of the vitamin in the diet.

Although the role of vitamin B_{12} in a wide variety of metabolic pathways in both animal and microbial systems has been reported, all the sites of function have probably not been discovered. The pivotal role of vitamin B_{12} in propionate metabolism has, however, been well-established as a result of several reports (1–5). Using the rat liver in vitro system, Gurnani et al. (3) have shown the requirement for vitamin B_{12} coenzyme in the enzymatic conversion of methylmalonyl-CoA to succinyl-CoA. Hartman and Dryden (6) have reported that dietary propionate intensified weight loss in vitamin B_{12}-deficient rats. In light of these observations and because of the significance of the methylmalonyl-CoA mutase reaction in the metabolism of propionate, branched-chain fatty acids, and isoleucine (7), and the report of a vitamin B_{12} coenzyme function in lysine metabolism in microorganisms (8), we have examined the effect of these compounds on the growth of chicks and the effect of propionate on the growth of rats in relation to their vitamin B_{12} status. The difficulties involved in inducing vitamin B_{12} deficiency in experimental animals have warranted the determination of a fast and uncomplicated method of achieving this goal; thus, the effect of various metabolites in enhancing the vitamin B_{12} deficiency condition in laboratory animals was studied.

EXPERIMENTAL PROCEDURE

Chicks hatched from eggs from hens maintained with a vitamin B_{12}-deficient diet were used (4). The diet used for the hens was based on the diet of Lillie et al. (9) and consisted of: (in %) yellow corn, 55.47; soybean oil meal, 30; alfalfa meal, 5; ground limestone, 5.5; dicalcium phosphate, 3.5; manganous sulfate, 0.03; iodized salt, 0.5; vitamin A and D oil (2000 USP units of vitamin A and 400 USP units of vitamin D), 0.2; and nicotinic acid, 1 mg /100 g diet.[4] The basal ration for the chicks was prepared according to Kokatnur et al. (10), omitting vitamin B_{12} and *p*-aminobenzoic acid and including vitamin E; it consisted of: (g/100 g basal diet) glucose, 68.18; 35.3 g isolated soybean protein; DL-

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methionine, 0.75; glycine, 0.3; choline chloride, 0.2; calcium carbonate, 2.166; potassium phosphate (monobasic), 1.05; calcium phosphate (dibasic), 0.94; sodium chloride, 0.8; magnesium sulfate, 0.25; ferrous sulfate, 0.03; manganous sulfate, 0.02; zinc carbonate, 0.01; cupric sulfate, 0.002; potassium iodide, 0.001; sodium molybdate, 0.001; (in milligrams) thiamine·HCl, 10; niacin, 10; riboflavin, 1.6; calcium pantothenate, 2.0; pyridoxine·HCl, 0.6; biotin, 0.006; folic acid, 0.4; α-tocopheryl acetate, 2.0; menadione, 0.5; and vitamin A acetate, 1000 IU and vitamin D_3, 60 IU. The normal chicks were given 10 g of vitamin B_{12} intraperitoneally every week. The compounds tested included sodium formate, L-lysine, sodium propionate, sodium butyrate and L-isoleucine and were incorporated in the diet at the levels specified at the expense of the total diet. The chicks were weighed periodically and their weight gains over a period of 3 weeks were recorded.

Rats of the Sprague-Dawley strain, weighing 45 to 50 g, were maintained with a soy-lactose vitamin B_{12}-free diet patterned after that of Cuthbertson and Thornton (11) consisting of: (in %) full-fat soy flour, 72; lactose, 22; salts 446 (12), 3; choline chloride, 0.5; DL-methionine, 0.1; and vitamins (1000 g diet): (in milligrams) thiamine·HCl, 2.5; riboflavin, 1; calcium pantothenate, 4; nicotinic acid, 10; pyridoxine·HCl, 0.6; biotin, 0.06; folic acid, 0.4; menadione, 0.1; vitamin A, 2000 IU; vitamin D, 200 IU; and α-tocopheryl succinate, 0.5. The control animals received vitamin B_{12} supplementation at a level of 50 g/kg diet.

In other rat experiments a purified diet of the following composition was used: isolated soy protein,[5] 26.8; cornstarch, 30.0; sucrose, 30.0; salts 446, (12) 4; corn oil, 8.1; methionine, 0.1; sodium propionate, 10; plus vitamins as in the previous diet. Propionate was tested at a level of 1 and 2% of the diet and was incorporated as the sodium salt. The animals were killed at the end of 6 weeks and the methylmalonyl-CoA mutase activities of their livers were assayed by measuring the incorporation of $^{14}CH_3$-labeled methylmalonyl-CoA into the permanganate-stable reaction product, succinic acid (3). Protein was estimated by the biuret method (13). The $^{14}CH_3$-labeled methylmalonyl-CoA was prepared by the two-step process starting with ^{14}C-methylmalonic acid (14–16).

RESULTS AND DISCUSSION

Data on weight gain for 3 weeks of chicks maintained with different rations are presented in table 1. As the metabolites were not tested simultaneously, a separate control group for each was used. Of the compounds tested, sodium formate and sodium propionate at 5% levels intensified growth depression in deficient animals while having no effect on the vitamin-supplemented animals. The other compounds had either no effect or some effect probably unrelated to the vitamin. Sodium propionate at 2% of the diet showed a similar effect in the case of rats (table 2). These observations are in general agreement with those of Hartman and Dryden (6) and of Hogue ond Elliot (17). The latter workers had observed only a partial growth response to vitamin B_{12} when included in propionate-containing diets, whereas our data show a more complete reversal of the growth-depressing effect due to propionate by vitamin B_{12}. Stokstad et al. (17, 18) have recently reported no ap-

TABLE 1

Effect of certain metabolites on induction of vitamin B_{12} deficiency in the chick

Metabolite added to basal diet	Wt gain, 3 weeks	
	Without vitamin B_{12}	With vitamin B_{12}
	g	g
—	152.0 ± 13.00 [1]	219.0 ± 16.74
Formate, 1%	109.7 ± 12.40	172.4 ± 19.23
—	158.0 ± 10.75	212.0 ± 16.50
Formate, 5%	113.5 ± 8.63	210.0 ± 10.28
—	120.0 ± 16.28	184.0 ± 18.94
Lysine, 1%	125.0 ± 8.99	186.0 ± 14.00
—	79.0 ± 9.40	128.0 ± 16.17
Sodium propionate, 5% [2]	43.0 ± 3.60	122.0 ± 9.70
—	122.0 ± 8.43	238.8 ± 11.22
Butyrate, 1%	117.4 ± 10.25	179.9 ± 7.05
—	96.0 ± 9.09	169.0 ± 7.10
Isoleucine, 1%	95.0 ± 7.27	210.0 ± 10.20

[1] Averages ± SEM for not less than 6 chicks.
[2] 17-day growth.

[5] Alpha Protein, Nutritional Biochemicals Corporation, Cleveland.

preciable protection by vitamin B_{12} against growth depression caused by 2 or 4% calcium propionate. In addition, using formiminoglutamic acid (FIGlu) excretion as the criteria of vitamin B_{12} deficiency, these authors could find no evidence for an enhancement of vitamin B_{12} deficiency by dietary calcium propionate in the rat. On the contrary, FIGlu excretion was decreased in the presence of propionate in the diet. However, vitamin B_{12} influences FIGlu excretion only indirectly by altering folate metabolism (19). We, therefore, studied liver methylmalonyl-CoA mutase activity as a specific criteria of vitamin B_{12} deficiency. The results are shown in table 3. The mutase activity was found to be significantly reduced as a result of the deficiency condition at the end of 6 weeks. The inclusion of 2% sodium propionate in the diet markedly aggravated the severity of the deficiency, as shown by the mutase levels. However, the enzyme activity was still reduced by propionate even when the diet contained more than a normally adequate amount of vitamin B_{12}. Although the vitamin counteracted the growth-depressing effect of propionate almost completely, at the level used it only partially restored the mutase activity.

TABLE 2

Effect of propionate on induction of vitamin B_{12} deficiency in the rat

Diet	No. of rats	Weekly body wt gain for 5 weeks	Liver wt/ body wt
		g	
Series 1, soy flour lactose diet (11) [1]			
Without vitamin B_{12}	9	21.75 ± 3.38 [2]	0.043
Without vitamin B_{12} + propionate 2%	4	13.10 ± 3.45	0.045
With vitamin B_{12}	8	33.30 ± 1.91	0.040
With vitamin B_{12} + propionate 2%	5	34.30 ± 3.35	0.040
Series 2, soy protein, starch, sucrose, 1% propionate purified diet [3]			
Without vitamin B_{12}	22	11.7 ± 3.5	
With vitamin B_{12}	13	30.1 ± 2.4	

[1] Rats fed diet at mean weight of 43 g.
[2] Averages ± SEM.
[3] Rats fed diet at mean weight of 95 g.

TABLE 3

Effect of propionate (2%) on rat liver methylmalonyl-CoA mutase activity [1]

Diet	Methylmalonyl-CoA mutase activity
	mmoles/mg protein
Without vitamin B_{12}	45.62 ± 4.94 [2]
Without vitamin B_{12} + propionate, 2%	8.03 ± 4.33
With vitamin B_{12}	88.37 ± 5.30
With vitamin B_{12} + propionate, 2%	35.2 ± 5.83

[1] Mutase assay was carried out by incubating ^{14}C-methyl-labeled methylmalonyl-CoA (0.8 mole, sp. act. 2.15×10^4 cpm/mole) for 1 hour at 37° in a medium containing Tris-buffer, 0.05 M, pH 7.4; $MgCl_2$, 0.003 M; ATP, 0.003 M; glutathione, 0.005 M; and 0.2 ml of 10% liver homogenate in a total volume of 2 ml.
[2] Averages of 3 rats/group ± SEM.

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Assessment of Nutritional Status of Men: Protein

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ABSTRACT Eight healthy young men were used as experimental subjects in a study to learn whether a deficiency of tryptophan, lysine, or methionine affects the constituents of urine and blood plasma. A simple method by which an early deficiency of protein or of any essential amino acids might be detected was evaluated. Nitrogen balance generally became negative after deprivation of any of the essential amino acids. After a short-term deficiency, as in the present experiment, excretion of creatinine remained constant and the excretion of urea and 17-ketosteroids varied in a normal range. On day 8 of tryptophan deprivation the excretion of N-MNA decreased, and that of pyridone decreased in 2 subjects and increased in one subject. In the deprivation of lysine or methionine, there was no significant trend of change. The concentration of plasma amino nitrogen remained normal. The plasma aminogram did not show any specific pattern that could be related to a deficiency of protein or of an essential amino acid. However, the free amino acid ratio in plasma increased significantly during the deficiency period, and this might be of value in detecting subclinical levels of protein deficiency.

A number of methods have been introduced for estimating protein nutrition, but few of them are simple and useful for early detection. In our laboratory, these methods have been re-examined comparatively and efforts were concentrated on establishing a better procedure for use in the evaluation of protein nutritional status. In the present experiments, the effect of omission of an essential amino acid from a basal (control) diet on the state of protein nutrition was studied by following changes in the metabolic products in blood and urine.

EXPERIMENTAL PROCEDURE AND RESULTS

As shown in table 1, eight healthy young men who had been living in the same dormitory served as experimental subjects. The basal diet (tables 2 and 3) was fed throughout the experimental period of 12 days. After subjects had consumed the basal diet for 4 days, they were divided into 3 groups of three, three and two; one group of three was fed the tryptophan-deficient diet, maintaining the total nitrogen at a constant level by the substitution of isonitrogenous glycine; the other group of three was fed the lysine-deficient diet; and the group of two was fed the methionine-deficient diet. Nitrogen in the diet and urine was determined by the semimicro-Kjeldahl method. Other determinations were made according to the following methods: urinary creatinine, Peters (1); urea, Van Slyke and Archibald (2), N-methyl-2-pyridone-5-carboxamide (pyridone), Price (3) and Walters et al. (4); 17-ketosteroids by our modification of the Zimmermann reaction method; amino acid-nitrogen in the blood plasma was also measured by the ninhydrin method of Moore and Stein (5); and the free amino acid ratio in plasma by the method of Whitehead (6). In addition, a plasma aminogram was examined by the use of an automatic amino acid analyzer.

1. *Effect on the constituents of urine.* Following use of the basal diet for 4 days,

TABLE 1

Age, height, weight and energy intake of subjects

Subject	Age	Body height	Body wt	Avg daily energy intake [1]
	years	*cm*	*kg*	*kcal/kg*
K.Z.	19	174.7	61	32
N.T.	19	167.8	51	31
S.H.	19	162.8	55	40
I.D.	20	157.7	51	41
W.B.	19	173.8	57	32
H.Y.	19	160.0	50	41
H.D.	19	168.5	51	38
S.I.	19	159.7	61	45

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

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TABLE 2

Composition and daily intake of experimental basal diet

	Daily intake	Calories
	g	*kcal*
Cornstarch	210	696
Sucrose	110	422
Butter	42	303
Corn oil	180	1590
Salt mixture [1]	4.7	
Vitamin mixture [2]	1.0	
Baking powder	7.0	
Sodium chloride	13	
Cellulose powder (carboxymethyl cellulose)	12	
Candy	13	49
Total		3060

[1] In grams, $Ca(H_2PO_4)_2 \cdot H_2O$, 4.00 (including 2.86 derived from baking powder); $CaCO_3$, 1.00; $KHCO_3$, 1.30; $MgSO_4 \cdot 7H_2O$, 0.65; $FeC_6H_5O_7 \cdot 6H_2O$, 0.10; and (in milligrams) $MnSO_4 \cdot 4H_2O$, 3; $CuSO_4 \cdot 5H_2O$, 8; KI, 0.4; $ZnCl_2$, 5; $CoCl_2 \cdot 6H_2O$, 0.2; $(NH_4)_3PO_4$, 12; and $MoO_3 \cdot 6H_2O$, 0.2.

[2] Thiamine, 10.0 mg; riboflavin, 5.0 mg; vitamin B_6, 10.0 mg; vitamin B_{12}, 10.0 μg; niacin, 10.0 mg; folic acid, 0.5 mg; Ca D-pantothenate, 20.0 mg; vitamin C, 50.0 mg; vitamin K_1 diacetate, 0.2 mg; retinyl palmitate, 2500 IU; vitamin D_2, 200 IU; α-tocopherol, 3.0 mg.

TABLE 3

Composition and daily intake of amino acid mixture

	Daily intake	Nitrogen content
	g	*g*
Essential amino acids		
L-Isoleucine	1.40	0.150
L-Leucine	2.20	0.236
L-Lysine	1.60	0.306
L-Methionine	2.20	0.206
L-Phenylalanine	2.20	0.186
L-Threonine	1.00	0.118
L-Tryptophan	0.50	0.068
L-Valine	1.60	0.192
Nonessential amino acids		
L-Alanine	8.59	1.350
L-Arginine	10.06	3.236
L-Aspartic acid	10.01	1.054
L-Glutamic acid	10.02	0.954
L-Sodium glutamate	11.63	0.963
Glycine	7.11	1.321
L-Histidine	0.68	0.184
L-Proline	4.29	0.523
L-Serine	6.44	0.858
Total (essential + nonessential)		11.905

the tryptophan-deficient diet was given and the results are shown in table 4. All subjects maintained almost constant body weight throughout the experimental period. The balance between the nitrogen intake and the total urinary nitrogen on day 8 of

TABLE 4

Urinary excretion of total nitrogen, urea, creatinine and 17-ketosteroids in tryptophan-deficient diet

Subject	Diet	Period no.	Body wt	Daily N intake	Daily urinary N	Difference	Urea N	Creatinine N	Urea N/creatinine N	N-MNA	Pyridone	17-ketosteroids
			kg	*g*	*g*	*g*	*g*	*mg*		*mg*	*mg*	*mg*
K.Z.	Basal	1	61.0	12.08								
		4	62.0	12.08	11.75	0.33	8.96	706	127	9.4	9.3	5.63
	Deficient	1	61.5	12.08								
		8	59.0	12.08	12.98	−0.90	10.99	701	157	9.1	5.5	5.47
N.T.	Basal	1	51.0	12.07								
		4	51.5	12.07	9.60	2.47	7.73	571	135	7.3	5.7	6.93
	Deficient	1	52.0	12.07								
		8	52.0	12.07	12.50	−0.43	10.36	566	183	5.4	7.5	4.77
S.H.	Basal	1	55.0	12.09								
		4	56.0	12.09	11.40	0.69	8.61	716	120	5.4	8.4	11.49
	Deficient	1	56.0	12.09								
		8	55.0	12.09	13.19	−1.10	10.82	663	163	5.0	4.2	9.43

TABLE 5

Urinary excretion of total nitrogen, urea, creatinine and 17-ketosteroids in lysine-deficient diet

Subject	Diet	Period no.	Body wt	Daily N intake	Daily urinary N	Difference	Urea N	Creatinine N	Urea N/creatinine N	N-MNA	Pyridone	17-keto-steroids
			kg	*g*	*g*	*g*	*g*	*mg*		*mg*	*mg*	*mg*
I.D.	Basal	1	51.0	12.09								
		4	51.0	12.09	8.19	3.18	5.81	580	100	4.1	7.9	4.26
	Deficient	1	51.2	12.09								
		8	50.0	12.09	11.54	0.55	8.75	629	139	6.8	10.0	4.51
W.B.	Basal	1	57.0	12.08								
		4	57.0	12.08	11.56	0.52	9.10	653	139	10.9	12.4	5.68
	Deficient	1	57.0	12.08								
		8	57.0	12.08	14.06	−1.98	11.48	609	189	5.9	8.5	4.30
H.Y.	Basal	1	50.0	12.09								
		4	50.5	12.09	11.12	0.97	8.19	609	134	7.3	8.7	7.28
	Deficient	1	50.0	12.09								
		8	48.0	12.09	14.46	−2.37	12.22	580	211	11.5	6.6	7.55

deprivation was negative. When the nitrogen in the feces is taken into consideration, nitrogen balance should become more negative. The excretion of creatinine was maintained at a constant level throughout the experimental period. In the period of deficiency, the excretion of urea increased and that of 17-ketosteroids decreased. The excretion of pyridone decreased in 2 subjects, but increased in one subject. However, the excretion of N-MNA was not significant.

The results in the experiment on lysine deficiency were almost the same as those obtained in the above experiment with tryptophan (table 5). In one subject (I.D.), the difference between nitrogen intake and the total urinary nitrogen did not become negative, but, when the nitrogen in the feces is taken into consideration, the nitrogen balance probably would become negative. The excretion of N-MNA and pyridone did not show a definite trend in change; an increase in some cases, but a decrease in other ones. The excretion of 17-ketosteroids was rather constant.

Results obtained in the methionine deficiency were also the same as those in the previous 2 experiments (table 6).

2. *Effect on the constituents of blood plasma.* On day 4 of the basal diet period, and on days 4 and 8 of the deficiency period, blood was withdrawn from the anticubital vein of the subjects, who had been without food for 12 to 14 hours, and collected into a tube which had been rinsed with heparin solution. It was promptly centrifuged to separate the plasma, and amino acid nitrogen in the plasma and the aminogram were determined.

In tryptophan deficiency (table 7) the total amino acid level remained almost unchanged, and varied in a normal range. The free amino acid ratio in plasma, determined by the method of Whitehead,[1] increased considerably in tryptophan deficiency. However, in plasma aminograms determined for two of three subjects, withdrawal of tryptophan from the diet resulted in a reduction of plasma lysine, as judged by comparison with that during the basal diet period (table 8 and fig. 1).

[1] Whitehead (6) observed, using chromatographic methods, that the nonessential amino acids are reduced in concentration while the essential ones remain almost unchanged.

TABLE 6

Urinary excretion of total nitrogen, urea, creatinine and 17-ketosteroids in methionine-deficient diet

Subject	Diet	Period no.	Body wt	Daily N intake	Daily urinary N	Difference	Urea N	Creatinine N	Urea N/ creatinine N	N-MNA	Pyridone	17-keto-steroids
			kg	*g*	*g*	*g*	*g*	*mg*		*mg*	*mg*	*mg*
H.D.	Basal	1	51.0	12.08								
		4	53.0	12.08	11.17	0.91	7.28	580	128	11.7	14.1	4.42
	Deficient	1	53.0	12.08								
		8	49.0	12.08	12.80	−0.78	12.43	556	224	10.1	10.9	4.45
S.I.	Basal	1	61.0	12.09								
		4	61.5	12.09	9.72	2.37	6.16	725	85	7.3	7.9	6.13
	Deficient	1	61.5	12.09								
		8	61.0	12.09	14.41	−2.32	11.17	701	159	16.6	16.9	6.05

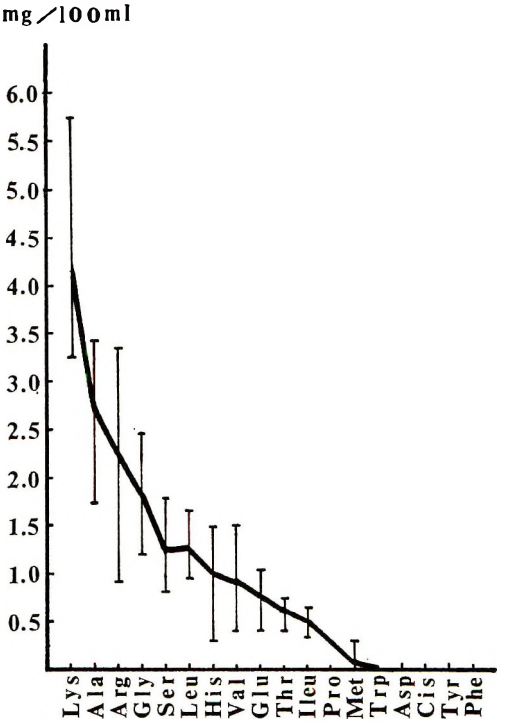

Fig. 1 Plasma amino acid pattern for 6 subjects, with maximal and minimal deviation, in basal diet including amino acid mixture as protein source.

Tryptophan was not detected in any of the aminograms even during the control diet period. In any case, the character of the patterns was not specific for tryptophan deficiency nor for the establishment of protein deficiency.

In the deprivation of lysine (table 8), plasma amino nitrogen did not show any definite change, but remained at a normal level. The free amino acid ratio in plasma increased markedly during the period of deficiency as in the tryptophan experiment. In plasma aminograms determined for two of three subjects, withdrawal of lysine from the diet resulted in an increase of valine, threonine, and glutamic acid, and a decrease in plasma lysine and arginine (table 8). However, the character of the pattern did not show the deficiency of lysine or protein with only one test.

With the withdrawal of methionine from the diet (table 8), the same result as in the experiment with tryptophan and lysine deficiency was obtained. Total amino

TABLE 7

Albumin N, amino acid N and free amino acid ratio in blood plasma

Subject	Diet	Period no.	Albumin N	Amino acid N	Free amino acid ratio
			mg/100 ml	*mg/100 ml*	
K.Z.	Basal diet	4	855	6.3	1.4
	Tryptophan-deficient	4	716	5.5	1.7
	Tryptophan-deficient	8	699	5.9	3.3
N.T.	Basal diet	4	846	6.3	2.0
	Tryptophan-deficient	4	651	5.9	1.6
	Tryptophan-deficient	8	868	5.5	2.9
S.H.	Basal diet	4	530	4.8	1.9
	Tryptophan-deficient	4	725	6.4	2.5
	Tryptophan-deficient	8	720	6.1	2.4
I.D.	Basal diet	4	833	5.7	1.6
	Lysine-deficient	4	734	6.4	2.0
	Lysine-deficient	8	964	6.0	3.1
W.B.	Basal diet	4	907	4.6	1.6
	Lysine-deficient	4	760	5.7	2.2
	Lysine-deficient	8	786	5.2	2.8
A.Y.	Basal diet	4	777	4.6	1.6
	Lysine-deficient	4	751	6.3	1.9
	Lysine-deficient	8	799	5.5	2.4
H.D.	Basal diet	4	820	4.7	2.0
	Methionine-deficient	4	777	6.8	1.8
	Methionine-deficient	8	846	5.7	3.1
S.L.	Basal diet	4	881	6.4	2.2
	Methionine-deficient	4	755	6.7	2.2
	Methionine-deficient	8	850	6.0	3.5

acid nitrogen in plasma remained at an almost normal level, but the free amino acid ratio of all subjects increased during the deficiency period. Plasma amino acid pattern showed an increase of threonine and a decrease of arginine and alanine (table 8).

DISCUSSION

In a deficiency of either tryptophan, lysine, or methionine, nitrogen balance became negative on day 8 of deprivation. It has been known for many years that kwashiorkor is associated with reduced excretion of urea nitrogen and a reduced ratio of urea and creatinine nitrogen, but our experimental results showed instead an increase in these values. There should be a substantial difference between results of the present study and those observed in kwashiorkor. In the latter, effects in children are due not only to the deficiency of both quality and quantity of protein, but also to deficiencies of calories and vitamins. On the contrary, in our study with adults, the subjects ate a diet which was adequate in all nutrients with the exception of one essential amino acid. Therefore, it may not be appropriate to compare directly the results of our study with those obtained in children with kwashiorkor. In our experiment, in which the total nitrogen intake remained at a constant level throughout the period, the negative nitrogen balance during the deficiency period was due to the decomposition of body protein, resulting in increased excretion of total nitrogen. Therefore, since urea nitrogen accounts for the majority of urinary nitrogen, and creatinine-nitrogen remained at a constant level throughout the experimental period, the ratio of urea and creatinine nitrogen should naturally increase. However, in a slight degree of protein deficiency as in our experiment, the urea nitrogen or the ratio of urea to creatinine may not be used as an index for protein nutrition. It has been reported that

TABLE 8

Plasma amino acids in basal diet and in tryptophan-, lysine-, and methionine-deficient diet

	Tryptophan				Lysine				Methionine			
	K.Z.		S.H.		H.Y.		W.N.		H.D.		S.I.	
	Basal	Deficient	Basal	Deficient	Basal	Deficient	Basal	Deficient	Basal	Deficient	Basal	Deficient
	mg/100 ml plasma				*mg/100 ml plasma*				*mg/100 ml plasma*			
Lysine	5.78	3.73	3.25	2.59	3.86	1.63	3.67	1.50	5.66	3.86	4.33	7.11
Histidine	1.51	1.33	0.30	0.42	0.72	0.66	1.33	0.78	1.14	0.78	0.96	1.50
Arginine	2.95	1.20	0.90	1.02	2.59	—	1.63	—	2.47	1.02	3.37	2.89
Threonine	0.48	0.72	0.66	0.78	0.42	0.96	0.48	0.72	0.78	0.96	0.60	1.20
Serine	0.96	4.34	6.57	1.57	0.84	1.20	1.81	0.87	0.84	0.96	1.33	0.96
Glutamine	1.08	0.48	0.66	—	0.42	1.02	0.66	0.72	0.90	—	0.84	1.27
Proline	—	—	1.08	—	—	—	—	—	—	—	0.48	—
Glycine	1.51	1.45	2.28	1.63	1.33	2.29	1.20	1.02	2.46	1.81	2.41	2.35
Alanine	1.75	1.45	3.43	2.47	2.77	2.53	1.75	1.27	2.89	1.45	3.37	2.05
Valine	1.33	—	1.08	1.08	0.48	1.75	0.66	0.90	1.51	1.14	0.36	1.14
Methionine	—	—	0.30	0.30	—	0.24	—	—	—	—	—	—
Isoleucine	0.60	—	0.66	0.54	0.54	0.48	0.36	0.54	0.54	0.30	0.36	0.36
Leucine	1.20	0.60	1.14	1.14	1.46	1.20	0.96	0.90	1.69	0.90	1.08	1.08

protein deficiency resulted in a reduced excretion of 17-ketosteriods. But in our experiment there was no significant change in its excretion, except for the small decrease during the tryptophan deficiency period. In a previous report (7), it was shown that the excretion of N-MNA and pyridone increased when any of the essential amino acids were excluded and that the nitrogen balance then became negative. In the present experiment, although the period of deprivation was longer, these constituents in urine were determined only on day 8 of the deprivation, and not day-by-day, and such a definite conclusion as that above cannot be drawn.

Plasma amino acid nitrogen may not be a good criterion for estimating a slight degree of protein deficiency, as in our experiment. Changes in plasma amino acid patterns in children with kwashiorkor have been reported, and Snydermann et al. (8) suggested the use of these in the diagnosis of protein deficiency. In our experiment, the character of the plasma aminogram was not specific enough to be used as a test for determining a slight degree of protein deficiency. However, it cannot be denied that an aminogram may be of value in detecting a serious deficiency such as in kwashiorkor. On the other hand, the free amino acid ratio in plasma increased significantly when the nitrogen balance became negative. Therefore, it is suggested that this test could be used for detecting subclinical levels of protein deficiency, although it would not indicate the deficiency of any one of the essential amino acids.

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Effect of Dietary Fat and Cholesterol on the in vitro Incorporation of Acetate-^{14}C into Hen Liver and Ovarian Lipids [1]

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ABSTRACT A study was made of the incorporation of acetate-1-^{14}C into the lipid fractions of liver slices and ovarian tissues from hens fed a basal diet and diets containing either safflower oil (30%), animal fat (30%), cholesterol (1%), or cholesterol (1%) and safflower oil (29%). When safflower oil was fed, acetate incorporation into total liver lipids decreased. The distribution of ^{14}C (ratio of ^{14}C in a particular fraction to the ^{14}C in total lipids) increased in the cholesterol and phospholipid fractions, decreased in triglycerides, and remained the same in diglycerides and cholesterol esters. When animal fat was fed, the distribution of ^{14}C increased in the cholesterol fraction and decreased in triglycerides, but total lipid synthesis was depressed further than it was when safflower oil was fed. When cholesterol was fed, acetate incorporation into liver cholesterol decreased, while incorporation into triglyceride increased slightly. When both safflower oil and cholesterol were included in the diet, synthetic control by cholesterol predominated. Regulation of the pathways of lipid biosynthesis from acetate was observed in ovarian tissue only when the diet containing both cholesterol and safflower oil was fed. The implications of these results on the synthesis of egg yolk lipids are discussed.

We reported previously that the cholesterol content of egg yolk increased when hens were fed diets containing various fats at a level of 30% (1, 2).

A number of investigators (3–14) have observed an increase in cholesterol synthesis from acetate in rat liver when various fats were fed. There is disagreement, however, on whether the nature of the dietary fatty acids affects cholesterogenesis. The decrease in lipogenesis, which occurs when dietary fat levels are increased (15), has been correlated by some workers with the increase in cholesterol synthesis. Increased cholesterol synthesis may represent an increase in an alternate pathway for acetate utilization (3, 13, 16) or may be related to the utilization of fat for energy (14).

The membranes (zona granulosa and theca interna) surrounding the growing ova in the hen are capable of synthesizing in vitro fatty acids and cholesterol from acetate (17, 18). In the present experiment, the effect of feeding diets containing either safflower oil or animal fat at a level of 30% on the pathways of lipid synthesis from acetate, was studied using hen liver slices and ovarian tissue. An attempt was made to determine whether there are similar mechanisms for the control of lipid synthesis occurring in the hen as there are in the rat. A possible explanation for the increase in egg cholesterol content, observed when a large amount of fat such as safflower oil was fed to hens, would be an increase in cholesterol synthesis in hen liver, ovarian tissue, or both. The egg cholesterol concentration was not increased to the same extent when animal fat was fed as when safflower oil was fed.

The effect of dietary cholesterol on the incorporation of acetate into the lipid fractions of liver and ovarian tissue was also studied. The cholesterol negative feedback

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system has been demonstrated in young (19) and adult (20) male chickens.

EXPERIMENTAL

White Leghorn hens were fed the following diets: a low fat control diet, a low fat diet containing cholesterol (1%), a high fat basal diet containing either safflower oil or animal fat at levels of 30%, a high fat diet containing safflower oil (29%) and cholesterol (1%). Fifty hens (10 per treatment) were fed each diet. Details on the composition of these diets and the sources of fats used have been published previously (2).

Birds in active egg production were killed by exsanguination and the livers and the membranes surrounding the larger growing ova were removed and placed in cold buffer. Liver slices (1 mm) were prepared with a Stadie-Riggs slicer. Aproximately 0.5 g of liver slices or 0.15 g of ovarian tissue strips were weighed in 50 ml erlenmeyer flasks containing 5 ml of Krebs-Henseleit bicarbonate buffer (21). Incubations of liver tissue were carried out in duplicate, but single incubations of ovarian tissue were used because of the small amount of ovarian tissue available from one hen. Tissues from hens fed the experimental diets were paired with tissues from hens fed a basal diet. Two or three experiments were performed for each experimental diet. The length of time the hens were fed the diet and the amount of isotope added are indicated in the tables.

After the flasks were equilibrated to 37°, one milliliter of Na acetate-1-^{14}C solution (4μCi) was added. The flasks were incubated in a Dubnoff metabolic shaker at 37° for 3 hours in a 5% CO_2/95% O_2 atmosphere. The reactions in the paired control flasks were stopped immediately after addition of the isotope by adding 20 ml of methanol containing 0.08 g sodium acetate. After adding the methanol solution and a porcelain chip at the end of the incubation, the flasks were heated at 60° for 10 minutes. Ten milliliters of chloroform were then added and the flasks heated again for 10 minutes. The contents were transferred to centrifuge tubes, the flasks rinsed with chloroform-methanol (2:1) and the washings were added to the centrifuge tube. After centrifugation, the residue was homogenized in a Potter-Elvehjem homogenizer with 2:1 chloroform-methanol (containing acetate, 0.4 g/100 ml methanol). Extraction of the residue was continued 3 times with ethanol-ether (3:1) at 50° for 10 minutes. The pooled extracts were evaporated in a rotary evaporator and the residue redissolved in 10 ml of petroleum ether (b.p. 35–65°). The petroleum ether solution was washed 3 times with a sodium acetate solution (200 mg/100 ml) and then 3 times with distilled water. After filtering through sodium sulfate, the solvent was evaporated and the residue dissolved in approximately 0.5 ml of chloroform. Acetone (15–20 ml) was added (with a sufficient quantity of carrier phospholipid added to the ovarian tissue extracts to induce precipitation) and the phospholipids were precipitated overnight at 4°. The acetone precipitation was repeated twice. The liver neutral lipids and phospholipids were finally dissolved in 25 ml of chloroform, and the ovarian neutral lipid and phospholipid fractions were dissolved in 5 ml.

A total of 150 to 300 μliters of lipid solution was spotted or streaked on a 20 × 5 cm thin-layer plate (30 g Silica Gel G [2] in 65 ml of 0.02% aqueous 2′,7′ dicholoroflourescein for 20 plates). The neutral lipid chromatograms were developed in 2 stages. Benzene-ethyl acetate (7:3) was used for the first stage, allowing the solvent front to advance 10 cm. The plates were air-dried to remove the solvent and development was continued in the same direction with hexane-benzene (7:3) for one hour beyond the time the solvent front reached a line 15 cm from the origin. The second solvent system separates the cholesterol esters from the triglycerides. The phospholipid fractions were chromatographed separately in the benzene-ethyl acetate system. The lipid fractions (fatty acids plus monoglycerides, cholesterol, diglycerides, triglycerides, cholesterol esters, phospholipids) were visualized under ultraviolet light and the areas containing lipid were scraped into polyethylene vials. Sixteen milliliters of scintillation mixture (200 ml dioxane, 16 g naphthalene, 1.0 g

[2] Brinkmann Instruments, Inc., Great Neck, New York.

2,5-diphenyloxazole,[3] 10 mg α-naphthylphenyloxazole,[4] 9.2 g Cap-O-Sil,[5] 30 ml methanol) were added to each vial and the radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer. The radioactivity in total lipid aliquots, which had been spotted on adsorbent, but not fractionated, was determined.

RESULTS AND DISCUSSION

As the female chicken approaches maturity, a change occurs in its lipid metabolism to meet the demands of increased lipid synthesis for egg production. The laying hen has a greater ability to incorporate acetate into liver lipids than the cockerel, and the synthesis of cholesterol appears to be preferentially stimulated in laying hens (22). The liver of the laying hen is capable of synthesizing greater quantities of triglycerides from palmitic acid than the liver of the immature bird (23). The effects of dietary factors on the lipid metabolism of various non-egg-laying experimental animals (including immature female birds) may differ from the effect of diet on the lipid metabolism of the laying hen, because of the metabolic differences necessitated by the egg-laying capacity of the hen. Egg-laying provides an excretory mechanism for the elimination of large amounts of dietary lipid, which are not a usual part of the hen's diet. Hypercholesterolemia, induced by feeding cholesterol in conjunction with agents that increase its absorption, results in increased levels of egg cholesterol (2). It has been observed often that egg yolk fatty acids reflect the fatty acids in dietary triglyceride (24).

Popjak and Tietz (17) demonstrated that ovarian tissue could synthesize cholesterol and fatty acids from acetate in vitro. The present experiment shows that the ovarian membranes are capable of synthesizing the major lipid classes that occur in the egg. The question of how much of the lipid deposited in the egg is synthesized in the liver and how much is contributed by ovarian synthesis remains, however. In the present experiment, acetate incorporation into lipids per weight of tissue was 2 to 4 times higher in liver slices than in ovarian tissue when hens were fed a basal diet (tables 1–3). The normal liver weight is approximately 400 times the weight of the membranes surrounding the growing ovum, but most of the lipid synthesized in the membranes would be expected to be deposited in the developing ovum. Whether the in vivo activity of the membranes is substantially different is not known. It has been proposed that egg cholesterol arises mainly from the plasma and ultimately originates in the liver. When labeled cholesterol was administered to hens, the specific activity of egg cholesterol was similar to that of plasma cholesterol (25, 26). The hypothesis that the liver is largely responsible for egg lipid synthesis is supported by the work of Schjeide (27), which indicates that lipoproteins, formed in the liver through estrogen induction, are transferred to the developing ova via the circulation.

Acetate incorporation data are presented in the figures of this paper as distribution of ^{14}C, which will be defined as the ratio of ^{14}C in any lipid fraction to the ^{14}C incorporated into total lipids $\times$ 100. The ratio of cholesterol-^{14}C to total lipid ^{14}C has been used to estimate cholesterol biosynthesis from acetate in rats fed various levels of fat and calories (10, 14). The use of this ratio eliminates much of the variation inherent in tracer studies using acetate-^{14}C, such as that related to pool size, degree of mixing of the label, and loss of isotope during administration during in vivo studies. Calculation of the distribution of incorporated acetate into the various lipid fractions (figs. 1–6) provides an indication of the pattern of lipid synthesis. Any changes in the pattern of lipid synthesis due to dietary factors would indicate regulation of the pathways of acetate metabolism. The pattern of acetate distribution in the various lipid fractions of hen liver and ovarian tissue was observed to be quite constant for a particular diet because individual variations in the absolute incorporation of acetate into lipids are eliminated. The average deviation of ^{14}C distribution is indicated in the figures for each lipid class.

[3] Packard Instrument Company, LaGrange, Illinois.
[4] See footnote 3.
[5] Cabot Corporation, Boston.

TABLE 1

Effect of a diet containing 30% safflower oil on the incorporation of acetate-1-^{14}C into liver and ovarian tissue lipids

Lipid class	Acetate incorporation [1]			
	Liver tissue		Ovarian tissue	
	Basal diet	Safflower oil diet	Basal diet	Safflower oil diet
	mμmoles/g tissue			
Monoglycerides + fatty acids	13.5	9.7	15.2	14.0
Cholesterol	10.3	14.7	33.1	35.9
Diglycerides	2.1	0.6	6.0	5.4
Triglycerides	110.0	18.7	5.6	7.4
Cholesterol esters	1.2	0.4	1.8	1.8
Phospholipids	14.4	9.1	3.7	4.2
Total	152.5	54.2	69.6	74.6

[1] Data are mean of 6 experiments (3 hens with duplicate samples) for liver tissue and mean of 3 experiments (3 hens with single sample) for ovarian tissue. Hens had been fed diets for 15 days or more before tissues were removed and incubated with 4 μCi (sp. act. 21 mCi/mmole) of acetate-1-^{14}C.

TABLE 2

Effect of a diet containing 30% animal fat on the incorporation of acetate-1-^{14}C into liver and ovarian tissue lipids

Lipid class	Acetate incorporation [1]			
	Liver tissue		Ovarian tissue	
	Basal	Animal	Basal	Animal
	mμmoles/g tissue			
Monoglycerides + fatty acids	28.5	5.3	18.0	25.4
Cholesterol	32.7	17.6	41.8	72.0
Diglycerides	2.8	0.7	9.3	14.6
Triglycerides	141.0	18.9	10.1	14.8
Cholesterol esters	0.7	0.2	2.9	3.8
Phospholipids	40.2	7.2	1.8	5.3
Total	247.5	50.1	87.0	141.0

[1] Data are mean of 4 experiments (2 hens with duplicate samples) for liver tissue and mean of 2 experiments (2 hens with single sample) for ovarian tissue. Hens had been fed diets for 15 days or more before tissues were removed and incubated with 4 μCi (sp. act. 5.3 mCi/mmole) of acetate-1-^{14}C.

TABLE 3

Effect of diets containing cholesterol (1%) or cholesterol (1%) and safflower oil (SO) (29%) on the incorporation of acetate-1-^{14}C into liver and ovarian tissue lipids

Lipid class	Acetate incorporation [1]					
	Liver tissue			Ovarian tissue		
	Basal diet	Cholesterol diet	Cholesterol + SO diet	Basal diet	Cholesterol diet	Cholesterol + SO diet
	mμmoles/g tissue			*mμmoles/g tissue*		
Monoglycerides + fatty acids	27.0	31.2	15.6	17.2	9.1	10.1
Cholesterol	25.5	2.6	0.2	33.0	16.3	8.1
Diglycerides	3.4	2.6	0.5	9.9	5.4	2.7
Triglycerides	153.0	199.5	52.2	8.1	4.1	8.9
Cholesterol esters	1.0	1.4	0.4	2.4	1.7	1.9
Phospholipids	35.9	31.9	25.2	4.9	2.0	4.3
Total	250.5	268.5	95.5	79.4	38.2	37.9

[1] Data are mean of 4 experiments (2 hens with duplicate samples) for liver tissue and mean of 2 experiments (2 hens with single sample) for ovarian tissue. Hens had been fed diets for 15 days or more before tissues were removed and incubated with 4 μCi (sp. act. 5.3 mCi/mmole) of acetate-1-^{14}C.

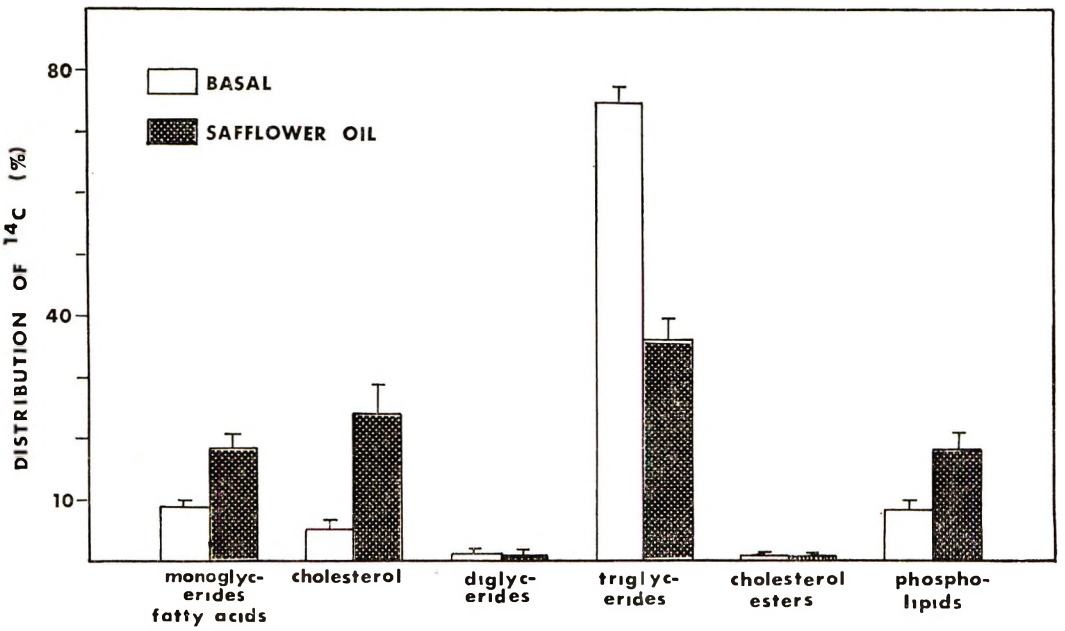

Fig. 1 Effect of a diet containing 30% safflower oil on the distribution of acetate-1-^{14}C in the lipid fractions of liver slices. Values are averages from experiments in which hens were fed the diet for 30, 130, and 150 days; average deviation is shown.

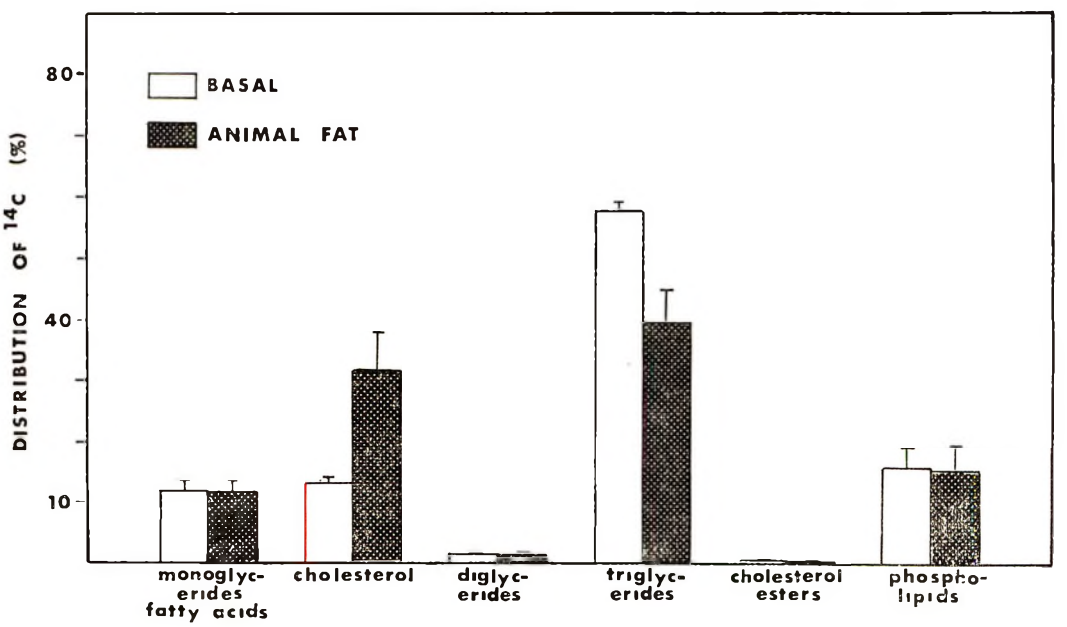

Fig. 2 Effect of a diet containing 30% animal fat on the distribution of acetate-1-^{14}C in the lipid fractions of liver slices. Values are averages from experiments in which hens were fed the diet for 17 and 30 days; average deviation is shown.

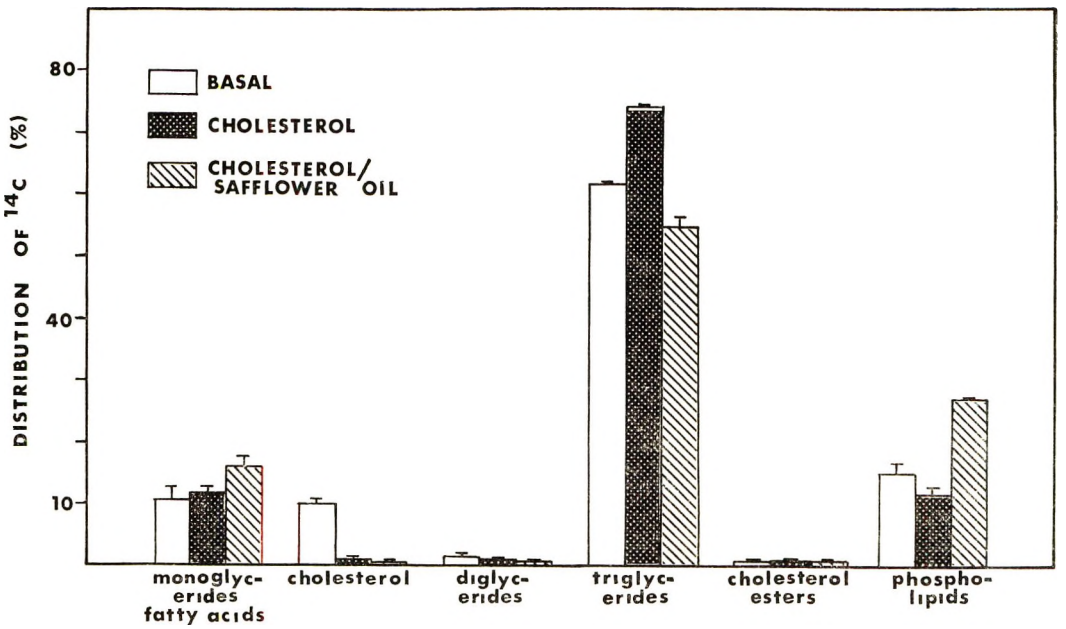

Fig. 3 Effect of diets containing cholesterol (1%) or cholesterol (1%) and safflower oil (29%) on the distribution of acetate-1-^{14}C in the lipid fractions of liver slices. Values are averages from experiments in which hens were fed the diets for 15 and 23 days; average deviation is shown.

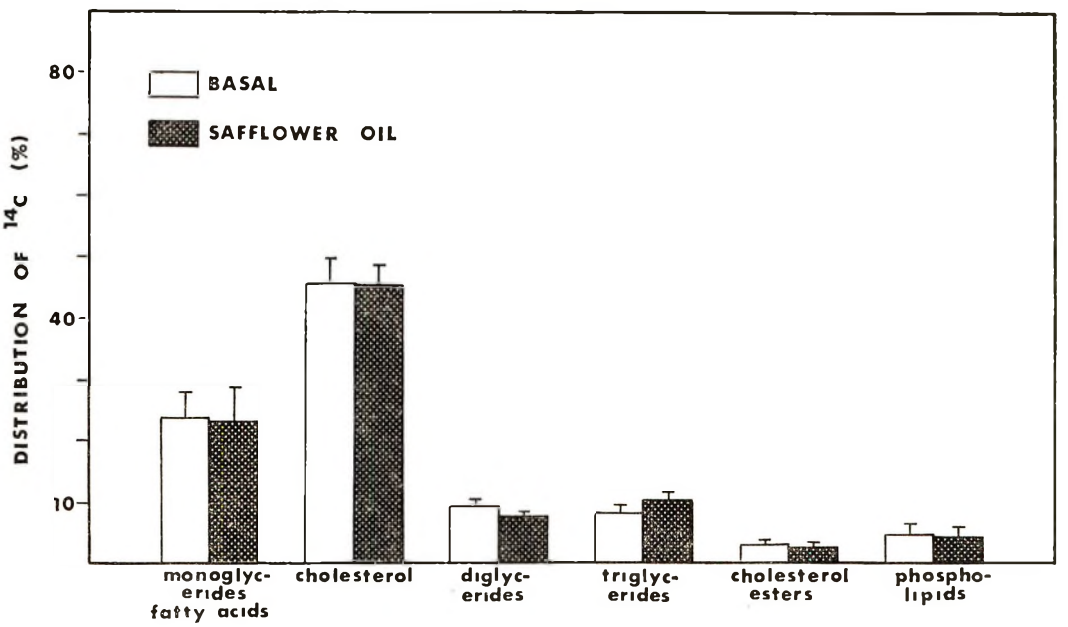

Fig. 4 Effect of a diet containing 30% safflower oil on the distribution of acetate-1-^{14}C in the lipid fractions of ovarian tissue. Values are averages from experiments in which hens were fed the diet for 30, 130, and 150 days; average deviation is shown.

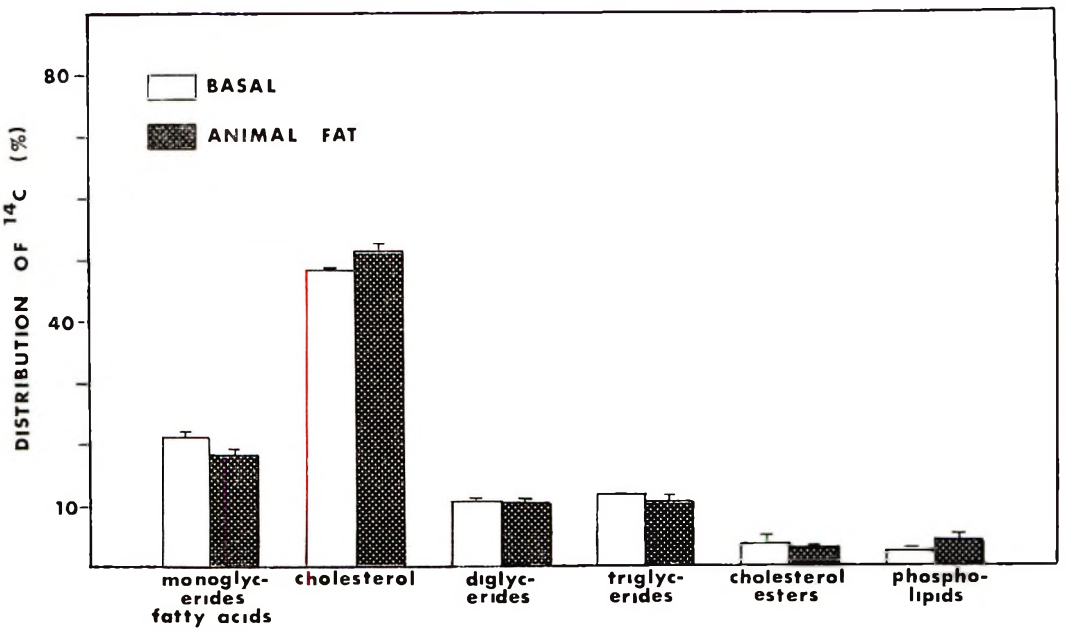

Fig. 5 Effect of a diet containing 30% animal fat on the distribution of acetate-1-^{14}C in the lipid fractions of ovarian tissue. Values are averages from experiments in which hens were fed the diet for 17 and 30 days; average deviation is shown.

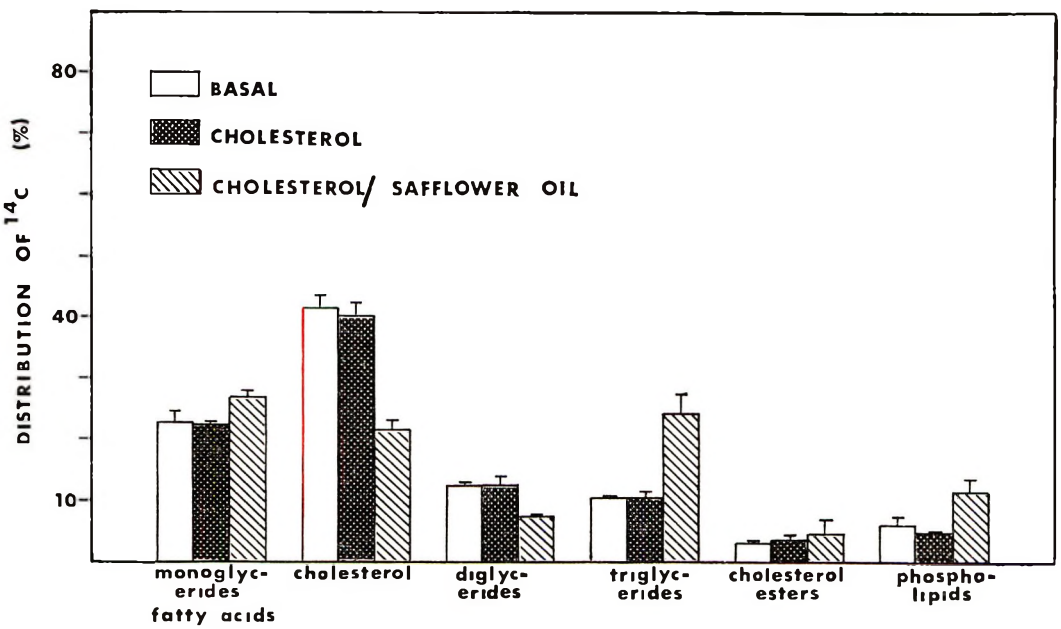

Fig. 6 Effect of diets containing cholesterol (1%) or cholesterol (1%) and safflower oil (29%) on the distribution of acetate-1-^{14}C in the lipid fractions of ovarian tissue. Values are averages from experiments in which hens were fed the diets for 15 and 23 days; average deviation is shown.

The distribution of incorporated acetate in the lipid fractions of liver slices and ovarian tissue differs. Synthesis of triglycerides predominates in the liver (figs. 1–3). Approximately 60 to 70% of the incorporated acetate is located in the triglyceride fraction. The predominant synthesis of cholesterol in ovariain tissue is indicated by the approximately 40 to 50% distribution of ^{14}C in the cholesterol fraction (figs. 4–6). Ovarian tissue is also relatively more active in the incorporation of acetate into cholesterol esters. The distribution of acetate-^{14}C in liver cholesterol, observed in these experiments, was similar to the values obtained by Husbands and Brown (22) in an in vivo study of acetate incorporation into the liver of hens. The value for the distribution of ^{14}C in ovarian tissue cholesterol is much higher than the approximately 4% cholesterol in yolk lipid. This may indicate that the egg cholesterol content would be higher than it actually is if much of the egg cholesterol originated from ovarian synthesis.

Effect of dietary fat on liver synthesis. When either safflower oil or animal fat was fed to hens at a level of 30%, total liver lipid synthesis was depressed (tables 1 and 2). The distribution of ^{14}C was decreased in the liver triglyceride fraction and increased in the cholesterol fraction when either fat was fed (figs. 1 and 2). The effects on acetate distribution were more pronounced, however, when safflower oil was fed. The decrease in lipogenesis and increase in cholesterogenesis is in agreement with studies in which rats were fed high levels of fat (3, 9, 10, 13, 14). Distribution of ^{14}C in the diglyceride and cholesterol ester fractions did not change when dietary fat was increased. Safflower oil feeding resulted in an increased acetate distribution in phospholipids (fig. 1), while no change was observed in the liver phospholipid fraction when animal fat was fed (fig. 2). Dietary regulation of liver lipid synthesis was observed when hens were fed the high fat diets for periods of time ranging from 15 days to 150 days.

The data in tables 1 and 2 can be summarized and expressed as the percentage change in acetate incorporation when the experimental diets were fed compared with the basal diet. The changes in the absolute incorporation of acetate-^{14}C when safflower oil was fed (average of all experiments) were the following: total lipids, -62%; cholesterol, $+51\%$; triglycerides, -82%; phospholipids, -10%. When animal fat was fed, the changes in acetate incorporation were the following: total lipids, -80%; cholesterol, -48%; triglycerides, -87%; and phospholipids, -79%.

There was a greater depression of the absolute incorporation of acetate-1-^{14}C into total lipids when animal fat was fed. Since total lipid synthesis was greatly diminished when animal fat was fed, the absolute incorporation of acetate into cholesterol was not increased over the basal value, even though there was a relative increase in the cholesterol synthetic pathway when either fat was fed (figs. 1 and 2). When safflower oil was fed, the absolute acetate incorporation tended to decrease in all the liver lipid fractions except the cholesterol fraction.

These results give some indication why egg cholesterol is increased when a large amount of fat, such as safflower oil, is fed to hens. If a large part of the cholesterol deposited in the egg is synthesized in the liver, an increase in liver synthesis, which appears to occur when safflower oil is fed, could result in increased egg cholesterol levels. It has been noted that hens can eliminate excess cholesterol via the egg, as evidenced by increased egg cholesterol levels when cholesterol is fed to hens. This experiment indicates that animal fat does not cause an absolute increase in liver cholesterol synthesis in vitro. This is one possible reason why the cholesterol content of egg yolk is not increased to the same extent when animal fat is fed to hens as when safflower oil is fed. It is possible that the effects of various dietary fats on cholesterogenesis are more similar than their effects on the transport of cholesterol. Transport of liver cholesterol to the ova also might be affected by the nature of the dietary fatty acids. Phospholipids, which are essential components of lipoproteins, appeared to be more readily synthesized when safflower oil was fed (table 1, fig. 1) than when animal fat was fed (table 2, fig. 2). The observation that the fatty acids of egg yolk phospholipids are not very susceptible to change as a result

of feeding a highly saturated fat (24) suggests that saturated fatty acids are less readily utilized for the synthesis of phospholipids in the liver of the hen.

In the present experiments, a decrease in total liver lipid content was noted when high levels of fat were fed to hens. This decrease in liver lipid, which occurs when a high fat diet is fed to laying hens, has been reported to involve mainly the liver triglycerides (28). If the results of this study were reported as acetate incorporation per weight of total liver lipid, the same conclusions would be reached. A large decrease in liver lipids would not be expected when high fat diets are fed to rats. When high fat diets were fed to rats, cholesterogenesis increased and lipogenesis decreased in the presence of a much larger pool of lipids in the liver, associated with fat feeding (10). However, in the present experiment, similar effects were observed using hen liver, in which the pool of lipids was decreased due to fat feeding.

The livers of hens fed a practical laying ration normally contain a large amount of fat because of the rapid fat synthesis needed to meet the demands of egg production. When large amounts of dietary fat are fed to hens, some of the dietary fatty acids may be deposited intact in the egg. Most investigators have not observed an increase in total egg yolk lipids when high fat diets were fed to hens (29). The decrease in liver lipid with fat feeding may be correlated with the decrease in lipogenesis, which is able to proceed at a slower rate when a high fat diet is fed.

From the results obtained in this study, two different effects of dietary fat on lipid synthesis from acetate can be postulated. It appears there is a tendency for the synthesis of each lipid class to be depressed when a high fat diet is fed. However, each lipid class is not affected in the same way. Brodie et al. (30) have postulated an alternate pathway for cholesterol synthesis using pigeon liver as an enzyme source. Rather than the condensation of 2 molecules of acetyl-CoA to give acetoacetyl CoA, which then condenses with another molecule of acetyl-CoA to ultimately give mevalonic acid, it involves utilization of a malonyl-CoA intermediate, formed by the carboxylation of acetyl-CoA. In the hypothesized pathway, enzyme-bound malonate condenses with acetyl-CoA to give enzyme-bound acetoacetate. In this scheme acetoacetate is postulated to be the focal point for mechanisms controlling the relation between the pathways for fatty acid and cholesterol synthesis. Ichihara et al. (31) concluded from their work with rat liver cells that malonyl-CoA may be a physiological intermediate for cholesterol synthesis, as well as fatty acid formation, but condensation of 2 molecules of acetyl-CoA may become an important shunt when formation of malonyl-CoA is disturbed, as in the diabetic state. Bortz et al. (32) have shown that the block in fatty acid synthesis, which occurs upon fat feeding, is due to the inhibition of the carboxylation reaction, in which malonyl-CoA is formed.

Assuming this is an active pathway, when acetate-^{14}C is used as a substrate for lipid synthesis under in vitro conditions, dietary fat would tend to depress acetate incorporation into all lipid classes by causing a depression of malonyl-CoA formation. It is possible that feeding different dietary fats results in a different degree of homeostatic regulation at this step. Possibly, when animal fat was fed in the present experiment, there was a greater inhibition of this reaction resulting in a greater depression of acetate incorporation into total lipids. Even if this alternate pathway exists, acetate-^{14}C could still be incorporated into cholesterol by condensation with another molecule of acetate or with acetoacetate.

Effect of dietary cholesterol on liver synthesis. It has often been demonstrated that feeding cholesterol results in a depressed cholesterol synthesis in the livers of experimental animals, including the male chicken (19, 20). The major site of the cholesterol feedback system is believed to be located where the reaction occurs that is responsible for the conversion of β-hydroxy-β-methylglutarate to mevalonate (33). In this study, cholesterol feeding at a level of 1% resulted in an approximately tenfold decrease in liver cholesterol synthesis from acetate (table 3 and fig. 3). When 1% cholesterol was fed, the distribution of ^{14}C increased about 10% in the triglyceride fraction. There was a slight increase in the absolute incorporation of acetate into the

triglyceride fraction although incorporation into total lipids did not change appreciably (table 3). This is further evidence of the interrelationship of the pathways of cholesterol and triglyceride biosynthesis.

When a diet containing both cholesterol (1%) and safflower oil (29%) was fed, cholesterol synthesis in the liver was virtually eliminated (table 3 and fig. 3). This is probably due to increased absorption of cholesterol and transport to the liver. That absorption is increased is borne out by the observation of increased blood and egg cholesterol levels when the cholesterol-oil diet was fed to hens when compared with the cholesterol levels observed when only cholesterol was added to the diet (2). The incorporation of acetate into total lipids was depressed when the cholesterol-oil diet was fed as it was when the oil alone was added to the diet. The distribution of ^{14}C in the phospholipid fraction increased when this diet was fed. Neither the distribution of ^{14}C in the triglyceride fraction (fig. 3) nor the absolute incorporation into this fraction (table 3) was depressed to the same extent when the cholesterol-oil diet was fed compared with the decreases when the oil alone was fed (table 1 and fig. 1). It appears that in this case the negative feedback control of cholesterol synthesis is greater than the regulation of lipogenesis by dietary triglyceride. More acetate-^{14}C is being diverted to triglyceride synthesis, and here again the relationship of the pathways can be seen.

Ovarian lipid synthesis. Lipid synthesis in ovarian tissue appears to be less susceptible to dietary influence than is liver lipid synthesis. The distribution of ^{14}C in the various lipid fractions of ovarian tissue of hens fed the basal diet was similar to that in hens fed diets containing either safflower oil or animal fat (figs. 4 and 5). There is a possibility of a slight increase in total lipid synthesis in the ovarian membranes when animal fat was fed (table 2), although the pattern of acetate distribution was not changed. A compensatory increase in ovarian lipid synthesis might occur if a smaller amount of cholesterol or other lipid is transported from the liver to the ova. Cholesterol feeding at a level of 1% did not affect appreciably the distribution of acetate-1-^{14}C in the ovarian lipid fractions (fig. 6). Metabolic control in ovarian tissue is indicated by the change in the pattern of ^{14}C distribution in ovarian lipids when the cholesterol-oil diet was fed (fig. 6). This is probably the result of increased absorption of cholesterol and its transport to the ova. When this diet was fed to hens, the cholesterol content of egg yolk increased 2.5 times (2). Such a large increase resulted in the suppression of cholesterol synthesis in both the liver and the ovaries. This is the only case where the distribution of acetate was observed to change in ovarian tissue. The distribution of acetate-^{14}C decreases from approximately 40% to 20% in the cholesterol fraction, whereas it increases in the triglyceride and phospholipid fractions. The absolute incorporation of acetate into total lipids was depressed slightly when the cholesterol-oil diet was fed.

Even if most of the egg cholesterol and lipid originates in the liver, rather than the ovarian tissue, it appears that in a situation where a drastic change is made from the usual diet of the hen, ovarian synthesis is adaptable. It seems possible that ovarian synthesis also provides a reserve lipid source, so that there is a minimal amount of nutrients and calories available for the proper development of the chick embryo.

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Effect of Dietary Fat and D-Thyroxine on the Incorporation of Acetate-1-^{14}C into Egg Yolk Lipids [1]

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ABSTRACT The effect of high fat diets and D-thyroxine treatment on the distribution of ^{14}C and the absolute acetate incorporation into the yolk lipid fractions of eggs from hens given acetate-1-^{14}C orally was determined. Acetate incorporation into total yolk lipids was decreased when either safflower oil or animal fat was fed at a level of 30%, but incorporation into the cholesterol fraction was not changed. The distribution of acetate-^{14}C (ratio of ^{14}C in a particular fraction to the ^{14}C in total lipids) increased in the cholesterol fraction and decreased in triglycerides; ^{14}C distribution increased slightly in the phospholipid fraction when safflower oil was fed and decreased slightly when animal fat was fed. D-Thyroxine injection resulted in an increased recovery of the isotope, with most of the increased radioactivity located in the triglyceride fraction. Regardless of treatment, the distribution of ^{14}C increased with time in the cholesterol fraction and decreased with time in the triglyceride fraction, whereas it was constant with time in the phospholipid fraction.

Eggs laid by hens that had received labeled acetate were found to have radioactivity in the shell, protein and yolk lipids, including cholesterol, fatty acids and the glycerol moiety (1–4).

In the present investigation, the distribution of acetate-^{14}C (ratio of ^{14}C in a particular fraction to the ^{14}C in total lipids) in the major lipid fractions of egg yolk was studied as a function of time. Acetate incorporation into the yolk lipids of eggs from hens fed diets containing either safflower oil or animal fat at a level of 30% was determined to gain further knowledge on the effect of dietary fat on the disposition of yolk lipids. The effect of D-thyroxine injection on the incorporation of acetate was also studied. D-Thyroxine administration, like safflower oil feeding, results in an increased egg yolk cholesterol concentration (5).

EXPERIMENTAL

The data reported here were obtained using 4 hens, each subjected to a succession of treatments: 30% safflower oil diet (2 hens) or 30% animal fat diet (2 hens); low fat control diet; and D-thyroxine injection. The composition of the diets used has been described in a previous publication (5). The individually caged White Leghorn hens were kept under a laboratory hood as a precautionary measure. Sodium acetate-1-^{14}C (either 0.10 or 0.18 mCi) was administered to the hens after they had been fed the high fat diets for at least 2 weeks and had become accustomed to the laboratory environment, and their egg-laying record was favorable over a period of time. A solution of the isotope was administered orally by means of a gelatin capsule on a day when the hen had laid an egg. After about 7 radioactive eggs had been collected, the hens were transferred to a low fat control (basal) diet (5). Preliminary studies had shown that the effect of a previous diet on egg yolk lipid composition was negligible after 10 to 12 days, which corresponds with the time required for ova growth and development. Hence, after 2 weeks, when the level of radioactivity in the eggs had diminished, another dose of labeled acetate was administered.

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After a sufficient number of radioactive eggs had been collected from the 4 hens when fed the basal diet, they were treated with D-thyroxine. Hen no. 1 was injected subcutaneously with 80 μg/100 g body weight/day of D-thyroxine [2] in saline for 2 weeks and another dose of isotope was administered. At this time, however, the hen started to molt and egg production ceased. At the time of isotope administration, thyroxine injection was reduced to 40 μg/100 g body weight every second day. After 3 weeks another dose of isotope was administered. A week earlier the hen had resumed egg production. Hen no. 2 was used as a control and was injected with 1.5 ml of saline for 2 weeks before isotope administration. Hen no. 3 was injected for 2 weeks with 80 μg/100 g body weight/day before isotope administration. Hen no. 4 was injected with the same dosage for a week preceding isotope administration. Hens 2, 3 and 4 were injected every second day after isotope administration.

The egg yolks were weighed and the lipid extracted with chloroform:methanol (2:1). The extract was filtered through a sintered glass funnel. The extract solvent (about 150 ml) was removed by rotary evaporation and the residue dissolved in chloroform. The lipid extract was filtered through glass wool, and made to volume in a 100-ml volumetric flask. One-milliter aliquots were taken for the determination of radioactivity in the total lipids. The original extract was diluted 5 times and a total of 150 μliters was chromatographed on thin-layer plates. Thin-layer chromatography and counting procedures were carried out as previously (6) with the following changes. Phospholipids were separated from neutral lipids by chromatography of the total lipid extract on Silica Gel G [3] plates, without dichlorofluorescein indicator, in acetone. The phospholipid fraction remains at the origin. Total lipids were chromatographed in 2 stages as previously, except that the ratio of benzene to ethyl acetate in the first solvent system was 8:2. When total lipids are chromatographed by the preceding method, monoglycerides, fatty acids and phospholipids do not separate into distinctive fractions. The radioactivity counts in the phospholipid fraction, which was separated from neutral lipids by chromatography in acetone, were subtracted from the counts in the phospholipid, monoglyceride, fatty acid fraction to determine incorporation in monoglycerides and fatty acids. The egg laid before the isotope was administered was used to determine background counts. Acetate incorporation into the yolk lipids of the six most radioactive eggs was determined. It was difficult to determine acetate incorporation into the yolk lipid fractions of less active eggs because of the limitations on sample size imposed by the use of thin-layer chromatography.

RESULTS AND DISCUSSION

In hens that normally laid every second day, the first egg laid after isotope administration contained radioactivity (2 days after isotope administration). If an egg was laid on the day following isotope administration, it contained very little or no radioactivity. For the sake of uniformity in procedure, the first egg that contained a significant amount of radioactivity is termed egg no. 1 in all the figures. The peak radioactivity, regardless of treatment, was usually located in either egg no. 2 or 3. However, the peak radioactivity of the various lipid fractions did not always coincide. The total radioactivity recovered in the various yolk lipid fractions of the six most radioactive eggs from each treatment group is shown in table 1. The data are expressed as the sum of millimicromoles of acetate incorporation into the 6 eggs.

When either the basal diet or the high fat diets were fed, the distribution of acetate-^{14}C in the cholesterol fraction increased with time (figs. 1 and 2), while it decreased in the triglyceride fraction (figs. 3 and 4). The distribution of ^{14}C in the cholesterol ester fraction also increased with the time. The distribution of acetate-^{14}C in the phospholipid fraction did not change considerably with time after isotope administration (figs. 5 and 6).

In a study by Husbands and Brown (7) of acetate-^{14}C incorporation in vivo into the liver lipids of the laying hen, the specific activity of cholesterol (and acetate distri-

[2] Sigma Chemical Company, St. Louis.
[3] Brinkmann Instruments, Inc., Great Neck, New York.

TABLE 1

Effect of safflower oil (SO), animal fat (AF) and D-thyroxine on the incorporation of acetate-1-^{14}C into egg yolk lipids

Hen no.	Dosage of acetate	Diet or treatment	Total lipids	Acetate incorporation [1]					
				Cholesterol	Triglycerides	Phospholipids	Cholesterol esters	Monoglycerides + fatty acids	Diglycerides
	mCi			*mμmoles/6 eggs*					
1	0.18 (sp. act. 3.5 mCi /mmole)	30% SO	1156.0	396.0	407.0	324.0	19.0	14.9	11.5
		basal diet	5549.0	376.0	3884.0	1125.0	16.2	44.8	58.9
		D-thyroxine	7194.0	376.0	5201.0	1337.0	22.9	88.8	121.0
2	0.10 (sp. act. 10.0 mCi /mmole)	30% SO	293.0	81.9	133.0	65.0	5.9	2.9	2.2
		basal diet	772.0	72.4	516.0	173.0	6.0	6.7	13.0
		saline	757.0	65.4	517.0	165.0	12.0	13.8	17.8
3	0.10 (sp. act. 10.0 mCi /mmole)	30% AF	399.0	105.0	213.0	69.0	8.2	8.4	4.6
		basal diet	780.0	97.2	494.0	176.0	6.2	4.3	11.6
		D-thyroxine	1053.0	53.6	776.0	193.0	9.0	6.9	21.6
4	0.18 (sp. act. 10.0 mCi /mmole)	30% AF	570.0	154.0	282.0	102.0	9.5	6.4	4.9
		basal diet	1365.0	129.0	903.0	299.0	11.0	17.3	15.7
		D-thyroxine	2781.0	145.0	2064.0	526.0	12.0	59.7	30.1

[1] Values are the sum of the acetate incorporated into the lipid fractions isolated from the 6 most radioactive eggs laid during the period from the second to the fourteenth day after isotope administration.

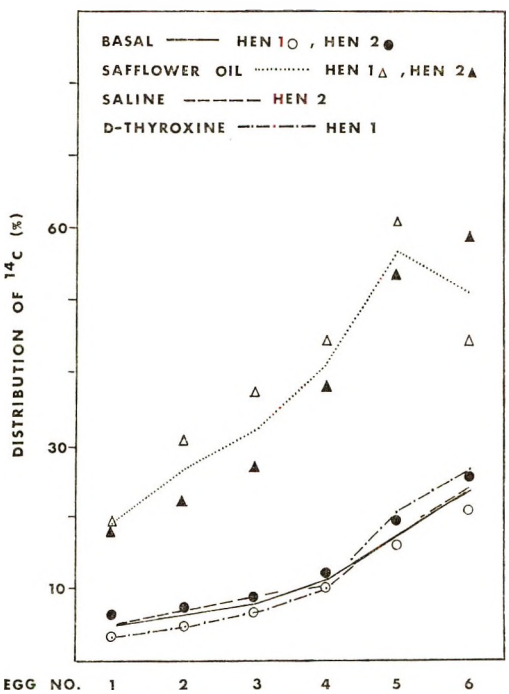

Fig. 1 Effect of safflower oil and D-thyroxine on the distribution of acetate-1-^{14}C in yolk cholesterol; hens 1 and 2; see text for details of treatment.

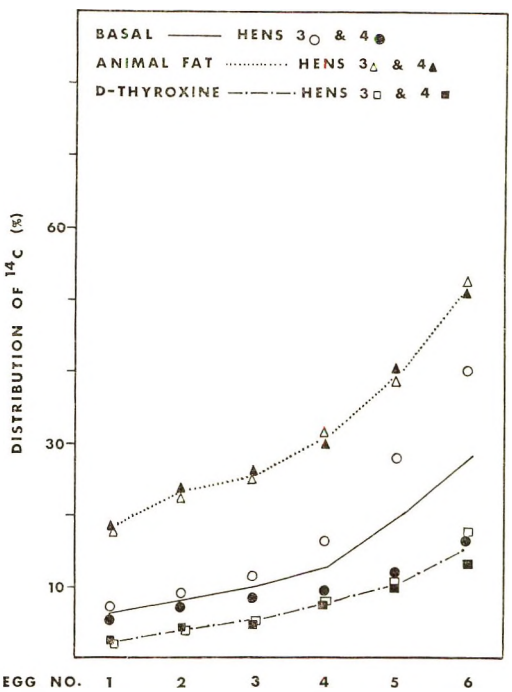

Fig. 2 Effect of animal fat and D-thyroxine on the distribution of acetate-1-^{14}C in yolk cholesterol; hens 3 and 4; see text for details of treatment.

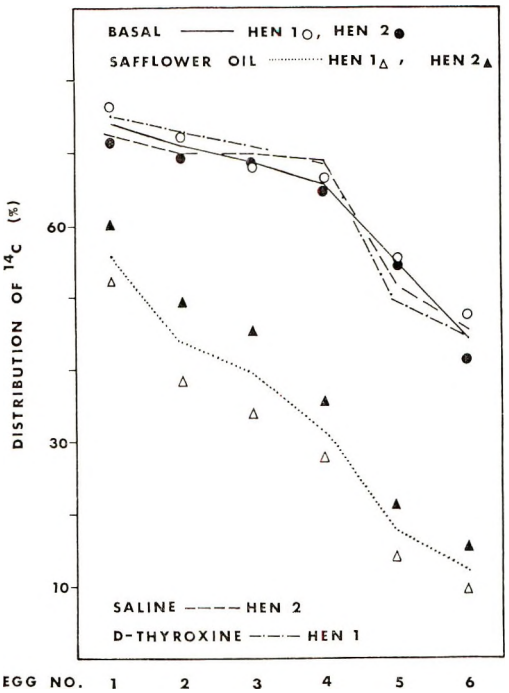

Fig. 3 Effect of safflower oil and D-thyroxine on the distribution of acetate-1-^{14}C in yolk triglyceride; hens 1 and 2; see text for details of treatment.

bution in cholesterol) increased with time, while the activity in the triglyceride fraction decreased. Their data suggested that cholesterol was continuously synthesized from fatty acids, which were derived from labeled acetate. The change in acetate distribution with time, observed in our experiments, may occur because the cholesterol not synthesized immediately could be synthesized later in the liver from labeled fatty acids. Another possibility is that labeled substrates could reach the ovary at a later time, and be used for the synthesis of cholesterol, which is the lipid preferentially synthesized by the ovarian membranes (6). The ovarian tissue surrounding the smallest developing ova at the time of isotope administration would, in this case, utilize a greater proportion of labeled substrate for cholesterol synthesis as time progressed. The difficulties in arriving at conclusions from this type of experiment are apparent because of the use of acetate as the primary precursor.

Dietary fats. When the hens were fed either safflower oil or animal fat, recovery of the isotope (figs. 7 and 8) or acetate incorporation into total yolk lipids (table 1) was reduced. The percentage change in acetate incorporation when the experimental diets were fed compared to the basal diet was calculated. Values are averages of the changes in acetate incorporation in the two most radioactive eggs from each hen. As noted earlier, the relative amount of the isotope incorporated in the cholesterol and triglyceride fractions changes with time. In experiments of this type, it is obvious that the choice of egg taken for analysis can determine the results obtained. The changes in the absolute incorporation of ^{14}C when safflower oil was fed were as follows: total lipids, − 71%; cholesterol, + 9%; triglycerides, − 82%; phospholipids, − 50%. The changes in acetate incorporation when animal fat was fed were these: total lipids, − 54%; cholesterol, + 14%; triglycerides, − 69%; phospholipids, − 64%. Even

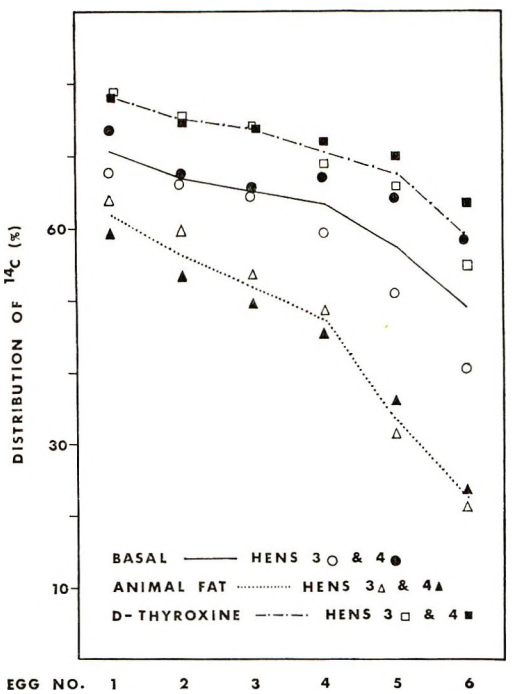

Fig. 4 Effect of animal fat and D-thyroxine on the distribution of acetate-1-^{14}C in yolk triglyceride; hens 3 and 4; see text for details of treatment.

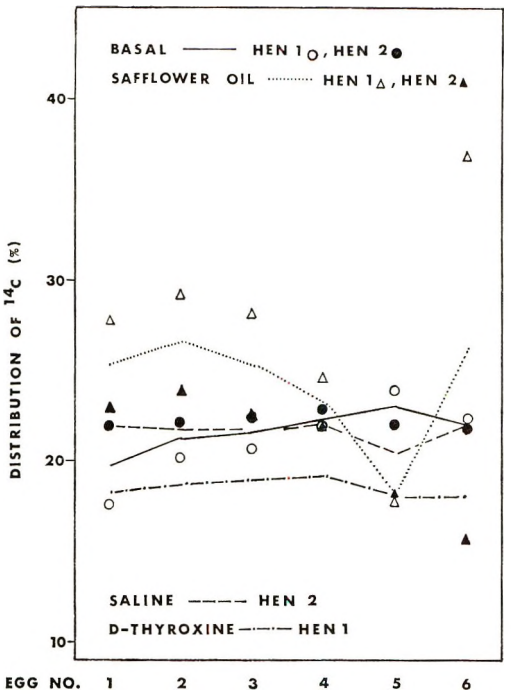

Fig. 5 Effect of safflower oil and D-thyroxine on the the distribution of acetate-1-^{14}C in yolk phospholipid; hens 1 and 2; see text for details of treatment.

though the absolute acetate incorporation into total egg yolk lipids was depressed when high levels of fat were fed, the incorporation into cholesterol was increased over the basal values. This increase, although sometimes slight, suggests the possibility that even though total lipid synthesis is decreased, cholesterol synthesis is increased leading to an increase in the egg cholesterol content.

Murty and Reiser (4) injected acetate-1-^{14}C into hens fed either trilinolein or trilinolenin at a level of 5%. There was a higher degree of incorporation of acetate into yolk fatty acids when linoleic acid was fed, but the incorporation into cholesterol was the same with both. The addition of tallow at a level of 5% to the diets containing unsaturated fatty acids markedly depressed the incorporation of acetate-^{14}C into fatty acids and cholesterol, in which case the incorporation into both fatty acids and cholesterol was higher with linolenic acid ingestion. The present paper and our previous work (5) indicate that much higher dietary fat levels are required to stimulate cholesterol biosynthesis and egg yolk cholesterol content.

There was not as great a depression in total lipid synthesis from acetate when animal fat was fed in this study, as there was in the in vitro study using liver slices (6). The effect of the high fat diets on the absorption of the orally administered acetate must be considered. If the isotope were more readily absorbed when animal fat was fed, an increase in isotope recovery in the egg would occur. The results would be a proportionate increase in the absolute acetate incorporation into all of the lipid fractions. Animal fat is less readily absorbed by the hen, but the effect this would have on the absorption of nonlipid components of the diet is not known. In the present experiment, presentation of the results as distribution of ^{14}C may be of more value, since any differences in loss of isotope during administration due to diet or treatment would be eliminated. The question of the effect of different dietary

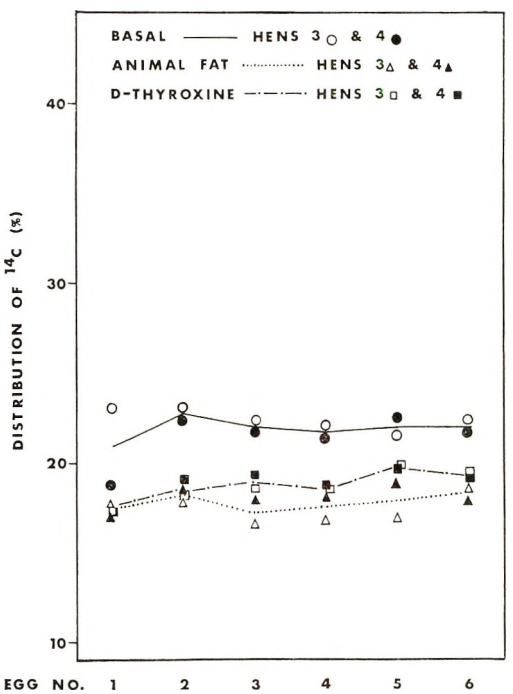

Fig. 6 Effect of animal fat and D-thyroxine on the distribution of acetate-1-^{14}C in yolk phospholipid; hens 3 and 4; see text for details of treatment.

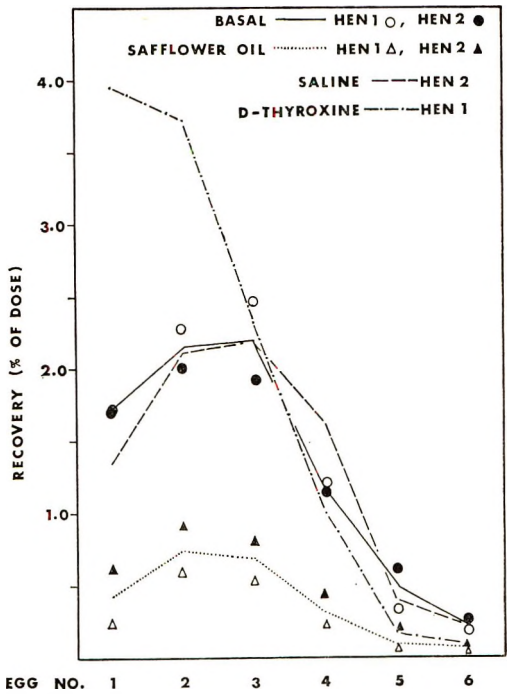

Fig. 7 Effect of safflower oil and D-thyroxine on the recovery of acetate-1-^{14}C in total yolk lipids; hens 1 and 2; see text for details of treatment.

fats on the transport of cholesterol from the liver to the egg is not adequately answered by the present experiments.

The ingestion of high levels of fat resulted in an increased distribution of acetate in the cholesterol fraction, while the distribution of ^{14}C in triglycerides decreased (figs. 1–4). These effects were more pronounced when safflower oil was fed and are similar to the results obtained using hen liver slices (6). The distribution of the label in cholesterol and triglycerides in the first radioactive egg laid by the hens fed either the basal diet or the high fat diets was similar to the distribution in liver slices, in contrast with that in ovarian tissue (6). This suggests that most of the egg lipid originates from liver synthesis.

When safflower oil was fed, there was a slight increase in the distribution of ^{14}C in the phospholipid fraction (fig. 5), whereas there was a slight decrease in acetate distribution in phospholipids when animal fat was fed (fig. 6). The relative increase in the pathway of phospholipid synthesis from acetate, when safflower oil was fed to hens, was also suggested by the results obtained in the in vitro studies (6). The relative stability of phospholipid synthesis with time gives some indication about the differences between triglyceride and phospholipid pathways. The ingestion of fat is believed to cause decreased lipogenesis by exerting biosynthetic control at an early stage of fatty acid synthesis (8). It is possible that a control is also exerted at a later stage of phospholipid and triglyceride synthesis from α-glycerophosphate. The availability of fatty acids required for either triglyceride or phospholipid formation can be an important factor at this stage. In most studies on the effect of fat feeding on lipogenesis in rats, incorporation of substrate into fatty acids has been used as a measure of lipogenesis. This does not distinguish the effect of lipogenic control factors on fatty acid synthesis from any effect on fatty acid esterification, so that the relative effect of this phenomenon on the synthesis of triglyceride and the phosphatides is not clear.

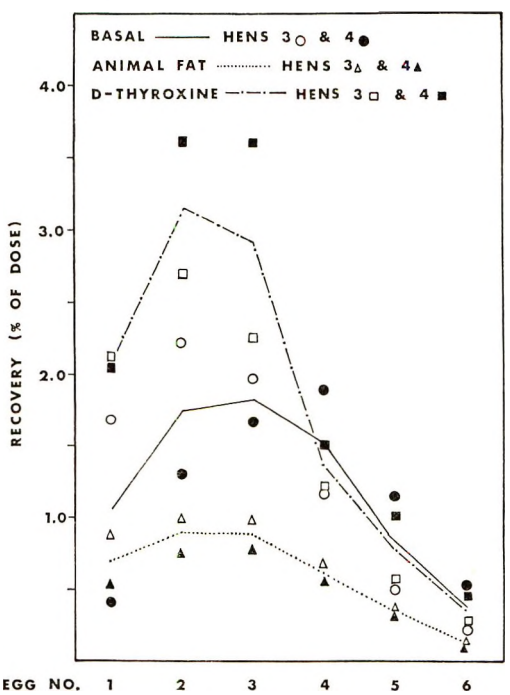

Fig. 8 Effect of animal fat and D-thyroxine on the recovery of acetate-1-^{14}C in total yolk lipids; hens 3 and 4; see text for detail of treatment.

One possibility for the relative stability of the phospholipid pathway is that phospholipids may not be utilized readily as precursors for cholesterol synthesis. This may be the case in the ovaries. Budowski et al. (9) administered tripalmitin, labeled in both the glycerol and fatty acid moieties, to hens. Their data indicate that triglycerides can be hydrolyzed in the ovary. Phospholipids, however, did not appear to undergo any hydrolysis as they passed from the plasma to the ova. These workers concluded, from the higher specific activity of unsaponifiable matter in the ova as compared with other tissues and organs, that synthesis of sterols occurs in the ovary from an acetate pool whose activity is also higher than that in any other tissue.

D-Thyroxine. Hen no. 1 began to molt during treatment with D-thyroxine and ceased egg production. D-Thyroxine treatment at a lower dosage was continued and possibly shortened the molting period. Hens 3 and 4 were also affected by D-thyroxine treatment when the hens were injected daily. They began to lose feathers but this was accompanied by simultaneous growth of new feathers. Thyroid hormone is believed to be involved in egg production, to promote feather growth, and to produce molting (10). These observations appear contradictory, but may be dependent on thyroxine concentration. The decreased egg yolk weight and increased yolk cholesterol resulting from thyroxine treatment has been discussed. It was suggested that thyroxine treatment may cause both premature ovulation and increased deposition of cholesterol in the ova (5).

A major effect of D-thyroxine treatment appears to be exerted on total lipid synthesis. The recovery of the label in the yolk lipids was increased when thyroxine was injected (figs. 7, 8). Acetate incorporation increased into the triglyceride and phospholipid fractions (table 1). Apparently there is an early rapid synthesis of fatty acids from the labeled acetate. Even when the decrease in yolk weight and lipid content is taken into consideration, these increases are pronounced. D-Thyroxine treatment did not result in an overall increase in acetate incorporation into cholesterol in all hens. The effect of D-thyroxine on the absolute incorporation of acetate into the lipid fractions was highly variable. The recovery of acetate-^{14}C in the cholesterol fraction was not changed in the eggs from hen no. 1, increased in the eggs from hen no. 4, and decreased in the eggs from hen no. 3, which was not injected with D-thyroxine for as long as the other 2 hens. The pattern of acetate distribution during D-thyroxine treatment changed only slightly from the pattern of ^{14}C distribution in the egg lipids when the hens were not injected. The distribution of ^{14}C in triglycerides increased slightly, whereas it decreased slightly in the cholesterol and phospholipid fraction. Again, the acetate-^{14}C distribution in the phospholipid fraction was constant with time. Saline injection of the control hen had no effects on total acetate incorporation or the pattern of acetate-^{14}C distribution in yolk lipids.

The effect of D-thyroxine injection on the absorption of the isotope is not known. The shape of the curves describing isotope recovery (figs. 7 and 8) obtained when hens were injected with D-thyroxine is different from the shape of the curve when either the basal diet or the high fat diets were fed. The recovery of the isotope increased rapidly in the eggs when D-thyroxine was injected, but decreased to the level of ^{14}C recovery in the eggs of hens fed the basal diet by the time the third or fourth eggs were laid. This indicates that D-thyroxine treatment increases the turnover of lipid in the hen. The effect of D-thyroxine is probably not due only to increased absorption of labeled acetate, since the curves describing isotope recovery would be expected to be the same shape in that case. The hypothesized increase in the deposition of lipid in egg yolk is in accord with the overall increase in metabolic activity caused by thyroxine.

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Upper Intestinal Tract Infection Produced by *E. acervulina* and Absorption of ^{65}Zn and ^{131}I-labeled Oleic Acid [1,2]

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ABSTRACT A series of trials was conducted to determine the effects of upper intestinal tract damage upon the absorption of labeled zinc and oleic acid used as examples of mineral and fatty nutrients. The protozoan parasite *Eimeria acervulina*, which attacks the anterior portion of the chicken intestine, was used to produce the intestinal damage. The infection caused growth retardation on the seventh through tenth days post-inoculation and visible damage to the lining of the upper one-third of the intestinal tract during the period from 5 to 10 days post-inoculation. Increased absorption of orally administered ^{65}Zn and ^{131}I-labeled oleic acid occurred on day 1 and from days 14 through 21 post-inoculation, whereas greatly decreased absorption of both labeled nutrients was observed on days 5 through 10 post-inoculation. Both oleic acid and zinc absorption were severely and equally depressed by *E. acervulina* infection. This was contrasted with *E. necatrix* infection in which zinc absorption was much more severely affected than oleic acid absorption.

Infections of the mid-small intestine produced by *Eimeria necatrix* have been shown to decrease absorption of orally ingested zinc (1) and oleic acid (2) during the acute stages of the infection, but to enhance the absorption of these nutrients during early infection and recovery phases (1, 2). Cecal infections produced by *Eimeria tenella* had little effect on zinc absorption (3). Erasmus et al. (4) demonstrated a marked decrease in liver storage of vitamin A one week after administration of oocysts of *E. tenella* and *Eimeria acervulina*. Since *E. tenella* infection appeared to have little effect on nutrient absorption (3), it seemed worthwhile to determine the effects of *E. acervulina* infection upon nutrient absorption.

In chicks, *E. acervulina* infection causes damage to the anterior one-half of the small intestine. The trials in the present report were designed to determine the effects of *E. acervulina* infection upon the absorption of ^{65}Zn and ^{131}I-labeled oleic acid throughout the disease cycle.

PROCEDURE

In each of the trials reported, broiler-type male chicks were raised in electrically heated, wire-floor battery brooders with ad libitum access to a corn-soy broiler starter diet and tap water. At 4 weeks of age, the birds were divided at random into 3 groups containing equal numbers of birds, and were weighed and wingbanded. Each bird in one group was inoculated via the crop with 1 ml of a suspension containing 1,000,000 sporulated oocysts of *E. acervulina*. A second group received 0.5 ml of this suspension, or approximately 500,000 oocysts per bird, and the third group was left as uninfected controls.

At the intervals after inoculation shown in the tables, 5 birds from each group were weighed, placed in individual wire cages and orally administered a gelatin capsule containing 50 μCi of ^{65}Zn absorbed on sucrose, and concurrently, a second capsule containing 20 μCi of ^{131}I-labeled oleic acid.[3] The intervals chosen to determine nutrient absorption rates were chosen to represent critical stages of the disease cycle. At intervals of 0.5, 1, 2, 4, and 8 hours after the administration of the tracers, 1 ml of blood was drawn from

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[2] Published with the approval of the Director of the S. C. Agricultural Experiment Station as technical contribution no. 654.
[3] Abbott Laboratories, Oak Ridge, Tennessee.

each bird by cardiac puncture. Radioactivity of all blood samples was determined in a well-type crystal scintillation detector fitted with a dual channel spectrometer adjusted for the photopeaks of ^{131}I and ^{65}Zn. Following the removal of the last blood sample, the birds were killed, and the anterior half of the small intestine was examined for visible damage. All intestines were assigned visual damage scores based on a scale of zero for a normal intestine, to 4 for a severely damaged, hemorrhagic intestine. Intermediate numbers signified intermediate amounts of damage. Weight gains and intestinal damage scores shown in the tables are the average of the 5 birds in each group used in the tracer trial at the given time interval post-inoculation.

Data for the 5 birds in each treatment group for the 5 time intervals have been averaged in the tables. The amount of each tracer in the bloodstream of the infected groups of birds is expressed as a percentage of the amount appearing in the bloodstream of the uninfected birds at the same time interval.

RESULTS AND DISCUSSION

In both trials, highly significant decreases in weight gains were observed between 7 and 10 days post-inoculation (tables 1 and 2). Weight gains were significantly decreased only on day 7 in trial 1, but smaller decreases were observed until day 14. In trial 2 (table 2), weight gains were significantly decreased only on day 10 at the lower level of infection, but were

TABLE 1

Effect of Eimeria acervulina infections on weight gain and intestinal damage (trial 1)

Days, post-inoculation	Weight gain [1]			Intestinal damage score [2]	
	Control	0.5 M [3]	1 M [3]	0.5 M [3]	1 M [3]
	g	*g*	*g*		
1	55	58	64	0	0
3	126	113	109	0	0
5	164	150	120	1.8 **	2.2 **
7	237	144 **	142 **	1.2 **	1.8 **
10	336	327	292	0.4 *	0
14	480	440	443	0	0
21	686	662	729	0	0
28	959	971	992	0	0
35	1267	1234	1130	0	0

[1] Average weight gain for the 5 birds in each group from day of inoculation to day of tracer trial.
[2] Scoring system: 0 = normal intestine, 4 = severely damaged intestine.
[3] 0.5 M = 500,000 sporulated oocysts of *E. acervulina*/bird; 1 M = 1,000,000 sporulated oocysts of *E. acervulina*/bird.
* Significantly ($P < 0.05$) greater or less than corresponding control value.
** Highly significantly ($P < 0.01$) greater or less than corresponding control value.

TABLE 2

Effect of Eimeria acervulina infections on weight gain and intestinal damage (trial 2)

Days, post-inoculation	Weight gain [1]			Intestinal damage score [2]	
	Control	0.5 M [3]	1 M [3]	0.5 M [3]	1 M [3]
	g	*g*	*g*		
1	9	7	9	0	0
3	43	45	48	0	0
5	121	126	111	2.6 **	2.6 **
7	181	193	150 *	1.8 **	1.2 **
10	270	214 **	186 **	0	0.2
14	438	423	268 **	0	0
21	579	580	475	0	0
28	728	822	649	0	0
35	921	1067	788	0	0

[1] Average weight gain for the 5 birds in each group from day of inoculation to day of tracer trial.
[2] Scoring system: 0 = normal intestine, 4 = severely damaged intestine.
[3] 0.5 M = 500,000 sporulated oocysts of *E. acervulina*/bird; 1 M = 1,000,000 sporulated oocysts of *E. acervulina*/bird.
* Significantly ($P < 0.05$) greater or less than corresponding value.
** Highly significantly ($P < 0.01$) greater or less than corresponding control value.

decreased on days 7 to 14 with smaller decreases through day 35 post-inoculation, when an infecting dose of 1,000,000 sporulated oocysts of *E. acervulina* was administered. Visible intestinal damage was observed on days 5 through 10 in infected birds in both trials. Observed intestinal damage was more severe in the second trial than in the first trial.

In the first trial (table 3) an increase in the absorption of both zinc and oleic acid was observed one day post-infection. In the case of zinc, absorption was graded according to the size of the infecting dose, as the larger number of oocysts caused the greatest increase in absorption. This effect was not observed in oleic acid absorption in either trial, but may have occurred with zinc in the second trial (table 4). On the third day post-inoculation, there was no significant deviation of either zinc or oleic acid absorption from that observed in the uninfected birds.

By the fifth through seventh days post-inoculation, highly significant decreases in absorption of both zinc and oleic acid had occurred in both trials. The magnitude of these decreases in nutrient absorption did not appear to be related to the number of oocysts in the infecting dose. Oleic

TABLE 3

Effect of Eimeria acervulina infection upon absorption of orally administered ^{65}Zn and ^{131}I-labeled oleic acid (trial 1)

Days, post-inoculation	^{65}Zn activity in blood		^{131}I activity in blood	
	0.5 [1]	1 M [1]	0.5 [1]	1 M [1]
	% of control [2]		*% of control* [2]	
1	136 *a3	187 *b	117 *	117 *
3	85	89	77	88
5	36 **	40 **	22 **	30 **
7	273 *a	54 *b	83 *a	66 *b
10	69 **	56 **	76 **a	59 **b
14	220 **	256 **	94	115
21	104	69	97	97
28	125	74	107	86
35	87	128	126	106

[1] 0.5 M = 500,000 oocysts/bird; 1 M = 1,000,000 oocysts/bird.
[2] The amount of each tracer appearing in the bloodstream of birds in each treatment group at the day shown is expressed as a percentage of that appearing in the uninfected controls. Each entry is an average of 5 birds at 5 time intervals or 25 determinations for each of the infected and uninfected groups.
[3] Values followed by * and ** are significantly different from corresponding control values at $P < 0.05$ and $P < 0.01$, respectively. Means under each nutrient followed by different letters are significantly different from each other and corresponding control means at $P < 0.05$.

TABLE 4

Effect of Eimeria acervulina infection upon absorption of orally administered ^{65}Zn and ^{131}I-labeled oleic acid (trial 2)

Days, post-inoculation	^{65}Zn activity in blood		^{131}I oleic acid activity in blood	
	0.5 [1]	1 M [1]	0.5 [1]	1 M [1]
	% of control [2]		*% of control* [2]	
1	115	130	89	97
3	161	92	80	92
5	63 *	54 *	71 **	56 **
7	27 **	61 **	40 **	31 **
10	87	116	84 **	78 **
14	135 *	149 *	108	127 *
21	192 **	238 **	79	142 **
28	79	79	104	89
35	60 **	43 **	76	99

[1] 0.5 M = 500,000 oocysts/bird; 1 M = 1,000,000 oocysts/bird.
[2] The amount of each tracer appearing in the bloodstream of birds in each treatment group at the day shown is expressed as a percentage of that appearing in the uninfected controls at the same time interval on that day.
* Significantly ($P < 0.05$) different from uninfected controls.
** Highly significantly ($P < 0.01$) different from uninfected controls.

acid absorption, on the average, tended to decrease at least as severely, if not more severely, than zinc absorption. This is in contrast with the pattern observed in *E. necatrix* infection (2), where oleic acid absorption was much less affected than was zinc absorption. This effect is probably due to the different areas of the intestine attacked by the 2 organisms.

An anomaly is seen in trial 1 (table 3) on the seventh day post-inoculation in which the absorption of zinc was much greater than normal, rather than decreased below normal as would be expected. A period of greatly increased absorption of zinc lasting approximately 18 to 24 hours, and occurring immediately after the acute stage of the infection, has been observed several times in *E. necatrix* infections.[4] Immediately preceding and following this period of enhanced absorption, were periods of severe absorptive impairment. The enhanced zinc absorption noted on day 7 of the first trial is believed to represent this same phenomenon. Since the disease cycles were not perfectly synchronized between infection levels or between trials, it would be possible for one group to be passing through the brief period of enhanced absorption at the same time as the other groups are at either an earlier or later stage.

By the tenth day post-inoculation, healing of the intestine in infected birds was well advanced (tables 1 and 2) and absorption rates of both zinc and oleic acid returned toward normal levels. On day 14 in trial 1 and days 14 through 21 in trial 2, a period of greatly increased zinc absorption occurred. Oleic acid absorption, however, returned only to levels observed for the uninfected birds, except in chicks given one million oocysts each in trial 2, in which oleic acid absorption was enhanced during days 14 through 21. This period of absorptive enhancement has been observed previously in *E. necatrix* infection (1, 2). This absorptive enhancement, particularly in the case of zinc, is probably due to a combination of factors such as: 1) increased permeability of the cells of the absorptive surfaces of the intestine due to swelling and slightly damaged or incompletely formed cell membranes; and 2) increased protein synthesis in healing tissues which also results in increased amounts of transport carriers being available for absorption and transport of the metal.

Following the period of enhanced zinc absorption, absorption rates of both zinc and oleic acid returned to levels comparable to those observed in uninfected birds. In trial 2, however, zinc absorption decreased significantly on day 35 post-inoculation. This has not been observed in other infections (1, 2) was not observed in trial 1, and is believed to be an anomaly occurring in this trial.

The delaying of zinc absorption observed in *E. necatrix* infections of the mid-small intestine (1) was not observed within the 8-hour period used in this study (fig. 1). Decreased zinc absorption at all time-intervals during the tenth day post-inoculation is shown, however. A marked delaying of the absorption of oleic acid (fig. 2) was observed in both trials in which *E. acervulina* was the infecting agent. This may be attributed to damage to sites directly involved in the absorption of this fatty acid.

We believe that the changes in zinc and oleic acid absorption observed in these trials are a function of damage to the upper one-third of the small intestine. Absorptive changes occurring with other nutrients will depend upon the portion of the intestine where the nutrient is absorbed and upon the portion of the intestine damaged by the infecting organism used.

The changes in the absorption of both zinc and oleic acid tended to parallel and are probably responsible for the changes

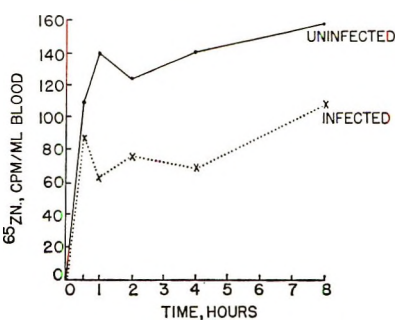

Fig. 1 Effect of *E. acervulina* infections upon absorption of orally administered ^{65}Zn from the intestine into the bloodstream.

[4] Turk, D. E., unpublished data.

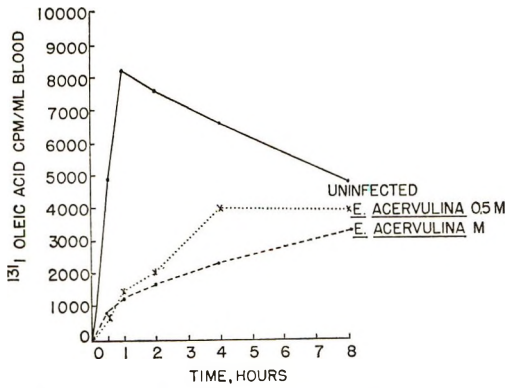

Fig. 2 Effect of *E. acervulina* infections upon absorption of orally administered ^{131}I-labeled oleic acid from the intestine into the bloodstream; 0.5 M = infecting dose of 500,000 sporulated oocysts of *E. acervulina*/bird; 1 M = infecting dose of 1,000,000 sporulated oocysts of *E. acervulina*/bird.

in growth rate observed in birds suffering from *E. acervulina* infections. There was normally a decrease in growth rate on days 5 to 10 post-inoculation (tables 1, 2), followed by an increase in growth rate in the following 2-week period. These changes in growth rate were paralled by increases in absorption above that experienced in uninfected birds during the next 2 weeks. Following this, both growth rate and absorption rates tended to approach those of uninfected birds of the same age.

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Effect on the Young Rat of Maternal Protein Restriction

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ABSTRACT The effects on the young of restricted maternal protein intake and the duration of these effects were studied. Pregnant rats fed a diet containing 6% casein as the sole source of protein produced litters of pups with decreased total body weight and length, decreased liver and kidney weights and increased heart, brain, and thymus weights in relation to total body weight. There were no macroscopically observable congenital abnormalities or differences in various indexes of maturation. Pair-bred protein-restricted and control litters were then matched in number, weight, and sex. Half of each litter was given at birth to the other dam of the pair to suckle. Lactation failure in the dam is the apparent cause of death in pups suckled by protein-restricted dams. Pups from litters in the protein-restricted group suckled by control dams showed an increased mortality rate and a decreased growth rate which persisted to the age of 76 days. It is suggested that these young lack sufficient vigor at birth to obtain adequate nourishment by suckling. There was almost complete mortality of protein-restricted pups suckled by control dams when no control pups were available to stimulate lactation. The postnatal effects of prenatal protein restriction are, therefore, the sum of effects observed in the newborn and their sequelae plus the consequences of inability to suckle adequately postnatally. It was demonstrated that reduction of total maternal food intake does not affect the young since young in pair-fed litters were indistinguishable from controls.

It is now generally accepted that maternal intake of some nutritionally deficient diets during pregnancy has severe effects on the offspring (1). In studies of the effects of feeding protein-deficient diets to pregnant and lactating rats, decreased litter size, decreased weight of individual pups (2–5) and decreased survival rates up to weaning age (6, 7) have been demonstrated. However, the reasons for the high mortality rate have not been investigated.

The studies cited were terminated at parturition or at weaning. It has been reported that reduction in the total food intake during pregnancy results in permanent growth stunting (8). It appeared likely that protein restriction in pregnant rats would also have long-term consequences. Therefore, the present work was undertaken to determine some of the effects on the offspring of restricted maternal protein intake and the duration of these effects.

Protein restriction has been demonstrated to quantitatively affect lactation in the rat (9–11). This results in a lack of adequate food for the young postnatally and is an obvious source of difficulty in studies of the duration of the effects of prenatal diet. In an attempt to eliminate this problem, adequately fed foster mothers were provided to nurse the young of protein-restricted dams.

EXPERIMENTAL

Female rats of the Sprague-Dawley strain, weighing 180 to 200 g, were housed in single wire-bottom cages and bred with a normal male. Pregnancy was assumed to have established when vaginal plugs or sperm were found. The day following the night of mating was considered day zero of pregnancy.

Part 1. Twelve pregnant rats were divided into 2 groups of six and fed a semipurified diet ad libitum starting on day zero of pregnancy. The control diet consisted of dextrose,[1] 62%; casein,[2] 24%; corn oil, 8%; and salt mix,[3] 6%. The restricted protein diet contain 80% dextrose and 6% casein. Each animal in both

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[1] Cerelose, Corn Products Refining Company, New York.

[2] Nutritional Biochemicals Corporation, Cleveland.

[3] Salt content of diet: (g/kg) $CaCO_3$, 18.0; K_2HPO_4, 19.5; $CaHPO_4$, 3.6; NaCl, 10.08; $FeSO_4 \cdot 7H_2O$, 1.5; $MgSO_4 \cdot H_2O$, 0.0075; KI, 0.015; $ZnCO_3$, 0.048; $CuSO_4 \cdot 5H_2O$, 0.018; and $MnSO_4 \cdot H_2O$, 0.138.

groups received a vitamin supplement mixture [4] 3 times weekly. Both diets were continued throughout pregnancy and lactation.

Following parturition, pups in each litter were weighed, counted, and distributed in random fashion as follows. All pups in excess of seven were preserved in Bouin's solution and examined for malformations using the method of Wilson (12). Of the remaining seven, crown-rump length of 3 pups from each litter was measured as well as the length of the tail, right forelimb from the axilla and right hindlimb from the groin. Each animal was then decapitated and blood was collected for hematocrit determination and for determination of hemoglobin by the acid hematin method (13). Liver, spleen, kidneys, adrenals, heart, brain, and thymus were removed and weighed. The remaining 4 pups were left with the dam to be used for study of weanlings and adults. However, all but two of the pups in the experimental group died. Consequently, work on all animals in part 1 was discontinued.

Part 2. Most of the pups that died had no milk in their stomachs, had failed to gain weight, and appeared dehydrated. This suggested that dehydration and starvation caused by inability of the dam to lactate or inability of the pups to suckle might be the primary cause of death. As a result, the next part of the work was planned to include a study of the relative importance of these factors.

Weanling and adult offspring for study of the duration of the effects found in part 1 were produced by 32 female rats, sixteen in the control group and sixteen in the experimental group. Feeding and mating procedures were the same as in part 1. In addition, the females of approximately equal weight were pair-bred so that both litters were produced in one day. The mother to be in the experimental group was fed the 6% casein diet, and the one in the control group was given the 24% casein diet. Both diets were fed ad libitum and food intake was measured daily.

As each litter arrived, it was reduced to 8 pups, out of which 2 groups of four were matched in sex and weight. Then one of the matched groups of the dam in the experimental group was given to the dam in the control group and vice versa. Food intake was not measured during lactation.

During the suckling period, each surviving pup was examined daily for evidence of growth and maturation. The ages at which ears and eyes opened and at which upper and lower incisors erupted were noted. Beginning at the age of 7 days, each pup was tested for ability to right itself in 5 seconds or less when placed on its back on a solid surface. The ability of each pup to right itself in air when dropped from a height of 30.5 cm was tested beginning at the age of 10 days.

At the age of 22 days, half the surviving animals in each group was dissected by the same procedure used with the newborns. The remaining animals were fed the control diet and water ad libitum and dissected at the age of 76 days. Body lengths of the 76-day-old rats were not obtained.

Part 3. Pups produced by dams fed the control diet in part 2 appeared to be more vigorous than those produced by the females fed low protein. Consequently, it seemed possible that low protein young suckled by control dams failed to receive sufficient milk because of competition within the litter. To evaluate this factor, part 3 of this work was undertaken. In addition, the effect of the altered pattern of food intake exhibited by the rats in the low protein group in part 2 was investigated.

Female rats in part 3 consisted of 8 rats in group A, fed the control diet, 22 rats in group B, fed the 6% casein diet, and 8 rats in group C, pair-fed an amount equal to the intake of each of the first 8 females in group B which became pregnant. In addition, another female was pair-bred for each animal in groups B and C to provide a nursing dam for the young of these rats.

At birth, all young in excess of 8 were dissected as in part 1. Then, the remaining young, 4 males and 4 females, chosen

[4] Calculated on a per day basis, each pregnant or lactating animal received: (in milligrams) choline 20.0; inositol, 10.0; ascorbic acid, 2.0; Ca pantothenate, 1.0; and (in micrograms) *p*-aminobenzoic acid, 200.0; pyridoxine, 600.0; nicotinic acid, 600.0; riboflavin, 200.0; thiamine, 600.0; menadione, 500.0; biotin, 5.0; folic acid, 12.0; vitamin B_{12}, 0.6; and vitamin A palmitate, 300 IU; vitamin D, 30 IU; and *dl*-α-tocopherol, 2.2 IU.

at random, were given to pair-bred females to nurse.

Survival rate and weight gain of pups were noted daily for 5 days and at 5-day intervals thereafter. At the age of 22 days, half of each surviving litter was dissected as above. The remainder were dissected at the age of 40 days.

The data were analyzed using analysis of variance for newborn young and Fisher's *t* test for weanling and 40-day-old young.

RESULTS

Part 1. The reproductive performance of the female rats is shown in table 1. Animals fed the 6% casein diet had significantly lower weight gains over the course of the pregnancy. There was a significant decrease in total litter weight and mean weight of the individual young. Survival rate of fetuses in utero was slightly less when the 6% casein diet was fed throughout pregnancy than when the control diet was fed but the difference was not statistically significant.

Results of examination and dissection of the newborn young are listed in table 2. At birth there were no significant differences between the sexes in any of the parameters measured; therefore, the data for both sexes were combined. Table 2 shows that pups produced by pregnant rats fed restricted protein diets were shorter in length, but the data did not indicate any disproportion of limb length. Relative organ weights, however, were not all affected in the same way or to the same degree. The relative weights of spleen and of adrenals remained unchanged. The heart, brain, and thymus were an increased proportion of body weight, while the liver and kidney weights were a decreased proportion. Examination of cross sections of newborn pups preserved in Bouin's solution failed to reveal any abnormalities. There were no differences in hemoglobin levels or hematocrits.

TABLE 1

Reproductive performance in pregnant rats fed diets containing 24% or 6% casein [1]

	24% Casein diet	6% Casein diet
Wt gain, g	138.0 ± 28.8 [2]	33.0 ± 15.0 [3]
No. of pups/litter	10.0 ± 2.80	8.6 ± 3.13
Litter wt, g	58.9 ± 9.47	42.0 ± 7.17 [4]
Mean wt of pups, g	5.8 ± 0.51	4.65 ± 0.69 [4]

[1] Six rats/group.
[2] Mean ± one SD.
[3] $P = < 0.001$.
[4] $P = < 0.01$.

TABLE 2

Relative organ weights at birth [1,2]

	24% Casein diet	6% Casein diet
Crown-rump length, mm	41.91 ± 2.15 [3]	38.03 ± 2.76 [4]
Relative organ weights		
Liver, %	5.010 ± 0.618	4.53 ± 0.448 [5]
Kidneys, %	1.014 ± 0.088	0.788 ± 0.135 [4]
Heart, %	0.517 ± 0.073	0.595 ± 0.090 [6]
Brain, %	3.877 ± 0.332	4.771 ± 0.656 [5]
Thymus, %	0.163 ± 0.046	0.199 ± 0.632 [6]

[1] Eighteen rats/group.
[2] % of body weight.
[3] Mean ± one SD.
[4] $P = < 0.001$.
[5] $P = < 0.05$.
[6] $P = < 0.01$.

Part 2. Reproductive performance of the females was not significantly different from that of those in part 1; however, there were marked differences in survival rate of the 4 groups of offspring. The highest survival rate, 81%, was in the group born to and suckled by dams receiving the control diet, whereas the survival rate of rats subjected to protein restriction during gestation only was 52%. None of the rats subjected to protein restriction during both gestation and during the suckling period survived more than 7 days. Those subjected to protein restriction during the suckling period only, but not during gestation, survived 11 days or less.

Mean feed intake of the 2 groups of pregnant animals was 17.7 ± 0.86 g/day for the controls and 16.0 ± 0.98 g/day for the protein-restricted group. The difference was not significant. However, examination of the daily food intake showed a difference in the intakes during the first and last halves of pregnancy (fig. 1). Food intake of the low protein group exceeded that of the controls for the first 10 days. During the last half of pregnancy, the food intake of the low protein group fell below that of the controls. There was a sharp decrease in intake in both groups as pregnancy progressed. This decrease began on day 18 of gestation in the control group and day 16 in the low protein group in which the decrease was more severe.

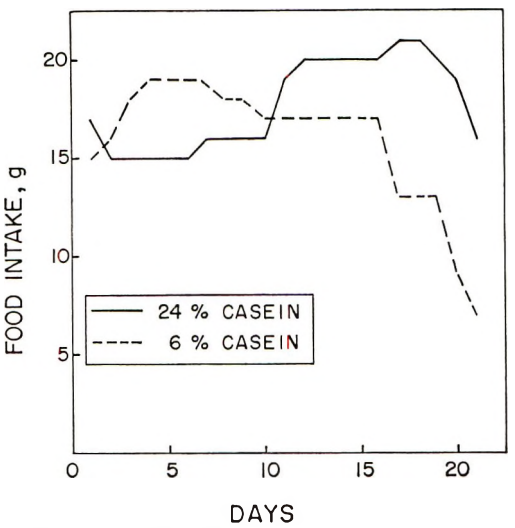

Fig. 1 Food intake of pregnant rats fed semi-purified diets containing 24% casein or 6% casein.

Data on ages of opening of ears and eyes, eruption of teeth, and development of ability to turn over and of air righting showed no difference between the 2 groups.

Body weights of animals that survived to weaning age, 22 days, and of adult animals, at 76 days, are shown in table 3. Data were not obtained for adult females since most of that group developed a viral respiratory infection and were destroyed. The data indicate that offspring of animals fed the 6% casein diet during pregnancy had a significantly lower body weight at both ages. The former differences in crown-rump length and organ weight-to-body weight ratios no longer existed at either age.

The ratio of crown-rump length to body weight was calculated giving the results shown in table 4. The data show a difference in body proportions. Newborn pups from low protein-fed dams were longer in proportion to body weight than were the pups in the control group. However, in general appearance they did not appear emaciated. Thus, it appears that prenatal protein deficiency affects body weight to a greater degree than it affects crown-rump length and the deficient animals are leaner than are the controls.

Part 3. There was no significant difference in any parameter between young of the pair-fed group and the control group at newborn, weaning, or 40-day ages. In litter size, survival rate, body weight, and organ weight-to-body weight ratios, young

TABLE 3

Effect of low protein diet during pregnancy on postnatal growth of offspring

	24% Casein diet		6% Casein diet	
	Males	Females	Males	Females
		Weanling rats		
No. of rats	15	11	10	6
Body wt, g	56.4 ± 6.81 [1]	49.2 ± 4.97	49.5 ± 4.36 [2]	44.5 ± 3.51 [3]
		Adults (76 days)		
No. of rats	15	—	10	—
Body wt, g	368 ± 29	—	323 ± 36	—

[1] Mean ± one SD.
[2] $P = < 0.01$.
[3] $P = < 0.05$.

TABLE 4

Crown-rump length-to-body weight ratios at birth and at weaning age

	24% Casein diet		6% Casein diet	
	Males	Females	Males	Females
	mm/g	*mm/g*	*mm/g*	*mm/g*
Newborn	7.10 ± 0.86 [1]	7.56 ± 0.62	8.37 ± 2.32 [2]	9.47 ± 1.14 [3]
Weanling	1.96 ± 0.14	1.89 ± 0.20	2.01 ± 0.10	2.03 ± 0.17

[1] Mean ± one SD.
[2] $P = < 0.05$.
[3] $P = < 0.001$.

of pair-fed females were indistinguishable from controls.

At birth the young of low protein-fed females showed essentially the same differences from the control group as indicated in part 1. However, the survival rate of these young was much reduced, compared with the animals in part 2. It was possible to rear only 2 survivors to weaning from 22 litters of 8 pups each.

DISCUSSION

It is commonly accepted that rats fed protein-deficient diets decrease their total food intake. This has the effect of producing a protein-calorie deficit. In the present study, however, the decrease in food intake in the restricted group occurred only after 10 days of pregnancy. This decrease was not sufficiently marked to produce a statistically significant reduction in total food intake over the course of the pregnancies. In any case, since litters produced by pair-fed dams in part 3 of this study were indistinguishable from control litters by all criteria used, it is believed that the small difference in total food intake was without effect on the young. It is concluded, therefore, that the effects on the young of the diet used are the results of protein restriction, not of a protein- calorie deficit.

In view of the weight loss in the pair-fed females, it appears that maternal tissue was used to compensate for the slightly decreased food intake. However, despite marked weight loss, pregnant rats fed the restricted protein diet were unable to provide adequately for their young even at the expense of their own body tissue.

The data on reproductive performance of the dams are in general agreement with those of Nelson and Evans (4). The results indicate that a 6% casein diet maintains normal reproduction in the rat if number of litters produced and number of pups per liter are the criteria. However, the offspring have a lower mean body weight and a severely decreased survival rate.

The evidence indicates a pronounced effect of maternal protein restriction on the young during gestation. These effects are obvious in the newborn. The pups were less active, were darker in color, and showed multiple and extensive subcutaneous hematomas at birth. The hematomas were localized mainly on the crown of the head, the muzzle, and the shoulders.

The effect of the protein restriction during gestation is also shown by the effects on organ weight-to-body weight ratios at birth. If the effect of the protein restriction on body size were the same on all parts of the body, it would be expected that the organ weight-to-body weight ratios would not be significantly different from those for the controls. However, in newborn offspring of protein-deficient females in this study, the liver and kidneys were a smaller proportion of total body weight than in the controls, while the heart, brain, and thymus were a larger relative proportion. This indicates that the protein-restricted diet during pregnancy has a greater effect on the liver and kidney and lesser effect on the heart, brain, and thymus, than on the body as a whole. Another effect is depressed skeletal development as evidenced by the decreased crown-rump length.

The relationship of the difference in size to possible differences in degree of maturation and functional ability is, at present, unknown. Potter and Thierstein (14) and Hammond (15) state that organ size is related to degree of maturation. It is interesting to speculate whether the sizes of organs such as the kidneys and liver of the newborn animals in this study are related to the functional ability of these organs and to survival rate. Studies on kidney and liver morphology and function presently underway should help clarify this point.

Maternal protein restriction appears to affect the young postnatally in several ways. The 100% mortality in the young of both control and protein-restricted dams that are suckled by deficient females indicates that lactation failure in the dam is the primary cause of early postpartum death in these groups. However, it seems clear that lactation failure is not the basic problem when deficient young are suckled by females fed the control diet since pups produced by control females and suckled in the same litters have much better survival and growth rates.

The mortality rate and decreased growth rate in this group might be attributed to the effects of competition in the litter. The presence of pups from both control and low protein dams in a single litter during the suckling period may have acted to deprive the low protein pups of milk. Since these pups were smaller and less vigorous, they were presumably less prepared to compete with their littermates for milk. It was postulated, therefore, that when low protein pups did not have to compete with larger, stronger littermates, their survival rate and growth rate would improve. It was unexpected, therefore, to find that, on the contrary, the survival rate dropped sharply.

One possible reason for this effect is that the dam rejected the litter. However, it has been our experience that, with rats, foster mothers accept another litter with no difficulty if the transfer is made soon after parturition. It has also been observed that when a dam rejects a litter, the dam either eats the litter or makes no attempt to make a nest, and hence the pups remain scattered throughout the cage. These young are not fed and invariably die in 48 hours or less. This type of behavior occurred in approximately 12% of the litters in part 3 of this study and occurred with equal frequency in control dams suckling their own young and those suckling foster young.

The behavior of the remainder of the foster mothers was similar to that of normal controls. They were observed to make a nest and gather the young into it. Milk could usually be detected in many of the young for varying periods. Usually, however, the pups died over a period of about a week.

It is suggested, therefore, that the low protein pups are unable to suckle sufficiently to maintain the flow of an adequate milk supply. In the mixed litters, then, it appears that the presence of control young improves survival and growth rate of their deficient littermates by stimulating adequate milk flow from the dam. While competition within the litter may be a factor, the condition of the young at birth, consequent to restricted prenatal nutrition, affects the postnatal development by affecting the ability of the pups to obtain nourishment.

It may be considered, therefore, that the effects on the young of maternal protein restriction during pregnancy may be the sum of those effects noted at birth and their direct sequelae, which we might call "primary effects," plus those effects consequent to the inability of the pup to suckle sufficiently, or "secondary effects."

It is probably safe to assume that, with adequate feeding postnatally, the secondary effects could be prevented. Under these circumstances it would then be possible to study the relative reversibility or permanence of the primary effects. The use of the artificial feeding techniques described by Dymsza et al. (16) may hold promise provided other variables inherent in the separation of pups from the dam can be adequately controlled. In the absence of assured adequate feeding postnatally, it cannot be said unequivocally that the growth failure and other effects reported in this paper are irreversible.

It has been reported, in studies of general malnutrition, that recovery is less likely when the malnutrition occurs very early (17, 18). Winick and Noble (18) suggest that malnutrition interferes with cell division. If we assume that this also occurs in protein deficiency, then it can be expected that newborn rats subjected to protein deprivation during gestation are permanently retarded in growth.

It is also possible that prenatal protein deficit results in malabsorption or metabolic aberrations in the young which interfere with growth. It has been suggested that this is the case in prenatal restriction of total food intake (19).

Further investigation now in progress should help clarify the mechanism of growth retardation produced by prenatal protein deficit.

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Intestinal Carbohydrase Activity and Carbohydrate Utilization in Mature Sheep [1]

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ABSTRACT Studies were undertaken to determine the levels of maltase, lactase, sucrase, amylase, and cellobiase in the mucosa of the duodenum, jejunum, and ileum and the rate of glucose absorption and maltose and starch utilization in the duodenum, jejunum, ileum, cecum, and colon of mature sheep. Carbohydrase assays show that maltase, amylase, sucrase, lactase, and cellobiase activities were higher in the jejunum than in the ileum and duodenum, and that the maltase activity was higher than the rest of the enzymes in each area. Glucose absorption from isolated segments of the jejunum, ileum, and colon of mature, anesthetized sheep increased for the first 7 minutes after substrate injection into the intestinal lumen, while the rate of glucose absorption from the cecum decreased. After 7 minutes, the rate of glucose absorption from the 4 intestinal areas was relatively constant. Rate of glucose absorption was most rapid in the jejunum, > cecum, > ileum, and > colon. Maltose utilization was greater in the jejunum than in the ileum and no detectable maltose utilization occurred in the cecum and colon. No starch utilization was detected in the isolated intestinal segments void of pancreatic amylase.

It has been established that microbial activity in the rumen constitutes the major digestion of carbohydrates in the mature ruminant and that digestion of carbohydrates in the small intestine is of secondary importance. Recent evidence shows that large amounts of free sugars and polysaccharides enter the abomasum when an animal is maintained with a high concentrate diet (1, 2). Barhydt and Dye (3) reported that the abomasal contents of calves fed a 5:1 hay-to-grain ration contained 108 mg of hydrolyzable polysaccharides per g dry matter of which 60% were pentose hemicelluoses and 40% were hexose polysaccharides.

The present research was conducted to determine: 1) the levels of maltase, lactase, sucrase, cellobiase, and amylase in the duodenum, jejunum, and ileum; 2) the rate of glucose absorption in the jejunum, ileum, cecum, and colon; 3) the utilization of maltose and starch in the jejunum, ileum, cecum, and colon of the mature sheep.

EXPERIMENTAL PROCEDURE

Enzyme assay. Segments of the small intestine from the duodenal (first 20 cm caudal to the pylorus), jejunal (middle of the small intestine), and ileal (first 20 cm cranial to the ileo-cecal junction) areas were obtained from 7 yearling, crossbred wethers (43 kg body weight) within 5 minutes after slaughter. The lumen of the intestine was carefully washed free of intestinal contents with cold saline and processed according to the general procedure of Dahlqvist (4). Substrate solutions, buffer solutions, and incubation procedure used were also those of Dahlqvist (4). A soluble starch suspension was prepared using a quantity of starch equivalent in weight to the quantity of maltose used to prepare a 0.056 M maltose solution.

Since all substrates yielded at least one molecule of glucose per molecule of substrate hydrolyzed, the glucose yield was used to determine enzymatic activity.

Glucose oxidase [4] was prepared (4) and used to determine the glucose produced during substrate hydrolysis. The color concentration resulting from the enzyme action on glucose was determined on a Beckman DU spectrophotometer and compared with glucose standards containing zero to 50 μg of glucose.

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[4] Obtained from Worthington Biochemical Company, Freehold, New Jersey.

Surgical preparation. The animals used in these experiments had been maintained with 227-g concentrates daily and hay ad libitum. After fasting for approximately 12 hours, the wethers were anesthetized using pentobarbital sodium or an electrical anesthesia device. A 0.154 M NaCl solution containing glucagon-free insulin [5] was administered intravenously at the rate of 0.75 units insulin/kg of body weight/hour.

A 20- to 30-cm incision was made in the right abdominal wall about 15 cm lateral to the midline. The sections of intestine to be used were flushed with 30 ml of warm saline with a hypodermic needle and syringe. Isolated intestinal segments were prepared by placing ligatures approximately 75 cm apart along the flushed section of intestine. The mesenteric vein was separated from its associate artery and 2 ligatures were placed loosely around the vein. (All veins which did not carry blood from the isolated segment, but which joined the mesenteric vein below the point selected for cannulation were ligated to prevent the blood from entering the selected vein.)

The animal was then heparinized with an injection of 10 cm^3 of heparin [6] into the right jugular vein. The posterior ligature on the mesenteric vein (distal to the intestine) was tied tightly and a small incision was made in the vein between the tightened ligature and the anterior loose ligature. A cannula made from PE 200 polyethylene tubing, flared on one end by heating, was placed through the incision and into the lumen of the vein. The flared end was pushed through the lumen, toward the intestinal segment, past the loose ligature and that ligature was tied tightly to hold the cannula in place to prevent leakage of blood around the cannula. This preparation (fig. 1), with minor adjustments, was made in all parts of the intestinal tract.

Glucose absorption and carbohydrate utilization. Four observations for glucose absorption were made in the jejunum and ileum with only 2 observations made in the cecum and colon, and each observation represented a different sheep. Three observations were made on the effects of ouabain and iodoacetate on glucose absorption in the jejunum; 2 observations were made on the effects of ouabain and iodoacetate on glucose absorption in the ileum; and one observation was made on the effect of iodoacetate on glucose absorption in the cecum. Maltose and starch utilization was determined by 4 observations in each of the areas, jejunum, ileum, cecum, and colon.

Glucose substrate solutions for the absorption studies were prepared by combining 3 parts KCl solution (12.8 g/liter) with 2 parts glucose solution (54 g/liter) and 5 parts NaCl solution (10 g/liter). Maltose substrate solution was prepared by combining 3 parts KCl solution (above) with 2 parts maltose solution (54 g/liter) and 5 parts NaCl solution (11.8 g/liter). Starch substrate was made by combining 3 parts KCl solution (above) with 2 parts soluble starch suspension (54 g/liter) and 5 parts NaCl solution (13.8 g/liter). The osmotic pressure of all three substrate solutions was equivalent to 320 milliosmols which is isosmotic with sheep blood.

Ten milliliters of a substrate solution were injected into the lumen of the isolated segment used for the absorption or utilization study of that particular substrate. One hundred microcuries of uniformly labeled ^{14}C-glucose were injected simultaneously with the glucose substrate solution.

Immediately after the substrates were placed into the isolated segments, blood from the cannulated veins was collected continuously for 30 minutes, allowing 3 minutes for each sample. One-half milliliter of ouabain (7×10^{-2} M) and 0.5 ml of iodoacetate (4×10^{-2} M) were injected into the lumen of the segments containing glucose in 5 sheep and in these cases collections of blood were continued as long as an hour.

Blood analysis. Plasma proteins were precipitated by a conventional procedure using $ZnSO_4$ and NaOH. The protein precipitate was filtered out using Whatman no. 42 filter paper.

The level of glucose was determined by either glucose oxidase analysis or by ra-

[5] Obtained from Eli Lilly Company, Indianapolis, Indiana.

[6] Obtained from Abbott Laboratories, North Chicago, Illinois.

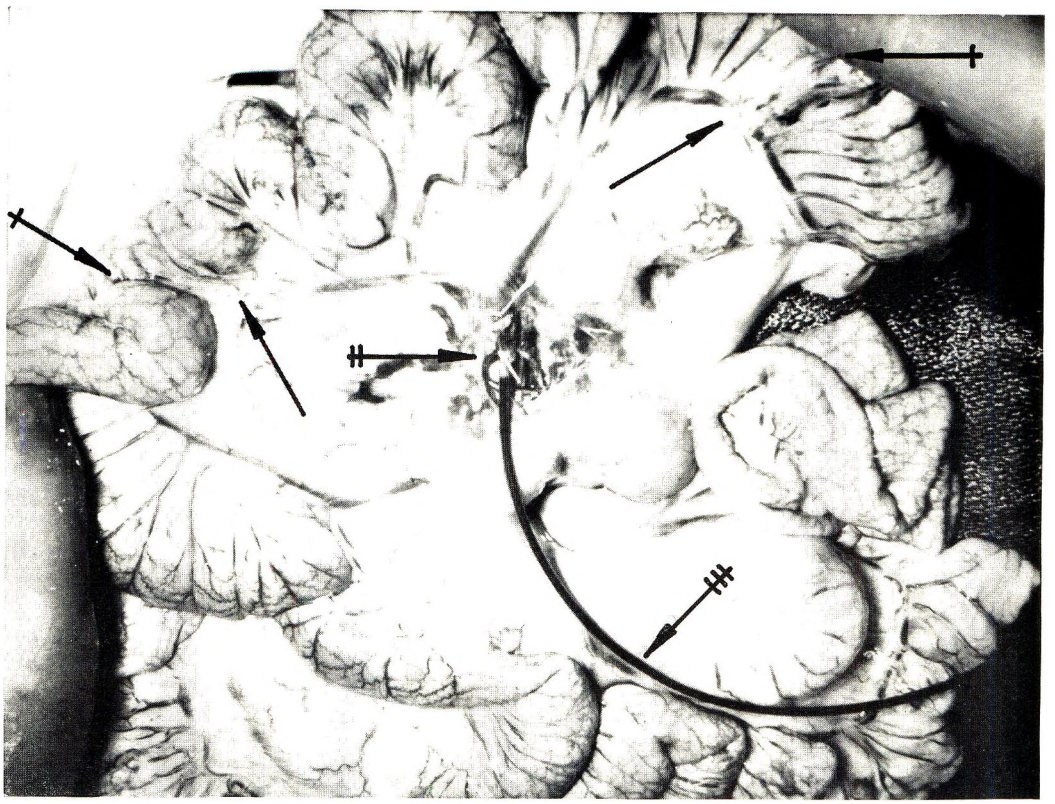

Fig. 1 Surgical preparation of the small intestine. Legend: ⟶ ligatures isolating mesenteric vein draining ligated intestinal segment; ⟶ ligatures isolating intestinal segment; ⟶ point of cannula entry into mesenteric vein; ⟶ PE 200 polyethylene tubing (cannula).

diochemical analysis for glucose in the plasma. The difference in the glucose level or ^{14}C activity in the plasma draining an intestinal segment containing a glucose injection and the glucose level or ^{14}C activity in the plasma draining an intestinal segment containing only an NaCl solution was considered a measure of glucose absorption. Increased glucose level in plasma draining the isolated segments containing maltose and starch was used to determine maltose and starch utilization.

Radiochemical analysis was made by combining 1 ml of the plasma filtrate and 14 ml of dioxane scintillator fluid and counting in a 2-channel liquid scintillation counter. The ^{14}C activity of the unknown samples was compared with plasma samples "spiked" with known quantities of ^{14}C-labeled glucose. Results of the radiochemical and enzymatic analyses were expressed in milligrams of glucose absorbed and milligrams of substrate used in the case of maltose and starch substrates.

The identity of the major compounds containing ^{14}C in the plasma was determined by the combined techniques of paper chromatography and autoradiography. After drying, the paper strips were placed on x-ray film for 16 days. Position of the labeled compounds on the film strips was compared with the location of stable compounds chromatographed in the same way.

RESULTS AND DISCUSSION

Enzyme assay. The mucosa of the jejunum of the mature sheep contained the greatest concentration of all enzymes assayed (table 1) and the duodenum contained the least of all with the exception

TABLE 1

Enzyme activity in the mucosa of the duodenum, jejunum, and ileum of mature sheep [1]

Intestinal segment	Maltase	Amylase [2]	Lactase	Sucrase	Cellobiase
		enzyme units [3] $\times$ *10*			
Duodenum	2.15[b]	0.65[b]	1.10[a,b]	0.25	0.20
Jejunum	8.05[a]	3.23[a]	2.07[a]	0.05	0.45
Ileum	5.00[a,b]	1.43[a,b]	0.70[b]	0.55	0.25
Significance	$P < 0.05$	$P < 0.05$	$P < 0.05$	ns	ns

[1] All means underlined not significantly different $P < 0.05$.
[2] Amylase expressed on a maltase activity basis.
[3] Enzyme unit = 1 μmole substrate hydrolyzed/min/g mucosal cells (wet weight).
[4] All means in column with same superscript are not significantly different.

of lactase activity which was higher in the duodenum than in the ileum. Maltase activity was greater in each of the areas than the activities of the other enzymes and amylase was the next most abundant enzyme in the jejunum and ileum. The hydrolytic enzymes from the jejunal mucosa make this area of the intestine more important than the duodenum and ileum for the hydrolysis of intestinal carbohydrates (table 1). A relatively large amount of maltose is produced by the amylase activity and the amylase assay and complete hydrolysis of starch in the intestine are both dependent on maltase activity. However, the quantity of maltase present indicated that maltase was not a limiting factor in the amylase assay. The importance of pancreatic amylase, which was not assayed, in the hydrolysis of starch to maltose cannot be denied. Since the pancreatic duct enters the small intestine caudal to the point of duodenal enzyme assay, it was not unexpected to find the greater concentrations of maltase caudal to the opening of the duct because that area of the small intestine would contain the greater quantity of maltose resulting from starch hydrolysis. Since the assays were made in the cranial 20 cm of the duodenum, this may not be a true picture of the activities in other portions of the duodenum.

The higher activities of maltase and amylase compared with lactase in the jejunum and ileum observed here are in agreement with the work of Dollar and Porter (5, 6) and Walker (7). These results along with those of Dollar and Porter (5, 6) indicate an adaptation in the hydrolytic enzyme synthesis resulting from increasing age or change in diet (higher maltose and starch levels in the small intestine) or both. Substrate levels in the intestine appear to have more influence on hydrolytic enzyme synthesis since the lactase level of the intestine decreases and the levels of maltase and amylase increase when a milk diet is replaced with a concentrate diet (5, 7). Another factor lending support to the theory is that starch, maltose, and sucrose are present in much higher levels in the intestinal tract of the nonruminant than the ruminant and the corresponding hydrolytic enzyme activities are higher in the nonruminant (7). The enzyme activities found in the small intestine of these mature sheep are much lower (0.8 units compared with 13–54 units of maltase, 0.1 unit compared with 6–17 units of sucrase, etc.) than the activities of the jejunum and ileum of humans observed by Dahlqvist (4).

Glucose absorption. Plasma glucose levels of the sheep used for the in vivo studies increased continuously from the initiation of anesthesia to the completion of the study on each animal (fig. 2). Although the effects of the high glucose level in the plasma on glucose absorption from the intestine was not investigated, a level of glucose in the plasma which approached normal physiological conditions was considered advantageous. Adrenalectomy of the sheep lowered the plasma glucose level slightly (fig. 2); however, it was obvious that other factor(s) played a much greater role in increasing plasma glucose levels

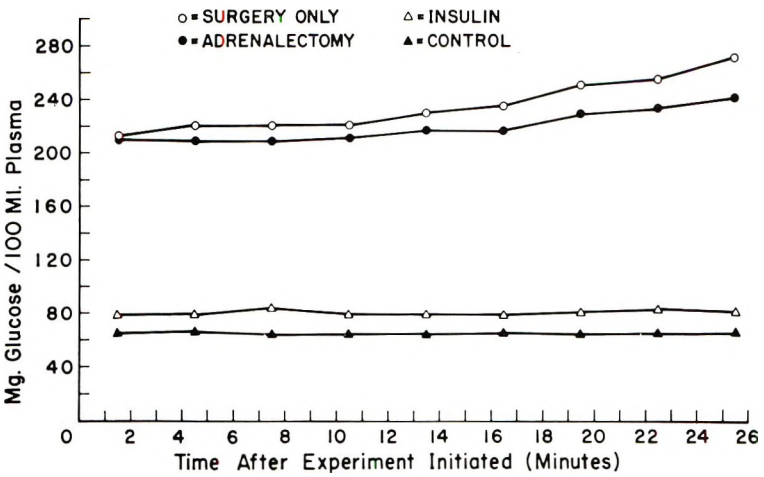

Fig. 2 Effect of adrenalectomy and insulin treatment on hyperglycemic conditions resulting from anesthesia and surgical shock. Abdominal surgery was performed on all groups except the controls.

under surgical conditions. Galansino et al. (8) suggested that more than one type of hyperglycemic substance may be produced in the area supplied by the pancreatic artery. Since removal of the pancreas was considered too intricate, an insulin injection was used to alleviate the hyperglycemic condition. An insulin injection of 0.75 units/kg body weight/hour held the plasma glucose level near normal (fig. 2). The effect, if any, of insulin injection on glucose absorption appears slight since Sols et al. (9) obtained an increase in glucose absorption rate of 8% in the first 30 minutes after a single injection of 2 units of insulin/100 g body weight in rats. Only 0.15 units of insulin/100 g body weight were used during the entire experimental period in this investigation.

At no time during the absorption studies did plasma glucose reach a level which was considered a limiting factor for glucose absorption. The intestines were constantly bathed with warm saline to prevent dehydration and vasoconstriction caused by loss of heat. Although blood flow decreased during the 30-minute collection periods, the decline in flow was small and considered relatively unimportant. At no time during the experimental period did the intestines appear to be in an anoxic condition and the PE 200 tubing was considered sufficient to maintain normal blood drainage. Blood flow from the cecum and colon per unit serosal area was greater than the flow from the jejunum and ileum.

Direct collection of the blood flowing from the isolated intestinal segments was used since the glucose absorbed did not reach the liver and there was no dilution from blood draining from other areas of the body. A quantitative evaluation of the absorption was made, and the glucose level of the arterial blood supplying the intestinal area remained constant.

By injecting a constant quantity and concentration of glucose (108 mg of glucose in 10 ml of solution), expressing the absorption per unit area, and not approaching the limiting capacity of the plasma, the rate of glucose absorption was calculated (table 2). The rate of glucose absorption from all areas of the intestinal tract was less variable from the 7.5- to the 16.5-minute sampling period. Although there was a tendency toward a slight decline in some instances, the absortpion rates remained nearly the same after 10.5 minutes.

Glucose absorption from the jejunum was about 3 times faster than absorption from the ileum and colon. Larsen et al. (10) showed that glucose was absorbed more rapidly from the jejunum than the

TABLE 2

Maltose hydrolysis and absorption of glucose in intestinal segments

Time after injection	From glucose				From maltose	
	Jejunum	Ileum	Cecum	Colon	Jejunum	Ileum
min	*μg glucose absorbed/100 cm²/min*					
1.5	62 ± 31 [1]	18 ± 7	332 ± 189	16 ± 1	20 ± 8	1 ± 0
4.5	146 ± 67	29 ± 12	237 ± 86	26 ± 14	27 ± 8	1 ± 0
7.5	150 ± 63	40 ± 28	147 ± 38	27 ± 14	26 ± 18	3 ± 1
10.5	157 ± 67	47 ± 36	121 ± 38	20 ± 20	21 ± 11	5 ± 1
13.5	152 ± 87	51 ± 37	140 ± 89	28 ± 10	21 ± 19	6 ± 5
16.5	148 ± 97	58 ± 40	102 ± 54	27 ± 10	20 ± 14	5 ± 5
19.5	160 ± 110	59 ± 40	103 ± 5	8 ± 1	19 ± 10	7 ± 5
22.5	136 ± 107	54 ± 38	80 ± 22	15 ± 20	19 ± 16	8 ± 5
25.5	124 ± 81	54 ± 36	83 ± 2	15 ± 38	19 ± 14	7 ± 5

[1] Mean ± SD.

ileum of isolated gut segments in 8-month-old calves; however, no plateau in absorption rate from the jejunum was reached in their studies. Fisher and Parsons (11) calculated that the rate of glucose absorption per unit area of mucosal surface was 3 times greater in the jejunum than in the ileum of the rat. Since the absorption rates in the present study were not based on mucosal surface area, a discrepancy between the data obtained and the data of Fisher and Parsons (11) could result if the mucosal surface area per unit serosal area in the jejunum was different from that in the ileum.

The rate of glucose absorption from the colon was low which agrees with the data of Larsen et al. (10). That the absorption rate from the cecum decreased rapidly during the first 7 minutes while the absorption rate increased in the other 3 areas during the same period of time is not understood. With the exception of the jejunum, the rate of glucose absorption from the cecum was greater than the rate from the other intestinal areas. This is in contrast with other data (11) which indicate that absorption from the ileum is greater than absorption from the cecum. There may have been a rapid movement of water across the cecal wall which carried the glucose into the blood at a rapid rate under the experimental conditions. However, similar conditions in the colon did not lead to similar results. Another possibility is that the cecal wall is more permeable than the walls of the ileum and colon.

Starch and maltose utilization. During the 30-minute experimental period no increased plasma glucose level resulting from starch hydrolysis was detected in any area of the intestinal tract. Huber et al. (12) reported that starch utilization from the ruminant intestine was slow. It is possible that a small amount of hydrolysis took place and went undetected. Since the isolated segments were flushed before the substrate injection, no pancreatic amylase was present and any hydrolysis of starch was dependent only on intestinal mucosal amylase. Starch was subjected to pancreatic amylase in the experiments of Huber et al. (12) and this may account for the difference in the results of the 2 experiments.

Maltose utilization was more rapid in the jejunum than in the ileum (table 2). In vivo utilization of maltose in the jejunum was about twice that in the ileum and corresponded closely with the enzyme assay of 62% maltase/g of mucosal cells in the ileum compared with the activity in the jejunum. Increased blood glucose due to the presence of maltose in the small intestine of the older ruminant has been demonstrated (5, 12, 13); however, no distinction was made between the jejunum and ileum. No maltose utilization was observed in flushed segments of the cecum and colon.

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A Controlled Environment System for New Trace Element Deficiencies [1,2]

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ABSTRACT An all-plastic, controlled environment system is described for animals such as rats. The system, consisting of an air filter assembly, isolator, caging, and accessories, minimizes extraneous trace element contamination. The isolator, a modification of germfree isolator models, can hold 25 rats in groups of 5/cage. Weanling rats fed laboratory ration in the controlled, but not germfree, environment grew as well as control animals maintained outside of the isolator in metal cages under conventional conditions. A new trace element deficiency was observed in rats fed amino acid diets supplemented with the known required trace elements *inside* the controlled environment. Inhibition of growth, seborrhea, and pronounced alopecia developed within 1 to 3 weeks. Death occurred within 2 to 6 weeks. Conventional outside controls fed the same diet grew more rapidly and showed no deficiency symptoms. Supplementation of 0.4% yeast ash prevented the deficiency symptoms and improved growth. The results indicate that the amino acid diet lacks unidentified inorganic trace factors which are supplied by the environment under conventional conditions of animal experimentation. The described technique coupled with modern, sensitive methods of analysis permits rigorous and definitive experiments on the essentiality of specific trace elements.

After detecting unexpected nutritional roles for the elements selenium (1) and chromium (2), it was decided to develop a new, systematic approach to the production of trace element deficiency diseases in laboratory animals.[4] Progress in the recognition of new trace element requirements depends largely on experimental designs which prevent access of the animal to the agent being studied. Some elements are physiologically effective at microgram or sub-microgram levels; for these the term ultra-trace element has been suggested (3, 4). Inadvertent supplementation of such elements to experimental animals can occur not only from impurities of diet and water, but also from the environment. The attempt, therefore, to demonstrate effects of elements for which biological functions have not yet been discovered requires the exclusion as possible sources of impurities of caging, food cups, water bottles, handling, and air.

To avoid trace element contamination from the air and through handling we have taken advantage of recent developments in germfree incubator techniques. An *all plastic* isolator was devised. Plastics also were chosen as fabricating materials for cages and accessories, after elimination of galvanization by platinum, polonium, or other precious metals from consideration as a means of avoiding trace element contamination. Plastics are rather easily handled and can be obtained in relatively pure form. Even though they contain small and often irreducible amounts of trace elements, these are considered to be tightly bound. They are not readily liberated and presumably biologically inert.[5] Plastic cages, water bottles, food cups, and

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[2] Some of these data were presented to the American Institute of Nutrition at the 50th annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1966, (Federation Proc., 25: 432, 1966, and to the VIIth International Congress of Nutrition at Hamburg, Germany (abstracts of papers, 1966, p. 297).

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[4] Initial planning and preliminary experiments were carried out 1961–63 at the former Section of Experimental Liver Diseases, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

[5] However, there are large differences between plastics; some of them contain considerable amounts of certain metallic elements. Others, like polyvinyl chloride, contain organometallic compounds such as organotins as stabilizers. These may be biologically active; they even can exert toxic effects.

a simple weighing device are described below. The resulting trace element-controlled environment system is free of metal, glass, and rubber components, with the exception of the air filter which contains glass fiber media.

In standard, semipurified diets such as casein rations the protein is a main source of contaminants. It has the capacity to bind small amounts of metals and other elements strongly; convenient methods for their removal do not exist. Starch is another dietary ingredient often contaminated with trace factors, while commercial sucrose is in certain respects a material of great purity. Diets used in our studies were based on a mixture of L-amino acids instead of protein, and contained sucrose as the source of carbohydrate.

In addition to protein and carbohydrate, all other components of a diet, of course, must also be taken into consideration as potential sources of trace element impurities. For example, reagent grade inorganic chemicals, the constituents of salt mixtures, are often heavily contaminated with trace elements of biological interest. No provisions were made to purify commercially obtained chemicals or other dietary constituents for the experiments reported in this paper.

Rats develop a new trace element deficiency disease when maintained with amino acid diets in the trace element-controlled system. Animals fed the same diets kept under conventional conditions outside of the incubator are rather normal. Inside the isolator, growth is lacking, and signs of seborrhea and pronounced alopecia develop. Death from the deficiency can occur within 2 to 6 weeks.

The present paper describes the design and operation of the trace element-con-

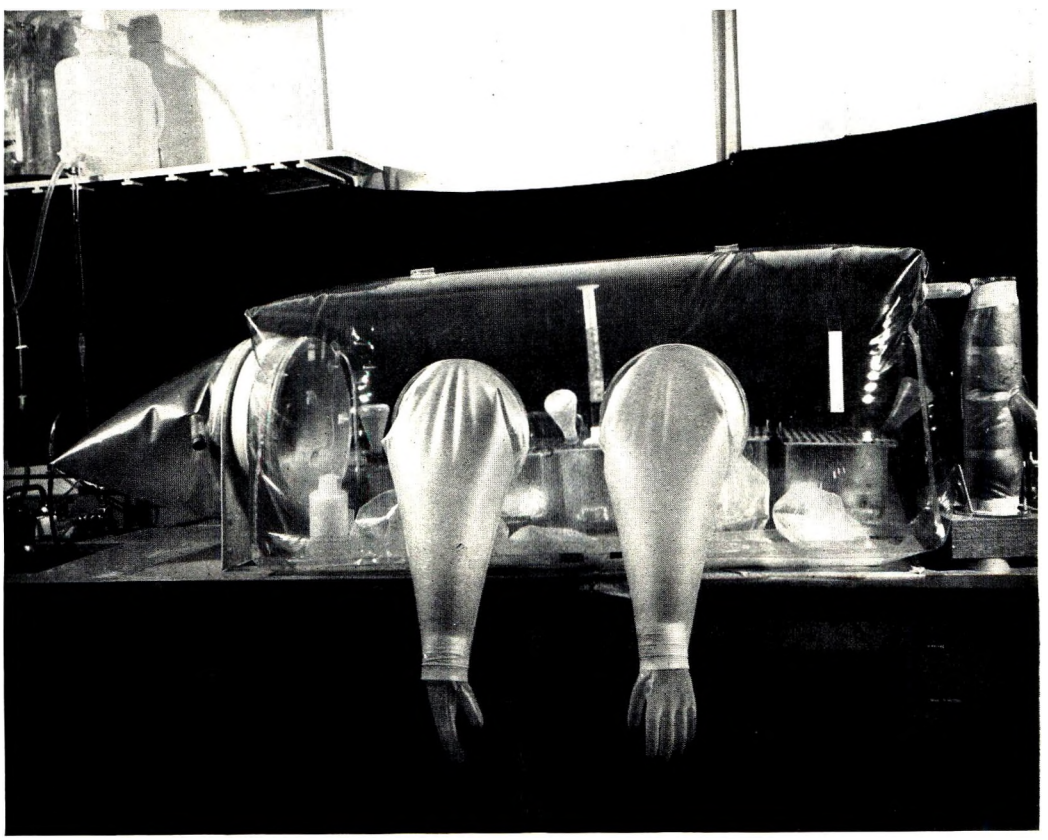

Fig. 1 Controlled environment plastic isolator.

trolled system and the new deficiency disease.

METHODS

Controlled environment isolator. To provide a barrier against airborne contamination, a thin film isolator was designed which is free of metal, rubber, and glass (fig. 1). The basic isolator is a variation of that described by Trexler (5). The dimensions are 61 × 61 × 152 cm. Thin vinyl sleeves are thermally sealed directly to the isolator. They are fitted with replaceable wrist length vinyl gloves. The gloves are attached to the sleeves by vinyl tape. Molded fiberglass is used for the door housing, and methyl-metacrylate plastic for the door proper.[6] Vinyl tubing serves as a gasket for the door. Nylon bolts fasten the door to the housing.

Air supply. The air supply assembly consist of a blower and a filter. A slight positive air pressure (< 12 mm water) is maintained within the isolator to assure proper ventilation and to keep the isolator in shape. A small metal with fractional-horsepower motor can be used after spraying of all metal surfaces with a plastic coating. Also satisfactory was a non-metallic blower assembly from a laboratory hood which was adapted to supply air to the incubator.[7] It was connected to the air filter by a vinyl coated vacuum hose.

A circular plastic frame forms the core of the air filter assembly (fig. 2). Glass fiber media[8] was wrapped 4 layers thick around this frame. The filter material was taped in place and covered by plastic screen material. A vinyl cover completed the unit and made it air-tight. Air passing through such a filter is free of dust particles larger than 0.35 μ in size as well as all demonstrable organisms, including bacteria, yeasts, and molds.

Animal cages. The most difficult part of this project was the construction of completely plastic animal cages which would permit free circulation of air, prevent condensation, and provide an open bottom for the elimination of feces and urine. Commercial acrylic plastic cages[9] having a floor area of 450 cm² were modified as shown in figure 3. A false bottom was fabricated from 5-mm methyl-metac-

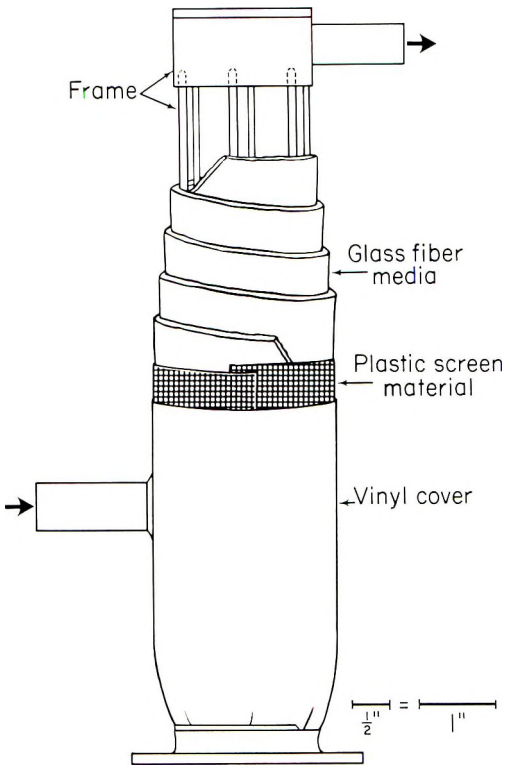

Fig. 2 Air filter assembly.

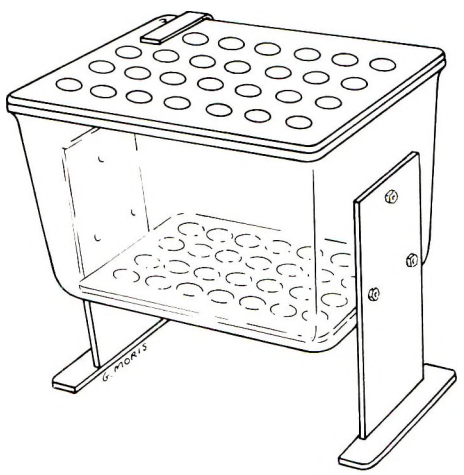

Fig. 3 Plastic animal cage.

[6] Manufactured by the Standard Safety Equipment Company, Palatine, Illinois. Their assistance in technical details is acknowledged.
[7] Labconco Hood Model 28, Laboratory Construction Company, Kansas City, Missouri.
[8] FG 50, Standard Safety Equipment Company, Palatine, Illinois.
[9] Model 110, Keystone Plastics, Media, Pennsylvania.

rylate plastic (Plexiglas), and fitted inside the cage. A series of 1.3-cm holes were drilled as close together as possible in the false bottom. The lid was made in a similar fashion. Nylon screws hold the top in place. The cage is raised approximately 10 cm by bolting adjustable plastic legs to both ends. A foot is cemented to the bottom of each leg to stabilize the cage and prevent puncture of the bottom of the isolator. A suitable cement is prepared by dissolving chips of acrylic palstic in chloroform. Each cage houses 5 animals. The isolator easily accomodates 5 such cages.

Food cups. Food cups were made by cutting off the neck of 250–ml polypropylene Erlenmeyer flasks, yielding cups 5 cm high and 5 cm wide at the top. The sloping sides of these cups aid in avoiding waste. To further present spillage, five 1.3-cm holes were drilled in round plastic discs which were placed on top of the food in the cup.

Water bottles. Polypropylene Erlenmeyer flasks (250-ml) served as water containers. Vinyl stoppers with polypropylene drinking tubes completed the unit.

Weighing device. A simple and sufficiently accurate weighing device was made using 2 polycarbonate plastic graduated cylinders (fig. 4). A 100-ml cylinder was stoppered, inverted, and placed into a 250-ml cylinder. Sufficient water was added to the larger cylinder to allow the inverted cylinder to float freely. The base of the smaller cylinder served as a perch for the animal being weighed. The weight of the rat was read directly from the larger graduated cylinder since a floating body displaces its own weight of water. The device weighs accurately to the nearest gram.

Cleaning. Before introduction of the animals, the isolator was washed thoroughly with a detergent, followed by spraying with a 2% peracetic acid solution. The system, with filter in place, remained closed overnight and was then rinsed several times with distilled, deionized water having a conductivity greater than 1 million ohms. The supporting equipment was cleaned in the same manner.

Animal experiments. Although the isolators received filtered air and were chemically sterilized the experiments were not carried out under germfree conditions. Neither the animals nor the diet were sterile. Weanling male rats of the Fischer 344 strain were used in all experiments. They are highly inbred and grow at a relatively slow rate. The basal amino acid diet used in these studies is shown in table 1. This ration is based on a mixture of L-amino acids as nearly optimal as feasible at the present state of knowledge. The composition of this mixture is patterned after those described more recently as optimal or near optimal (6–8). The diet contains the known required trace elements in sufficient and balanced amounts and also fluorine. It is deficient, however, in Factor G, a newly discovered organic agent under investigation (9).

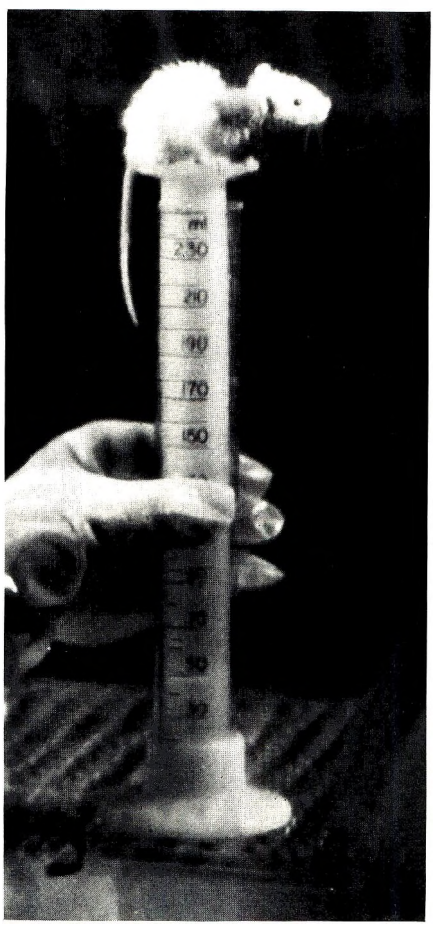

Fig. 4 Weighing device.

TABLE 1

Composition of basal diet for Factor G assay

	g
Amino acid mixture S-7 [1]	17.7
Sucrose	61.2
Stripped lard [2]	10
Cottonseed oil [3]	5
H.M.W. salts [4]	2
Salt supplement [5]	3
Trace supplement mixture [6]	0.1
Vitamins [7]	1
$NaHCO_3$	1.5
Cellulose [8]	2

[1] Composition: (in g/kg of diet) arginine·HCl, 13.0; histidine·HCl, 7.0; isoleucine, 10.0; leucine, 15.0; lysine·HCl, 16.0; methionine, 8.0; phenylalanine, 10.0; threonine, 8.0; tryptophan, 3.0; valine, 10.0; alanine, 4.0; aspartic acid, 3.0; cystine, 3.0; glutamic acid, 40.0; glycine, 5.0; proline, 5.0; serine, 5.0; tyrosine, 6.0; and asparagine, 6.0.

[2] Vitamin E-free animal fat, stripped by molecular distillation (Distillation Products Industries, Eastman Kodak Co., Rochester, New York).

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

[4] Hubbel et al. J. Nutr., *14:* 273, 1947; obtained from Nutritional Biochemicals Corporation, Cleveland.

[5] Composition: (in grams/kg diet) $Ca(H_2PO_4)_2 \cdot H_2O$, 24.52; $CaCO_3$, 1.825; $MgSO_4$, 1.95; Na_2HPO_4, 0.62; KH_2PO_4, 1.054; and $MnSO_4 \cdot H_2O$, 0.14.

[6] Composition: (in grams) $Zn(CH_3COO)_2 \cdot 2H_2O$, 3.37; MoO_3, 0.152; $CoSO_4 \cdot 7H_2O$, 1.1925; and lactose to make 50 g.

[7] Composition: (in grams) choline dihydrogen citrate, 21.15; niacin, 1; (and in milligrams) lactose, 76.532; thiamine·HCl, 40; riboflavin, 25; pyridoxine·HCl, 20; Ca D-pantothenate, 200; menadione (2-methyl-1,4-naphthoquinone), 10; folic acid, 20; biotin, 10; and vitamin B_{12}, 1. Selenium was added as sodium selenite (15 μg selenium/100 g diet, dissolved in 1 ml water). The following vitamins were added in 1 ml absolute ethanol/100 g diet: (in mg/100 g diet) crystalline vitamin A acetate, 2; vitamin D (Drisdol, Winthrop Laboratories, New York), 0.002; and *d*-α-tocopheryl acetate, 10.

[8] Whatman. Col. Chromedia CF1, fibrous cellulose powder.

RESULTS AND DISCUSSION

To test the system for adverse effects, 5 rats maintained in the ultraclean controlled environment were fed laboratory ration.[10] Animals on the outside served as controls. They received the same diet but were kept under conventional conditions in stainless steel cages with glass water bottles and food cups. The growth rate of the 2 groups fed laboratory ration was similar (exp. 1, table 2). The physical appearance of the animals in the controlled environment was superior. It thus appeared that the trace element-controlled system, per se, exerted no adverse effect upon the animals fed an optimal diet.

Rats maintained under conventional conditions with the purified amino acid ration on the outside grew less than those fed laboratory ration, but they appeared well and fairly normal in contrast with those fed the amino acid diet inside the isolator system. Animals inside developed a severe pathological condition within 1 to 3 weeks (fig. 5). The deficiency documented itself by lack of growth, seborrhea, shaggy fur, and loss of hair leading at times to complete alopecia. The deficiency caused death after 2 to 6 weeks. Except for the changes in the integument, no gross pathology was seen at autopsy. More detailed studies will be necessary to determine which tissues and pathophysiological mechanisms are involved in this disease.

[10] Lab Blox, Allied Mills, Inc., Chicago.

TABLE 2

Comparison of rats maintained in conventional and controlled environments [1,2]

	Apperance		Daily wt gain [3]	
	Conventional	Controlled	Conventional	Controlled
			g/day	*g/day*
	Experiment 1			
Basal amino acid diet	normal	alopecia seborrhea	1.5 ± 0.07 [4]	0.8 ± 0.07
Laboratory ration	normal	excellent	3.4 ± 0.50	3.5 ± 0.08
	Experiment 2			
Basal amino acid diet	normal	alopecia seborrhea	1.7 ± 0.10	1.2 ± 0.20
Basal amino acid diet + 0.4% ash of Torula yeast	normal	normal	2.0 ± 0.11	1.7 ± 0.02

[1] The experiments shown here have been carried out repeatedly over the past 18 months, with very similar results.

[2] Fischer 344 weanling male rats, 5 animals/group.

[3] Exp. 1: 21 days; exp. 2: 19 days.

[4] Average ± SE of mean.

Fig. 5 Comparison of rats fed the same amino acid diet in conventional and controlled environments. *Left:* animal fed basal diet after 2 weeks in the control environment; *right:* control animal fed basal diet kept outside in metal cage with glass water bottle and food cup.

To prove that the impaired performance of animals in the trace element controlled system was due to lack of a trace element, the basal diet was supplemented with the ash of Torula yeast (exp. 2, table 2). Ashing was carried out by combustion of dry yeast and heating of the ash in an open dish at 600° for 4 hours. The resulting ash was added to the basal amino acid diet at a level of 0.4%, which is equivalent to 5% whole, dry yeast. The ash prevented the deficiency symptoms. Alopecia or seborrhea were not evident in animals fed this diet, and growth of the ash-supplemented group inside the incubator was identical with that observed on the outside in the unsupplemented control. The slight difference seen between animals with and without ash supplement on the outside was not statistically significant and was not confirmed in subsequent studies. The effect of ash supplements inside the isolator is highly significant. It has been observed consistently in several trials. The same effect was produced by purified concentrates derived by fractionation from yeast ash.[11]

These experiments demonstrate that conventional caging, housing, and handling of rats contributes inorganic trace factors which are missing in our amino acid diets, but present in laboratory ration and in yeast ash. The active elements may be supplied by dust in the air.[12]

Current studies are aimed at the identifications of the unknown trace element, or elements, which prevent the new deficiency. The importance of a complete microflora was stressed by Greever et al.[13] who reported that germfree rats receiving a chemically defined diet developed pancreatic fibrosis and atrophy. In contrast, conventional, that is, non-germfree, controls fed the same diet were largely unaffected. Our animals are not maintained under germfree conditions, but it it felt that the observed effects are not mediated by the microflora of the gastrointestinal tract.

It is noteworthy that a single, relatively easily constructed barrier suffices to exclude trace contamination effectively. Our plans initially called for a second barrier in the form of a plastic ultraclean room to house four of the trace element control isolators. This precaution proved to be unnecessary.

The trace element-controlled system opens up a number of new possibilities in trace element research. In conjunction with modern, sensitive methods of trace element determination, it permits us to perform rigorous and definitive determina-

[11] To be published as a separate study.

[12] In 20 consecutive experiments with the trace element-controlled system we have encountered one breakthrough of dust through the filter. The animals inside the isolator reverted within one week from a severely deficient status to normal.

[13] Geever, E. F., F. S. Daft and S. M. Levenson 1965 Pancreatic atrophy and fibrosis in the germfree rat on a chemically defined diet. Federation Proc., 24: 246.

tions of trace element requirements on laboratory animals. Biological functions have been postulated but not proved for several elements, for example, arsenic, nickel, and fluorine. It is likely that for rigorous, environment-controlled studies on these elements specially purified inorganic and organic chemicals will have to be used as dietary constituents.

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Destruction of Molar Teeth in Rats Fed Amino Acid Diets [1]

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ABSTRACT Serious molar breakdown was found in rats receiving free glutamic acid contained in either amino acid or casein based diets for only 4 weeks. Gross appearance of the lesions was similar to that of typical caries except for an occlusal erosion frequently observed. Also, a favorable response resulted from diet supplementation with cariostatic materials, including sodium phosphate, penicillin and fluoride, and from decreasing the sucrose content of the diet. Diet acidity (pH 5.0) was shown to be a causal factor since increasing the pH by adding sodium bicarbonate or by replacing glutamic acid with casein or monosodium glutamate decreased tooth damage. However, extensive molar breakdown was also noted with a nearly neutral (pH 6.7) amino acid diet. The importance of bacteria in this problem was indicated by penicillin supplementation to the acid diets which nearly eliminated damage except for relatively minor occlusal erosion. Fluoride, provided in the rat's drinking water at 50 ppm, was more effective than penicillin in protecting the teeth from erosion when the acid diets were fed.

Purified diets containing free amino acids as the source of nitrogen have been used in recent years to produce acceptable weight gains in young rats (1–3). Free glutamic acid was included in these diets to the extent of 2 to 12% of the total diet to provide nonessential amino acid nitrogen.

Of several amino acid mixtures fed to weanling rats by Salmon (1) one of the most satisfactory, in terms of growth produced, contained 7% L-glutamic acid. A diet in which the amino acid composition was closely patterned after Salmon's mixture was fed to young rats in our laboratory for the purpose of studying the effect of level of dietary lysine on incidence of tooth decay. However, examination of the teeth of these rats after 30 days showed that serious destruction had occurred to the molars regardless of the amount of lysine used. It was therefore apparent that one or more other factors in the diet were contributing to a rapid tooth breakdown. The present studies were undertaken to provide information about the causes of this rampant molar destruction, heretofore unreported for rats fed amino acid diets.

METHODS

Male weanling rats of the Sprague-Dawley strain, about 21 days of age and 40- to 50-g weight, were obtained from a commercial breeder for these studies. They were earmarked and sorted into nearly equal weight treatment groups which were then assigned at random to treatments. Weights were recorded at 7-day intervals and the average gains were determined for a 28-day experimental period. Rats were housed in suspended, screen-bottom cages and given distilled water and food ad libitum. Diets were fed dry, fresh food being given at least twice weekly, except when water was added to diets as a variable in the dietary treatment, in which case fresh food was provided daily. When water was added to the amino acid diet, it was necessary to restrict the water content to 15% and add the corn oil component after mixing in water, in order to provide an oily coating on food particles and prevent excessive stickiness of the high sugar diet. The diet was then stored at − 20° until fed.

The basal diets are described in table 1, the amino acid composition of the basal amino acid diet being essentially the same as Salmon's mixture L (1). Diet pH was measured after vigorously mixing 5 g of

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TABLE 1
Composition of the diets

	Diet 300	Diet 100
	g	*g*
Amino acid mixture [1]	18.6	—
Vitamin-free casein [2]	—	20.0
Confectioner's sugar	50.0	50.0
Cornstarch	20.7	21.3
Sodium bicarbonate	2.0	—
Minerals [3]	3.5	3.5
Vitamins [4] in glucose monohydrate [5]	1.0	1.0
Choline chloride	0.2	0.2
Corn oil + vitamins A, D and E [6]	4.0	4.0
Total	100	100

[1] The amino acid mixture supplied the following amounts of amino acid per 100 g diet: (in grams) L-arginine·HCl, 0.8; L-cystine, 0.3; L-glutamic acid, 7.0; glycine, 0.6; L-histidine HCl·H_2O, 0.7; DL-isoleucine, 1.4; L-leucine, 1.0; L-lysine·HCl, 1.4; L-methionine, 0.6; DL-phenylalanine, 1.2; DL-threonine, 1.2; DL-tryptophan, 0.2; DL-valine, 1.8; and L-tyrosine, 0.4.

[2] Amino acids and vitamin-free casein were obtained from Nutritional Biochemicals Corporation, Cleveland, or General Biochemicals, Inc., Chargin Falls, Ohio.

[3] The salt mixture contained: (in percent) $CaCO_3$, 20.71; $CaHPO_4$, 32.28; Na_2HPO_4, 18.6; KCl, 20.86; $MgSO_4$, 6.57; $MnSO_4 \cdot H_2O$, 0.0044; $CuSO_4$, 0.00037; $FeC_6H_5O_7 \cdot 5H_2O$, 0.0043; $ZnCO_3$, 0.0006; and KIO_3, 0.000028. University of California salt mixture UCB-1Rb, based on a report by G. M. Briggs and M. A. Williams, Federation Proc., 22: 261, 1963, and modified by the authors.

[4] The vitamin mixture provided per kg of diet: (in milligrams) menadione, 2.5; thiamine, 25; riboflavin, 25; pyridoxine·HCl, 8; *p*-aminobenzoic acid, 75; folic acid, 1.25; biotin, 0.25; niacin, 100; Ca pantothenate, 100; and inositol, 250.

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

[6] The corn oil supplement furnished 5000 IU of vitamin A, 4000 IU of vitamin D, and 75 mg of *dl*-α-tocopheryl acetate/kg of diet.

diet with 250 ml of water for 5 minutes with a magnetic stirrer.

Since the diet was originally formulated to be "cariogenic," 50% confectioner's sugar was incorporated (4). This ingredient proved very satisfactory as a major carbohydrate source since the resulting diet was acceptable to the rat and absorbed very little moisture even when left in food cups for one week.

Twenty percent casein (diet 100) was used to replace amino acids to make a control diet for a standard of growth rate and also of normal, undamaged molars since little or no decay developed in rats fed this ration over the 28-day period. Diets were stored at 4° and usually fed within 30 days from date of mixing.

Rats were decapitated after being fed the experimental diet for 28 days and the mandibles were excised and evaluated for molar damage with the aid of a dissecting microscope and a dental explorer. Cleaned mandibles were stored for future reference in a 70% solution of isopropyl alcohol. Maxillary molars were not evaluated since damage to them was much less noticeable. Data were analyzed statistically by analysis of variance (5) and Duncan's (6) multiple range test was used to test for significance of differences between treatment means.

RESULTS

The appearance of normal molars from rats fed casein diets (fig. 1) may be compared with severely damaged molars of a rat fed an amino acid diet containing 7% glutamic acid (fig. 2).

Two types of lesions have been observed in molars of rats subsequent to feeding acid diets: occlusal erosion of minor or more serious extent, and deeply penetrating cavities that may involve only a small cusp or an entire molar. This second type of damage is similar to the caries associated with high sugar diets and is usually found in cases of more severe molar damage when the acid diets are fed. We have avoided use of the term "caries" in describing the condition of teeth resulting from the feeding of acid diets, however, since we were uncertain whether the conditions responsible for such rapid deterioration of the teeth were analogous to the situation existing with "natural" diets. In consideration of these factors, the scale in table 2 was developed to provide a numerical scoring system against which molar damage might be evaluated.

To facilitate and standardize the scoring procedure, molar damage was rated relative to 6 "standard" mandibles previously selected to typify 6 degrees of deterioration and stored in alcohol for that purpose. To avoid bias in scoring, each mandible was tagged with a metal wing band,[3] and then rated without the scorer knowing the dietary history of the teeth.

After the observation of extreme molar deterioration in all rats regardless of the level of lysine fed, a number of diet modifications were prepared and tested with the results summarized in table 3. These observations indicated that free glutamic acid was the major cause of molar break-

[3] National Band and Tag Company, Newport, Kentucky.

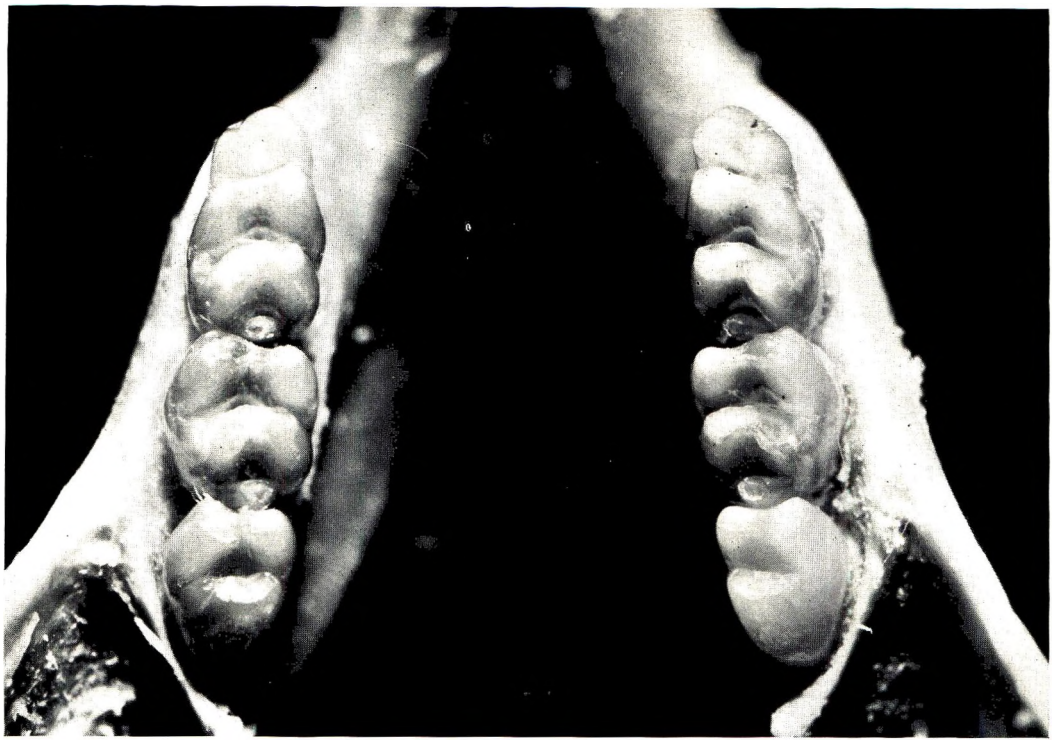

Fig. 1 Intact molar teeth in rat fed 20% casein diet (no. 100).

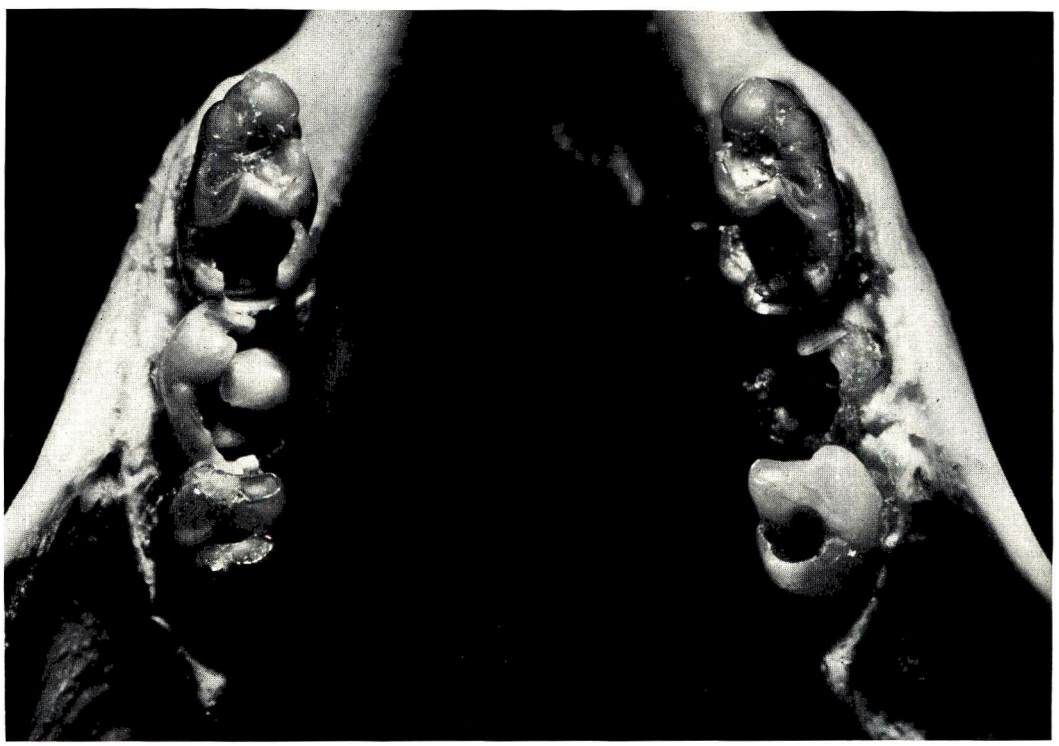

Fig. 2 Damaged molars in rat fed amino acid diet no. 300 for 28 days.

TABLE 2
Molar scoring system [1]

	Score
No detectable molar damage	0
First definite evidence of erosion or decay	10
Deeper surface erosion ("cupping" or decay)	20
Deep penetration through crown equivalent total destruction of 1 molar	30
Damage equivalent to total destruction of 2 molars	40
Damage equivalent to total destruction of 3 molars or more	50

[1] Half scores (25, 35, etc.) given for borderline cases.

down, since replacing it with its monosodium salt sharply reduced tooth damage. The pH of the diet rose from 4.9 to neutrality with the inclusion of monosodium glutamate, suggesting that acidity of the diet was a significant factor in the breakdown of the teeth of rats fed these amino acid diets.

Procaine penicillin, added to the diet at 100 ppm, did not significantly reduce tooth destruction ($P < 0.05$). This indicated that bacterial action was not the major factor in the breakdown of molars under the conditions of this experiment. However, subsequent experiments showed that higher concentrations of penicillin could partially prevent molar destruction.

A marked decrease in molar damage occurred when 50 ppm fluoride as sodium fluoride was administered in the drinking water of rats fed the diets containing 7% glutamic acid. Occlusal erosion, characteristically observed in rats fed acid diets in these studies, was minor or nonexistent in rats given fluoride, indicating that a protective action of the fluoride was to decrease the solubility of molar surfaces to acid attack, a principle previously demonstrated by in vitro (7, 8) and in vivo (9, 10) techniques.

Phosphate compounds, known for their cariostatic qualities (11), were added to the basal amino acid diet in the form of dibasic sodium phosphate and tricalcium phosphate. Calcium phosphate was not beneficial in this study although the sodium phosphate was responsible for a significant reduction in molar scores ($P < 0.05$). With orthophosphates, Harris and Nizel (12) reported that the cariostatic activity depended on the cation, sodium being much more effective than calcium.

To determine whether free glutamic acid would also have a destructive effect

TABLE 3
Effect of fluoride, phosphate and penicillin on molar damage in rats fed 7% glutamic acid diets

Treatments	pH of diet	No. of rats	Gain/day	Molar score
			g	
Experiment 1 [1]				
20% casein diet (no. 100)	6.5	6	6.0 ± 0.2 [2]**	0 **
Basal amino acid diet (no. 300)	4.9	6	5.1 ± 0.3	38 ± 2.8
Modifications of diet 300				
50 ppm fluoride [3]	4.9	6	4.5 ± 0.2	5 ± 2.2 **
100 ppm procaine penicillin	4.9	6	5.0 ± 0.4	28 ± 6.6
2% dibasic sodium phosphate	5.6	6	4.5 ± 0.1	24 ± 5.6 *
2% tricalcium phosphate	5.5	6	4.5 ± 0.2	34 ± 4.9
7% monosodium glutamate [4]	7.1	6	5.0 ± 0.3	13 ± 4.2 **
Experiment 2 [1]				
20% casein diet with 7% glutamic acid	4.3	12	6.0 ± 0.1	35 ± 1.2
Modifications of casein-glutamic acid diet				
50 ppm fluoride [3]	4.3	12	5.3 ± 0.1 *	14 ± 2.6 **
100 ppm procaine penicillin	4.3	12	5.9 ± 0.2	34 ± 1.2

[1] Data in experiments 1 and 2 were analyzed separately.
[2] Mean ± SE of each treatment.
[3] Sodium fluoride added to drinking water.
[4] Replaced glutamic acid.
* Significantly ($P < 0.05$) different from unmodified glutamic acid treatment.
** Significantly ($P < 0.01$) different from unmodified glutamic acid treatment.

in diets containing intact protein, 7% glutamic acid was added to the 20% casein diet with a compensating decrease in cornstarch. The nonprotein components of the amino acid and casein diets were identical except that sodium bicarbonate was omitted from the latter and more cornstarch was contained in the former. Mean molar scores summarized in table 3 indicate that similar damage occurred in either case. Results with fluoride and penicillin supplementation were also similar when the casein diet was fed, the indicated decrease in molar protection by fluoride probably being due to a more acid diet, pH 4.3 compared with pH 4.9 for diet 300.

Average weight gains in table 3 are typical of those we have obtained with weanling rats fed amino acid or casein based diets for 28 days from weaning. Amino acid diet 300 has produced about 5 g of gain per day, which is significantly less ($P < 0.01$) than the 6 g or more by rats fed either 20% casein diets or the amino acid diet supplemented with 5% casein (table 4).

The significance of glutamic acid as a component of amino acid diet 300 in promoting tooth breakdown was compared with other organic acids in treatments summarized in table 4. Equimolar amounts of either succinic or aspartic acid resulted in approximately equal damage scores, indicating that diet acidity, and not glutamic acid per se, was the significant, destructive factor. Further indication of the importance of dietary acidity was implied by the relatively minor amount of molar damage found when neutral diets were fed, the result of either omitting the free organic acid or replacing it by monosodium glutamate. In this series 5% casein was added to the amino acid diets to provide supplementary protein and thus make up for changes in nitrogen level between the different treatments. Superior growth generally accompanied 5% casein supplementation, although rats appeared to dislike the food containing succinic acid, spilled an excessive amount, and gained significantly less weight ($P < 0.01$).

An inverse relationship between dietary pH and molar damage scores is indicated in table 4, whether the alteration in pH was caused by addition of glutamic acid or by different levels of sodium bicarbonate. On a molar basis, about 4% sodium

TABLE 4

Effect of adding free organic acids, monosodium glutamate and sodium bicarbonate to a 5% casein-amino acid diet [1]

Treatment no.	Diet variables: Free organic acid or monosodium glutamate	Diet variables: $NaHCO_3$	Diet pH	Gain/day	Molar [2] score
		%		g	
		Experiment 1			
1	None	2	7.1	6.2 ± 0.2 [3]	2 ± 1.0 [a]
2	3% glutamic	2	5.4	6.3 ± 0.2	16 ± 3.5 [c]
3	7% glutamic	2	4.9	6.6 ± 0.1	29 ± 3.3 [b]
4	6.3% aspartic	2	4.8	6.2 ± 0.2	27 ± 2.8 [b,c]
5	5.6% succinic	2	4.9	4.2 ± 0.3 [4]	28 ± 2.7 [b]
		Experiment 2			
1	None	—	6.5	5.4 ± 0.4	5 ± 2.6 [a]
2	None	2	7.0	5.4 ± 0.1	7 ± 3.9 [a]
3	7% glutamic	—	4.5	5.8 ± 0.2	29 ± 1.9 [b]
4	7% glutamic	2	4.9	6.1 ± 0.3	24 ± 3.6 [b,c]
5	7% glutamic	4	5.9	5.5 ± 0.2	12 ± 4.7 [a,c]
6	8.05% monosodium glutamate	—	6.6	6.0 ± 0.2	9 ± 3.4 [a]
7	8.05% monosodium glutamate	2	7.0	5.9 ± 0.2	2 ± 1.3 [a]

[1] Diet was identical with no. 300 except for addition of 5% casein and variations of free organic acids, monosodium glutamate, $NaHCO_3$ and cornstarch.

[2] Data in experiments 1 and 2 were analyzed separately. Mean scores with different superscript letters (*a,b,c*) differ significantly ($P < 0.01$); with same superscript letter (c) differ significantly ($P < 0.05$).

[3] Mean ± SE/treatment of 10 rats.

[4] Significantly ($P < 0.01$) lower than other treatments in experiment 1.

bicarbonate is required in amino acid diet 300 to convert 7% free glutamic acid to its monosodium salt. Increasing the bicarbonate to this level decreased molar damage significantly ($P < 0.05$), the resulting scores being similar to those obtained when 8.05% monosodium glutamate was fed.

Fluoride, while effectively inhibiting molar breakdown when provided in the drinking water at 50 ppm, was also observed to decrease slightly the growth rate of rats fed 20% casein diets. However, no consistent effect of fluoride on weight gain was found for rats receiving amino acid diet 300 and given 3 levels of fluoride: 5, 15 or 50 ppm (table 5). Although 5 ppm did result in a highly significant reduction ($P < 0.01$) in tooth damage scores, a further significant improvement occurred with 50 ppm fluoride.

The importance of bacteria in breakdown of molars induced by the feeding of free glutamic acid is shown by data in table 6, which summarizes the results of adding 200 ppm penicillin to the dry diets. A lower concentration of 100 ppm had not proven to be effective (table 3). In this series of treatments, rats consuming amino acid diets containing equimolar amounts of either monosodium glutamate or glutamic acid exhibited about the same amount of tooth damage when sodium bicarbonate was omitted from the rations, despite the wide difference in dietary pH (4.3 vs 6.7). These data confirm unpublished results found previously with smaller number of rats which indicated that rapid destruction of molars could occur with the amino acid diets whether they had an acid reaction or not.

TABLE 5

Molar protection with three levels of fluoride in rats fed amino acid diet 300 [1]

Fluoride added to drinking water [2]	Gain/day [3]	Molar score [4]
ppm	*g*	
—	4.6 ± 0.2 [5]	36 ± 2.0 [a]
5	4.4 ± 0.3	23 ± 3.7 [b]
15	5.0 ± 0.2	17 ± 2.8 [b]
50	4.6 ± 0.1	7 ± 2.6 [c]

[1] Diet 300 contained 4.8 ppm fluoride. Assayed by Curtis and Tompkins, Ltd., San Francisco.
[2] Sodium fluoride.
[3] Daily weight gains were not significantly different ($P < 0.05$).
[4] Mean scores with different superscript letters (a,b,c) differ significantly ($P < 0.01$); with same superscript letter (b) are not significantly different ($P < 0.05$).
[5] Mean ± SE of 16 rats/treatment.

Bacterially induced decay appeared to be a factor in the destruction of teeth of rats fed monosodium glutamate, since penicillin effectively prevented damage with those diets. Although damage was also reduced when penicillin was added to the glutamic acid diets, occlusal erosion was general, probably the result of acid demineralization. Deep lesions which penetrated into the pulp of the molars were observed only in rats that had not received penicillin.

The data also indicate that supplements of either 5% casein (table 4) or 2% sodium bicarbonate (table 6) to the monosodium glutamate-amino acid diet changed conditions so that only minor damage

TABLE 6

Effect of adding 200 ppm penicillin to amino acid diets

Treatment no.	Diet variables [1]				Diet pH	Gain/day	Molar score
	Glutamic acid	Monosodium glutamate	Penicillin G potassium	$NaHCO_3$			
	%	%	*ppm*	%		*g*	
1	7	—	—	2	5.0	5.1 ± 0.2 [2]	29 ± 4.2
2	7	—	200	2	5.0	5.1 ± 0.2	10 ± 2.7 **
3	7	—	—	—	4.3	4.7 ± 0.1	34 ± 2.9
4	7	—	200	—	4.3	5.2 ± 0.2	16 ± 1.8 **
5	—	8.05	—	—	6.7	4.7 ± 0.2	32 ± 3.3
6	—	8.05	200	—	6.7	5.4 ± 0.2	0 **
7	—	8.05	—	2	7.2	4.4 ± 0.2 *	6 ± 2.7 **
8	—	8.05	200	2	7.2	4.7 ± 0.3	2 ± 1.3 **

[1] Ingredient changes made at expense of cornstarch.
[2] Mean ± SE of 10 rats/treatment.
* Significantly ($P < 0.05$) different from treatment 1 (diet 300).
** Significantly ($P < 0.01$) different from treatment 1 (diet 300).

occurred. A comparison of similar treatments in tables 4 and 6 show a tendency for lower scores in the casein-supplemented rats, as well as an improved growth rate. A similar situation is not apparent for treatments with sodium bicarbonate, however, since growth depression was sometimes indicated when additions of this substance were made to the diet.

Amino acid diets developed in recent years to produce satisfactory growth in rats have sometimes been mixed with water before feeding (1–3) to take advantage of improved rate and efficiency of growth associated with wet diets (13). Diet 300 and variations of it were fed dry in the present experiments because of the high sugar content and the insolubility of some of the ingredients. For treatment 3, table 7, a limited amount of water was incorporated into the diet to make a thick slurry before corn oil was added, a technique which largely overcame the problems of adhesiveness and diet separation. The addition of water to diet 300 produced a significant improvement ($P < 0.05$) in molar damage scores, although growth rate was not similarly affected.

The type of carbohydrate, found to be of major importance in determining the cariogenic nature of animal diets (14–16), also appears to be a vital factor affecting tooth damage when amino acid diet 300 is fed. Molar scores were highest with sucrose, intermediate with glucose, and minor with starch when these 3 substances were compared as the major carbohydrate source. Even greater differences might have resulted if the glucose and starch diets had not contained 15% sucrose.

DISCUSSION

Tooth breakdown observed in rats fed amino acid or casein diets containing 7% glutamic acid was found to be similar in many respects to tooth decay generally associated with cariogenic diets. This was true for the gross appearance of the lesions (except for the less serious occlusal surface "cupping" sometimes observed with acid diets) and also in the nature of response to such cariostatic materials as fluoride, penicillin and sodium phosphate. Type of carbohydrate was of major importance in the amino acid diets, the damage observed to be extensive with sucrose and minor with cornstarch when these materials were compared as primary dietary components, a situation very much like that reported for cariogenic diets fed to hamsters (14) and rats (15, 16).

Microorganisms are ultimately responsible for tooth decay, since it does not occur in their absence (17). The importance of bacteria in tooth breakdown with diet 300 was demonstrated by the limited damage—a relatively minor degree of surface occlusal erosion—found when supplementation was made with 200 ppm penicillin. Serious penetration and undermining of tooth structure was therefore indicated to be *decay* caused primarily by penicillin-sensitive bacteria. Further, this breakdown also was observed with the

TABLE 7

Effect of further modifications of amino acid diet no. 300 on tooth breakdown

Treatment no.		Diet pH	Gain/day	Molar score
			g	
1	20% casein diet (no. 100)	6.2	6.2 ± 0.3 [1] **	0 **
2	Amino acid diet (no. 300)	5.0	5.1 ± 0.2	29 ± 4.2
	Modifications of diet no. 300:			
3	Diet fed wet [2]	5.0	5.4 ± 0.2	19 ± 4.2 *
4	4% $NaHCO_3$	6.0	4.9 ± 0.2	11 ± 3.4 **
5	50% cornstarch [3]	5.2	5.3 ± 0.2	9 ± 3.3 **
6	50% glucose monohydrate [3,4]	5.1	5.2 ± 0.2	17 ± 1.9 **
7	50% dextrin [3]	5.0	5.0 ± 0.1	9 ± 3.4 **

[1] Mean ± SE of 10 rats/treatment.
[2] Diet contained 15% water.
[3] Diet also contained 15% sucrose; confectioner's sugar omitted.
[4] Cerelose, Corn Products Company, Argo, Illinois.
* Significantly ($P < 0.05$) different from treatment 2.
** Significantly ($P < 0.01$) different from treatment 2.

feeding of neutral amino acid diets whereupon it could be completely eliminated with penicillin treatment. This suggested that bacteria, nurtured by available sugar and free amino acids, were capable of effectively destroying molars in only 4 weeks' time without the aid of demineralizing dietary acid. Chelation occurring with the feeding of EDTA has been found to decalcify molar surfaces of rats (18, 19) and is a possibility with free amino acids in solution at a relatively neutral pH. However, chelation probably was not a factor for molar erosion in these studies since none was apparent when the neutral amino acid diets were supplemented with penicillin.

Damage with rats fed EDTA was also influenced by the amount of sucrose in the diet (20) and largely prevented by antibiotic supplementation (19). However, in contrast with our experience with glutamic acid, EDTA was reported to affect the smooth surfaces to a greater degree than the occlusal areas of the molar teeth (19, 20). Studies of acid damage to rat teeth from acid beverages (21, 22) have also shown that the smooth surfaces of the molars were affected, but unlike the results for either glutamic acid or EDTA, lesions were exclusively in the nature of erosion and therefore did not resemble typical caries.

The necessary presence and activity of certain oral microorganisms for serious damage when either acids or chelators are included in diets indicate that the destructive action of these factors, like many others, are determined by the way they influence the attack of decay-causing bacteria. Demineralization of molar surfaces may allow easy initial penetration of tooth structure by microorganisms, but a further, direct effect on bacteria by free amino acids and EDTA is also possible. Availability of amino acids and an acid pH will favor the growth of certain microorganisms. Chelation, from the addition of EDTA to the medium, improved the growth of *Escherichia coli* in experiments by Shipe and Fields (23).

Additional investigation is needed to elucidate the mechanism of tooth breakdown with free amino acid diets as well as the role of bacteria in this destructive process. The influence of dietary ingredients such as chelating agents and organic acids are of practical importance in helping to explain rampant caries that sometimes affect children. Highly acid foods commonly ingested are soft drinks and fruit juices, which may, in combination with dry food in the mouth, create a situation not unlike that found with the acid diets fed to rats in these studies.

Fluoride, provided in the rat's drinking water during the same period when the acid diets were fed, proved highly beneficial in reducing damage scores. Although occlusal erosion was evident in most rats fed penicillin-supplemented acid diets, it was rarely observed in those given 50 ppm fluoride. Effective in vivo protection by fluoride against acid demineralization of molar surfaces was therefore indicated.

An obvious conclusion from this work is that diets containing free amino acids can cause serious damage to the teeth of recipient animals in a relatively short time. Restriction of acid components such as glutamic and aspartic acid, neutralization with sodium bicarbonate or other base, feeding the diet mixed with water and keeping sucrose content minimal are ways to help overcome this problem. That amino acid diets need not be injurious to the teeth was indicated by the work of Greenstein et al. (24) who fed a water-soluble amino acid diet in a 50% solution with water (pH 4.5 to 5.5) and detected no lesions on the molars of rats that had consumed this diet for over a year from weaning.

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Site and Rate of Active Transport of L-Lysine in the Intestine of the Fowl [1,2]

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ABSTRACT A study was made to determine whether the avian small intestine was capable of actively transporting L-lysine from the mucosal to the serosal surface against a concentration gradient; and if it was, whether the rate of transport varied along the length of the intestine. The rate of active transport of L-lysine across the intestinal wall of the fowl was determined at 6 levels of the small intestine; the upper and lower halves of the duodenum, of the jejunum and of the ileum. Transport rates were calculated both as the amount of lysine transported per unit weight of dried intestine and per length of live intestine. These data indicated no active transport of lysine by the duodenum but active transport by the jejunum and ileum. Based on weight, the jejunal and ileal levels transported 7.0 μmoles of lysine/g of dried intestine per hour. Based on length, the rate of active transport progressively decreased significantly from the upper jejunal to lower ileal regions of the small intestine. It was concluded that rates of L-lysine transported across the intestinal wall based on unit length represented true physiological differences between the various intestinal segments studied.

Kratzer (1) concluded that all amino acids were absorbed by simple diffusion. He measured the rate of absorption of 15 amino acids (including L-lysine) from the intestinal tract of White Leghorn chicks using the Cori method. In this procedure a measured volume of solution was introduced into the crop of an unanesthetized chicken, and at the end of the experimental period the animal was killed, and the unabsorbed gastrointestinal tract contents were analyzed.

Hagihira et al. (2), using everted sacs of intestine and an initial 1 mM amino acid concentration in the mucosal and serosal fluids, successfully demonstrated that L-lysine is actively transported across the intestinal wall of the hamster against a concentration gradient. According to Wilson (3), Hagihira showed that the rate of active L-lysine transport is approximately the same in the upper, mid, and lower small intestine of the hamster. Larsen et al. (4) obtained similar results using everted sacs of rat small intestine.

Since the Kratzer study, Paine and Newman (5), and Lin and Wilson (6) have found evidence that the L-isomers of methionine and histidine, and the L-isomer of tyrosine, respectively, are transported across the intestinal wall of the chick by a selective process.

Imondi and Bird (7) investigated the site of nitrogen absorption in the fowl and found that the percentage of dietary nitrogen which disappeared from each intestine level progressively decreased from the upper jejunum to the lower ileum.

The present investigation was undertaken to gain a more thorough understanding of the physiology of absorption in the avian intestine. The specific aims of the investigation were to determine whether the avian intestine was capable of actively transporting L-lysine from the mucosal to the serosal surface against a concentration gradient; and if it was, at what rate the lysine was transported across the various levels of the intestine (upper and lower halves of the duodenum, of the jejunum, and of the ileum).

EXPERIMENTAL PROCEDURE AND MATERIALS

The procedure and the radiometric techniques used have been presented in

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[1] This paper was taken from a thesis submitted by J. R. Fearon to The Graduate School, University of Maine, in partial fulfillment of the requirements for the degree of Master of Science.

[2] This study was supported in part by grants from American Cyanamid, Princeton, New Jersey; Agway, Inc., Syracuse, New York; Merck and Company, Inc., Rahway, New Jersey; H. K. Webster Company, Lawrence, Massachusetts; and Wirthmore Feeds, Inc., Waltham, Massachusetts.

detail in a previous paper (8). A modification of the Wilson and Wiseman (9) in vitro everted sac technique was used. The mucosal and serosal fluid contained an initial 1 mM lysine concentration and an initial 1.78 mM glucose concentration (8). The salts and glucose used to prepare the mucosal and serosal fluid were A.C.S. certified.

Chromatograms of the L-lysine hydrochloride and radiograms of the L-lysine-^{14}C hydrochloride (hereafter lysine) (specific activity: 130 μCi/mmole) indicated both compounds to be pure. Chromatograms and radiograms of the postincubation serosal fluids from all 6 levels of intestine studied showed that lysine was the only ninhydrin-sensitive and radioactive compound present. It was assumed, therefore, that the results of all radioactivity analyses were direct measurements of the amount of lysine present. All chromatograms and radiograms were developed on Whatman no. 1 chromatographic paper with *n*-butanol:acetic acid:water (4:1:5) solvent mixture, and sprayed with ninhydrin. A chromatogram scanner was used to locate the lysine-^{14}C following chromatography.

The intestinal segments were obtained from young adult White Leghorn cockerels maintained with the modified 1965 New England College Conference broiler starting diet.

The statistical analysis for all data was performed according to Snedecor's approximate method of analysis of variance for block design with disproportionate subclass numbers (10).

RESULTS

1. *Lysine transport based on weight of dried intestinal sac.* The milligrams of lysine actively transported per gram of dried sac by the upper and lower levels of the duodenum after 40, 60, and 80 minutes of incubation are shown in table 1. Since there were no appreciable differences in the amount of lysine transported across the duodenal wall for the three incubation periods, and since the amounts transported were very close to zero, it was concluded that the duodenum did not actively transport lysine. The finding [3] that glucose was actively transported by both levels of the duodenum under like conditions demonstrated the integrity of the duodenal active transport system.

The upper and lower levels of both the jejunum and the ileum did transport lysine against a concentration gradient (table 2). There were no significant dif-

TABLE 1

Milligrams [1] of L-lysine actively transported across the upper and lower levels of the duodenal wall of the avian intestine after 40, 60 and 80 minutes of incubation at 37°

Incubation time	Intestinal levels			
	Upper duodenum		Lower duodenum	
min	*mg/g*	*mg/8 cm*	*mg/g*	*mg/8 cm*
40	−0.104	−0.049	−0.083	−0.035
60	−0.164	−0.073	−0.067	−0.031
80	−0.113	−0.073	−0.007	−0.003

[1] Each value is the average of 2 observations and is expressed per gram of dried sac or per 8 cm of intestine.

[3] Unpublished results by J. R. Fearon and F. H. Bird.

TABLE 2

Milligrams [1] of L-lysine per gram of dried sac actively transported across the upper and lower levels of the jejunal and the ileal walls of the avian intestine after 40, 60 and 80 minutes of incubation at 37°

Incubation time	Jejunum		Ileum	
	Upper level	Lower level	Upper level	Lower level
min				
40	0.407 ± 0.107 [2] (4) [3]	0.576 ± 0.117 (6)	0.774 ± 0.179 (8)	0.560 ± 0.141 (9)
60	0.686 ± 0.199 (5)	0.750 ± 0.176 (10)	1.006 ± 0.215 (7)	1.077 ± 0.233 (7)
80	1.722 ± 0.200 (6)	1.330 ± 0.265 (7)	1.923 ± 0.483 (8)	1.494 ± 0.336 (7)

[1] There were no significant differences among these 4 levels with respect to their abilities to actively transport lysine.
[2] Means ± SE of mean.
[3] Numbers in parentheses are number of observations.

ferences among these 4 levels with respect to their abilities to transport lysine. The rate of transport across these levels for 40, 60, and 80 minutes of incubation was linear ($P < 0.01$) and there was no significant interaction between intestinal level and incubation time. Consequently, one straight regression line calculated from the data in table 2 represents the average rate of active lysine transport across these four intestinal levels (fig. 1). The average rate of active lysine transport under the conditions of these studies, was calculated to be 1.028 mg or 7.03 μmoles/g of dried sac per hour. Also presented in figure 1 is the mean amount of lysine transported by each level during each of the three incubation intervals.

2. *Lysine transport based on length of live intestinal sac.* The milligrams of lysine activity transported by an 8-cm length of live intestine from each of the 6 levels of the small intestine during the three incubation periods are tabulated in tables 1 and 3. These data indicate that the upper and lower levels of the duodenum (table 1) did not transport lysine, whereas the other 4 levels did (table 3). There were no significant differences among the lower four intestinal levels with respect to their ability to actively transport lysine. Their rate of transport with time was linear ($P < 0.01$).

There was a significant interaction ($P < 0.05$) between intestinal level and incubation time. This variation in rate of transport with time is shown in figure 2.

DISCUSSION

1. *Lysine transport based on weight of dried intestinal sac.* The results of the lysine transport study based on a unit of weight of dried tissue (table 1 and fig. 1) indicate there is a high degree of similarity between the avian and mammalian classes.

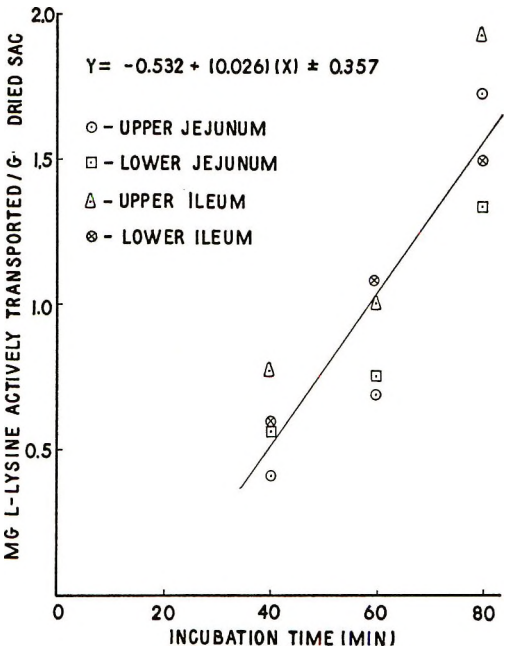

Fig. 1 Average rate per gram of dried sac of active transport of L-lysine across the upper and lower halves of the jejunal and ileal walls of the avian intestine; each point represents a mean of 4–10 determinations.

TABLE 3

Milligrams[1] *of* L-*lysine per 8 cm of intestine actively transported across the upper and lower levels of the jejunal and the ileal walls of the avian intestine after 40, 60 and 80 minutes of incubation at 37°*

Incubation time	Jejunum		Ileum	
	Upper level	Lower level	Upper level	Lower level
min				
40	0.130 ± 0.043 (6)	0.176 ± 0.051 (9)	0.213 ± 0.036 (8)	0.137 ± 0.036 (9)
60	0.294 ± 0.077 (7)	0.297 ± 0.067 (10)	0.260 ± 0.065 (8)	0.279 ± 0.060 (7)
80	0.842 ± 0.308 (5)	0.634 ± 0.190 (7)	0.567 ± 0.136 (8)	0.398 ± 0.087 (7)

[1] There were no significant differences among these 4 levels of intestine with respect to their abilities to actively transport lysine although there was a significant time × level interaction ($P < 0.05$).
[2] Means ± SE of mean.
[3] Numbers in parentheses are number of observations.

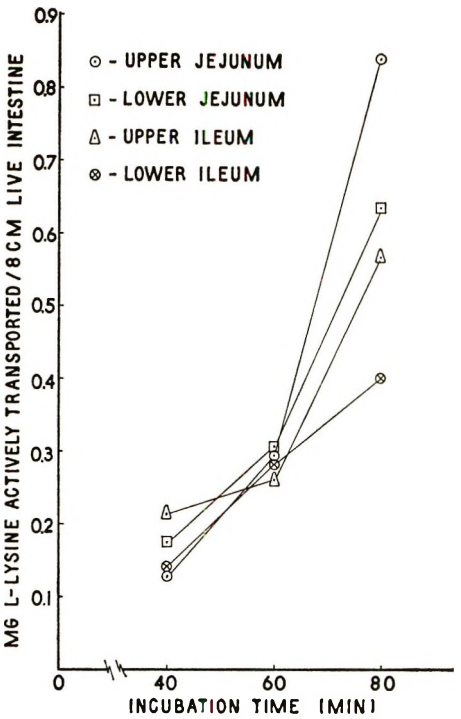

Fig. 2 Rate per 8 cm of live intestine of active transport of L-lysine across the upper and lower halves of the jejunal and ileal walls of the avian intestine; each point represents a mean of 5–10 determinations.

In the present investigation no significant differences were found in the ability of the upper and lower levels of the avian jejunal and ileal intestine walls to actively transport lysine. According to Wilson (3), Hagihira showed that the upper, mid, and lower levels of the hamster small intestine actively transport lysine at the same rate against a concentration gradient. Larsen et al. (4) observed similar activity by the rat small intestine.

There was no lysine transport in the avian duodenum (table 1) in the present investigation. It is not clear whether Hagihira et al. (2) and Larsen et al. (4) made studies of this region.

The rate of active lysine transport across the upper and lower portions of the avian jejunal and ileal intestine walls in the present investigation was calculated to be 7.0 μmoles/g of dried sac per hour. This value is in close agreement with Hagihira's value, 7.7 μmoles/g of sac per hour in the hamster.

2. *Lysine transported based on length of live intestinal sac.* The data tabulated in table 3 and plotted in figure 2 indicate that the rate of lysine transport across the intestinal wall per unit length of intestine decreased progressively from the upper jejunum to the lower ileum. Although these results may appear to contradict the results based on tissue weight, they do not.

The dry weight of 8-cm lengths of live intestine is shown in table 4. Although a unit length of the upper level of the jejunum transported almost twice as much lysine as a unit length of the lower level of the ileum at the end of 80 minutes of incubation (table 3), the rate of transport based on tissue weight is similar for the 2 levels, because the upper jejunum has approximately twice the weight of the lower ileum per unit length.

Both Verzar and McDougall (11) using the pigeon, and Imondi and Bird (12), using the chick, demonstrated a progressive decrease in the length of the villi from the duodenum through the ileum. Consequently, there must be a corresponding decrease in the mucosal absorptive area per unit length of intestine. These findings probably explain the observed decrease in the rate of transport from the upper jejunum to lower ileum when the rates are based on intestinal length. It is not known why the differences in transport rate were not manifested at 40 and 60 minutes of incubation. Possibly individual variation among the birds overshadowed any existing rate differences.

From the transport rates based on intestinal length, the observations of Verzar and McDougall (11), and those of Imondi and Bird (12), it is concluded that the rate of lysine absorption and the

TABLE 4

Dry weight of 8-cm lengths of various regions of the avian small intestine

Intestinal level	Dry wt
	g/8 cm
Upper jejunum	0.4395
Lower jejunum	0.4309
Upper ileum	0.3040
Lower ileum	0.2592

area of mucosal absorptive surface of the avian small intestine levels below the duodenum are closely correlated. Thus, the progressive decrease in the rate of lysine transport per unit length from the upper jejunum to the lower ileum noted in this study is probably a reflection of the corresponding decrease in mucosal absorptive surface. Consequently, the transport rates based on intestinal length appear to be more nearly a true representation of the physiological differences among the various levels of the avian intestine to actively absorb lysine than the values based on intestinal weight.

The results reported by Imondi and Bird (7) add further support to this conclusion. They showed, by means of an in vivo nonabsorbable marker technique, that the percentage of dietary nitrogen which disappeared per intestinal level decreased progressively from the upper jejunum to the lower ileum. Since their results were presented as absolute values instead of being based on intestinal weight or intestinal length, they are true values within the limitations of their experimental procedure.

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Effects of Dietary EDTA and Cadmium on Absorption, Excretion and Retention of Orally Administered ^{65}Zn in Various Tissues of Zinc-deficient and Normal Goats and Calves [1,2,3]

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ABSTRACT The effects of dietary EDTA (300 ppm) and cadmium (350 ppm) on absorption, excretion and tissue distribution of ^{65}Zn were determined following a single oral dosing in zinc-deficient and normal calves and goats. The feeding of EDTA had no significant effect upon fecal excretion or tissue distribution of ^{65}Zn. However, there was a marked increase in urinary ^{65}Zn excretion among the EDTA-fed animals which persisted throughout the experimental period. Dietary cadmium decreased absorption and tissue concentration of ^{65}Zn. When ^{65}Zn was calculated as percentage of absorbed dose most tissues of cadmium-fed animals contained amounts comparable to those of other animals. However, livers of cadmium-fed calves, but not goats, had more ^{65}Zn as a percentage of absorbed dose than livers of other animals. Cadmium had little effect on urinary excretion of ^{65}Zn. In general the effects of dietary EDTA and of cadmium in zinc-deficient animals were comparable to those in normal animals.

When added to diets containing phytic acid, orally administered ethylenediaminetetraacetic acid (EDTA) can aid in the utilization of zinc by rats (1, 2), turkey poults (3, 4), chickens (5, 6) and pigs (7). This increased utilization may be due to increased absorption of the zinc (4, 5). No published information has been found concerning the effects of dietary EDTA upon the absorption and tissue distribution of orally administered ^{65}Zn in normal or zinc-deficient ruminants.

Cadmium and zinc are related in the metabolism of mice (8, 9), rats (8, 10–12), rabbits (13), turkey poults (14), chicks (15) and calves (16). The oral administration of cadmium can cause symptoms which resemble a zinc deficiency (14, 16). The exact mechanism by which cadmium, an antimetabolite of zinc, causes these symptoms has not been elucidated. Cadmium may compete with zinc and replace it at active metabolic sites thereby rendering them inactive (15).

In a previous publication (16), the cadmium toxicity syndrome in the male bovine was described and the Cd-Zn relationship studied. However, that study was not designed to determine the effect of dietary Cd on absorption and tissue distribution of ^{65}Zn and did not involve animals in a zinc-deficient condition.

The objective of the present study was to determine the effect of dietary EDTA and cadmium on absorption, excretion and tissue distribution of orally administered ^{65}Zn in normal and zinc-deficient calves and goats.

EXPERIMENTAL PROCEDURE

Eighteen male Holstein calves and 17 male goats were fed a practical-type diet including commercial milk replacer and

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[2] This report is taken from a thesis submitted to the Graduate faculty of the University of Georgia by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

[3] Appreciation is extended to the Kraft Foods Company, Garland, Texas, for dried whole whey; to the Chas. Pfizer Company, Terre Haute, Indiana, for antibiotics and vitamins; to Hoffmann-LaRoche, Inc., Nutley, New Jersey, for the biotin; to Commercial Solvents, New York, for choline; to Merck and Company, Rahway, New Jersey, for pyridoxine and vitamin B_{12}; to Distillation Products, Inc., Rochester, New York, for *d*-α-tocopheryl acetate; to American Cyanamid, Princeton, New Jersey, for folic acid; to Abbott Laboratories, North Chicago, Illinois, for menadione; to Basic, Incorporated, Cleveland, for magnesium oxide; and to the Allied Chemical Company, Atlanta, Georgia, for urea.

calf starter until 2 months of age. Using diets similar to those described previously (17, 18) (the only change from diets listed in reference 17 was a 30% increase in vitamin A potency), they were then fed either a zinc-deficient or a control diet. The zinc-deficient animals were fed ad libitum and the normal ones (within replications) restricted to about the same level of intake. By analysis the zinc-deficient basal diet contained 4.0 ppm zinc. The control diet was identical to the zinc-deficient diet except for the addition of 40 ppm supplemental zinc as ZnO.

After the animals receiving the deficient diet developed typical symptoms of a zinc deficiency (19–21), they and the controls were assigned at random to one of the following treatment diets: (a) basal, (b) basal + 300 ppm EDTA (as disodium EDTA), or (c) basal + 350 ppm cadmium (as $CdCl_2$). (In each replication, initially 3 animals were assigned to the control diet and the same number to the deficient diet. During the time required for the deficiency to develop a number of animals were lost but generally not due to reasons associated with the treatments. In such instances, arbitrarily the cadmium treatment was left out in the randomization of animals to dietary treatments.) All of the basal diets were zinc-deficient so as to avoid confounding the metabolic effects of the zinc deficiency with the dietary effects due to differences in zinc level. Feeding of these diets began 7 days before oral dosing with ^{65}Zn.

The ^{65}Zn (as $ZnCl_2$ in HCl solution) was administered orally in gelatin capsules by the procedure described previously (22). The calves weighed from 56 to 91 kg (avg, 76 kg) and were 9 to 18 weeks of age (avg, 15 weeks) at dosing. The goats were 16 to 19 weeks of age (avg, 18 weeks) and weighed from 10 to 26 kg (avg, 15 kg) at dosing. All tissue concentration data were adjusted to a uniform body weight as discussed in a previous paper (22). Beginning 7 days before dosing, the animals were allowed to become accustomed to metabolism crates.

After dosing, fecal and urine output was determined by total collection daily. Blood samples were obtained from the jugular vein at 1, 4, 8, and 24 hours and 2, 4, 7, 10, and 13 days after dosing. Heparin was used to prevent coagulation and washed red blood cells were obtained as follows: 50-ml aliquots were centrifuged and the plasma was removed. The red blood cells were resuspended and centrifuged 3 times in 30 ml saline. Serum was obtained from blood samples which were collected without an anticoagulant.

Fourteen days following dosing with ^{65}Zn, the animals were anesthetized by injection of sodium pentobarbital and killed by cannulation of the carotid artery to remove blood from the organs and tissues. At this time, tissue samples were taken and frozen until analyses were made for stable zinc and ^{65}Zn. The tissues were sampled in the following manner: (a) liver, 1 g from lower center of reticular impression; (b) spleen, 2 g from cross section at one-fourth of the distance from the tip to the other end; (c) kidney, 1 g from the medulla; (d) tibia, 1.5 to 2.0 g (calves) and 0.5 to 1.2 g (goats) from the epiphyseal-diaphyseal junction; (e) testicles, 2-g sample from center cross section after the viceral layer and the tunica albuginea were removed; (f) muscle, 2-g section from the semitendinosus (round); (g) heart, 2 g from apex; and (h) lung, 2 g from center cross section avoiding major blood vessels.

Total zinc concentration was determined by atomic absorption spectroscopy with nitric-perchloric-sulfuric acid wet-ashing of samples (23). The ^{65}Zn activity of the samples was determined with either a whole-body counter designed for small samples and a 400-channel multichannel analyzer [4] or an automatic gamma test tube changer system with a NaI (T1) well crystal.[5]

RESULTS

The daily rate and accumulated total fecal excretion of ^{65}Zn for the goats and calves are presented in figure 1. In most instances, the peak rate of fecal excretion of ^{65}Zn from animals fed cadmium occurred at a later time after dosing than in those fed the basal and EDTA diets. The latter reached a peak at 2 or 3 days

[4] The whole-body counter was manufactured by Metrix, Inc., Deerfield, Illinois, and the multichannel analyzer by Technical Measurements, Inc., North Haven, Connecticut.

[5] Manufactured by Baird Atomic, Cambridge, Massachusetts.

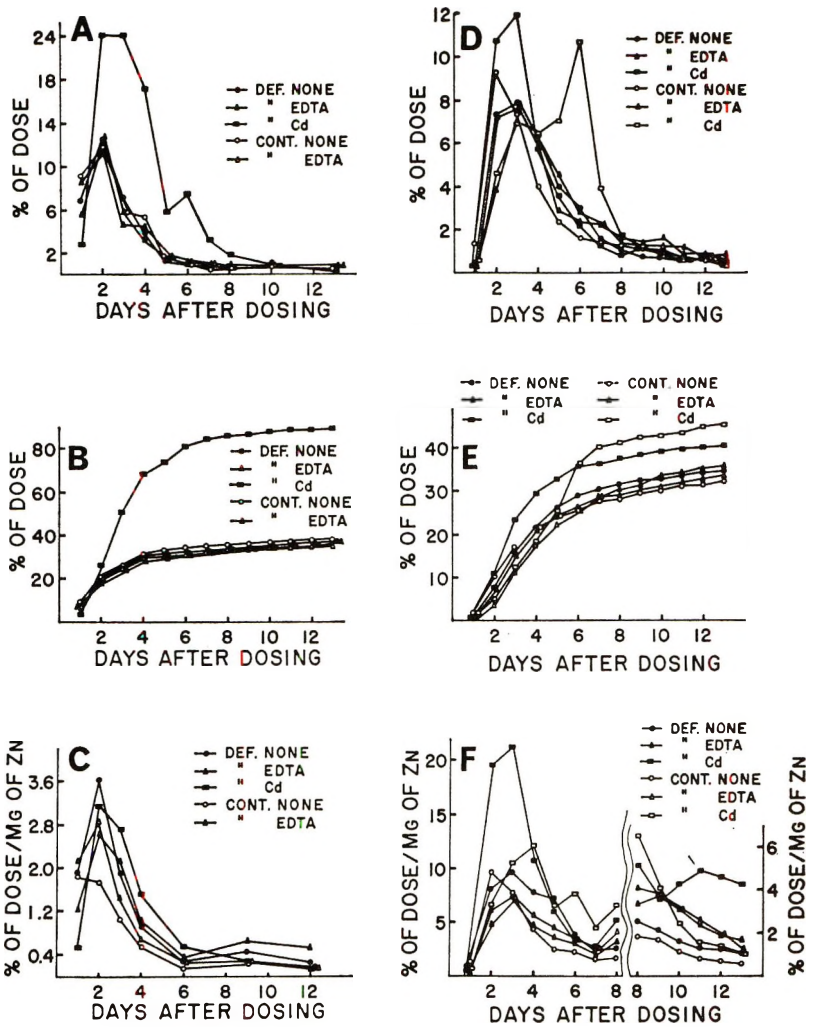

Fig. 1 Effects of dietary EDTA and Cd on ^{65}Zn excretion and specific activity in normal and zinc-deficient ruminants following a single dose of ^{65}Zn: (*A*) daily rate of fecal excretion by calves (4 animals/treatment except 3 for deficient-none and 2 for deficient-cadmium); (*B*) accumulated total fecal excretion by calves; (*C*) specific activity of calf fecal zinc; (D) daily rate of fecal excretion by goats (3 goats/treatment except 2 for control-EDTA); (*E*) accumulated total fecal excretion by goats; and (*F*) specific activity of goat fecal zinc.

after dosing which is comparable to previous results (22). Within species, there were no significant differences between the ^{65}Zn excretion of the animals given EDTA and the controls. Likewise the differences between zinc-deficient and control animals fed the basal diet were not significant. Both goats and calves fed cadmium excreted considerably more ^{65}Zn in the feces than did the others, with cadmium-fed calves excreting approximately twice as much as goats given cadmium. After 8 days, there were no large differences in daily excretion rate between any of the 6 treatments.

The specific activity data (% of the ^{65}Zn dose excreted/mg of zinc) of goat feces (fig. 1) tended to be similar to those of the percentage of dose per gram of feces. In most instances the specific activity of the feces from cadmium-fed goats was higher than that from the others. The

specific activity of the feces from the calves fed cadmium (fig. 1) was not higher than the other calves as cadmium-fed animals excreted considerably more stable zinc in the feces than the others. Fecal excretion of total zinc was 2.6 times dietary intake in cadmium-fed calves indicating considerable endogenous loss. For calves given the other treatments and goats on all treatments, feed intake of total zinc was about equal to or larger than fecal losses.

Dietary EDTA significantly increased urinary excretion of ^{65}Zn by the normal and deficient goats (fig. 2). A similar though somewhat less marked effect was observed in calves. Urinary ^{65}Zn excretion of goats fed EDTA reached a peak on the second day after dosing, whereas in other animals a sharp peak did not occur.

Level of ^{65}Zn in whole blood of both species (figs. 3-C and 4-A) increased very sharply for 8 hours with a further appreciable increase at 2 days. After that it appeared to increase slightly for the 14-day study period. The ^{65}Zn, as percentage of dose per gram, in the goat serum (fig. 3-A) and calf plasma (fig. 4-B) increased for the first 2 to 4 days following dosing and declined thereafter. The ^{65}Zn content of red blood cells in calves (fig. 4-C) increased rapidly for the first 24 hours and then advanced at a much slower rate until the end of the experimental period. Levels of ^{65}Zn were much lower in whole blood, plasma and red blood cells of calves fed cadmium and tended to be lower in whole blood and serum from goats given cadmium than in those fed the other diets. There were no important differences in ^{65}Zn content of whole blood, red blood cells, serum, or plasma among animals fed the EDTA and the basal diet.

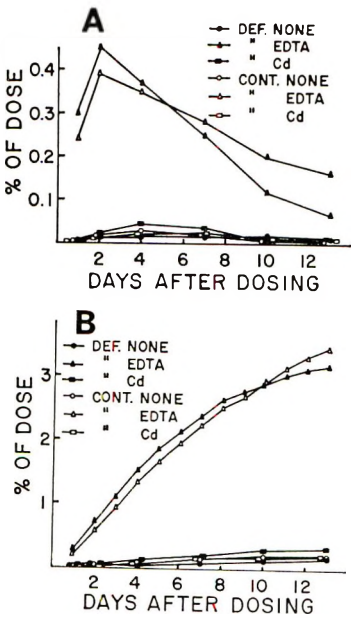

Fig. 2 Effects of dietary EDTA and Cd on ^{65}Zn excretion and specific activity in urinary zinc of normal and zinc-deficient goats following a single oral dose of ^{65}Zn: (*A*) daily rate of urinary excretion; (*B*) accumulated total urinary excretion. Three animals/treatment except 2 for control-EDTA.

Percentages of the ^{65}Zn dose retained in various goat and calf tissues are presented in tables 1 and 2. In all instances liver contained the highest concentration with spleen, kidney, lung and heart also having large amounts and usually in that order. The other tissues contained considerably less ^{65}Zn with the bone and skin having the least. The deficient animals retained a larger percentage of the dose in most tissues which is comparable to previous results (22). Bone was a notable exception to this effect. In general EDTA did not have large effects on ^{65}Zn distribution in tissues. However, there was a tendency for the normal goats and calves fed EDTA to have slightly more ^{65}Zn in their tissues than the normal animals fed no EDTA whereas the zinc-deficient animals given EDTA had slightly less tissue ^{65}Zn than the zinc-deficient ones given no EDTA. None of these differences was significant ($P = 0.05$).

The tissues from calves and goats fed cadmium generally contained a lower level of ^{65}Zn than those of the animals given no additives (tables 1 and 2). In calves this effect was usually significant ($P = 0.05$). However, in most instances the differences in goat tissues were not significant. When calculated as percentage of absorbed dose

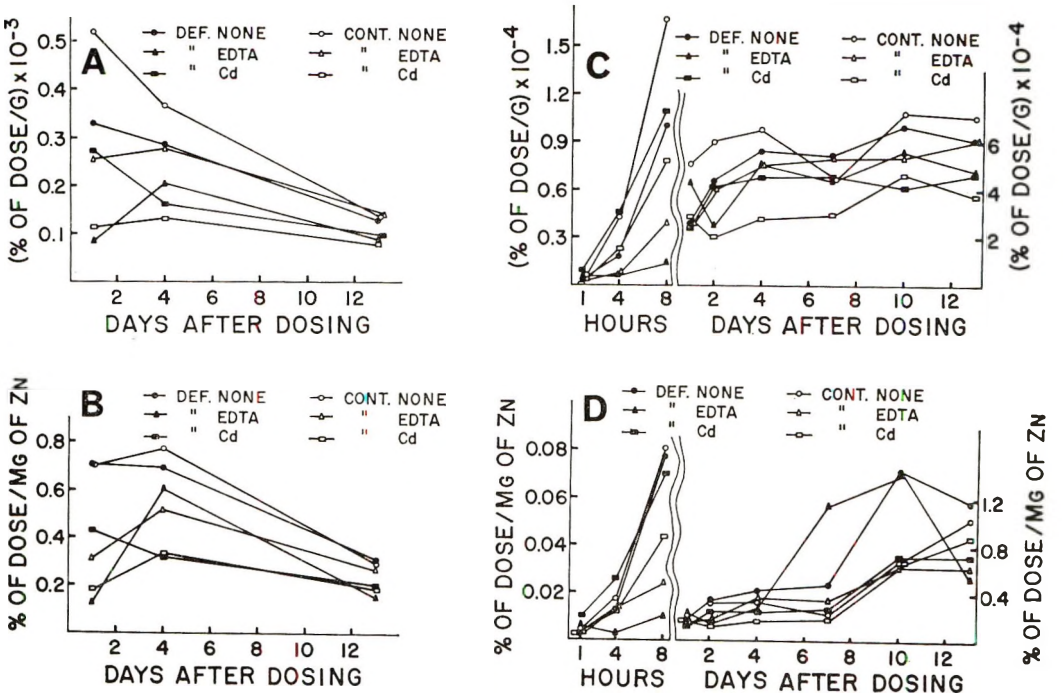

Fig. 3 Effects of dietary EDTA and Cd on ^{65}Zn content and specific activity of goat whole blood and serum following a single oral dose of ^{65}Zn: (*A*) ^{65}Zn content of serum (values presented as exponents to avoid awkward numbers, for example 0.4×10^{-3} should be read 0.0004); (*B*) specific activity of serum zinc; (C) ^{65}Zn content of whole blood; (*D*) specific activity of whole blood zinc. Three animals/treatment except 2 for control-EDTA.

TABLE 1

Effects of dietary EDTA and Cd on ^{65}Zn retention in tissues of zinc-deficient and control goats 14 days after oral dosing

Tissue	Control [1]			Zinc-deficient			SE [2]
	None (3 goats)	EDTA (3 goats)	Cd (3 goats)	None (3 goats)	EDTA (2 goats)	Cd (3 goats)	
	% of ^{65}Zn dose/kg of fresh tissue						
Heart	7.30^{ab} [3]	9.35^{a}	3.88^{b}	7.69^{ab}	6.80^{ab}	5.43^{ab}	1.14
Lung	8.18^{a}	9.70^{a}	6.91^{a}	7.83^{a}	6.31^{a}	6.37^{a}	1.36
Liver	17.68^{ab}	23.11^{a}	9.96^{b}	21.33^{a}	13.84^{ab}	18.10^{ab}	1.79
Spleen	10.10^{ab}	13.96^{a}	6.44^{b}	12.07^{a}	8.83^{ab}	8.95^{ab}	1.43
Kidney	8.45^{a}	12.87^{a}	6.02^{a}	11.92^{a}	8.15^{a}	9.21^{a}	1.88
Testicles	5.27^{ab}	5.96^{a}	2.63^{b}	4.20^{ab}	2.68^{ab}	3.24^{ab}	0.72
Scrotum	3.28^{ab}	3.85^{a}	1.55^{b}	3.17^{ab}	1.97^{ab}	1.39^{b}	0.53
Muscle (round)	5.12^{a}	3.64^{ab}	1.72^{b}	4.40^{a}	2.90^{ab}	3.67^{ab}	0.57
Bone (tibia)	2.56^{a}	1.88^{ab}	0.78^{c}	1.08^{bc}	0.83^{bc}	0.79^{c}	0.27
Skin	2.57^{b}	2.86^{b}	1.19^{a}	2.08^{ab}	2.13^{ab}	1.50^{a}	0.25

[1] Control animals were in a normal physiological condition. All animals given low zinc purified basal diet with either no additive (None), 300 ppm EDTA, or 350 ppm cadmium.

[2] SE = standard error of a treatment mean for 3 animals. When only 2 animals are in a treatment the SE should be multiplied by 1.225.

[3] Within each individual tissue those values not followed by the same letter are significantly different at the 5% probability level.

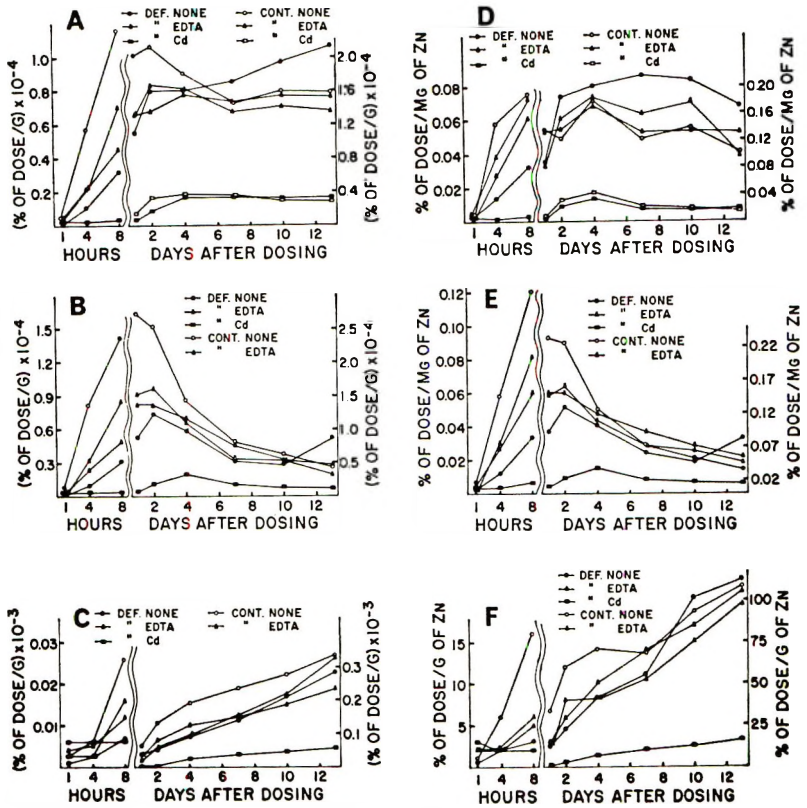

Fig. 4 Effects of dietary EDTA and Cd on ^{65}Zn content and specific activity of zinc following a single oral dose of ^{65}Zn: (*A*) ^{65}Zn content of whole blood (values presented as exponents to avoid use of awkward numbers, for example 0.4×10^{-4} should be read 0.00004); (*B*) ^{65}Zn content of plasma; (*C*) ^{65}Zn content of red blood cells; (*D*) specific activity of whole blood zinc; (*E*) specific activity of plasma zinc; (*F*) specific activity of red blood cell zinc. Four calves/treatment except; 3 for deficient-none, 2 for deficient-cadmium, and 1 for control-cadmium.

TABLE 2

Effects of dietary EDTA and Cd on ^{65}Zn retention in tissues of zinc-deficient and control calves 14 days after oral dosing

Tissue	Control [1]			Zinc-deficient			SE [3]
	None (4 calves)	EDTA (4 calves)	Cd (1 calf) [2]	None (3 calves)	EDTA (4 calves)	Cd (2 calves)	
	% of ^{65}Zn dose/kg of fresh tissue						
Heart	1.46[a,4]	1.67[a]	0.47	2.03[a]	2.06[a]	0.42[b]	0.19
Lung	1.60[b]	1.83[ab]	0.51	2.37[a]	2.10[ab]	0.46[c]	0.18
Liver	3.34[bc]	3.90[ab]	1.59	5.19[a]	5.55[a]	1.58[c]	0.41
Spleen	1.99[a]	2.33[a]	0.52	3.09[a]	2.74[a]	0.55[b]	0.33
Kidney	1.59[b]	1.91[ab]	0.58	2.56[a]	2.45[a]	0.56[c]	0.21
Testicles	1.12[a]	1.24[a]	0.35	1.50[a]	1.40[a]	0.25[b]	0.16
Scrotum	0.52[ab]	0.47[ab]	0.12	1.03[a]	0.77[ab]	0.09[b]	0.15
Muscle (round)	0.68[a]	0.62[a]	0.14	0.71[a]	0.68[a]	0.14[b]	0.08
Bone (tibia)	0.58[a]	0.45[ab]	0.08	0.38[ab]	0.26[ab]	0.05[b]	0.09
Skin	0.40[b]	0.42[ab]	0.13	0.62[a]	0.57[ab]	0.08[c]	0.49

[1] Control animals were in a normal physiological condition. All animals given low zinc purified basal diet with either no additive (None), 300 ppm EDTA, or 350 ppm cadmium.
[2] Values for this animal not included in statistical analysis.
[3] SE = standard error of a treatment mean for 4 animals. When only 2 or 3 animals are included in a treatment, the SE for that mean should be multiplied by 1.155 or 1.414, respectively.
[4] Within each individual tissue those values not followed by the same letter are significantly different at the 5% probability level.

TABLE 3

Effects of dietary EDTA and Cd on ^{65}Zn retention in tissues of zinc-deficient calves 14 days following oral dosing [1]

Tissue	Control [2]		Zinc-deficient		
	None (4 calves)	EDTA (4 calves)	None (3 calves)	EDTA (4 calves)	Cd (2 calves)
	% of absorbed ^{65}Zn dose/kg of fresh tissue				
Heart	2.4	2.6	3.2	3.2	3.7
Lung	2.6	2.8	3.8	3.2	4.1
Liver	5.4	6.0	8.2	8.5	13.9
Spleen	3.2	3.6	4.9	4.2	4.9
Kidney	2.6	2.9	4.1	3.8	4.9
Testicles	1.8	1.9	2.4	2.2	2.2
Scrotum	0.8	0.7	1.6	1.2	0.8
Muscle (round)	1.1	1.0	1.1	1.0	1.2
Bone (tibia)	0.9	0.7	0.6	0.4	0.4
Skin	0.6	0.6	1.0	0.9	0.7
Avg [3]	2.1	2.3	3.1	2.9	3.7
Whole blood [4]	0.26	0.21	0.34	0.23	0.27
Plasma	0.07	0.06	0.14	0.07	0.10
Red blood cells	0.55	0.36	0.45	0.50	0.52

[1] Calculated as percentage of dose not excreted in feces for 13 days after dosing.
[2] Control animals were in a normal physiological condition. All animals given low zinc purified diet with either no additive (None), 300 ppm EDTA, or 350 ppm cadmium.
[3] Does not include blood or its components.
[4] Blood samples taken 13 days following dosing.

TABLE 4

Effects of dietary EDTA and Cd on ^{65}Zn retention in tissues of zinc-deficient and control goats 14 days after oral dosing [1]

Tissue	Control [2]			Zinc-deficient		
	None (3 goats)	EDTA (3 goats)	Cd (3 goats)	None (3 goats)	EDTA (2 goats)	Cd (3 goats)
	% of absorbed ^{65}Zn dose/kg of fresh tissue					
Heart	10.5	15.6	7.1	11.7	10.6	9.2
Lung	11.7	8.6	12.7	12.0	9.9	10.8
Liver	25.3	38.5	18.2	32.6	21.6	30.7
Spleen	14.5	23.2	11.8	18.4	13.8	15.2
Kidney	12.1	21.4	11.0	18.2	12.7	15.6
Testicles	7.6	9.9	4.8	6.4	4.2	5.5
Scrotum	4.7	6.4	2.8	4.8	3.1	2.4
Muscle (round)	7.3	6.1	3.2	6.7	4.5	6.2
Bone (tibia)	3.7	3.1	1.4	1.6	1.3	1.3
Skin	3.7	4.8	2.2	2.2	3.3	2.5
Avg [3]	10.1	13.8	7.5	11.5	8.5	9.9
Whole blood [4]	1.04	1.00	0.68	0.92	0.76	0.79
Serum	0.20	0.23	0.14	0.20	0.14	0.17

[1] Calculated as percentage of dose not excreted in feces for 13 days after dosing.
[2] Control animals were in a normal physiological condition. All animals given low zinc purified basal diet with either no additive (None), 300 ppm EDTA, or 350 ppm cadmium.
[3] Does not include blood or components.
[4] Blood samples taken 13 days following dosing.

(table 3), the ^{65}Zn content in calf livers was increased by cadmium feeding. With the other tissues from animals given cadmium the ^{65}Zn level as percentage of absorbed dose, generally was not very different from that of other calves. As percentage of absorbed dose, tissue ^{65}Zn concentration of goats fed cadmium tended to be slightly lower than that of the others (table 4). The large increase in liver ^{65}Zn, as percentage of absorbed dose, observed with the calves as a result of cadmium feeding, was not evident in goats.

The specific activities of zinc for goat and calf tissues were calculated. In general the treatment effects on these data

were similar to those observed in ^{65}Zn content data. Thus the specific activity values [6] are not presented here.

DISCUSSION

In this study the addition of 300 ppm EDTA to a low zinc purified diet did not greatly influence absorption of ^{65}Zn in zinc-deficient or normal calves and goats. However, this does not prove that EDTA would not have such an effect in ruminants under different conditions. It is possible that the effect of EDTA on zinc absorption was masked by the extremely high percentage of the ^{65}Zn dose absorbed. It is reasonable to assume that the beneficial effect of EDTA on absorption might be observed only in cases where ^{65}Zn absorption is reduced, possibly as a result of an interfering dietary constituent. The increased utilization of zinc that occurs when EDTA is fed to other species may be due to EDTA removing dietary zinc from a zinc-phytate complex (4, 5) or from some other relatively unavailable form, thereby making it more readily absorbed from the gastrointestinal tract. The diets used in the present experiment would not be expected to contain very much phytic acid as none of the ingredients normally contain apreciable amounts. In pigs and chickens the zinc requirement is much less when casein or gelatin is the source of protein rather than soybean or sesame meals (7, 24, 25). The addition of EDTA to a diet containing a high level of zinc does not give an added response, indicating that EDTA itself is not a growth stimulant (26).

The large increase in urinary ^{65}Zn excretion in this study which resulted from EDTA is in agreement with earlier observations in humans by Spencer et al. (27). They reported that the infusion of Ca-EDTA following the intravenous injection of a tracer dose of ^{65}Zn resulted in a 100-fold increase in urinary ^{65}Zn. The downward trend, 2 to 4 days after dosing, in urinary ^{65}Zn excretion by the animals fed EDTA may be due to the more labile ^{65}Zn being excreted shortly after dosing. If such is the case, each subsequent day there might be less labile ^{65}Zn present for the EDTA to chelate. This declining effect of a chelating agent on urinary excretion of ^{65}Zn has been observed in humans (27). The total quantity of ^{65}Zn excreted via urine is small. Therefore, over a limited period of time, as in this study, it had a negligible effect on the total body burden of ^{65}Zn.

After dosing there is a continuing redistribution of ^{65}Zn among the various body tissues (22). Therefore, had the tissue samples in the present experiment been taken at a time other than 14 days following dosing the relative values for tissue ^{65}Zn content and specific activity probably would have been different.

In the present experiment dietary EDTA had no significant effect on tissue distribution of orally administered ^{65}Zn. Foreman (28) reported that only a small percentage of orally administered EDTA was absorbed and that this was rapidly excreted in the urine. Also, it appears that the EDTA which is absorbed from the gastrointestinal tract is not present in substantial amounts in most tissues but is primarily in the body fluids. It has been reported that the intraperitoneal injection of EDTA can greatly increase excretion of ^{65}Zn and thereby decrease tissue concentration.[7]

The specific activity of zinc was consistently and appreciably higher, even after 10 to 13 days following dosing, in feces than in the plasma or serum in calves and goats (figs. 1, 3 and 4). In earlier data specific activity was higher, after 2 or 3 weeks, in feces than in urine of animals given a single intravenous dose (17). Both of these observations are of interest and somewhat perplexing. Clearly some phenomenon is present which is not experienced to a comparable degree with a number of other elements (29). While no satisfactory explanation has been established, it may be that the cells of the small intestine accumulate appreciable amounts of ^{65}Zn soon after dosing and that this is excreted over a period of time as the cells are sloughed (30, 31).

The low levels of ^{65}Zn in the tissues and blood of the animals fed cadmium were primarily a result of these animals absorbing considerably less ^{65}Zn from the

[6] See footnote 2.

[7] Ilin, L. A., G. V. Arkhangeliskaya and T. A. Norets 1964 Comparative effectiveness of some complex-forming compounds on the acceleration of zinc-65 elimination from the organism. Radiobiologiva, 4(6): 926 (cited in Chem. Abstr., 62: 5664d, (1965).

gastrointestinal tract than the ones fed no cadmium (tables 3 and 4). The increase in ^{65}Zn content of the liver of calves fed cadmium, when calculated as percentage of absorbed dose, is in agreement with observations made with rats (32) and mice (33) injected with ^{65}Zn and given cadmium either orally or by injection. This effect was not observed in goat livers or in other tissues of calves or goats.

The delayed peak in fecal excretion of ^{56}Zn by some of the animals administered cadmium may be a result of erratic fecal excretion. Cadmium reduced palatability as determined in cafeteria-type experiments (34) and voluntary feed intake was lowered independent of palatability when the cadmium was administered by capsule.[8]

Dietary cadmium greatly decreased the apparent absorption of ^{65}Zn and increased the fecal excretion of stable zinc. The increase in fecal zinc is reflected in the difference in fecal specific activity data being smaller than those for ^{65}Zn content. Reduced net absorption may well be a part of the mechansims by which dietary cadmium can produce symptoms similar to a zinc deficiency. It is not known whether this is due to: (a) a cadmium-zinc interaction, (b) a cadmium-induced pathology in which altered zinc metabolism is only indirectly involved, or (c) to some other mechanism. No clinical symptom of cadmium toxicity other than reduced feed intake was observed during the experimental period (16). Likewise dry-matter digestibility was not greatly affected by dietary cadmium.[9] Perhaps a better understanding of the zinc absorption mechanism will be necessary before the reduced ^{65}Zn absorption in cadmium-fed ruminants can be explained satisfactorily.

ACKNOWLEDGMENTS

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[9] Unpublished data, J. M. Hiers, Jr., W. J. Miller and D. M. Blackmon, 1967.

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Relationships between Vitamin B_6-vitamer Content and the Activities of Two Transaminase Enzymes in Rat Tissues at Varying Intake Levels of Vitamin B_6 [1,2]

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ABSTRACT To study relationships between the vitamin B_6-vitamer content of rat tissues to the activities of 2 transaminase enzymes in those tissues, and the relative biological activity of 3 vitamin B_6-vitamers in the rat, we arranged to study, in depth 7 groups of rats that were fed diets containing (μg/g diet) 0, 0.5, 1.0, 2.0, and 10.0 of pyridoxol·HCl and 1.0 of pyridoxal·HCl or pyridoxamine·2HCl. Although a dietary level of 2.0 μg/g diet of pyridoxol was adequate to support maximal growth, higher tissue levels of total vitamin B_6 and of the individual vitamers were reached at the 10.0 μg/g diet level, in liver, kidney, brain, muscle, and heart. Of the 3 vitamin B_6 vitamers, pyridoxamine was depleted to the greatest extent, as percentage, at lower levels of dietary pyridoxol. In studies on glutamic-pyruvic (GPT) and glutamic-oxalacetic transaminase (GOT) activities in the above tissues plus plasma and red blood cells, enzyme activity increased with increased dietary pyridoxol and with increased tissue vitamin B_6 content. GPT was depleted to a greater extent than, and GOT to a lesser extent than, the percentage depletion for tissue vitamin B_6 at lower levels of pyridoxol intake. It appeared from studies on growth, tissue content of vitamin B_6 and vitamers, and tissue transaminase activity, that at the level of 1.0 μg/g diet, pyridoxal was essentially equivalent to pyridoxol for vitamin B_6 activity; the study on pyridoxamine required re-evaluation. A simplified medium was used for the microbiological portion of the assay of the vitamin B_6 vitamers.

Much work has been carried out on the effects of vitamin B_6-deficiency on the activity of tissue transaminases in a number of animal species (1–5) and conditions (6, 7). To our knowledge, however, little work has been reported on the relationships between tissue vitamin B_6 content and tissue transaminase activity (8). With the recent availability of a modification of the Toepfer and Lehmann assay (9) which was suitable for the estimation of the content of the vitamin B_6-vitamers in animal tissues (8), a study was undertaken to relate vitamer content to the enzyme activity of 2 transaminases in various tissues of rats maintained at several levels of vitamin B_6 intake. Another assay medium was used in these studies to further simplify the analytical procedure.

MATERIALS AND METHODS

1. A preliminary study was made in which plasma transaminase activity was measured in groups of female rats fed purified diets containing: (in µg/g diet) 0.1, 0.5, 1.0, and 2.0, respectively, of pyridoxol·HCl. The results (fig. 1) suggested that the highest level which was fed approached, but may not have reached, the amount of dietary pyridoxol necessary to maintain maximal activity of these enzymes in plasma.[3] This general range of dietary pyridoxol to include one higher level appeared to span the range of adequacy for the rat (10) and was therefore chosen for the purposes of the present study, as follows.

2. *Animals and diets.* Seven groups of 6 or 7 male weanling rats [4] were fed puri-

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[2] Presented in part at the meetings of the Federation of American Societies for Experimental Biology, Chicago, 1967.
[3] Preliminary report presented at the annual meeting of the American Institute of Nutrition, Chicago, 1960 (Federation Proc., *19*: 321, 1960).
[4] Purchased from Carworth, Inc., New City, New York.

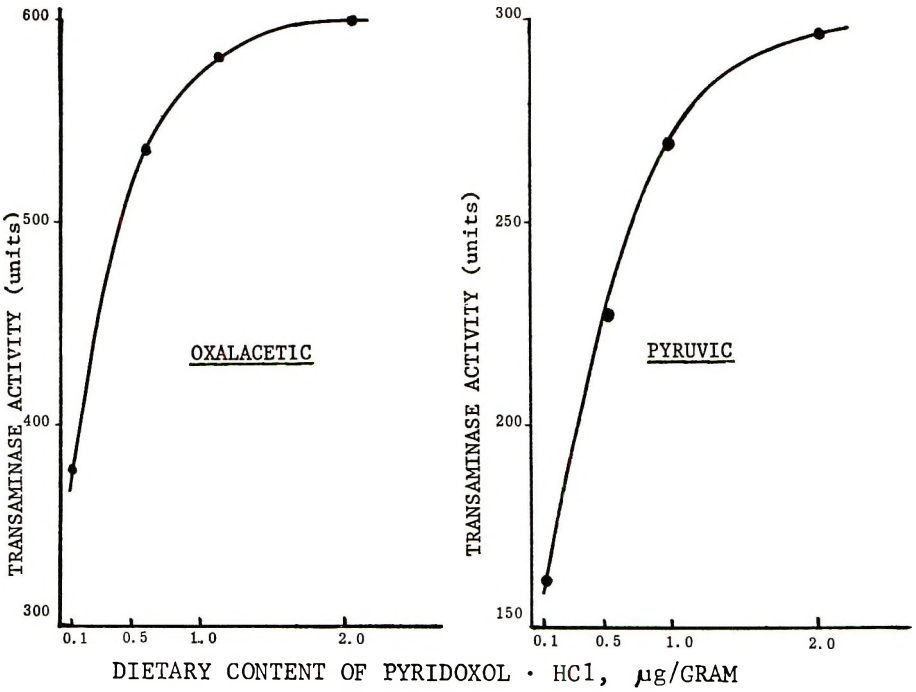

Fig. 1 Relationship between the activities of 2 plasma transaminase enzymes in rats to the dietary pyridoxol·HCl, after a 2-week experimental feeding period. Transaminase activity is expressed in micrograms of pyruvate formed per milliliter of plasma per hour.

fied diets for 14 days which varied in vitamin B_6 content (µg/g diet) as follows: group 1, 0; group 2, 0.5 pyridoxol·HCl;[5] group 3, 1.0 pyridoxol·HCl; group 4, 2.0 pyridoxol·HCl; group 5, 10.0 pyridoxol·HCl; group 6, 1.0 pyridoxal·HCl; group 7, 1.0 pyridoxamine·2HCl. The basal purified diet contained: (in percent) glucose, 73; devitaminized casein, 18; corn oil, 4; Hegsted salt mixture (11),[5] 4; cod liver oil, 1. The vitamins added were: (µg/100 g diet) thiamine·HCl, 400; riboflavin, 800; Ca pantothenate, 2500; niacin, 4,000; and choline chloride, 100,000. Food and water were available ad libitum. Each of the 3 vitamin B_6 vitamers was fed individually at a level of 1.0 µg/g diet. This dietary level was suboptimal on the basis of transaminase activity (fig. 1) and it was considered desirable to be on the ascending portion of that curve for better comparison of the equivalence of vitamin B_6 activity. Animals were killed, and tissues were taken for analysis after the 2-week feeding period.

3. *Vitamin B_6 assay.* a. Tissue samples were hydrolyzed, and the hydrolysates were chromatographed as described previously (8). However, the microbiological procedure was modified as follows: To the tubes containing 0.5, 1.0, and 2.0 ml of column eluates obtained from the tissue hydrolysates, water was added to make a total of 5 ml/tube.

b. The assay was made by adding 5 ml of double strength "Pyridoxine-Y-Medium," [6] to each tube, shaking each to mix the 2 phases, and steaming in the autoclave for 10 minutes at 100°. The use of this medium eliminated the need for the preparation of separate nutrient solutions as described by Toepfer and Lehmann (9) and for sterile filtering as needed previously with the vitamin-free yeast base (7). Maintenance of the organism (*Saccharomyces carlsbergensis*, ATCC no. 9080) and the preparation of inoculum was as described previously (8).

[5] Purchased from Nutritional Biochemicals Corporation, Cleveland.
[6] Purchased from Difco Laboratories, Detroit.

c. Standard solutions were made as follows: Solution A contained 50 mg of pyridoxol·HCl in 500 ml of a 25% solution of ethanol in water. A similar solution was made for each of the other 2 vitamers. Solution B, as made for each vitamer, contained 2 ml of solution A in 1 liter of a 25% solution of ethanol in water. Then solutions C, as made for pyridoxol·HCl and pyridoxal·HCl vitamer contained 1 ml of solution B in 100 ml of distilled water. Solution C for pyridoxamine·2HCl contained 3 ml of solution B. The final concentrations of the B_6 vitamers in solutions C were: (mμg/ml) pyridoxol·HCl, 2; pyridoxal·HCl, 2: pyridoxamine·2HCl, 6.

d. Standard curves were prepared by pipetting into duplicate tubes, the following amounts of each vitamer: (mμg) pyridoxol·HCl, 2, 3, 4, 5, 6, 8, 10; pyridoxal·HCl, 1, 2, 3, 4, 6, 8; and pyridoxamine·2HCl, 6, 9, 12, 15, 12, 24, 30.[7] Water was added to make a total volume of 5 ml of each tube.

4. *Transaminase activity.* Glutamic-pyruvic (GPT) and glutamic-oxalacetic (GOT) transaminases were assayed according to procedures described previously (12, 13) and applied to studies on pyridoxol deficiency (5, 14).

RESULTS AND DISCUSSION

1. *Effects of varying the levels of dietary vitamin B_6 vitamers on growth of rats.* The growth curves for the 7 groups of rats studied are presented in figure 2. The greatest weight gain was attained by the group receiving 2 μg of pyridoxol/g diet. Of the groups receiving varying levels of vitamin B_6, the least gain was shown by those receiving (in μg/g diet) 1.0 pyridoxamine and 0.5 pyridoxol, although none of these differences was statistically significant for the 2-week experimental period.

2. *Distribution of the 3 vitamin B_6 vitamers in rat tissues at varying levels of pyridoxol intake.* The distribution of the vitamin B_6 vitamers in rat tissues are presented in tables 1, 2, and 3 for the content of pyridoxol, pyridoxal and pyridoxamine,[8]

[7] Hereafter, in this report, the vitamer hydrochlorides will be referred to as the vitamers per se.

[8] By "content of pyridoxol, pyridoxal, and pyridoxamine" is meant the activity of each of these as elicited by the microbiological assay of the appropriate chromatographic eluates, as described in the text.

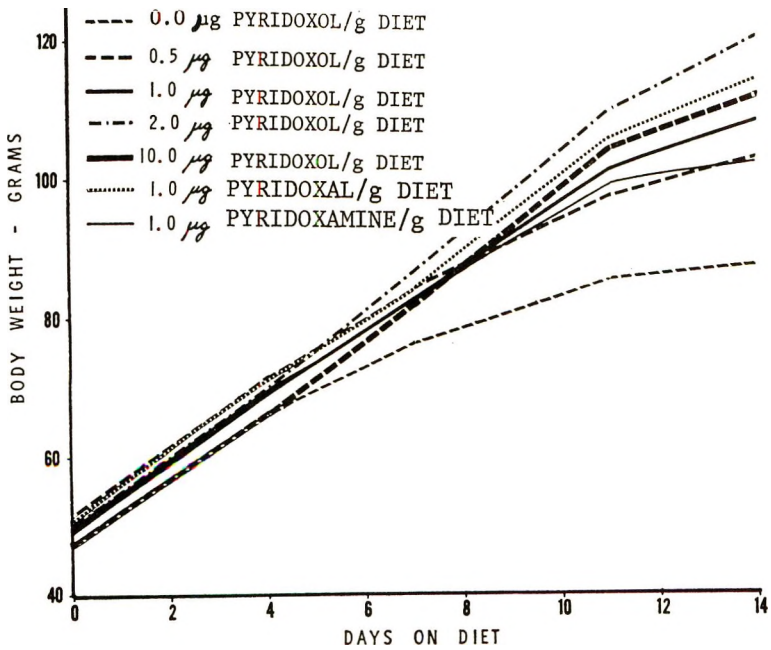

Fig. 2 Growth curves for body weights of rats maintained with a vitamin B_6-deficient diet, diets containing varying levels of pyridoxol·HCl, and diets containing single levels of each of the vitamin B_6 vitamers.

TABLE 1

Pyridoxol·HCl content of rat tissues

Diet	Liver	Kidney	Brain	Muscle	Heart
μg vitamer/g diet			*μg/g tissue*		
— [1]	0.48 ± 0.08	0.49 ± 0.04	0.07 ± 0.02	0.18 ± 0.07	0.28 ± 0.02
0.5 pyridoxol [2]	0.44 ± 0.05	0.54 ± 0.07	0.11 ± 0.02	0.17 ± 0.06	0.25 ± 0.01
1.0 pyridoxol [2]	0.56 ± 0.05	0.76 ± 0.15	0.14 ± 0.05	0.19 ± 0.05	0.26 ± 0.01
2.0 pyridoxol [2]	0.65 ± 0.08	0.73 ± 0.05 **	0.11 ± 0.04	0.21 ± 0.06	0.41 ± 0.07
10.0 pyridoxol [2]	0.69 ± 0.06	1.18 ± 0.20 **	0.13 ± 0.03	0.34 ± 0.05	0.40 ± 0.05
1.0 pyridoxal [1]	0.47 ± 0.03	0.70 ± 0.06 *	0.10 ± 0.03	0.20 ± 0.08	0.30 ± 0.03
1.0 pyridoxamine [1]	0.51 ± 0.07	0.48 ± 0.07	0.09 ± 0.02	0.27 ± 0.10	0.33 ± 0.04

[1] Mean of 6 rats and SE of mean.
[2] Mean of 7 rats and SE of mean.
* Significantly different from the deficient mean, $P < 0.05$.
** Significantly different from the deficient mean, $P < 0.01$.

TABLE 2

Pyridoxal·HCl content of rat tissues

Diet	Liver	Kidney	Brain	Muscle	Heart
μg vitamer/g diet			*μg/g tissue*		
— [1]	1.60 ± 0.16	0.70 ± 0.08	0.53 ± 0.03	1.07 ± 0.06	0.66 ± 0.05
0.5 pyridoxol [2]	1.62 ± 0.07	0.39 ± 0.10 *	0.56 ± 0.08	1.30 ± 0.10	0.66 ± 0.00
1.0 pyridoxol [2]	2.45 ± 0.24 *	0.65 ± 0.21	0.60 ± 0.06	1.62 ± 0.10 **	0.79 ± 0.09
2.0 pyridoxol [2]	2.49 ± 0.32 *	1.14 ± 0.23	0.78 ± 0.08 *	2.41 ± 0.11 **	0.66 ± 0.08
10.0 pyridoxol [2]	2.40 ± 0.29 *	1.28 ± 0.32	0.83 ± 0.08 **	2.34 ± 0.16 *	0.76 ± 0.10
1.0 pyridoxal [1]	1.67 ± 0.26	0.63 ± 0.10	0.63 ± 0.05	1.35 ± 0.12	0.58 ± 0.09
1.0 pyridoxamine [1]	1.54 ± 0.14	0.89 ± 0.21	0.44 ± 0.07	0.88 ± 0.11	0.55 ± 0.10

[1] Mean of 6 rats and SE of mean.
[2] Mean of 7 rats and SE of mean.
* Significantly different from the deficient mean, $P < 0.05$.
** Significantly different from the deficient mean, $P < 0.01$.

TABLE 3

Pyridoxamine·2HCl content of rat tissues

Diet	Liver	Kidney	Brain	Muscle	Heart
μg vitamer/g diet			*μg/g tissue*		
— [1]	4.04 ± 0.05	1.57 ± 0.03	1.12 ± 0.11	0.37 ± 0.04	1.93 ± 0.08
0.5 pyridoxol [2]	3.99 ± 0.31	1.74 ± 0.07	1.31 ± 0.13	0.43 ± 0.03	2.10 ± 0.35
1.0 pyridoxol [2]	5.60 ± 0.04 *	2.56 ± 0.14 **	1.59 ± 0.16 *	0.64 ± 0.06 **	2.65 ± 0.22 *
2.0 pyridoxol [2]	6.91 ± 0.24 **	3.36 ± 0.20 **	1.57 ± 0.12 *	1.05 ± 0.15 **	3.38 ± 0.19 **
10.0 pyridoxol [2]	6.33 ± 0.26 **	3.91 ± 0.23 **	1.60 ± 0.16 *	1.22 ± 0.15 **	3.79 ± 0.35 **
1.0 pyridoxal [1]	4.53 ± 0.30	3.08 ± 0.30 **	1.20 ± 0.06	0.54 ± 0.06 *	3.05 ± 0.11 **
1.0 pyridoxamine [1]	4.12 ± 0.22	2.04 ± 0.15 *	1.10 ± 0.04	0.48 ± 0.06	2.45 ± 0.27

[1] Mean of 6 rats and SE of mean.
[2] Mean of 7 rats and SE of mean.
* Significantly different from the deficient mean, $P < 0.05$.
** Significantly different from the deficient mean, $P < 0.01$.

TABLE 4

Total vitamin B_6 content of rat tissues

Diet	Liver	Kidney	Brain	Muscle	Heart
μg vitamer/g diet			*μg/g tissue*		
— [1]	6.12	2.77	1.72	1.62	2.87
0.5 pyridoxol [2]	6.05	2.67	1.98	1.90	3.01
1.0 pyridoxol [2]	8.61	3.97	2.33	2.45	3.70
2.0 pyridoxol [2]	10.05	5.23	2.46	3.67	4.45
10.0 pyridoxol [2]	9.42	6.37	2.56	3.90	4.95
1.0 pyridoxal [1]	6.67	4.41	1.93	2.09	3.93
1.0 pyridoxamine [1]	6.17	3.41	1.63	1.63	2.94

[1] Mean of 6 rats.
[2] Mean of 7 rats.

respectively, and in table 4, for total tissue vitamin B_6 content. Generally, tissue pyridoxol content was affected the least and pyridoxamine the most, by varying the pyridoxol content of rat diets. Of all the tissues studied, brain was the most refractory to changes in vitamer content, with varying pyridoxol intake. However, in all cases the tissue content of vitamin B_6 vitamers increased with increasing levels of dietary pyridoxol.

At the maximal level of pyridoxol fed, 10.0 µg/g diet, the tissue pyridoxal content was from 1.1 times (kidney) to 6.9 (muscle) times the pyridoxol content. Also, at that dietary level, the tissue pyridoxamine content was from 3.3 times (kidney) to 12.3 (brain) times the pyridoxol content. Also, the pyridoxamine content was from 0.5 times (muscle) to 5.0 (heart) times the pyridoxal content. It was evident, therefore, that as the dietary availability of pyridoxol increased, the increase in tissue pyridoxamine was generally greater than that for pyridoxal (except for muscle) which in turn, was greater than that for pyridoxol.

As dietary pyridoxol increased, so did total vitamin B_6 content (table 4). In most cases tissue vitamin B_6 content increased somewhat as the dietary pyridoxol level was increased fivefold from 2.0 to 10.0 µg/g diet, although the differences for each tissue were generally not as great as between the dietary pyridoxol levels of 1.0 and 2.0 µg/g diet. It might be suggested, on the basis of these data, that tissue saturation for vitamin B_6 was virtually achieved at a dietary level of 10.0 µg/g diet in rats.

3. *Effects of feeding varying levels of pyridoxol on the activities of GPT and GOT in rat tissues.* Transaminase activities in various tissues of rats fed varying levels of pyridoxol are presented in tables 5 and 6 for the GPT and GOT enzymes, respectively. For all tissues except kidney, GPT levels in rats fed 2.0 and 10.0 µg pyridoxol /g diet were significantly different from the vitamin B_6-deficient group. Although there were no statistically significant differences in GPT activity between rats fed those 2 higher dietary levels, there was a slight increase in GPT activity in three of

TABLE 5

Glutamic pyruvic transaminase activity (GPT)

Diet	Liver	Kidney	Brain	Muscle	Heart	Plasma	Red blood cells
µg vitamer/g diet	*mg pyruvic acid/g/hr*			*mg pyruvic acid/g/hr*		*µg pyruvic acid/ml/hr*	
— [1]	53 ± 7	4 ± 0.6	9 ± 0.4	5 ± 0.5	3 ± 0.3	107 ± 10	229 ± 41
0.5 pyridoxol [2]	57 ± 8	3 ± 0.3	10 ± 0.3	6 ± 0.5	4 ± 0.5	152 ± 17	302 ± 27
1.0 pyridoxol [2]	111 ± 30	4 ± 0.4	11 ± 0.5	8 ± 0.8 *	6 ± 0.5 **	177 ± 18 **	315 ± 26
2.0 pyridoxol [2]	145 ± 31 *	4 ± 0.6	11 ± 0.4 **	23 ± 2.0 **	14 ± 0.5 **	255 ± 9 **	629 ± 77 **
10.0 pyridoxol [2]	171 ± 37 *	4 ± 0.4	11 ± 0.3 **	27 ± 2.0 **	15 ± 0.7 **	370 ± 38 **	628 ± 81 **
1.0 pyridoxal [1]	102 ± 20 *	3 ± 0.4	11 ± 0.2 **	7 ± 0.6 *	6 ± 0.4 **	243 ± 63	304 ± 37
1.0 pyridoxamine [1]	74 ± 18	2 ± 0.2 *	11 ± 0.3	5 ± 0.8	3 ± 0.4	130 ± 33	200 ± 36

[1] Mean of 6 rats and SE of mean.
[2] Mean of 7 rats and SE of mean.
* Significantly different from the deficient mean, $P < 0.05$.
** Significantly different from the deficient mean, $P < 0.01$.

TABLE 6

Glutamic oxalacetic transaminase activity (GOT)

Diet	Liver	Kidney	Brain	Muscle	Heart	Plasma	Red blood cells
μg vitamer/g diet	*mg pyruvic acid/g/hr*			*mg pyruvic acid/g/hr*		*μg pyruvic acid/ml/hr*	
— [1]	487 ± 20	290 ± 13	409 ± 31	104 ± 5	424 ± 16	553 ± 90	3263 ± 264
0.5 pyridoxal [2]	487 ± 17	300 ± 7	416 ± 22	120 ± 7	451 ± 19	800 ± 76	3328 ± 381
1.0 pyridoxol [2]	632 ± 23 **	344 ± 12 *	427 ± 22	162 ± 7 **	518 ± 30 *	807 ± 78	3970 ± 333
2.0 pyridoxol [2]	616 ± 35 *	372 ± 11 **	445 ± 30	228 ± 12 **	610 ± 32 **	1403 ± 278 *	4690 ± 430 *
10.0 pyridoxol [2]	644 ± 17 **	378 ± 9 **	451 ± 25	258 ± 15 **	601 ± 9 **	1664 ± 334 *	3906 ± 368
1.0 pyridoxal [1]	567 ± 22 *	315 ± 9	441 ± 27	163 ± 10 **	475 ± 32	927 ± 238	3832 ± 246
1.0 pyridoxamine [1]	564 ± 33	278 ± 11	430 ± 28	130 ± 8 *	465 ± 23	715 ± 200	3094 ± 253

[1] Mean of 6 rats and SE of mean.
[2] Mean of 7 rats and SE of mean.
* Significantly different from the deficient mean, $P < 0.05$.
** Significantly different from the deficient mean, $P < 0.01$.

the tissues at the highest dietary pyridoxol level.

For the GOT enzyme (table 6) increased activity resulted in all tissues from increasing dietary pyridoxol and statistically significant differences were shown in all tissues except brain. Although tissue enzyme activity was slightly higher in most cases at the 10.0-μg dietary pyridoxol level than at the 2-μg level, these differences were not statistically significant.

With the observation that transaminase activity continued to increase with a fivefold increase of dietary pyridoxol above 2.0 μg/g diet, although the effect was not statistically significant, it appeared worthwhile to make plots relating tissue vitamin B_6 content with tissue transaminase activity. These data are shown in figures 3 and 4 for the GPT and GOT enzymes, respectively. The generally linear relationship between tissue levels of vitamin B_6 and transaminase activity is evident from an inspection of these charts. Of interest, however, was the tapering off of GOT activity at higher levels of vitamin B_6 in kidney, liver, and heart. By plotting a similar set of GOT charts for each vitamin B_6 vitamer, it was observed that the described effect in figure 4 was due largely to variations in the pyridoxamine content of heart, kidney, and liver. This suggested that the tissue level of pyridoxamine, which was necessary to maintain maximal GOT activity, may have been exceeded in those 3 tissues at the 10.0 μg pyridoxol/g diet feeding level.

4. *Relative vitamin B_6 activity of 3 vitamin B_6 vitamers when fed to rats.* To compare the relative value of the 3 vitamin B_6 vitamers for the maintenance of tissue vitamin B_6 vitamer levels, and for the support of transaminase activity, each of the 3 vitamers was fed at a dietary level of 1.0 μg/g of diet.

a. Effects on tissue levels of vitamin B_6 vitamers. The feeding of pyridoxal resulted in approximately similar tissue levels of pyridoxol (table 1) but slightly lower levels of pyridoxal (table 2) pyridoxamine (table 3) and total vitamin B_6 (table 4) than the feeding of the same level of pyridoxol. The differences for tissue pyridoxal and pyridoxamine were not statistically significant, however. Pyridox-

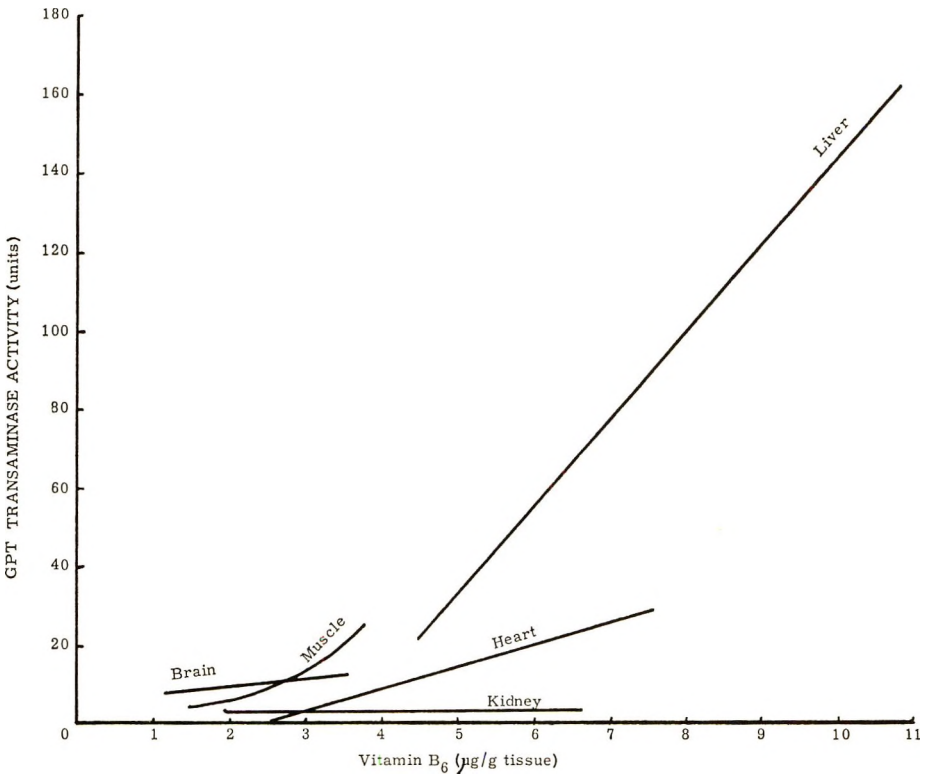

Fig. 3 Relationship between the total vitamin B_6 content of rat tissues and the activity of glutamic-pyruvic transaminase in those tissues. Transaminase activity is expressed in micrograms of pyruvate formed per milliliter of plasma per hour.

amine appeared to be less effective than both pyridoxol and pyridoxal in supporting tissue levels of vitamin B_6 when all were fed at the same dietary level, however.

b. Effects on tissue transaminase activity. For the support of tissue GPT activity (table 5) pyridoxal was as effective as pyridoxol for all of the tissues studied, but pyridoxamine was less so in all tissues except brain. Tissue GOT activity (table 6) was somewhat less when pyridoxal was fed than when the supplement was pyridoxol, although not significantly so. In all cases, GOT activity was lower when pyridoxamine was fed.

The lower effectiveness of pyridoxamine, in supporting tissue vitamin levels and transaminase activity, raised some questions concerning its availability when given by the oral route, or possibly its stability in the purified diet. Analyses of the appropriate diets for the vitamin B_6 vitamers, when the discrepancy became evident, showed that neither pyridoxal nor pyridoxamine was completely stable in the purified, even though refrigerated, diet, but that pyridoxamine was far more sensitive to storage than pyridoxal. Unfortunately the analyses were made on the diet residues about 2 months after the experiment was terminated, thereby making it impossible to establish what the effective pyridoxamine level was during the experimental period. The data support the conclusion, however, that pyridoxal, when fed at the same dietary level, was as effective a source of vitamin B_6 as pyridoxol. The effectiveness of pyridoxamine as compared with the other 2 vitamin B_6 vitamers, is being reinvestigated by administering the 3 forms daily as an oral supplement.

5. *Relative effects of vitamin B_6 deficiency on tissue vitamin B_6 and vitamer content and on tissue transaminase activity.* Data for these variables are pre-

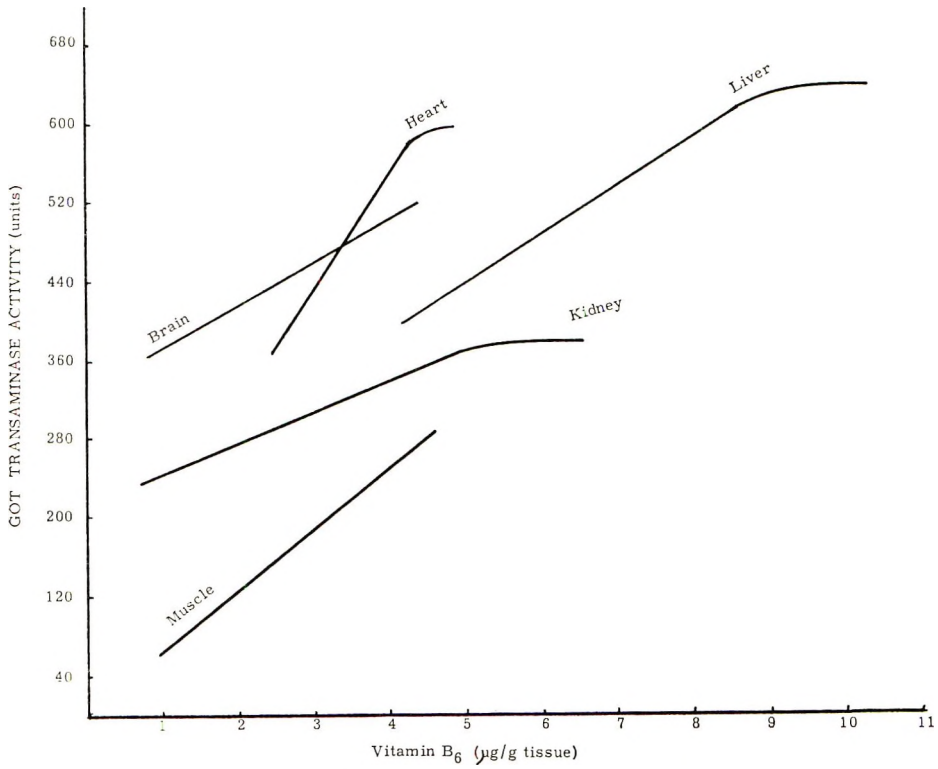

Fig. 4 Relationship between the total vitamin B_6 content of rat tissues and the activity of glutamic-oxalacetic transaminase in those tissues. Transaminase activity is expressed in micrograms of pyruvate formed per milliliter of plasma per hour.

TABLE 7

Extent of depletion of various factors in vitamin B_6-deficient rat tissues [1]

Factor	Tissue							
	Liver	Kidney	Brain	Muscle	Heart	Plasma	Red blood cells	Average
	% depleted							
Pyridoxol	30.4	58.5	46.2	47.1	30.0	—	—	42.4
Pyridoxal	33.3	45.3	36.1	54.3	13.2	—	—	36.4
Pyridoxamine	36.2	59.8	30.0	69.7	49.1	—	—	49.0
Total vitamin B_6	35.0	56.5	32.8	58.5	42.0	—	—	37.4
Glutamic-pyruvic transaminase	69.0	0	18.2	81.2	80.0	71.1	63.5	54.7
Glutamic-oxalacetic transaminase	24.4	23.3	9.3	59.7	29.5	66.5	16.5	32.7

[1] Calculated as: $\frac{10\ \mu g/g\ \text{diet value} - \text{deficient value}}{10\ \mu g/g\ \text{diet value}} \times 100.$

sented in table 7, as calculated from tables 1 through 6. Kidney and muscle were more severely depleted of vitamin B_6 than liver, heart or brain. Generally, for each tissue, the 3 vitamers were depleted about equally, and with the exception of brain, the tissues were more severely depleted of pyridoxamine than of the other 2 vitamers.

On the average, GPT enzyme activity was depressed in vitamin B_6-deficient tissues to a greater degree than the GOT enzyme. This was in accord with other studies in which the GPT was shown to be more sensitive to physiologic change, whether vitamin B_6 deficiency or other (2, 5, 15). It was also noted that in most tis-

sues the depression in GPT activity was much greater than the depression in vitamin B_6 content, while the opposite was true for GOT activity. This suggested that vitamin B_6 levels nearer to tissue saturation were required to support maximal GPT activity.

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Influence of Carbohydrate and Nitrogen Sources on the Rumen Volatile Fatty Acids and Ethanol of Cattle Fed Purified Diets

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ABSTRACT Three experiments were conducted to study the influence of various carbohydrate and nitrogen sources on the volatile fatty acid and ethanol content in the ruminal ingesta of cattle fed purified diets. Molar percentages of isobutyric and isovaleric acids were significantly greater when cattle were fed a natural diet compared with purified diets containing either urea or isolated soy protein. Furthermore, the molar percentages of these same acids were significantly greater when isolated soy protein was compared with urea. Molar percentages of isobutyric and isovaleric acids were similar when urea phosphate, uric acid, biuret, and urea were compared as nitrogen sources in purified diets. Cattle fed the biuret diet had the greatest ruminal concentrations of butyric, valeric and caproic acids and also ethanol. Major sources of carbohydrate studied and the molar percentages of acetic, propionic, butyric, and valeric acids in ruminal fluid were: wood pulp, 73.7, 18.3, 4.8, 0.8; starch, 60.4, 24.7, 10.4, 2.7; starch plus glucose, 57.1, 28.9, 9.9, 1.9; sucrose, 49.6, 23.2, 20.2, 4.4; and glucose, 38.0, 22.3, 25.8, 10.4.

Considerable research has been conducted to study the dietary factors which influence the volatile fatty acids (VFA) resulting from ruminal fermentation. Generally, the proportion of acetic acid in the ruminal fluid is correlated positively with the fiber content of the diet (1). The effect of diet on the proportions of propionic and butyric acids is less well understood. In some experiments decreased acetic acid proportions have resulted in increased proportions of butyric acid (2, 3). Recently, the longer chain VFA's (valeric and caproic) were detected in large quantities in the ruminal ingesta of cows fed purified diets (4). The branched chain VFA's found in ruminal ingesta are derived mainly from the breakdown of dietary protein and the deamination of the branched chain amino acids (5–7). Recently, only traces of isobutyric (8) and isovaleric acids (8, 9) were detected in the ruminal ingesta of sheep fed purified diets containing urea as the source of nitrogen. Valine, leucine, isoleucine and phenylalanine were present in significantly smaller quantities in the blood plasma of steers fed urea compared with isolated soy protein in purified diets (10) and a relationship was suggested between the branched-chain VFA's and the branched-chain amino acids. The branched-chain VFA's have also been shown to be essential for some cellulolytic rumen bacteria (11, 12) and to influence feed intake (13).

The present experiments were conducted to study the ruminal fermentation patterns of cattle fed purified diets containing protein and nonprotein-nitrogen (NPN) sources and carbohydrate sources varying from simple monosaccharides (glucose) to the more complex polysaccharides (celluloses).

EXPERIMENTAL PROCEDURE

Experiment 1. Twenty-three bull and heifer calves were weaned at 14 weeks of age and were assigned to one of three treatment groups. Group 1 (7 animals) received a natural diet containing: (in percent) cracked corn, 50.0; chopped alfalfa hay, 15.0; chopped timothy hay, 15.0; soybean meal, 10.0; molasses, 9.0; and trace mineralized salt, 1.0. Group 2 (10 animals) received a purified diet containing urea as the dietary source of nitrogen; and group 3 (6 animals) received a

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TABLE 1

Percentage composition of experimental diets with combinations of carbohydrate sources

	Exp. 1		Exp. 2				Exp. 3									
Wood pulp [1]	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	63.8	74.0
Cornstarch	34.8	28.6	28.4	28.3	27.0	27.7	28.6	34.8	46.6	56.8	12.8	12.8	12.8	12.8	12.8	12.8
Glucose monohydrate [2]	22.0	18.0	28.4	28.3	27.0	27.7	18.0	22.0	—	—	33.8	44.0	—	—	—	—
Sucrose	—	—	—	—	—	—	—	—	—	—	—	—	33.8	44.0	—	—
Isolated soy protein [3]	—	14.9	—	—	—	—	14.9	—	14.9	—	14.9	—	14.9	—	14.9	—
Urea [4]	4.7	—	4.7	—	2.5	—	—	4.7	—	4.7	—	4.7	—	4.7	—	4.7
Biuret [5]	—	—	—	4.9	—	—	—	—	—	—	—	—	—	—	—	—
Urea phosphate [6]	—	—	—	—	5.0	—	—	—	—	—	—	—	—	—	—	—
Uric acid	—	—	—	—	—	6.1	—	—	—	—	—	—	—	—	—	—
Mineral mix [7]	6.4	6.4	6.4	6.4	6.4 [8]	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4
Refined soybean oil	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Choline chloride	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Vitamins [9]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[1] Grade CBR-20. International Filler Corporation, North Tonawanda, New York (82% hemicellulose and cellulose, 17% lignin and 1% ash).
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] Assay Protein C-1, Skidmore Enterprises, Cincinnati, Ohio.
[4] Feed grade.
[5] Biuret, 85.6%; triuret, 10.2%; urea, 2.7%; cyanuric acid, 1.1%.
[6] Analyzed 17.7% nitrogen and 19.6% phosphorus.
[7] Mineral mixture contained: (in per cent) $CaHPO_4$, 49.987; K_2CO_3, 28.737; $MgSO_4$, 11.010; NaCl, 8.478; $FeSO_4$, 0.750; $MnSO_4 \cdot H_2O$, 0.112; $Na_2B_4O_7 \cdot 10H_2O$, 0.361; $ZnSO_4 \cdot 7H_2O$, 0.529; $CuCO_3$, 0.030; KI, 0.003; $CoCl_2 \cdot 6H_2O$, 0.001; MoO_3, 0.001; and Na_2SeO_3, 0.001.
[8] $CaCO_3$ replaced $CaHPO_4$ in mineral mixture.
[9] Contained 8,800 USP units vitamin A palmitate, 1,100 USP units vitamin D (D-activated plant sterol), and 21 IU *d*-α-tocopheryl acetate/kg diet.

purified diet containing isolated soy protein as the dietary source of nitrogen. The ingredients of the purified diets are shown in table 1. The animals were given feed and water ad libitum. Bedding was not used in this experiment nor in experiments 2 and 3.

Ruminal samples were taken when the cattle were approximately 1 year of age. The day before sampling, feed was withheld for 12 hours and then the cattle received half of their daily feed intake to minimize feeding pattern differences. The ruminal samples (1 to 2 liters) were taken by stomach tube 2 hours after feeding. The pH was determined on each sample immediately after collection. The ruminal ingesta was strained through cheesecloth and 25-ml samples were preserved in 0.5 ml of a saturated $HgCl_2$ solution. The collected samples were frozen until analysis was performed. Volatile fatty acid analyses were made using gas-liquid chromatography with a flame ionization detector. Ethanol analyses were made according to the procedure of Allison et al. (14). In the ethanol analysis the carrier gas was nitrogen, column temperature was 100° and injection temperature was 135°. The same analytical procedures were used in experiments 2 and 3. Experiment 1 was conducted to study the ruminal ingesta of cattle fed protein versus NPN (urea) in purified diets and also to compare purified diets with a natural diet.

Experiment 2. Four steer calves were assigned to a 4 × 4 Latin square and were fed the diets shown in table 1. The NPN sources studied were urea, urea phosphate, biuret and uric acid. The urea phosphate treatment consisted of both urea and urea phosphate to avoid possible adverse effects of excessive feeding of phosphorus. Periods were 6 weeks and the level of feeding was 1.3 kg/100 kg of body weight per day. The animals were fed twice daily in two equal feedings. Rumen samples were obtained by stomach tube 4 hours after feeding on the last day of each period. Experiment 2 was conducted to investigate the effect of NPN sources on the composition of ruminal ingesta and especially the concentration of the branched chain VFA's.

Experiment 3. One set of identical twin steers was used. Both steers were rumen-fistulated. Throughout the experiment one steer received isolated soy protein and the other received urea. The carbohydrate sources and combinations studied are shown in table 1. Each diet was fed for at least 14 days during which rumen samples were obtained on the twelfth and the last day. The sequence in which the carbohydrate variables were fed was: starch plus glucose, high starch, high glucose, high sucrose, and high cellulose (wood pulp containing 82% hemicellulose and cellulose, 17% lignin and 1% ash). The level of feeding was 1.3 kg/100 kg body weight which was given in two equal feedings per day. The rumen samples were taken 2 hours after the morning feeding. Small quantities of uneaten feed left 1 hour after feeding were placed into the rumen via the fistula. The diets used in the 3 experiments (table 1) were essentially isonitrogenous and analyzed about 14% crude protein equivalent on a dry basis. Experiment 3 was conducted to study the effect of various carbohydrate sources and protein versus NPN on ruminal VFA and ethanol concentrations.

RESULTS

Experiment 1. The treatment means and standard errors are shown in table 2. Cattle fed the isolated soy purified diet had lower ($P < 0.01$) ruminal pH values than cattle fed either the urea purified diet or the natural diet. The total concentration of VFA's was greater for the cattle receiving either the isolated soy purified diet ($P < 0.01$) or the urea purified diet ($P < 0.05$) than cattle receiving the natural diet. Acetic acid proportions were lower in the rumen when urea was fed than when either the isolated soy protein or the natural diets ($P < 0.05$) were fed. The isobutyric acid proportions were greater with the isolated soy than the urea diet ($P < 0.01$) and greater with the natural diet than either the isolated soy or the urea diets ($P < 0.01$). Butyric acid proportions were greater with the urea ($P < 0.05$) and natural ($P < 0.01$) diets than with the isolated soy diet. There was less isovaleric acid ($P < 0.01$) with both purified diets than the natural diet. The valeric acid proportion was greater with the urea diet than with either the natural or isolated

TABLE 2

Ruminal fermentation patterns of cattle fed natural or purified diets containing protein or NPN

Treatment	pH	VFA concn	Volatile fatty acids							Ethanol
			C_2	C_3	$IsoC_4$	C_4	$IsoC_5$	C_5	C_6	
		mEq/liter	*molar % of VFA's*							*mEq/liter*
Natural diet	5.7 ± 0.1 [1]	116.0 ± 15.3	54.5 ± 2.3	21.6 ± 2.3	1.3 ± 0.2	18.8 ± 1.4	1.6 ± 0.1	2.1 ± 0.1	0.1 ± 0.1	1.4 ± 0.6
Urea purified diet	5.9 ± 0.2	163.5 ± 14.2	45.7 ± 2.3	27.4 ± 2.1	0.1 ± 0.0	17.4 ± 1.4	0.6 ± 0.2	7.4 ± 1.0	1.4 ± 0.1	6.4 ± 1.2
Isolated soy purified diet	5.0 ± 0.1	193.7 ± 13.2	53.7 ± 3.8	25.2 ± 4.4	0.8 ± 0.1	10.9 ± 0.9	0.9 ± 0.1	4.2 ± 1.5	4.3 ± 1.5	4.1 ± 0.8

[1] SE of mean.

soy diet ($P < 0.01$), and also greater with the isolated soy diet than natural diet ($P < 0.05$). Caproic acid concentration was greater with the isolated soy diet than the urea diet ($P < 0.05$) or natural diet ($P < 0.01$). There were differences ($P < 0.01$) between each of the experimental diets in ethanol concentration.

Experiment 2. The effect of nitrogen source on the ruminal pH, VFA, and ethanol is shown in table 3. There were no significant treatment differences in ruminal pH or in the total concentration of VFA. The proportion of propionic acid was higher when either urea or urea phosphate were compared with either biuret or uric acid ($P < 0.01$). There were no significant treatment differences in the proportions of isobutyric, butyric and isovaleric acids. Valeric acid proportions were greater with biuret when compared with either urea or uric acid ($P < 0.05$) or urea phosphate ($P < 0.01$). A similar pattern was noted with caproic acid and ethanol although the differences were not significant.

Experiment 3. The effect of the two nitrogen sources (across carbohydrate sources) is shown in table 4. Ruminal pH was lower ($P < 0.05$) and total concentration of VFA was greater when isolated soy protein was the nitrogen source. The proportion of acetic acid was greatest in the urea-fed animal. There were no significant differences in the propionic acid proportions. The treatment differences in the proportions of isobutyric and isovaleric acids were highly significant ($P < 0.001$). Butyric acid ($P < 0.05$), valeric acid ($P < 0.01$), caproic acid ($P < 0.01$) and the ethanol concentrations ($P < 0.05$) were greater with the isolated soy diets.

The effect of the carbohydrate sources (across nitrogen sources) is shown in table 5. Ruminal pH tended to decline with increasing solubility of the tested carbohydrate. The pH differences between either cellulose or starch and the other three carbohydrate sources were all significant ($P < 0.05$).

The acetic acid proportion in the rumen contents when steers were fed the cellulose diets was greater than when they were fed the glucose ($P < 0.01$), sucrose ($P < 0.01$), starch plus glucose ($P < 0.05$) or starch ($P < 0.05$) diets. The proportions of acetic

TABLE 3

Effect of NPN sources on the ruminal fermentation patterns of cattle fed purified diets

Treatment	pH	VFA concn	Volatile fatty acids							Ethanol
			C_2	C_3	$IsoC_4$	C_4	$IsoC_5$	C_5	C_6	
		mEq/liter	*molar % of VFA's*							*mEq/liter*
Biuret	5.8	69.9	53.4	21.0	0.9	18.5	0.2	5.3	0.7	2.0
Urea	5.7	91.7	50.2	27.9	0.7	17.8	0.2	2.9	0.3	1.0
Urea phosphate	6.0	67.5	52.1	28.3	0.6	17.4	0.0	1.3	0.3	0.8
Uric acid	5.8	84.5	59.2	22.6	0.7	15.2	0.3	2.0	0.0	1.2
SE of means	±0.2	±8.2	±2.0	±2.0	±1.0	±2.5	±0.1	±0.6	±0.3	±0.6

TABLE 4

Ruminal fermentation patterns of steers fed isolated soy protein versus urea in purified diets

Treatment	pH	VFA concn	Volatile fatty acids							Ethanol
			C_2	C_3	$IsoC_4$	C_4	$IsoC_5$	C_5	C_6	
		mEq/liter	*molar % of VFA's*							*mEq/liter*
Isolated soy protein	6.0	110.9	52.7	22.2	1.4	15.6	1.4	5.3	1.4	4.5
Urea	6.6	90.1	58.7	24.7	0.2	12.9	0.6	2.8	0.1	2.9
SE of means	±0.1	±5.9	±1.8	±1.8	±0.1	±0.6	±0.1	±0.4	±0.1	±0.5

TABLE 5

Effect of high percentages of different carbohydrate sources on the ruminal fermentation patterns of steers fed purified diets

Treatment	pH	VFA concn	Volatile fatty acids							Ethanol
			C_2	C_3	$IsoC_4$	C_4	$IsoC_5$	C_5	C_6	
		mEq/liter	*molar % of VFA's*							*mEq/liter*
Celluloses	6.9	87.1	73.7	18.3	1.0	4.8	1.4	0.8	0.0	0.9
Starch	6.7	76.3	60.4	24.7	0.7	10.4	0.9	2.7	0.2	1.8
Starch + glucose	6.0	114.4	57.1	28.9	0.6	9.9	1.0	1.9	0.6	0.6
Sucrose	5.8	121.7	49.6	23.2	0.6	20.2	0.7	4.4	1.3	5.9
Glucose	5.7	102.6	38.0	22.3	0.9	25.8	1.0	10.4	1.6	9.3
SE of means	±0.2	±9.3	±2.8	±2.9	±0.1	±1.0	±0.2	±0.6	±0.1	±0.8

acid on the starch and the starch plus glucose diets were greater than with the glucose ($P < 0.01$) or sucrose ($P < 0.05$) diets. The propionic acid proportion increased from the cellulose diet to the starch and the starch plus glucose diets and decreased again on the sucrose and glucose diets. There were no significant treatment differences in the isobutyric or isovaleric acid fractions. In the butyric acid proportions, sucrose and glucose were different ($P < 0.01$) from any of the other rations. Also a difference ($P < 0.05$) was apparent between cellulose and the starch plus glucose and starch diets in the proportions of butyric acid. The valeric acid proportion was greater with the glucose diets than with any of the other diets ($P < 0.01$). Significant differences ($P < 0.05$) were detected between sucrose and both the cellulose or the starch plus glucose diets. The caproic acid proportion was greater ($P < 0.01$) with the sucrose and glucose diets than with the other diets and greater ($P < 0.05$) with the starch plus glucose diet than with the cellulose diet. Similarly, the concentration of ethanol in the rumen was greater with the glucose ($P < 0.01$) and sucrose ($P < 0.05$) diets and greater when the animals received glucose than sucrose ($P < 0.05$).

An intraction ($P < 0.05$) was apparent in the ethanol concentration. The ethanol concentration with the sucrose diet was greater with urea (9.4 mEq/liter) than with isolated soy protein (2.4 mEq/liter),

whereas in the overall means the ethanol concentrations were highest on the isolated soy diet.

DISCUSSION

The effect of the nitrogen sources on the proportions of the branched-chain VFA's agrees with recent results (8, 9) and supports the suggestion that these acids are derived mainly from the degradation of dietary protein and the deamination of the branched-chain amino acids (5–7). In the present studies small quantities of the branched-chain VFA's were nearly always detected after feeding in the rumen ingesta when the cattle were fed NPN. Quite possibly these small quantities could be the result of the breakdown of microbial protein at the rumen level. Source of NPN in the purified diets had little effect on the concentration of the branched-chain VFA's indicating the general lack of these acids at the ruminal level compared with protein-containing diets. Possibly, the dietary addition of the branched-chain VFA's to diets in which all of the nitrogen is supplied as NPN may have a beneficial effect on digestion and nitrogen retention (8) and on amino acid balance (10).

Purser et al. (15) reported that the concentration of isobutyric and isovaleric acid in the ruminal ingesta of sheep fed natural diets was not detectable 4 hours after feeding but was detected at levels of 3.0 and 6.0 molar percentages, respectively, before feeding. Possibly the branched-chain VFA's may have been detected in greater concentrations before feeding in the present experiments with both the NPN and protein-containing diets. Further research is needed in this area.

In experiments 1 and 3 the ruminal pH was highest when urea was compared with isolated soy protein as nitrogen sources. This was probably due to the rapid release of ammonia from the hydrolysis of urea which was apparent 2 hours after feeding when the ruminal samples were taken.

The effect of carbohydrate source on ruminal fermentation is intriguing and suggests the elucidation of some factors involved in VFA formation. Table 5 shows a decreased proportion of acetic acid going from the complex (celluloses in wood pulp) to the simple (glucose) carbohydrate sources. Propionic acid proportion increased from the celluloses to starch plus glucose and then actually lowered somewhat with the sucrose and glucose diets. Instead the butyric acid increased with the sucrose and glucose diets as did the proportions of valeric and caproic acids. It is likely that both glucose and sucrose and presumably also the other mono- and disaccharides undergo rapid fermentation and the present evidence suggests that the proportions of butyric and higher VFA's may be indicative of the rate of fermentation. The greatest concentration of the longer chain acids occurred when isolated soy protein was the nitrogen source (table 4) except with the starch plus glucose diet.

The ethanol concentrations were greatest with the diets where the largest quantities of butyric, valeric and caproic acids were observed. This agrees with the sugestion (4) and further evidence (16) that ethanol may be associated with the synthesis of longer chain acids but its precise role, if any, cannot be assessed from these studies.

The present studies may have an importance in raising the low butterfat percentage which occurs when cows are fed high concentrate diets. It has been shown that butyric acid, due to its composition of C_2 units, can participate in milk fat synthesis and thereby have a favorable effect on milk fat percentage (17, 18) while propionic acid, due to its glucogenic nature, in most cases depresses butterfat percentage (19).

Fibrous diets such as those containing large quantities of wood pulp or natural roughages produce a high proportion of acetic acid and generally promote a high butterfat percentage (1). However, diets with an intermediate solubility such as those high in starch are either escaping ruminal fermentation in large quantities and are being absorbed in the lower gut as glucose (20, 21) or are being converted in the rumen to large quantities of propionic acid or both, thereby depressing butterfat synthesis (19). Compounds containing lactose have been shown to raise the butterfat percentage in the milk of

cows fed restricted roughage diets.[2] In ruminal fermentation two acetyl CoA units, which act as electron acceptors, are condensed and reduced to form butyric acid while butyryl CoA and acetyl CoA are reduced to caproic acid. Propionyl CoA and acetyl CoA similarly yield valeric acid. Although a butyric acid fermentation is theoretically less efficient than a propionic acid fermentation (22) it is a more efficient process than when excess hydrogen derived from acetic acid production is being directed towards reducing CO_2 to CH_4 which would be essentially lost to the animal. The observed differences between the starch plus glucose and glucose or sucrose diets suggest that large quantities of sugars are required to produce the effect.

The reason that propionic acid formation might be depressed when the fermentation proceeds very rapidly is not clear. However, it is possible that the concentration of certain enzymes and co-factors are limiting and the result is a disposal of excess electrons via synthesis of longer chain acids.

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Pyridoxine Deficiency and Iron Metabolism in the Pregnant Rat: Maternal Responses [1]

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ABSTRACT Effects of pyridoxine deficiency on the normal increase in iron absorption in the pregnant rat and on the use of a large dose of iron administered orally during gestation were investigated. The deficiency was evidenced by poor reproductive performance and impaired erythropoiesis, including polycythemia and reduced mean corpuscular hemoglobin and mean corpuscular volume. Plasma iron concentration, total iron-binding capacity and unsaturated iron-binding capacity were not altered in deficient animals even in those receiving large doses of iron. During normal gestation iron was mobilized from spleen stores and small amounts from liver. Iron supplementation alleviated the reduction in liver stores but had little effect on spleen. Elevation in liver iron observed in the deficiency was more pronounced in nonpregnant than pregnant animals. Similarly iron supplements intensified elevations more in nonpregnant than pregnant animals. Differences were related to use of maternal stores for fetal needs. Spleen values elevated in the deficiency were not intensified by iron supplements or by pregnancy. Elevations were related in part to less utilization of iron in erythropoiesis. Hemosiderin-to-ferritin ratios were increased in liver, spleen and duodenal tissues of deficient animals. Although alterations in tissue iron stores were observed in the deficiency, no major impairment in iron absorption appeared evident. Deficient animals tolerated large doses of oral iron during gestation when iron absorption is normally increased.

Normal regulation of body iron stores is believed to be controlled by intestinal cells but factors associated with the regulation are not completely known. The possibility that pyridoxine is a factor in the regulation is controversial. Enhanced iron absorption has been reported for pyridoxine-deficient swine (1) and rats (2) despite replete body stores. These findings have been questioned by Yeh et al.[2] and by Neal and Pearson (3). However, the latter investigators observed increased iron absorption in deficient rats when a large dose of iron was fed. Recently Calloway and McMullen (4) have suggested that the absorption of iron by humans fed a ration high in iron was related to a marginal pyridoxine intake. Abnormal iron metabolism leading to varying degrees of iron overload has been a consistent observation in pyridoxine-responsive anemia in humans (5).

Iron is normally transported and stored in the body as protein complexes. Amounts of the mineral in excess of that utilized in hemoglobin and enzyme synthesis are deposited in liver, spleen and bone marrow as ferritin and hemosiderin. It is not known whether pyridoxal phosphate, an essential cofactor for many phases of protein metabolism, is necessary for the synthesis of the protein moiety of iron-protein complexes involved in iron transport and storage. Alterations in levels of these compounds, namely, transferrin, ferritin and hemosiderin, could affect the transport and storage of the mineral as well as its absorption.

A marked increase in iron absorption has been observed in normal pregnancy (6, 7); the increase is associated with elevated maternal and fetal needs but a complete knowledge of regulatory factors is lacking.

The purpose of the present study was to determine whether pyridoxine deficiency intensifies the normal increase in iron absorption in late pregnancy and whether a large intake of oral iron during gestation adversely affects reproductive performance and alters the iron composition of maternal blood and tissues. This paper will focus

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[1] Journal paper no. 2989 of the Purdue University Agricultural Experiment Station. This study was supported in part by the Nutrition Foundation, Inc.
[2] Yeh, S. O. J., B. Padella and B. F. Chow 1962 Iron absorption by vitamin B_6-deficient rats. Federation Proc., 21: 468 (abstract).

on responses of the maternal organism; the fetal data will be presented in a subsequent report.

EXPERIMENTAL

Eighty female, Sprague-Dawley strain rats, 80 days of age, were divided into 8 groups of 10 animals each. Animals were housed individually in wire-bottom suspended cages in a room maintained at 24° and 40% relative humidity and controlled for 12-hour periods of alternate lightness and darkness. Four groups of animals were fed ad libitum pyridoxine-deficient diets containing: (%) casein, 26; sucrose and vitamin mix, 18.85; cornstarch, 34; hydrogenated fat, 10; corn oil, 5; Jones-Foster salt mixture (8), 4; agar, 2; and L-cystine, 0.15. The composition of the vitamin mixture was as follows: (mg/100 g diet) thiamine, 2; riboflavin, 2; *p*-aminobenzoic acid, 200; niacin, 10; pantothenic acid, 8; biotin, 0.04; inositol, 400; vitamin B_{12} triturate (0.1% in mannitol), 4.0; folic acid, 0.4; choline, 400; and naphthoquinone, 1.0. Vitamins A, D and E were mixed in corn oil to contain 450 IU vitamin A acetate, 400 IU vitamin D_3 and 10 mg DL-α-tocopheryl acetate in 2 drops. Two drops were administered orally to each rat every 3 days. Four pyridoxine-fed groups received the above diet supplemented with 0.8 mg pyridoxine/100 g diet. Iron content of the diets calculated as elemental iron was 200 mg/kg. Iron requirements of the rat for reproduction have not been established definitely (9). Iron sufficiency has been reported with levels ranging from 50 mg Fe/kg diet (10) to 600 (11). McCall et al. (12) observed that growth and reproduction were supported through 3 generations of rats fed 240 mg/kg diet which is similar to the level used in this study.

After receiving the experimental diets for 3 weeks, two deficient and two pyridoxine-fed groups were mated. Mating was confirmed by the presence of sperm in the vaginal smear. Throughout gestation each rat in one deficient and one pyridoxine-fed group was given daily, by stomach tube, 1 ml of a solution of $FeSO_4 \cdot 7H_2O$ in 1% HCl equivalent to 2 mg elemental iron. The supplement approximated the amount provided by 10 g diet. All groups were maintained with the diets fed before mating for the 3-week gestation period. Four groups of nonpregnant rats received similar treatments for the same time.

On days 1 and 21, after a 16-hour fast, tail blood samples were obtained for the following measurements: hemoglobin; hematocrit; red blood cell count; and concentrations of plasma iron, iron-binding capacity and plasma total protein. Blood was always collected at the same time of day. To minimize the quantity of blood withdrawn, determinations were made on an ultramicro scale. Less than 0.4 ml of blood was required for all measurements.

On day 21, animals were killed by chloroform anesthesia. Liver and spleen were removed, blotted to remove traces of blood, weighed and frozen in foil bags. A 20-cm segment of duodenum was washed in deionized-distilled water, weighed and frozen. Before killing the anesthetized pregnant animals, the young were removed by abdominal section and implantation sites, and number and weights of live young and total litter weight were recorded.

Hemoglobin was determined as oxyhemoglobin using 0.02 ml blood and 6 ml 0.007 N NH_4OH. Absorbancy was measured at 545 mμ. Hematocrits were determined by a micro-technique described by Strumia et al. (13). Red cell counts were determined in duplicate in conventional counting chambers.

Plasma iron concentration was measured by an ultramicro adaptation of the method of Trinder (14) using a 20-μliter serum sample. Absorbancy was read at 545 mμ using a Beckman DU spectrophotometer. Total iron-binding capacity was determined by adding 30 μliters $FeCl_3$ (10 μg/ml in 0.005 N HCl) to a 20-μliter serum sample and allowing to stand for 5 minutes. To remove excess ferric ions, 4 mg $MgCO_3$ were added and mixed intermittently in a mechanical shaker during a 30-minute interval and then centrifuged at 2000 × *g* for 10 minutes. Iron concentration of 30 μliters supernatant was measured by the procedure described for plasma iron.

Plasma protein was determined by an ultramicro procedure (5 μliters plasma). The procedure was a modification of the method of Kingsley (15).

TABLE 1

Food consumption, body weight and tissue weights

	Pyridoxine-fed				Pyridoxine-deficient				Treatment
	Nonpregnant		Pregnant		Nonpregnant		Pregnant		Significant *F* values
	Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		
	0	2	0	2	0	2	0	2	$P < 0.01$
Food consumption,[1] g	260 [2]	236	317	303	188	186	236	227	vitamin, pregnant
Body wt,[3] g	269	258	376	371	218	211	265	263	vitamin, pregnant, vitamin × pregnant
Body wt gain,[1] g	10	10	130	125	−1	−7	+50	+42	vitamin, pregnant, vitamin × pregnant
Liver									
g/animal	8.09	7.34	10.80	10.58	5.77	5.73	7.50	6.58	vitamin, pregnant
g/100 g body wt	2.99	2.84	2.86	2.86	2.64	2.71	2.81	3.09	
Spleen									
g/animal	0.56	0.59	0.64	0.61	0.48	0.43	0.42	0.42	vitamin
g/100 g body wt	0.20	0.23	0.18	0.17	0.22	0.20	0.16	0.17	pregnant
Duodenum									
20-cm segment, g	1.12	1.18	1.25	1.19	1.31	1.05	1.26	1.24	

[1] Day 1 to 21.
[2] Averages for 10 rats.
[3] Day 21.

Tissue samples were dried at 110°. Samples were wet-ashed according to the method of Reitz (16). The iron content was determined spectrophometrically by the method of Sandell (17) using orthophenanthroline reagent.

Tissue samples were homogenized, diluted to 10 ml with deionized distilled water and centrifuged at 2000 × g. Storage iron was separated into water-soluble ferritin and water-insoluble hemosiderin by the method of Kaldor (18). Iron content of fractions was determined according to the method of Sandell (17).

The data were analyzed by analysis of variance techniques (19).

RESULTS

Food consumption, body weight, liver, spleen and duodenum weights are summarized in table 1. Oral iron supplementation did not influence food consumption, weight gains or tissue weights. Liver and spleen weights were less for deficient animals but not when values were calculated per 100 g body weight. Liver weights of pregnant animals were not significantly different from nonpregnant values when calculated on the basis of body weight but spleen weights were lower.

Number of resorptions, average number of live young, and fetal weights are shown in table 2. Pyridoxine-deficient animals had significantly more resorptions and the average number and size of live young was less than for vitamin-fed animals. Iron-supplemented groups tended to have more resorptions. This finding was not, however, statistically significant.

Values for hemoglobin, hematocrit, red blood cell count, mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) for days 1 and 21 are presented in table 3. Pyridoxine deficiency did not significantly influence hemoglobin concentration but lowered MCH values. Polycythemia and low MCV were also evident in deficient groups on days 1 and 21. Iron supplements did not significantly influence any of the hematological values reported here.

Pregnancy significantly decreased hematocrit, hemaglobin and red blood cell counts, but MCH and MCV were not changed. None of the hematological values were altered when the iron intake was approximately doubled by the oral supplement.

Plasma iron concentration, total iron-binding capacity (TIBC) and unsaturated iron-binding capacity (UIBC) were not influenced by the deficiency per se (table 4). However, at term pyridoxine-deficient pregnant animals had higher plasma iron and TIBC and lower UIBC compared with their pyridoxine-fed counterparts.

Pregnancy was accompanied by decreased plasma iron concentration, TIBC and UIBC. The differences were greater in pyridoxine-fed groups. Iron supplementation did not affect the concentrations of plasma iron, TIBC or UIBC in any group.

Plasma total protein was only slightly lower in pyridoxine deficiency (table 4). These differences were not evident among pregnant groups on day 21. In pyridoxine-fed pregnant animals a marked decrease in total protein was observed at term.

Total iron, non-heme, ferritin and hemosiderin fractions in liver are presented in table 5. Total iron concentration was elevated by the deficiency but total iron content (μg/liver) was not. The combined

TABLE 2

Reproductive performance

	Pyridoxine-fed		Pyridoxine-deficient		Treatment
	Fe suppl., mg		Fe suppl., mg		Significant *F* values
	0	2	0	2	$P < 0.01$
Implantations, avg no.[1]	12	13	11	12	
Resorptions, total no.[2]	3	20	35	55	vitamin
Live young, avg no.	12	11	7	6	vitamin
Fetal wt, avg, g	4.5	4.4	3.3	3.2	vitamin

[1] Averages for 10 rats.
[2] Totals for 10 rats.

TABLE 3

Hemoglobin, hematocrit, red blood cell count, mean corpuscular hemoglobin (MCH) [1] *and mean corpuscular volume (MCV)* [2]

	Pyridoxine-fed				Pyridoxine-deficient				Treatment
	Nonpregnant		Pregnant		Nonpregnant		Pregnant		Significant *F* values
	Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		$P < 0.01$
	0	2	0	2	0	2	0	2	
Hemoglobin, g/100 ml									
Day 1	15.5 [3]	15.2	14.7	14.8	14.9	14.9	14.2	14.6	
Day 21	15.6	15.4	12.2	12.3	14.7	15.0	12.3	12.4	pregnant
Hematocrit, %									
Day 1	45.2	44.7	43.0	46.0	44.0	44.1	44.5	44.7	
Day 21	46.0	45.2	35.9	37.9	44.3	45.6	41.0	38.4	pregnant
Red blood cells, $10^6/mm^3$									
Day 1	8.4	8.4	8.9	9.0	9.4	9.7	9.6	9.7	vitamin
Day 21	8.6	8.5	6.5	6.6	9.9	10.2	8.7	8.0	vitamin, pregnant
MCH, $\mu\mu g$									
Day 1	18.6	18.2	18.7	18.1	15.6	15.6	15.9	16.1	vitamin
Day 21	18.3	18.3	18.7	18.9	14.8	14.7	14.1	15.0	vitamin
MCV, μ^3									
Day 1	53.8	53.8	50.0	50.4	45.9	45.6	44.7	43.5	vitamin
Day 21	54.0	53.0	55.1	58.4	44.7	44.7	46.1	48.4	vitamin

[1] $MCH = \frac{\text{hemoglobin (g/liter blood)}}{\text{RBC} \times 10^6/mm^3 \text{ blood}}$.

[2] $MCV = \frac{\text{vol RBC (ml/liter blood)}}{\text{RBC} \times 10^6/mm^3 \text{ blood}}$.

[3] Average for 10 rats.

effects of pregnancy and the deficiency resulted in the elevation of both parameters. Both the concentration and percentage of total storage iron hemosiderin and hemosiderin-to-ferritin (H/F) ratios were higher in the deficiency, whereas both values for ferritin decreased. Iron supplementation increased liver iron concentration, total iron content, and storage iron concentration. Both ferritin and hemosiderin concentrations increased with no change in H/F ratios. Pregnancy resulted in decreased liver iron concentration especially in pyridoxine-fed groups. These groups also had low total iron content. In pregnancy both ferritin and hemosiderin iron concentrations decreased but the percentage of each fraction in storage iron and H/F ratios was not altered.

Total iron, non-heme, ferritin and hemosiderin iron fractions in spleen are reported in table 6. Pyridoxine deficiency resulted in higher iron concentration but total spleen iron (μg/spleen) was not significantly different from values for animals receiving the vitamin except for the pregnant groups. Concentrations of total storage iron, ferritin and hemosiderin iron increased in the deficiency. Storage iron calculated as a percentage of total iron was also slightly higher in deficient animals. In the deficiency the percentage of ferritin in storage iron decreased and hemosiderin increased, resulting in increased H/F ratios. Pregnancy led to decreased total iron concentration and total iron content in spleen. Differences were more pronounced in pyridoxine-fed groups. These animals also had a lower percentage storage iron. In pregnancy the percentage ferritin storage iron was less and hemosiderin was higher resulting in increased H/F ratios. Iron supplements did not cause significant changes in any of the values reported for total iron or storage fractions.

The iron content of the duodenum is summarized in table 7. Concentrations of total iron, storage iron, ferritin and hemosiderin were increased by the deficiency. The percentage ferritin storage iron was slightly less in deficient animals and hemosiderin was higher resulting in the ele-

TABLE 4

Plasma iron, total iron-binding capacity (TIBC), unsaturated iron-binding capacity (UIBC) and plasma total protein

	Pyridoxine-fed				Pyridoxine-deficient				Treatment
	Nonpregnant		Pregnant		Nonpregnant		Pregnant		Significant *F* values
	Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		$P < 0.01$
	0	2	0	2	0	2	0	2	
Plasma iron, μg/100 ml									
Day 1	271 [1]	269	260	261	274	277	269	272	
Day 21	277	281	288	105	278	275	135	149	pregnant, vitamin × pregnant
TIBC, μg/100 ml									
Day 1	564	571	563	569	553	563	564	549	
Day 21	570	572	319	309	560	563	345	352	pregnant, vitamin × pregnant
UIBC, μg/100 ml									
Day 1	293	296	304	308	279	287	296	275	
Day 21	303	293	231	224	282	289	210	203	pregnant, vitamin × pregnant
Plasma total protein, g/100 ml									
Day 1	6.62	6.49	6.32	6.50	5.80	6.12	6.00	6.01	vitamin
Day 22	6.80	6.90	5.08	4.88	6.34	6.02	5.40	5.30	pregnant, vitamin × pregnant

[1] Averages for 10 rats.

TABLE 5

Total iron, non-heme, ferritin and hemosiderin fractions in liver

	Pyridoxine-fed				Pyridoxine-deficient				Treatment
	Nonpregnant		Pregnant		Nonpregnant		Pregnant		Significant F values
	Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		$P < 0.01$
	0	2	0	2	0	2	0	2	
Total iron									
μg/g fresh tissue	351 ± 36 [1]	434 ± 41	193 ± 60	297 ± 70	443 ± 30	503 ± 47	355 ± 42	423 ± 45	vitamin, pregnant, Fe, vitamin × pregnant
μg/liver	2850 ± 147	3101 ± 127	2112 ± 144	2946 ± 162	2553 ± 125	2873 ± 144	2665 ± 158	3276 ± 143	Fe, vitamin × pregnant
Storage iron									
μg/g fresh tissue	287 ± 28	366 ± 26	137 ± 35	229 ± 26	393 ± 39	447 ± 35	310 ± 57	370 ± 49	vitamin, pregnant, Fe, vitamin × pregnant
% total iron	81.7 ± 1.03	84.3 ± 0.94	70.9 ± 1.21	77.1 ± 0.96	88.7 ± 1.12	88.9 ± 1.06	87.3 ± 0.98	87.4 ± 1.01	vitamin, vitamin × pregnant
Ferritin iron									
μg/g fresh tissue	261 ± 30	326 ± 18	124 ± 25	206 ± 21	337 ± 20	381 ± 38	265 ± 29	316 ± 26	vitamin, pregnant, Fe
% storage iron	90.9 ± 0.41	89.1 ± 0.58	90.5 ± 0.61	89.9 ± 0.82	85.2 ± 0.88	85.6 ± 1.02	85.6 ± 1.01	85.4 ± 1.14	vitamin
Hemosiderin iron									
μg/g fresh tissue	26 ± 1.20	40 ± 2.02	13 ± 1.33	23 ± 2.16	56 ± 2.24	66 ± 1.84	45 ± 1.63	54 ± 2.34	vitamin, pregnant, Fe
% storage iron	9.1 ± 0.51	10.9 ± 0.71	9.5 ± 0.94	10.0 ± 0.82	14.8 ± 1.01	14.4 ± 0.98	14.4 ± 0.82	14.6 ± 0.91	vitamin
H/F ratio	0.120 ± 0.008	0.123 ± 0.010	0.105 ± 0.009	0.112 ± 0.011	0.160 ± 0.008	0.173 ± 0.014	0.170 ± 0.016	0.171 ± 0.012	vitamin

[1] Averages for 10 rats ± SE of mean.

TABLE 6

Total iron, non-heme, ferritin and hemosiderin fractions in spleen

	Pyridoxine-fed				Pyridoxine-deficient				Treatment
	Nonpregnant		Pregnant		Nonpregnant		Pregnant		Significant *F* values
	Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		$P < 0.01$
	0	2	0	2	0	2	0	2	
Total iron									
μg/g fresh tissue	2250 ± 146 [1]	2333 ± 120	1303 ± 140	1566 ± 180	2640 ± 134	2830 ± 182	2661 ± 206	2740 ± 239	vitamin, pregnant, vitamin × pregnant
μg/spleen	1260 ± 65	1377 ± 82	843 ± 92	955 ± 101	1267 ± 95	1217 ± 98	1117 ± 113	1151 ± 169	pregnant, vitamin × pregnant
Storage iron (non-heme)									
μg/g fresh tissue	1950 ± 92	2069 ± 84	1080 ± 156	1323 ± 181	2376 ± 174	2575 ± 102	2341 ± 184	2441 ± 187	vitamin, vitamin × pregnant
% total iron	86.7 ± 0.93	88.7 ± 1.11	82.9 ± 0.98	84.5 ± 1.32	90 ± 1.14	91.0 ± 1.06	88.0 ± 1.26	89.7 ± 1.05	vitamin, vitamin × pregnant
Ferritin iron									
μg/g fresh tissue	1720 ± 63	1793 ± 45	909 ± 61	1103 ± 54	1906 ± 70	2075 ± 78	1817 ± 61	1831 ± 72	vitamin, vitamin × pregnant
% storage iron	88.2 ± 0.81	86.7 ± 0.58	84.2 ± 0.84	83.4 ± 0.61	80.2 ± 0.88	80.6 ± 1.09	77.6 ± 1.17	75.0 ± 1.04	vgitamin, pregnant
Hemosiderin iron									
μg/g fresh tissue	230 ± 31	276 ± 18	171 ± 21	220 ± 26	470 ± 30	500 ± 41	524 ± 40	610 ± 38	vitamin, vitamin × pregnant
% storage iron	11.8 ± 0.71	13.3 ± 0.54	15.8 ± 0.84	16.6 ± 0.90	19.8 ± 0.91	19.4 ± 1.01	22.4 ± 0.98	25.0 ± 0.82	vitamin, pregnant
H/F ratio	0.134 ± 0.008	0.154 ± 0.009	0.188 ± 0.010	0.200 ± 0.011	0.247 ± 0.014	0.241 ± 0.016	0.288 ± 0.012	0.333 ± 0.013	vitamin, pregnant

[1] Averages for 10 rats ± SE of mean.

TABLE 7

Total iron, non-heme, ferritin and hemosiderin fractions in duodenum

	Pyridoxine-fed				Pyridoxine-deficient				Treatment
	Nonpregnant		Pregnant		Nonpregnant		Pregnant		Significant F values
	Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		Fe suppl., mg		$P < 0.01$
	0	2	0	2	0	2	0	2	
Total iron									
μg/g fresh tissue	38.7 ± 2.85 [1]	49.5 ± 3.92	19.7 ± 3.61	36.0 ± 4.12	45.6 ± 2.90	58.4 ± 3.67	41.7 ± 4.31	60.6 ± 4.88	vitamin, pregnant, Fe, vitamin × pregnant
Storage iron (non-heme)									
μg/g fresh tissue	36.3 ± 3.61	47.1 ± 2.43	17.9 ± 2.22	33.3 ± 3.03	43.0 ± 2.25	56.9 ± 2.50	39.9 ± 2.74	58.5 ± 2.82	vitamin, pregnant, Fe
% total iron	93.9 ± 1.22	95.1 ± 1.01	90.9 ± 0.81	92.5 ± 1.48	94.4 ± 1.37	97.4 ± 1.20	95.6 ± 1.53	96.6 ± 1.41	vitamin × pregnant
Ferritin iron									
μg/g fresh tissue	30.0 ± 1.35	38.5 ± 0.91	14.3 ± 1.83	26.6 ± 1.62	33.8 ± 1.90	44.0 ± 2.03	30.6 ± 1.80	44.2 ± 2.21	vitamin, Fe, vitamin × pregnant
% storage iron	82.7 ± 0.56	81.7 ± 0.90	79.9 ± 0.81	80.0 ± 0.85	78.6 ± 1.07	77.4 ± 0.91	76.8 ± 1.04	75.5 ± 0.94	
Hemosiderin iron									
μg/g fresh tissue	6.3 ± 0.36	8.6 ± 0.41	3.6 ± 0.48	6.7 ± 0.51	10.4 ± 0.86	12.9 ± 0.92	9.3 ± 0.91	14.3 ± 0.89	vitamin, Fe, vitamin × pregnant
% storage iron	17.3 ± 1.01	18.3 ± 0.72	20.1 ± 1.43	20.0 ± 1.61	21.4 ± 1.44	22.6 ± 1.86	23.2 ± 1.45	24.5 ± 1.77	vitamin
H/F ratio	0.210 ± 0.010	0.223 ± 0.008	0.252 ± 0.010	0.252 ± 0.009	0.308 ± 0.012	0.293 ± 0.018	0.304 ± 0.020	0.324 ± 0.022	vitamin, vitamin × pregnant

[1] Averages for 10 rats ± SE of mean.

vated H/F ratio. Pregnant animals, except for the iron-supplemented deficient group, had decreased concentrations of total and storage iron. Reductions were pronounced in pyridoxine-fed groups. These groups had significant decreases in concentrations of ferritin and hemosiderin but no significant change in the percentage distribution of storage fractions. However, H/F ratios were slightly elevated. Iron supplements increased concentrations of duodenal total iron, storage iron, ferritin and hemosiderin but the percentage total storage iron and the proportions of ferritin and hemosiderin storage iron were not altered.

DISCUSSION

The time-period for producing pyridoxine depletion was limited so that animals would implant and maintain pregnancy. The period was sufficient, however, to result in impaired reproduction including increased fetal resorptions and decreased numbers and weights of live young. These observations have been reported previously in pyridoxine deficiency (20). A trend toward increased resorptions in all iron-supplemented groups suggested that the mineral intake may have been approaching an excessive level for successful reproduction. Iron supplementation per se did not produce more adverse impairments in reproductive performance in pyridoxine-deficient animals than observed in the vitamin-fed group. On the basis of this criterion, deficient animals appeared to tolerate an increased level of iron intake during gestation at least as well as animals receiving the vitamin.

In this study none of the parameters of plasma iron were influenced by pyridoxine deficiency per se. Elevated plasma iron and TIBC and low UIBC observed in deficient pregnant animals compared with values for animals receiving the vitamin appeared to reflect blood volume differences. In the deficiency normal physiological changes in pregnancy were less pronounced because of the reduced size of the litter. Reductions in plasma iron and TIBC levels in pregnant groups, even in animals receiving the vitamin and large oral iron supplements, were similar to observations by Morgan (21) in rats that had been iron-loaded before pregnancy. The findings of Morgan in conjunction with those of this study support the suggestion that these changes are a normal physiological phenomenon of pregnancy.

The essentiality of pyridoxine in the biosynthesis of hemoglobin is well established. Pyridoxal phosphate participates in the formation of an activated glycine derivative and also acts as a coenzyme in decarboxylations of α-amino-β-ketoadipic acid in the pathway of porphyrin synthesis. In this study hemoglobin concentration per se was not altered by dietary omission of the vitamin; however, polycythemia accompanied by significantly lower MCH and MCV were evidences of the effects of the deficiency on hematopoiesis. The addition of the oral iron supplement to the basal diet did not change the hematological findings. Bishop and Bettwell (22) suggested that excessive iron concentration at the site of hemoglobin synthesis could result in the formation of an iron pyridoxal phosphate complex which inhibited normal coenzyme function of pyridoxal phosphate in porphyrin synthesis. The level of iron supplement used in this study did not appear to be sufficient to intensify the effects of the deficiency on hematopoiesis.

Decreased hemoglobin, hematocrit and red blood cell counts observed in pregnant animals on day 21 of gestation are apparently physiological phenomena of normal pregnancy (21, 23). The physiological increase in blood volume is related to the changes. In support of this suggestion, MCH and MCV values were not changed in pregnancy. Oral iron supplementation had no significant effect on any of the hematological values reported. Morgan (21) reported that iron-loading rats before mating did not prevent the decrease in hemoglobin concentration in pregnancy.

Increased total storage iron, decreased ferritin and increased hemosiderin levels were consistent observations in tissues of deficient animals. The extent to which these changes in iron storage can be intensified by a more prolonged vitamin deficiency than used in this study is being investigated in nonpregnant animals.

Decreased iron concentrations and total iron content in liver and in spleen of pregnant animals are in accord with the results reported by Morgan (21). In liver, decreases in both ferritin and hemosiderin

iron concentrations were observed resulting in no significant alteration in the percentages of these storage fractions. However, in spleen, the percentage ferritin was decreased and hemosiderin increased, suggesting that a large proportion of iron for gestational needs was mobilized from the ferritin fraction of spleen. In the deficiency this pattern of tissue mobilization of iron did not appear to be altered even though less iron appeared to be mobilized from tissues.

Regardless of the treatments used, a larger percentage of total iron was stored as ferritin in liver than spleen, whereas hemosiderin was stored in larger proportions in spleen. These results are in agreement with those of Kaldor (18) and Reddy et al. (24).

Iron supplements resulted in increased liver iron concentration and in total iron content. The increases were distributed between the two storage fractions without a change in H/F ratios. Iron supplements did not result in large increases in spleen iron levels. These observations suggested that liver was the principal deposition site of the supplementary iron. The pattern of deposition appeared to be similar in vitamin-fed and deficient animals although the amounts deposited were either greater, or less was withdrawn in the deficiency.

In numerous hypothetical models of intestinal function in iron metabolism the iron content of absorptive epithelial cells has been suggested as an important mechanism controlling iron balance (25–27). Low and high concentrations of duodenal iron, particularly ferritin, have been associated with increased and decreased absorption of iron, respectively. In this study decreased duodenal iron concentration, especially ferritin, was observed in vitamin-fed pregnant groups. This may have allowed for increased iron absorption which has been well-established in pregnancy (6–7). It is also possible that the high level of iron in the duodenum of iron supplemented and deficient groups was part of a regulatory mechanism which blocked excessive absorption of iron. Both of these groups had elevated iron stores and therefore the need for iron was less.

Although deficient animals had elevated levels of iron in all tissues examined, no major impairment in iron absorption was evident. The animals tolerated a 2-mg supplement of elemental iron given daily throughout gestation even though this is a period during which absorption of iron is normally increased. The oral supplement did not alter plasma iron levels or greatly intensify the elevations of iron in tissues of deficient animals. In these animals MCH was decreased and in pregnant groups, the young were smaller in size and number. Thus tissue elevations in the deficiency appeared to be due largely to decrease utilization of iron in hematopoiesis and to reduced fetal demands.

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Effect of Gossypol on the Iron-binding Capacity of Serum in Swine [1,2]

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ABSTRACT The effect of iron and calcium ions, alone and in combination, on the toxicity of gossypol for swine fed cottonseed flour rations, was studied. Weekly data on the performance of the animals were kept for 15 weeks, and blood samples were taken every 15 days and analyzed for hemoglobin, hematocrit, glutamic-oxaloacetic transaminase, total iron-binding capacity and serum iron. The results indicated that, when both chemicals were added to the ration, the animals showed better weight gains and no changes in the hair coat. The addition of calcium alone resulted in low hemoglobin, hematocrit, iron-binding capacity and serum iron levels, but there was no change in the glutamic-oxaloacetic transaminase serum levels. Iron supplementation, however, resulted in hemoglobin, hematocrit and iron-binding capacity approaching those of the control group, but the levels of glutamic-oxaloacetic transaminase were significantly elevated, indicating some liver damage. The addition of both minerals resulted in low levels of the transaminase and hemoglobin, hematocrit and iron-binding capacity levels similar to those obtained with the addition of iron alone. The results suggest that gossypol binds iron, interfering with its absorption, and consequently iron-deficiency anemia develops. The addition of calcium increases the effectiveness of the gossypol-iron complex formation, resulting in full protection from gossypol toxicity, although probably the levels of iron used were not sufficiently high to maintain a normal blood picture.

Several workers [3] (1, 2) have shown that the addition of ferrous salts to swine rations containing cottonseed meal reduces the toxicity of gossypol to varying degrees. The added iron forms an insoluble complex with gossypol, decreasing its free concentration and consequently its toxicity. In vitro studies by Bressani and co-workers (3) showed that the addition of iron in the presence of calcium salts decreases the concentration of free gossypol to a low level in food mixtures containing cottonseed flour. Jarquín et al. (4) have shown recently that the addition of 1% $Ca(OH)_2$ and 0.1% $FeSO_4 \cdot 7H_2O$ completely eliminates or inactivates the toxicity of gossypol for pigs fed high levels of cottonseed meal. Furthermore, Braham et al. (5) observed that the high levels of serum glutamic-oxaloacetic transaminase observed in animals fed high levels of cottonseed meal decreased to normal values when the ration was supplemented with both iron and calcium.

The results of Jarquín et al. (4) with swine, and those of Danke and Tillman (6) with rats, showed that the feeding of high levels of gossypol resulted in decreased levels of hemoglobin and hematocrit. The latter authors showed that the anemia thus produced was microcytic, hypochromic in character.

The present study deals with the effect of the addition of calcium and iron, alone or in combination, on the performance and certain blood constituents of swine fed high levels of cottonseed meal.

MATERIALS AND METHODS

Experimental procedure. The experiment consisted of 7 treatments, one of which was a control. Eight pure-bred Duroc Jersey piglets, 4 males and 4 females, were used per group. The animals were from 6 to 8 weeks of age when placed on the experiment and weighed between 5.4 and 12.2 kg.

Composition of rations. The composition of the rations fed is described in

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[2] INCAP Publication I-420.

[3] Hale, F., and C. M. Lyman 1962 Effective utilization of cottonseed meal in swine rations. J. Animal Sci., 21: 998 (abstract).

table 1. A ration containing 50% of soybean meal was used as a control and all the experimental rations contained 42% of a high quality cottonseed flour. This material was produced by the pre-press solvent extraction process and contained: (in %) protein, 50; fat, 3.2; crude fiber, 5.1; free gossypol, 0.058; total gossypol, 0.943; available lysine, 3.10 g/16 g N. Ferrous sulfate ($7H_2O$) and calcium hydroxide were added, alone and in combination. The residue from the crude oil purification step contained: (in %) protein, 52.1; fat, 39.7; crude fiber, 2.9; free gossypol, 0.332; total gossypol, 1.228; available lysine, 2.36 g/16 g N, and was added to rations 6 and 7 to increase the gossypol level both in the presence and absence of the combination of ferrous sulfate and calcium hydroxide. All rations were analyzed for free and total gossypol by the methods of the AOCS (7).

Care and feeding of animals. Throughout the experimental period, which lasted 15 weeks, each group of 8 pigs was confined in a pen which measured 5 × 2 m. The pens had concrete floors and were washed daily. Free access to feed and water was provided at all times. All animals received 1 cm^3 of a 20% zinc sulfate solution every other day throughout the experimental period. Weight and feed consumption data were recorded at weekly intervals.

Blood chemistry. Blood samples from the jugular vein were obtained from each pig at the beginning and every 15 days thereafter until the fifteenth week, for the determination of hemoglobin (8), hematocrit (9), serum glutamic-oxaloacetic transaminase [4] and serum total iron-binding capacity and serum iron (10). From the latter values, the unsaturated iron-binding capacity and the amount of circulating transferrin were calculated.

RESULTS

The growth performance, mortality and observations on the discoloration of the hair of the pigs are presented in table 2; also shown is the free gossypol concentration of the ration fed. The animals fed the control ration gained significantly more than those fed the ration containing cottonseed flour with or without additional $Ca(OH)_2$ and $FeSO_4 \cdot 7H_2O$, alone or combined. The addition of ferrous sulfate and calcium hydroxide combined (ration 5) resulted in no greater response, with respect to growth and feed efficiency, than the addition of iron alone (ration 3). There was, however, a marked difference in the appearance of the animals. Those receiving the ration with the combined chemicals showed no discoloration of hair and 5 out of 8 pigs showed discoloration of hair in the group fed the ration supplemented with iron alone.

The results on the blood constituents determined are presented for groups 1 through 5 only, because results for groups 6 and 7 are similar to those for groups 2

[4] Sigma Chemical Company 1964 Tech. Bull. no. 505 (May), St. Louis.

TABLE 1

Composition of the experimental rations

	Ration no.						
	1	2	3	4	5	6	7
Soybean meal	50.0	—	—	—	—	—	—
Cottonseed flour [1]	—	42.0	42.0	42.0	42.0	42.0	42.0
Mineral supplement [2]	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Torula yeast	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cod liver oil	0.5	0.5	0.5	0.5	0.5	0.5	0.5
$FeSO_4 \cdot 7H_2O$	—	—	0.1	—	0.1	0.1	—
$Ca(OH)_2$	—	—	—	1.0	1.0	1.0	—
Ground yellow corn	45.3	53.3	53.2	52.3	52.2	46.2	47.3
Cottonseed residue [3]	—	—	—	—	—	6.0	6.0
Aurofac [4]	0.2	0.2	0.2	0.2	0.2	0.2	0.2

[1] Processed for human consumption.
[2] Salmina, Riverside Company, Guatemala, C. A.: contains (in %) calcium carbonate, 33; bone meal, 33; iodized sodium chloride, 33; and minor elements, 1.
[3] A cottonseed filter press cake containing 332 mg/100 g of free gossypol.
[4] 1.8 g chlortetracycline per gram. American Cyanamid Company, Wayne, New Jersey.

TABLE 2

Performance of pigs fed cottonseed meal supplemented with iron and calcium

Ration no.[1]	Free gossypol	Initial wt	Wt gain [2]	kg feed/kg gain ratio	Mortality	No. of pigs showing hair depigmentation
	mg/100 g	*kg*	*kg*			
1	—	8.1	54.6	3.23	0/8	0/8
2	30	8.1	31.2	3.42	0/8	6/8
3	20	8.1	38.8	3.24	0/8	5/8
4	26	8.2	31.0	3.41	1/8 [3]	1/7
5	19	8.1	42.4	3.44	0/8	0/8
6	26	8.2	37.2	3.36	0/8	1/8
7	44	8.1	24.9	2.97	0/8	8/8

[1] See table 1 for treatments.
[2] Differences between control and experimental groups statistically significant ($P < 0.01$); least significant difference: 16.5 kg.
[3] Death of one animal due to diaphragmatic hernia.

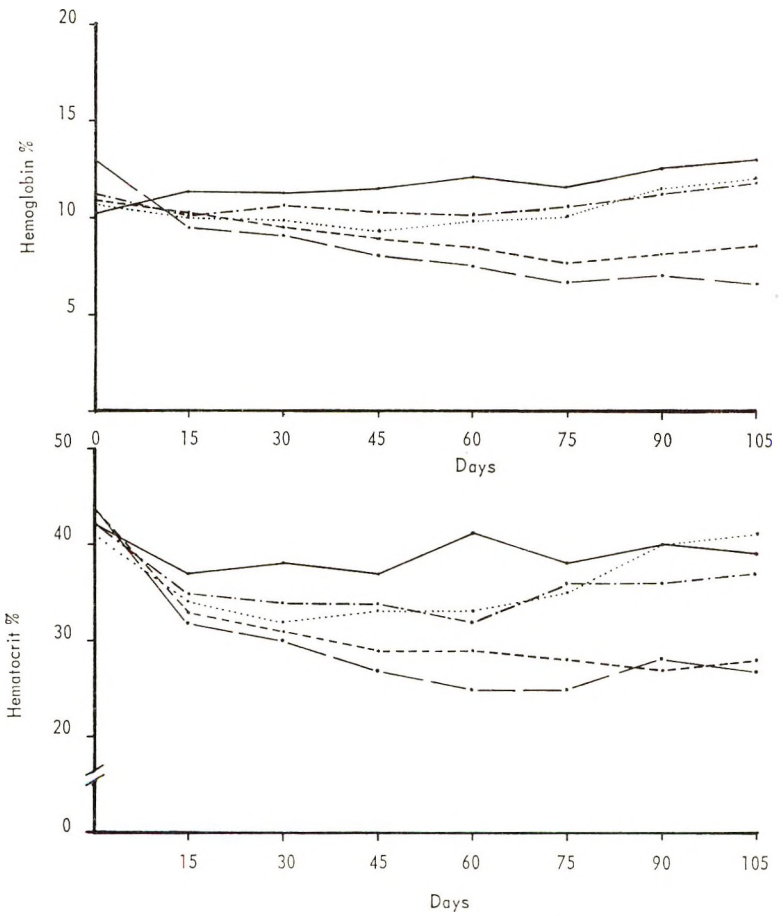

Fig. 1 Effect of the addition of calcium and iron on the levels of hemoglobin and hematocrit of pigs fed cottonseed meal; ——— control; – – – – – – cottonseed meal; —·—·— cottonseed meal + Fe; ·— —· cottonseed meal + Ca; ..... cottonseed meal + Fe + Ca.

and 3, respectively, except for higher levels of gossypol, and the blood values obtained were the same in trend and magnitude for each group and its counterpart.

Hemoglobin levels. Figure 1 shows the hemoglobin levels of the animals in the various groups. The results indicated a significantly lower hemoglobin concentration ($P < 0.01$) in all groups fed cottonseed meal with and without supplementation when compared with the controls. Iron supplementation alone, or the combination of calcium and iron increased the levels of hemoglobin but not quite to normal values and calcium supplementation alone resulted in the lowest levels throughout the experimental period.

Hematocrit values. The same trends were observed in the hematocrit values (fig. 1) as those observed for hemoglobin. The lowest values were observed in the group fed the ration supplemented with calcium alone. The effects of treatment and time were highly significant ($P < 0.01$).

Serum glutamic-oxaloacetic transaminase (SGO-T). The results for this determination are shown in figure 2. The groups fed cottonseed meal, alone or supplemented with calcium, or with calcium and iron, showed values similar to those obtained for the control group. The values obtained for the group supplemented with iron alone, however, showed a significant increase ($P < 0.05$) in the levels of this enzyme.

Serum total iron-binding capacity (TIBC) and serum iron. Figure 3 shows the results for these 2 determinations. There was a significant difference ($P < 0.01$) due to treatments and to time for both determinations; the interaction between time and treatments was also significant. The addition of iron alone maintained the levels of TIBC and serum iron within normal limits; the combination of calcium and iron in the ration was less effective than iron alone, and the addition of calcium alone resulted in the highest levels of TIBC and lowest levels of serum iron.

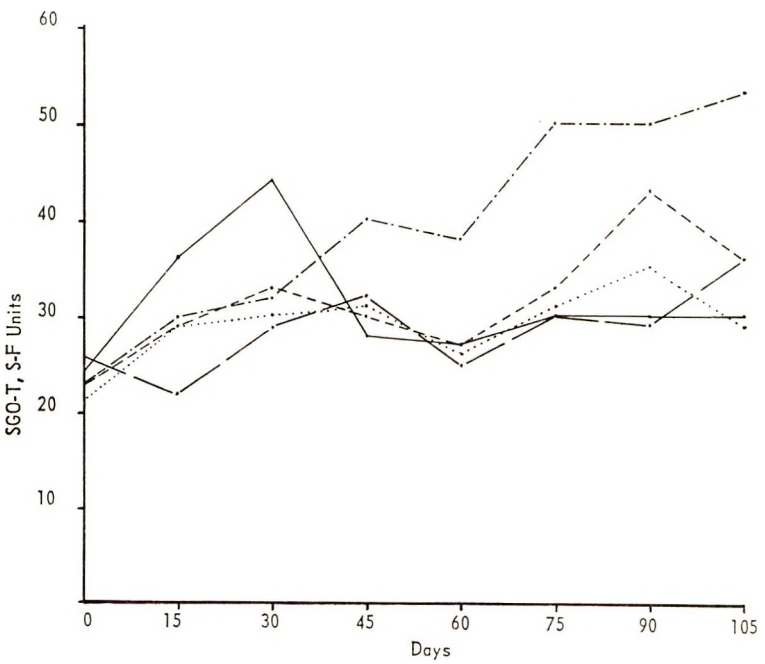

Fig. 2 Effect of the addition of calcium and iron on the levels of serum glutamic-oxaloacetic transaminase of pigs fed cottonseed meal; ——— control; – – – – – – cottonseed meal; —·—·— cottonseed meal + Fe; ·— —· cottonseed meal + Ca; ..... cottonseed meal + Fe + Ca.

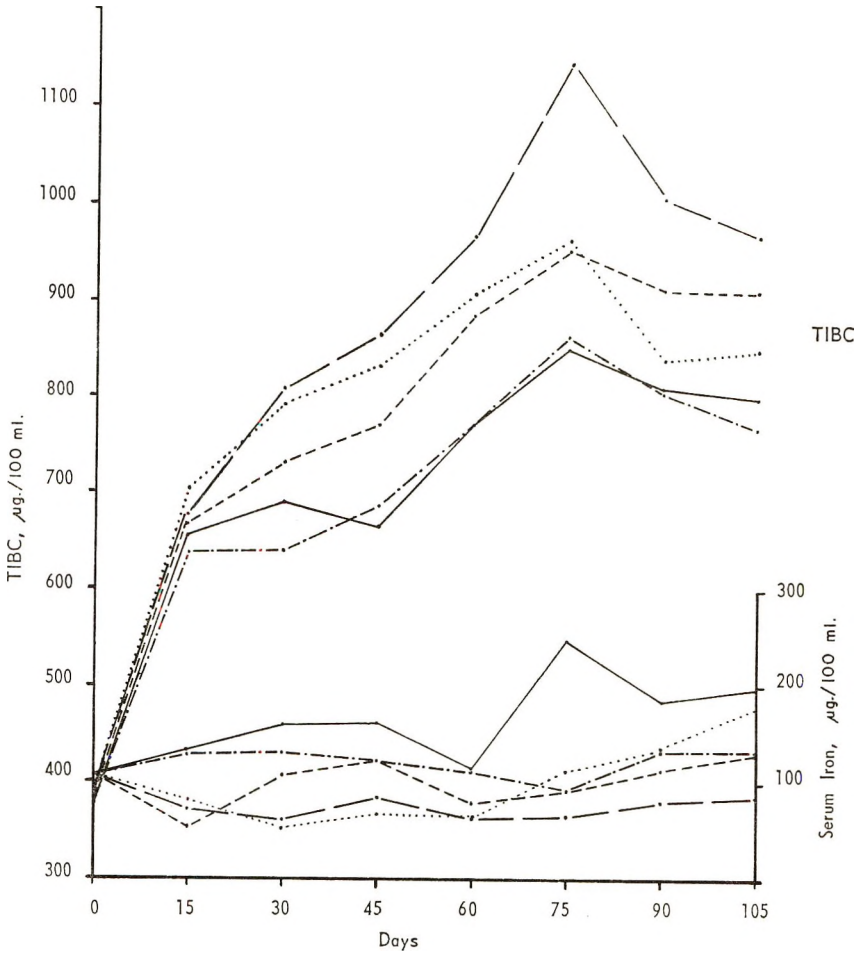

Fig. 3 Effect of the addition of calcium and iron on serum total iron-binding capacity and serum iron of pigs fed cottonseed meal; ——— control; – – – – – – cottonseed meal; —·—·— cottonseed meal + Fe; ·— —· cottonseed meal + Ca; ..... cottonseed meal + Fe + Ca.

The percentage of saturation of transferrin is shown in figure 4. As expected, the lowest values correspond with the group fed the ration supplemented with calcium alone, and iron alone was more efficient than the combination of both chemicals.

When iron alone was fed, the unsaturated iron-binding capacity values (UIBC) of the serum were almost as low as those obtained in the control group. Its combination with calcium was less effective in this respect, and the supplementation of calcium alone resulted in the highest UIBC. The amount of circulating transferrin was not actually determined. It was calculated from the molecular weight of transferrin (89,000), number of iron atoms bound by a molecule of this serum protein (2 atoms) and determined values of TIBC. The average figures obtained were 60.2, 65.8, 58.7, 74.1 and 67.8 g % of transferrin for groups 1, 2, 3, 4 and 5, respectively. Iron alone maintained normal levels, and its combination with calcium increased the levels of transferrin. Calcium alone increased the levels of the iron-binding protein to the highest values obtained within the experimental conditions used.

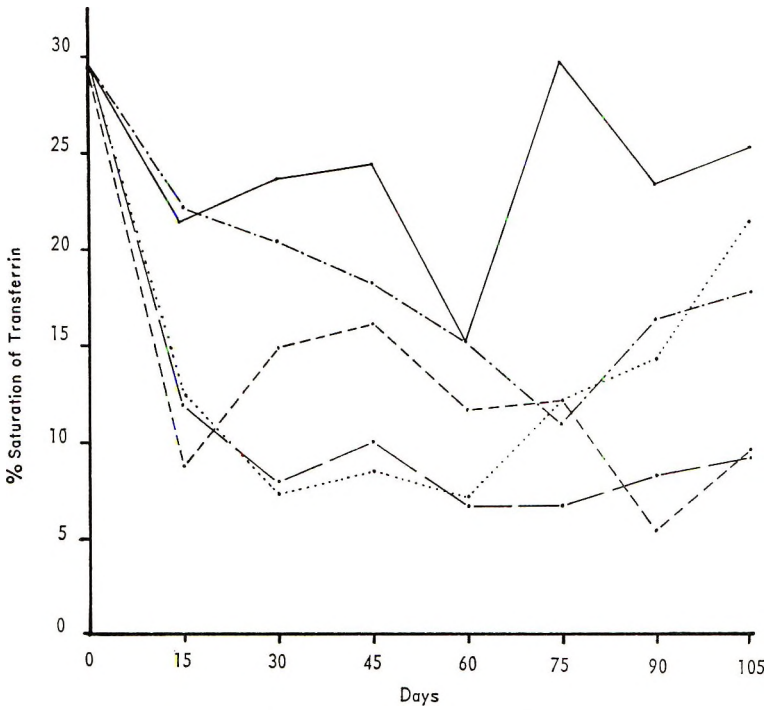

Fig. 4 Effect of the addition of calcium and iron on the saturation of serum transferrin of pigs fed cottonseed meal; ——— control; – – – – – – cottonseed meal; ·–·–·–· cottonseed meal + Fe; ·—— ——· cottonseed meal + Ca; ..... cottonseed meal + Fe + Ca.

DISCUSSION

The data on the performance of the pigs show the same trend as those reported previously from this laboratory (4). Neither iron nor the combination of iron and calcium resulted in weights comparable to those obtained with the control group, which may likely be due to some interference, by gossypol, with the utilization of calories. However, as the control group was fed a soybean meal ration, the soybean protein may have supported better growth than the cottonseed protein. When the cottonseed flour control ration (ration 2) is used as a basis of comparison, there is a definite effect of iron and of its combination with calcium; but not of calcium alone with respect to weight gain. This again corroborates the reports on the effect of iron and the detoxification of gossypol (1, 2).

As in previous studies (5), it was observed that some animals showed discoloration of the hair. The usual shiny, red hair coat of the animals turned a dull yellow and the smooth texture of the hair became coarse and kinky. As shown in table 2, gossypol in the ration appears to cause the hair changes. Iron alone in the ration did not have much protective effect, whereas calcium did. The combination of both protected the animals completely.

The role of gossypol in relation to color and structure of the hair is still obscure. Some minerals, such as zinc and copper, might be involved. Such possibilities are being studied in this laboratory.

The hematological findings characterized by low hemoglobin and hematocrit values, high iron-binding capacity, unsaturated iron-binding capacity and transferrin concentration, and low serum iron and percentage of saturation of transferrin, indicate a typical picture of iron-deficiency anemia (11). The results show that when calcium alone is added to the ration, the symptoms of anemia are made more acute than when iron was added alone or combined with calcium.

When 0.1% ferrous sulfate was added alone, there was a hematological response toward normal values which, however, never reached the values obtained with the group fed the soybean meal ration. The combination of iron and calcium was better than iron alone, and calcium alone resulted in the lowest hematological values obtained in this study.

The mechanisms through which iron-deficiency anemia is being produced may be as follows: when iron is added alone, in the absence of added calcium, part of it would be used to bind the gossypol present to the extent that the calcium present from the mineral mixture added and that from other components of the ration would allow. That this amount of calcium is not enough to result in significant binding of iron to gossypol is shown by the fact that enough iron is available to maintain a hematological picture approching normality. On the contrary, when calcium hydroxide alone was added, it exerted its stimulatory effect on the trapping of gossypol by the iron present from the other components of the ration so efficiently that very little iron was absorbed, as shown by the low levels of serum iron and the other hematological measurements. When the 2 compounds were added, the results were very similar to those obtained when iron alone was added. Evidently iron is being bound by gossypol in the presence of calcium, but the level of 0.1% ferrous sulfate used in this study is still not enough both to bind all the gossypol present and maintain a normal blood picture.

The results suggest that the toxic effect of gossypol is not the result of a systemic or specific action of the pigment on the hematopoietic mechanism. The binding of gossypol by iron probably takes place at the intestinal level, although it is clear from the results of Bressani et al.[5] that this binding is effective in vitro, and therefore already effective in the ration itself before it is consumed. Both mechanisms may be taking place, but whether in vitro or in vivo, the process appears to be very inefficient. This view is supported by the fact that, although chemically the ratio might be 1:1, biologically it is not. In this respect, Jarquin et al. (4), in experiments with swine, calculated that 5 moles of iron were needed to bind 1 mole of gossypol.

When a measure of the specific effect of gossypol at the organ level, such as the determination of serum glutamic-oxaloacetic transaminase as an indication of liver necrosis, was used, the results corroborated those discussed previously. This enzyme was significantly increased only when iron alone was supplemented, which indicates again that the level of iron used was not capable, in the absence of added calcium, of binding all the gossypol present. Enough iron was thus available for absorption, as judged by the hematological picture of these animals, and enough gossypol was also free for absorption, as indicated by the serum transaminase levels. Once calcium and iron were added, the levels of the enzyme dropped to normal. Calcium, when supplemented alone, maintained normal levels of transaminase, since all of the gossypol, or as much as the iron from the other components of the ration will allow, is being bound. We have no explanation for the low levels of transaminase obtained when cottonseed meal alone was fed.

In conclusion, the 2 effects of gossypol are unrelated. Animals whose rations are supplemented with iron alone will probably have normal hematology but enough gossypol may be absorbed, producing toxic effects, and calcium alone will result in an abnormal blood picture, but otherwise no toxic symptoms will be detected. The combination of the two will result in normal animals, as long as the amount of iron added can successfully bind all the gossypol present and still support normal hemopoiesis.

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