Dietary Production of Parathyroid Cysts [1]

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ABSTRACT Dietary supplements of dihydrotachysterol (DHT) and calcium acetate transform the parathyroids of the rat into sponge-like cystic organs. This lesion presumably represents "retention cyst" formation. It is tentatively ascribed to a selective blockade of the discharge of parathyroid secretion into the blood, without suppression of its formation within the gland. The advantages of the nutritional technique for the production of parathyroid cysts are discussed.

In the course of our work on calciphylaxis (1) it was noticed accidentally that, following simultaneous treatment with dihydrotachysterol (DHT)[2] and calcium acetate, rats regularly develop cystic parathyroids, associated with a singular form of muscular dystrophy (2). Essentially similar results were obtained in animals which received vitamin D_2, vitamin D_3 or parathyroid hormone instead of DHT. However, calcium acetate could not be replaced by an amount of dibasic sodium phosphate which was equally effective in augmenting the generalized soft-tissue calcinosis normally elicited by overdosage with vitamin D compounds or parathyroid extract (3).

In all these experiments, DHT, vitamin D_2 and calcium acetate were given by stomach tube, whereas parathyroid extract and vitamin D_3 were injected subcutaneously. The question arose whether further studies on the mechanism of this singular parathyroid reaction could be facilitated by a more continuous administration of the active agents (vitamin D compounds and calcium) as dietary supplements.

EXPERIMENTAL

Forty female Sprague-Dawley rats of the Holtzman strain with a mean initial body weight of 101 g (range 100 to 105 g) were subdivided into 4 equal groups, all of which were fed exclusively with ground commercial laboratory chow[3] to which we added supplements of DHT and calcium acetate.[4] Group 1 received a diet containing 75 μg of DHT and 200 mg of calcium acetate in each 5 g of the chow, while the remaining 3 groups received the same amount of DHT and calcium acetate in 10, 20 and 50 g of the chow, respectively. The DHT was dissolved in a few drops of ethanol, mixed with the calcium acetate powder and gradually added to the chow diet in a blender, to facilitate equal distribution. Thus, the 4 diets contained equal proportions of DHT and calcium acetate but in different concentrations.

The rats were maintained exclusively with these diets and tap water ad libitum for 21 days after which period all survivors were killed with chloroform. At autopsy, the thyro-parathyroid apparatus and the skeletal musculature were examined with a binocular loupe and specimens of them were fixed in Bouin's solution for subsequent embedding in paraffin and staining with hematoxylin phloxine and the periodic acid Schiff (PAS) procedure.

RESULTS

The mortality in group 1 (which received the highest concentration of DHT plus calcium acetate) was 60%, with a mean survival time of 17 days, and in group 2, 40%, with a mean survival time of 19 days. The rats of groups 3 and 4 (given the lowest concentrations of the dietary supplements) all survived.

In comparison with earlier observations on the experimental production of parathyroid cysts with different techniques, the most striking finding at autopsy was the virtually complete absence of muscle lesions, a fact subsequently confirmed by

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[1] This work was supported by the Canadian Arthritis and Rheumatism Society and the Medical Research Council of Canada.

[2] Calcamin, supplied by Dr. A. Wander, S.A., Berne, Switzerland.

[3] Purina Laboratory Chow, Purina Company of Canada.

[4] Fisher Scientific Company, Fair Lawn, New Jersey.

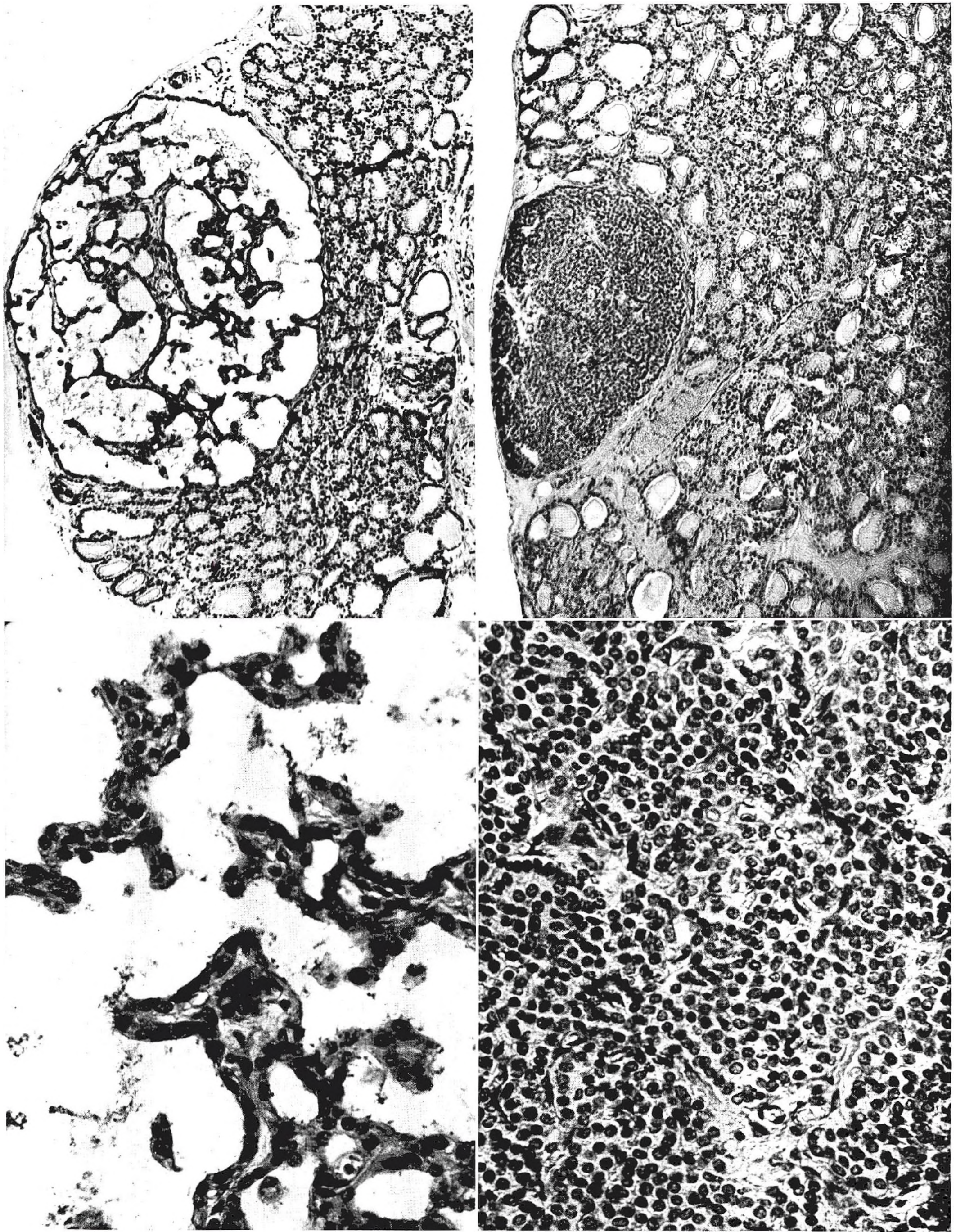

Fig. 1 *Top left:* Advanced cystic degeneration of the parathyroids in a rat of group 1 which received the largest supplements of dihydrotachysterol (DHT) plus calcium acetate. The cysts have perforated into each other so that only crests remain to indicate where the walls of individual follicles were. The number of epithelial cells is diminished and these form only a flat lining around the proliferating sclerotic connective-tissue stroma (hematoxylin phloxine, × 120). *Top right:* Essentially normal parathyroid of a rat from group 3 which received an ineffective dose of DHT plus calcium acetate. The solid structure of the parathyroid is preserved (hematoxylin phloxine, × 120). *Bottom left:* Higher magnification of a region from the picture above (hematoxylin phloxine, × 460). *Bottom right:* Higher magnification from the picture above (hematoxylin phloxine, × 460).

histologic examination. By macroscopic or even loupe inspection, parathyroid cysts are not detectable as such in the rat, but in groups 1 and 2 the parathyroids were noticeably enlarged and of a translucent watery aspect.

Upon histologic examination, no cysts were demonstrable in groups 3 and 4, but intense cyst formation was evident in all the rats of groups 1 and 2, including those that died prior to the termination of the experiment. The cysts differed from those previously described, mainly in that they were larger, more regular and more numerous. Presumably, cyst formation was facilitated because the dietary administration of DHT and calcium acetate is comparatively well tolerated and, hence, many of the rats survived up to 3 weeks, whereas previously we were unable to continue such experiments for more than 16 days. As a consequence of this increased survival, most of the cysts became so large that they perforated into each other, leaving mere crests of stroma covered by atrophic epithelial cells in what appeared to be a single, complex intercommunicating cavity. The fluid within this sponge-like structure was largely washed out during staining, but whatever was left consisted of a granular, slightly basophilic material which in part also stained with the PAS procedure. Since we had prepared step series through different levels of each parathyroid, it was possible to compare sections going through the widest parts of the cystic (groups 1 and 2) and non-cystic (groups 3 and 4) parathyroids. Such comparisons clearly indicate not only an atrophy but a great reduction in the number of the remaining epithelial cells within the cysts, whereas the connective tissue of the stroma proliferates. In contrast with these severe structural alterations in the parathyroids, the adjacent thyroid tissue was completely unaffected (fig. 1).

It remains to be seen whether further improvement of our technique, permitting still more prolonged survival, would result in a "chemical parathyroidectomy" owing to complete destruction of the epithelial elements with sclerosis, but evidently even 3 weeks of survival suffices to carry this process to a very advanced degree.

DISCUSSION

Our observations demonstrate the possibility of producing extensive cystic destruction of the parathyroids by purely dietary means. Apparently, the more continuous overdosage with DHT and calcium that is induced when these compounds were added to the food, is at least equally effective in this respect, and yet less toxic, than the repeated acute overdosage that results from daily injections or gavages.

The widespread lesions in the skeletal musculature that were elicited by the latter procedures in our earlier work were also missing in the present series of experiments. Although the reason for this difference is not yet clear, the absence of a severe muscular dystrophy was probably one of the factors ensuring longer survival.

It would be hazardous at this time to enter into far-reaching speculations concerning the possible pathogenesis of the experimentally produced parathyroid cysts. Serial examinations of the parathyroids in different stages of cyst development (not reported here in detail) gave little additional information apart from showing that the first histologically detectable traces of intercellular fluid accumulation appear between the seventh and tenth day, the process reaching its maximum towards the end of the third week. It is tempting to assume that we are dealing with "retention cyst" formation, presumably owing to a selective blockade of the discharge of parathyroid hormone secretion into the blood, without suppression of its formation within the gland. However, only future studies — perhaps with the sensitive immunochemical techniques that are now available — will be able to prove with certainty that the accumulated fluid actually contains parathyroid hormone or the more recently postulated calcitonin.

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Influence of Artificial Diet on Weight Gain and Body Composition of the Neonatal Rat [1]

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ABSTRACT Following an extensive analysis of rat's milk, a basal diet based upon non-fat dry cow's milk was developed for the artificial feeding by intubation of neonatal rats from birth to weaning at 23 days. Since this diet did not support optimal growth, it was supplemented with 1, 5, and 10% rat's milk. With the 5% rat's milk supplement, weight gains were improved to the point where they were equivalent to those of control rats raised by lactating mothers. However, carcasses of pups artificially fed this diet contained 35% more fat than the mother-raised controls. Conversely, rats intubated with the basal diet plus zero, 1, and 10% rat's milk supplements gained less weight than the mother-reared controls but had body compositions similar to those of mother-reared rats. These data indicate that weight gain alone is an inadequate index for evaluation of early diets. Lean body mass and body composition, expressed on an absolute basis appears to be more satisfactory. It is suggested that the technique used in this investigation is suitable for study of the effects of early nutrition on later life.

Advances in experimental techniques are now making it possible to undertake carefully controlled, detailed studies on the nutrition of artificially fed human infants (1–3) and some newborn animals (4). With the rat, however, studies during the preweaning period have been limited to effects of varying the milk supply offered to naturally suckling rat pups. Widdowson and McCance (5), Dickenson and Widdowson (6), Widdowson and Kennedy (7) and Heggeness et al. (8) have reported that the quantity of milk obtained by the rat during the suckling period profoundly influenced rate of growth and body development. One of the above studies (7) was extended to include life-span effects.

Recently, Miller and Dymsza (9) discussed the history of artificial feeding of laboratory animals and described a technique for the artificial feeding and rearing of neonatal rats. The present paper reports on a continuation of these studies. The development of a diet capable of promoting weight gains in preweaning rat pups is described. Data are also presented to show the influence of diet and supplements on body composition of the artificially fed rats at weaning age.

EXPERIMENTAL

To provide a basis for formulation of a diet, a composite sample of rat's milk was analyzed for the major constituents listed in table 1. The milk was obtained from 30 females during their 2- to 7-day lactation period by a previously described technique (9). Based upon this analysis, a number of diets, composed of various natural and purified ingredients, were formulated. These diets were tested for suitable physical properties such as solubility and ability to pass through a 24-gauge intubation needle. Promising formulations were then test-fed to small groups of infant rats.

As a result of the preliminary feeding trials, it was decided to initiate studies on the relatively simple diet based upon dry non-fat cow's milk shown in table 2. Although this formulation did not exactly simulate rat's milk, it was capable of supporting growth, but not at an optimal level (9). Since it appeared that certain factors found in rat's milk may have been

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TABLE 1

Composition of composite sample of rat's milk

	Per 100 ml milk
Moisture, g	74
Kilocalories, total, calculated	159
Protein Cal., %	23
Fat Cal., %	69
Carbohydrate Cal., %	8
Protein, g	9.2
As casein, %	73
As whey, %	10
As peptones and proteoses, %	17
Lipid, g	12.3
Carbohydrate, g	3
Ash, g	1.5
Calcium, mg	620
Phosphorus, mg	229
Iron, mg	0.61
Copper, mg	0.43
Potassium, mg	140
Sodium, mg	66
Chlorine, mg	176
Fluorine, mg	0.05
Iodine, mg	0.10
Cobalt, μg	1
Magnesium, mg	0.01
Zinc, mg	1
Vitamin A, IU	70
Thiamine, mg	0.11
Riboflavin, mg	0.89
Niacin, mg	2.2
Ascorbic acid, mg	5.3
Pyridoxine, μg	75.6
Vitamin B_{12}, μg	2.5
Folic acid, μg	2.5
Biotin, μg	7.5
Pantothenic acid, mg	0.84
p-Aminobenzoic acid, μg	10
Inositol, mg	62
Choline, mg	82

lacking or not in proper balance, 2 experiments, herein described, were conducted in which the basal cow's milk diet was fed at zero, 1, 5 and 10% levels of fresh rat's milk supplements.

Diets were prepared every 2 days by mixing all of the components in a Waring Blendor. Unused portions of diet were stored in a refrigerator. Before each feeding, the required amount of diet was warmed to about 32°. All diets were fed by intubation according to the technique and schedule previously published (9). In each experiment, all animals received the same number of feedings and were intubated with a maximal amount of diet commensurate with stomach capacity.

Day-old unsexed pups of the Charles River C.D. strain weighing 6 to 7 g each were used. In both experiments, the pups were derived from 4 litters and randomly assigned to experienced nonlactating foster mothers in groups of 8 or 10 pups. Individual groups of animals and their foster mother were maintained together on shavings in plastic tubs covered with a wire-mesh top which contained feed and water for the foster mother. The rat pups were individually weighed daily or every 2 days. All animals were kept under strict sanitary and isolation conditions in a temperature- and humidity-controlled room.

Experiment 1 was terminated at 8 days because of the onset of a respiratory infection in the infant rats. Except for minor dietary modifications, experiment 2 was a

TABLE 2

Composition of diets artificially fed to infant rats

	Basal		Basal + 1% rat's milk		Basal + 5% rat's milk		Basal + 10% rat's milk	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	%	%	%	%	%	%	%	%
Non-fat dry milk	15.0	15.0	15.0	15.0	13.5	13.5	12.0	12.0
Lactose	2.0	—	2.0	—	2.0	—	2.0	—
Corn oil	10.0	10.0	10.0	10.0	9.5	9.5	9.0	9.0
Vitamin mixture [1]	3.0	2.0	3.0	2.0	3.0	2.0	3.0	2.0
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Calcium carbonate	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Water	69.0	72.0	68.0	71.0	66.0	69.0	63.0	66.0
Rat's milk [2]	—	—	1.0	1.0	5.0	5.0	10.0	10.0

[1] The vitamin mixture was composed of the following: (in grams) vitamin A acetate and vitamin D_2 mixture (325,000 IU A/32,500 D), 0.6000; vitamin E acetate (25%), 8.0000; vitamin K (2-methyl-naphthoquinone), 0.0100; thiamine·HCl, 0.1060; riboflavin, 0.8850; niacinamide, 2.2000; ascorbic acid, 5.2500; pyridoxine·HCl, 0.0756; p-aminobenzoic acid, 0.0100; biotin, 0.0076; Ca pantothenate, 0.8360; folic acid, 0.0025; inositol, 62.3000; vitamin B_{12} (0.1%), 2.4800; and glucose, 917.2373. In experiment 1, lactose was used instead of glucose as the diluent, and the mixture contained one-half of the amount of vitamins A and D.

[2] Two- to 7-day milk.

replicate of experiment 1. At the termination of experiment 2 after 23 days, the animals were killed, selected organs weighed and carcasses analyzed for moisture, protein, fat and ash.

RESULTS

Eight-day (exp. 1) and 23-day (exp. 2) growth curves for groups fed the basal cow's milk diet alone and supplemented with 1, 5 and 10% of 2- to 7-day rat's milk are shown in figure 1. In this figure results from both experiments 1 and 2 are compared with a laboratory control curve consisting of average weights of 15 to 96 pups per point fed by lactating mothers.

A comparison of the weight gains in figure 1 indicates that optimal growth, as compared with lactating mother-maintained controls resulted only when the basal diet was supplemented with 5% rat's milk. Using 23-day weight gains, rats in experiment 2, receiving the 5% rat's milk supplement, gained an average 2.7 g/day as compared with 1.3, 1.2, and 1.1 g gain in body weight per day for rats fed the basal, basal plus 1% rat's milk and the basal plus 10% rat's milk diets, respectively. Mother-raised control rats gained 2.3 g/day.

Table 3 shows nutrient intake and efficiency of nutrient utilization of rats in experiment 2 from zero to 13 days of age. Data are limited to this period because after 13 days of age, the rats are able to see and start to consume a small amount of the foster mother's diet. As shown in table 3, food, protein and caloric intake was rather similar in all groups. However, animals that were intubated with the basal plus 5% rat's milk diet gained approximately 3 times more body weight and were approximately 3 times more efficient in food, protein and caloric efficiency than rats receiving the basal diet alone or supplemented with 1 or 10% rat's milk. With the basal diet, 13-day old rats gained 8.3 g at a food efficiency of 22%. In comparison, the rats that received the basal plus 5% rat's milk gained 24.2 g at a food conversion of 66%.

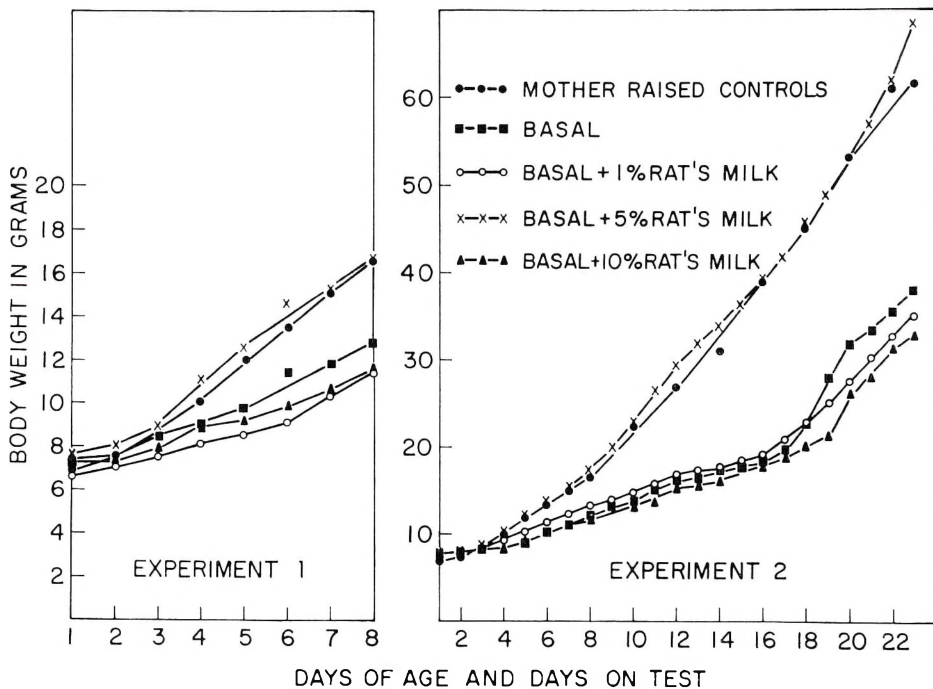

Fig. 1 Comparative growth curves of neonatal rats raised with lactating mothers and artificially fed rat pups intubated with a non-fat cow's milk basal diet supplemented with zero, 1, 5 and 10% rat's milk.

In general, organ weight, as expressed on a percentage of body weight basis, varied among the different experimental groups (table 4). Although these differences were generally statistically significant, their magnitude was usually small. With the exception of the spleen, pups intubated with the basal diet supplemented with 5% rat's milk had organ weights most similar to that of the controls. In the case of the spleen, there appeared to be a relationship between spleen size and feeding by intubation.

Carcass composition of one-day-old and 23-day old rats in experiment 2 is shown in table 5. From zero day to 23 days, all groups exhibited changes typical of maturation, characterized by decrease in moisture content and increases in the percentages of protein, fat and ash. The most striking

TABLE 3

Weight gain, nutrient intake and efficiency of nutrient utilization of 13-day old artificially fed rats in experiment 2 [1]

	No. of rats	Wt gain	Nutrient intake [2]			Nutrient efficiency [2,3]		
			Food	Protein	Calories	Food [4]	Protein [5]	Calories [6]
		g	*g*	*g*	*kcal*			*kcal*
Basal	8	8.3	38.3	1.89	57.5	21.7	5.39	14.4
Basal + 1% rat's milk	10	9.4	39.4	1.95	59.1	23.9	4.82	15.9
Basal + 5% rat's milk	8	24.2	36.5	1.80	54.8	66.3	13.44	44.2
Basal + 10% rat's milk	8	8.1	39.5	1.93	59.3	20.5	4.20	13.7

[1] Calculations based on a 13-day period prior to eye-opening and before any appreciable ad libitum food consumption could take place.
[2] Wet basis.
[3] See text.
[4] Grams gain of body weight/100 g diet intubated.
[5] Grams gain of body weight per gram protein intake.
[6] Grams gain of body weight/100 kcal consumed.

TABLE 4

Body weight and weight of selected organs at birth and 23 days of age of experiment 2 pups

Diet	No. of rats	Body wt	Percentage of body weight				
			Liver	Kidneys	Heart	Spleen	Adrenals
		g					
			Zero-day-old rats				
	10 [1]	6.2 ± 0.1 [2]	4.4 ± 0.24	0.94 ± 0.03	0.46 ± 0.02	0.17 ± 0.01	—
			23-Day-old rats				
Control [3,4]	22	62 ± 3	5.1 ± 0.12	1.2 ± 0.08	0.44 ± 0.03	0.4 ± 0.07	0.029 ± 0.001
Basal [5]	2	38 ± 6	6.2 ± 0.10	1.6 ± 0.02	0.67 ± 0.00	1.1 ± 0.04	0.035 ± 0.009
Basal + 1% rat's milk [6]	6	35 ± 3	5.8 ± 0.71	1.5 ± 0.08	0.58 ± 0.03	1.0 ± 0.22	0.020 ± 0.002
Basal + 5% rat's milk [7]	8	71 ± 4	5.1 ± 0.64	1.2 ± 0.08	0.50 ± 0.07	1.1 ± 0.22	0.019 ± 0.004
Basal + 10% rat's milk [8]	3	33 ± 3	6.6 ± 0.59	1.7 ± 0.13	0.62 ± 0.01	1.2 ± 0.15	0.030 ± 0.004

[1] Seven females and 3 males.
[2] SE.
[3] Animals raised by lactating mother.
[4] Fifteen females and 6 males.
[5] One male and one female.
[6] Five females and one male.
[7] Five females and 3 males.
[8] Three females.

TABLE 5

Carcass composition of day-old and 23-day-old rats in experiment 2

Diet	No. of rats	Body wt	Lean body mass[1]		Moisture	Protein	Fat	Ash	Total
		g	%	g	%	%	%	%	%
Zero-day-old rats									
	10	6.2 ± 0.1[2]	97.2 ± 0.2	6.1 ± 0.1	82.9 ± 0.1	12.1 ± 0.1	2.7 ± 0.1	2.3 ± 0.1	100.0 ± 0.1
23-Day-old rats									
Control[3]	10	62 ± 3	89.4 ± 1.0	55.3 ± 2.0	67.0 ± 0.9	18.9 ± 0.3	9.2 ± 0.6	3.4 ± 0.2	98.5 ± 0.8
Basal	2	38 ± 6	91.7 ± 2.1	35.1 ± 0.9	69.3 ± 3.0	18.5 ± 0.6	8.1 ± 2.2	3.6 ± 0.3	99.5 ± 1.1
Basal + 1% rat's milk	6	35 ± 3	89.9 ± 0.9	31.9 ± 1.8	67.4 ± 0.8	18.9 ± 0.2	9.5 ± 0.5	3.3 ± 0.2	99.4 ± 0.8
Basal + 5% rat's milk	8	71 ± 4	85.1 ± 1.5	58.1 ± 3.4	61.9 ± 0.7	21.1 ± 0.6	14.3 ± 1.1	3.0 ± 0.1	100.4 ± 0.6
Basal + 10% rat's milk	3	33 ± 3	89.3 ± 0.7	29.7 ± 2.5	68.9 ± 0.5	17.0 ± 0.6	8.4 ± 0.6	3.4 ± 0.1	97.7 ± 1.0

[1] Fat-free body consisting of moisture, protein and ash.
[2] SE.
[3] Animals raised by lactating mothers.

differences in body composition at 23 days appeared in the basal plus 5% rat's milk group. The carcasses of these rats contained significantly more fat ($P = 0.01$) and significantly less moisture ($P = 0.05$) than observed in mother-reared controls or the other experimental groups. In addition, the carcasses of pups of this group contained slightly but significantly more protein ($P = 0.05$). Furthermore, the lean body mass of the rats, expressed as a percentage of body weight, was slightly yet significantly less ($P = 0.05$) than that of the controls. Although other differences in carcass composition were observed, these appear to be generally less distinctive.

DISCUSSION

Development of a diet suitable for the artificial feeding of neonatal rats began with analysis of rat's milk because of the assumption that a correlation must exist between milk composition and nutritive requirements of the young of a particular species. Analyses of the pooled sample of rat's milk were in general agreement with the values reported by other workers which include Cox and Mueller (10, 11), Luckey et al. (12) Houston and Kon (13), and Glass.[2]

In actual practice, the formulation of a diet with suitable chemical, physical and physiological properties proved to be difficult. Common symptoms observed in infant rats intubated with unsuccessful diets included failure to grow, delayed stomach emptying, diarrhea, and dehydration. The extreme response of the infant rat to its diet may be a result of the immaturity and semi-embryonic state of the rat at birth. McCance and others (14) and Leeman[3] have called attention to the immature adrenal, gut and kidney function of the neonatal rat.

The basal diet used in these experiments represented a compromise between many desirable characteristics. For example, the diet supported a fair rate of growth but could not contain a level of protein greater than 5%. Higher concentrations of protein which approached the 9.3% protein

[2] Glass, R. L. 1956 Chemical, physical and biological studies of rat's milk and its components. Ph.D. Thesis, University of Minnesota, Minneapolis.
[3] Leeman, S. E. 1963 Delay in maturation of the stress response in newborn rats. Federation Proc., 12: 165 (abstract).

of rat's milk resulted in decreased stomach emptying time, and severely curtailed the number of possible intubations. However, improvement in weight gains up to the point of surpassing those of mother-reared controls occurred when the basal diet was supplemented with 5% rat's milk although its protein level was less than that of rat's milk. Since a 1% rat's milk supplement was not sufficient and 10% rat's milk was excessive, and, since it is obvious that rat's milk itself supports adequate weight gain, it must be assumed that at some point, increased additions of rat's milk greater than 10% would again bring the diet into nutritional balance.

The effect of rat's milk supplementation is a product of a complex interrelationship. To explain this effect on the basis of a simple deficiency of some essential factor is not possible when the opposing responses to 5 and 10% supplementation with rat's milk are considered.

In an effort to obtain some knowledge of the nature of this effect, preliminary studies have been made with rat γ-globulin and nucleotide supplementation. Unpublished observations in this laboratory have shown that rat γ-globulin had no effect, whereas supplementation with adenosine-3′,5′-cyclic phosphoric acid (cyclic AMP) generally, but not always, resulted in some improvement in weight gain.

Nucleotides were included in these studies because they are present in relatively high concentrations in early milk and because the composition of the nucleotide fraction is significantly different among different species (15, 16). Cyclic AMP was selected as the nucleotide for these studies because it has been shown to play a significant independent role in metabolism (17, 18). However, these observations, alone, do not provide a basis for explaining the unusual effect of rat's milk supplements.

As reported by Heggeness et al. (8), the nutritional state of the animals did not influence the rate of observable physical maturation as measured by eye-opening and hair appearance. It was observed, however, that 11-day-old pups from experiment 1 had an eye condition which appeared to be cataracts. As a result, all possible lactose in the diet was eliminated and the level of vitamin A in the diet increased. The eye condition was not observed in the pups in experiment 2.

When compared with the basal and other experimental diets, the beneficial effects of the 5% rat's milk supplement are best demonstrated by the threefold improvement in weight gain and food, protein and caloric efficiency. Also, the 13-day PER of 13.44 obtained with the 5% rat's milk addition indicates that the interpretation of PER, as generally used and accepted, would have to be modified for use as a measure of protein ultilization in the preweaning rat.

The data obtained also demonstrate that weight gain alone is not a good index of diet adequacy. In experiment 2, weight gains over the 23-day experimental period were statistically the same for mother-reared control rats and those intubated with the basal diet supplemented with 5% rat's milk. However, at 23 days of age the artificially fed animals receiving the basal plus 5% rat's milk had 35% more body fat than the controls. Conversely, groups of rats fed the 3 other intubated diets, and which gained much less weight than the mother-reared controls, had a body composition on a percentage basis similar to that of rats raised with a lactating mother. It appears that the most valuable index of the adequacy of a diet in the preweaning rat is probably lean body mass and proximate body analysis expressed in absolute terms. Some support for this contention can be found in the work of Sherman and Booher (19), Williams et al. (20), Wallace et al. (21), Filer et al. (4) and Heggeness et al. (8) who demonstrated that although fat and certain mineral components of the body can be changed by diet, gross body composition on a fat-free lean-body basis usually remains rather constant.

Since the animals possessed a high degree of genetic homogenicity, it may be assumed that the neonatal diet was the major influence in the differences obtained in weight gain and body composition. The meaning of these differences in the development of the neonatal rat is unknown. For example, were the fatter rats fed the basal diet supplemented with 5% rat's milk any less "healthy" than pups fed by lactating mothers even though both groups

were similar in body weight? On the other hand, were the animals fed the basal diet or the diets supplemented with 1 and 10% rat's milk, and which gained less weight but were rather similar to mother-raised controls in body composition, as "healthy" as the larger animals?

Since these experiments were terminated at weaning, it is not possible to attempt to answer the above questions or to correlate changes induced by the neonatal diet with any postweaning effects. However, the techniques used in these studies are suitable for such investigations.

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Reproduction and Lactation in Rats Fed Excessive Iodine [1,2]

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ABSTRACT In a series of experiments adult female rats were fed zero to 2500 ppm supplemental iodine from zero to approximately 35 days prepartum. Females were permitted to litter normally and the survival of the young was observed. Other females were killed either between 17 and 19 days of pregnancy or between 24 and 48 hours postpartum to observe ovulation rate, implantation rate, development of normal fetuses and histology of mammary tissue. Increasing mortality of young after birth occurred with increasing levels of iodine. Most young failed to survive for 24 hours and those that survived 48 to 72 hours postpartum usually survived until weaning. Those failing to survive had essentially no milk present in the stomach. Visual observation and histological examination of mammary tissue from females fed iodine revealed that secretion was absent or markedly diminished.

The role of iodine in thyroid function and the manifestations of iodine deficiency in various species have been well established and have been reviewed by Riggs (1) and Berson (2). The effect of excessive iodine, however, is less clearly defined. Morrison (3) reported that excessive intake of iodine is injurious to animals but did not describe the effects, indicate the levels of iodine required, or cite the experimental evidence. Malan et al. (4, 5) reported that 200 mg KI daily had detrimental effects on reproduction in sheep; however, the authors stated that the conditions under which reproductive failure occurred involved not only high levels of iodine but deficiences of both phosphorus and vitamin A. Other effects of excessive iodine on reproduction have not been reported, although reproductive abnormalities have been attributed to iodine deficiencies (6, 7).

The present studies were conducted to determine the effects of various levels of dietary iodine upon reproduction and lactation of rats.

PROCEDURE

A series of 5 experiments was conducted in which sexually mature, nonparous female, hooded rats of the Long-Evans strain were fed various levels of supplemental iodine for intervals of time before littering and during lactation. All females were bred to normal males of the same strain. A ground stock diet [3] served as the basal ration and potassium iodide was added to yield the desired levels of dietary iodine. Potassium carbonate was added to the control ration to provide a level of potassium equivalent to the highest level fed in the experimental rations. The rats were housed in individual metal cages with screen bottoms in an air-conditioned room maintained at 26°. Females were permitted to litter normally and observations were made at littering of number of young born and those born dead. Periodic observations were made for evidence of nursing, mothering instinct and survival of young.

The survival data were treated statistically by chi-square technique and the remaining data by analysis of variance (8).

RESULTS

Experiments 1 and 2. The results of feeding supplementary iodine from zero to 2500 ppm are shown in table 1 and figure 1. In both experiments, the female rats were fed supplemental iodine beginning 12 days before cohabitation with males and continuing until the young died

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[3] Ralston Purina Company, St Louis.

TABLE 1

Reproduction and survival of young rats from females fed iodine (exps. 1 and 2)

Treatment	No. rats	Avg feed consumed[1]	Avg wt gain[1]	No. litters	"Gestation" days[2]	Total young	Number of litters and number of young surviving: 24 hours, Litters	24 hours, Total	24 hours, %	5 days, Litters	5 days, Total	5 days, %
		g	g						%			%
Experiment 1												
Control	5	133	14.0	5	—	50	5	49	98	5	45	90
Iodine, 2500 ppm	5	126	9.0	5	—	44	3	12	27	1	3	7
Experiment 2												
Control	9	147	−2.1	9	24.0	84	9	80	95[3]	9	78	93[3]
Iodine, 500 ppm	9	146	−4.2	8	24.8	62	8	60	97	7	52	84
Iodine, 1000 ppm	9	144	−9.1	6	24.4	50	5	34	68	5	33	66
Iodine, 1500 ppm	9	132	−7.8	8	25.4	56	8	32	57	4	20	36
Iodine, 2000 ppm	9	137	−8.2	6	26.2	57	5	22	39	1	9	16

[1] Average total per female for 12 days before mating.
[2] Days between cohabitation with male and littering.
[3] Chi-square test indicated most of the variance except error variance which was accounted for by linear trend.

or were weaned. In experiment 1, which compared 2500 ppm iodine with no supplemental iodine, the female rats receiving the iodine consumed slightly less feed and gained slightly less body weight during the first 12 days of the trial. All 10 rats littered with an average of 10.0 and 8.8/litter for the control and 2500 ppm iodine group, respectively. Twenty-four hours after birth, 2 complete litters from high-iodine dams were dead and by 48 hours after birth there was only one surviving litter with 3 young (7% of total) in the high-iodine group. The 3 young survived the 18-day period and this number may be compared with 5 litters with a total of 45 young which survived (90% of total) in the control group.

In experiment 2, female rats were fed zero, 500, 1000, 1500 and 2000 ppm iodine. Although not significantly different, less feed was consumed and a greater weight loss occurred in the rats receiving the higher levels of iodine during the first 12 days. The number of full-term pregnancies per group and the length of time from cohabitation with males to time of littering, although varying from an average of 24.0 days for the control group to 26.2 days for the group receiving 2000 ppm iodine, were not significantly affected by treatment. The data shown in table 1 and illustrated in figure 1 show an increas-

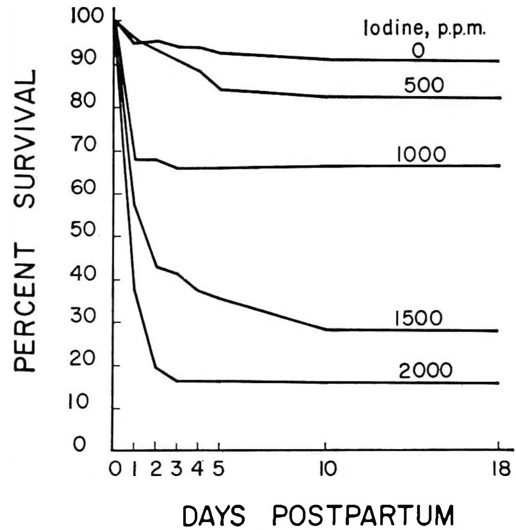

Fig. 1 Survival of young from females fed at various levels of dietary iodine (exp. 2).

ing mortality of the newborn rats with increasing levels of iodine. Significant differences in survival rate were evident within 24 hours postpartum and those that survived 48 to 72 hours postpartum usually survived until weaning. When the data were analyzed by the chi-square technique, most of the variance except for error variance was accounted for both at 1 and 5 days by linear trend. At the 2000 ppm level of iodine, all young from 5 of the 6 litters were dead within 72 hours. The 9 young in the remaining litter survived the entire 18-day period.

Neither the birth weights nor the 18-day body weights of surviving rats were affected by treatment (table 2). The young that died within the first few days after birth weighed less than at birth and showed no evidence of having nursed as determined by examination of the stomach for the presence of milk.

Experiment 3. The effect of feeding 2500 ppm iodine on ovulation rate, implantation rate and development of normal fetuses is shown in table 3. Twenty females each were fed either the control diet, 2500 ppm continuously beginning 12 days prior to mating, or 2500 ppm iodine for 12 days prior to mating and again following the 10-day mating period with iodine feeding continued until the end of the experiment. Thus in the last treatment the males were not forced to consume the high-iodine diet. Feed consumption was equalized within trios ex-

TABLE 2

Average body weight of young from females fed iodine (exp. 2)

Treatment	Birth		At death [1]		18 Days	
	No.	Weight	No.	Weight	No.	Weight
		g		*g*		*g*
Control	84	5.5	2	4.6	68	23.4
Iodine, 500 ppm	62	5.7	4	4.0	48	24.2
Iodine, 1000 ppm	50	5.3	7	5.1	33	25.9
Iodine, 1500 ppm	56	5.4	27	4.8	17	26.3
Iodine, 2000 ppm	57	5.2	28	4.3	9	22.3

[1] Weights obtained on those young dying within 48 hours after birth which were found intact.

TABLE 3

Corpora lutea and implantation rates for females fed 2500 ppm iodine and killed either between 17 and 19 days of pregnancy or between 24 and 48 hours postpartum (exp. 3)

Treatment	No. rats	No. litters	Avg no. corpora lutea	Implantation sites as % of corpora lutea	% corpora lutea represented by:		Young alive at killing
					Normal fetuses	Regressed sites or fetuses	
							%
17–19 days of pregnancy							
Control	10	7	10.9	74	64	10	—
Iodine, 2500 ppm (continuous)	10	6	11.5	67	57	10	—
Iodine, 2500 ppm (interrupted)	10	7	11.4	87	82	5	—
24–48 hours postpartum							
Control	10	8	13.2	61	43	18	87 [1]
Iodine, 2500 ppm (continuous)	10	7	12.7	80	65	15	35
Iodine, 2500 ppm (interrupted)	10	7	11.6	70	63	7	37

[1] Significant at the 1% level.

cept during the mating period. The pregnant females were killed either between 17 and 19 days of pregnancy or between 24 and 48 hours postpartum for examination of the reproductive organs and to obtain mammary tissue for histological examination.

The average number of corpora lutea per dam varied by treatment from 10.9 to 13.2 and the implantation rate from 61 to 87% with no suggestion of a treatment effect (table 3). Although large variations among treatment means were observed in the percentage of corpora lutea represented by normal fetuses or live young (43 to 82) or by regressed sites (5 to 18) the variability occurring within treatment was such that statistical differences were not evident. Eighty-seven per cent of the young from control dams were alive when killed 24 to 48 hours postpartum compared with 35 and 37% ($P < 0.01$) for the dams receiving iodine either continuously or on an interrupted basis. The average body weight gain for the surviving young was 0.60 g for the controls and −0.60 g and −0.25 g for those from dams fed iodine (table 4) and was significantly affected by treatment ($P < 0.01$). The stomachs, with contents, of young whose dams received iodine averaged 206 mg in weight compared with 336 mg for the stomachs of young from control dams ($P < 0.01$). Histological examination of mammary tissue from females fed iodine revealed epithelial development comparable to control rats. Mammary secretion, however, was absent or markedly diminished in the iodine treated group.

Experiment 4. Thirty-three female rats were fed 2500 ppm iodine from zero to 8 days prepartum to determine the length of feeding time required to adversely affect lactation. Survival of young from females fed supplementary iodine for 3 to 4 days prepartum or longer was similar to that shown for high levels of iodine in figure 1. Death of most of the young occurred within 24 hours postpartum with only limited change in survival after this period. When the females had received iodine only 1 to 2 days prepartum, there appeared to be a delay in death until about 3 days postpartum. Seventy per cent of the young were alive through the third day but within the next 2 days survival dropped to 42 and 23%, respectively.

Experiment 5. Twenty-four females, averaging 216 g body weight, were randomly assigned to 2 groups of 12 each and fed either the control ration or 2500 ppm iodine ration beginning 12 days before cohabitation with males. One-half of each litter from 7 high iodine dams was transferred to 7 control dams within a few hours after birth. Likewise, one-half of the control dam's litter was transferred to an iodine dam. The control litters were older at time of transfer and varied from a few hours to 24 hours.

During the 12 days prior to mating, the control females consumed an average of 167 g of feed compared with 133 g for the iodine group ($P < 0.01$) and gained an average of 10.4 g body weight compared with 3.0 g for the iodine groups ($P < 0.01$). The average length of time from cohabitation with males until parturition was 24.5 days for the control group and 26.3 days for the iodine group ($P < 0.01$). The latter data are similar to those obtained in experiment 2 and suggest that iodine at high levels prolonged gestation.

Twenty-one per cent of the young from iodine-fed dams survived until 10 days of age when left with their dams. This can

TABLE 4

Average body weights and stomach weights for young surviving 24–48 hours from females fed iodine (exp. 3)

Treatment	Body wt		Gain	Stomach wt [1]
	Birth	24–48 hr		
	g	*g*	*g*	*mg*
Control	5.46	6.06	0.60 [2]	336 [2]
Iodine, 2500 ppm (continuous)	5.71	5.11	−0.60	207
Iodine, 2500 ppm (interrupted)	5.73	5.48	−0.25	205

[1] Weight of stomach plus contents.
[2] Significant at the 1% level.

TABLE 5

Effect of exchanging young between control dams and dams receiving 2500 ppm iodine on survival of young (exp. 5)

Gestation treatment [1]	No. litters	No. young	Lactation treatment [2]	Young		
				Initial no.	Surviving 10 days	
					No.	%
Iodine	7	58	iodine	28	6	21.4
			control	30	10	33.3
Control	7	64	iodine	32	21	65.6
			control	32	30	93.8

[1] Gestation treatment significant at the 1% level.
[2] Lactation treatment significant at the 5% level.

be compared with 33% survival of the young when transferred to control dams (table 5). Ninety-four per cent of the controls survived to 10 days of age when left with their dams and 66% survived when transferred to iodine dams. The effect of treatment of females during gestation and lactation was significant at the 1 and 5% level, respectively. Average body weights for surviving young at 10 days of age were comparable for all groups. Many of the controls were several hours older and had visible milk curds in their stomachs at the time of transfer, whereas there was essentially no evidence of the iodine young having nursed prior to transfer. Thus the control young may have been stronger and this perhaps influenced the survival of those from iodine mothers whether nursing control females or their own iodine dam. The additional nursing efforts of the stronger controls may have stimulated greater milk flow in the mothers fed high iodine than had occurred in other experiments.

DISCUSSION

The mortality rate of young rats from females fed iodine beginning 12 days prior to cohabitation with males was increasingly greater with increased levels of supplemental iodine from zero to 2500 ppm. In a further study, high mortality of the young occurred when 2500 ppm iodine were fed from only 1 to 2 days preparatum. Examination of the stomachs of the young that died within the first few days postpartum indicated no milk to be present, and the stomach contents of those young which survived 24 to 48 hours were significantly less than the contents of young from control dams. Histological examination of mammary gland tissue from females fed iodine indicated that milk secretion was absent or markedly diminished. Mammary glands of control rats at the same stage following parturition contained milk which was readily observed upon incision. On the basis of these observations, the mortality of the young was attributed to lactation failure of the dam.

The mechanism by which high levels of dietary iodine may inhibit lactation is not known; however, it may be related in some way to thyroid activity. Iodine administered in single large doses to rats (9), or treatment of rats with high doses for extended periods (10, 11), has been shown to produce temporary inhibition of the organic binding of iodine by the thyroid. Following the temporary inhibition, adequate amounts of thyroid hormone were produced in spite of continued high level feeding of iodine (10–12); however, Galton and Pitt-Rivers (10) found the iodine binding capacity somewhat low even after 7 weeks. The importance of a normally functioning thyroid gland in lactation is not clear. Kon and Cowie (13), in reviewing data obtained with several species, suggested that the thyroid gland is not essential for milk secretion, but that in its absence the intensity and duration of milk secretion is reduced. In the present studies either the thyroid or other endocrine gland(s) may have been affected which in turn inhibited milk secretion but not the development of mammary tissue.

No adverse effects on reproductive performance were observed in male rats fed iodine. Four males from a dam receiving

2500 ppm iodine continued to be fed the high iodine ration until they were about 200 days of age. At this age, while receiving the high iodine ration, each male was proved fertile by successful mating with 2 control females.

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Effects of High Levels of Dietary Vitamin A Acetate on Tissue Tocopherol and Some Related Analytical Observations [1,2]

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ABSTRACT The total tocopherol content of liver tissue and plasma was determined after 2-week-old vitamin A-depleted chicks had received 0.5, 5, 50, 500, and 5,000 mg of vitamin A acetate/kg of diet for 5, 10, and 20 days. The tocopherol content in the tissues was markedly depressed especially at the highest levels of vitamin A intake. The depletion of tocopherol from the tissues at the highet levels of vitamin A intake was similar to that previously encountered by feeding a vitamin E-low basal diet. Decreasing liver tocopherol values with *time* at a dietary level of 17.6 mg of *dl*, α-tocopheryl acetate/kg may be indicative of a gradually increasing tocopherol requirement of the rapidly growing chick. Florex chromatography was unsatisfactory in removing the large amounts of vitamin A in the liver. Hydrogenation of the vitamin A followed by chromatography through Florex proved to be satisfactory.

That vitamin A has a detrimental influence on the tocopherol content of animal tissue has been suggested by the work of Irving and Bundtz-Olsen (1) and Irving (2). These workers presented evidence of the interrelationship between vitamin A and α-tocopheryl acetate when they observed that the protective action of the tocopherol upon the incisal enamel organ and tooth color of rats fed vitamin E-free diets was eliminated when either a high potency vitamin A hake liver oil or 15,000 units of a vitamin A concentrate from fish oil was fed. These observations were shown to be unrelated to unsaturation of the fish oil. The high intake of vitamin A also caused the dialuric acid hemolysis test to become positive, which is generally indicative of a low tocopherol status. Dicks et al. (3) observed a significant decrease in liver and plasma tocopherol concentration in calves as the vitamin A intake was increased from 0.01 to 1 mg/454 g live weight per day. More recently, Edwin et al. (4) showed a similar trend of liver, heart, and kidney tocopherol with rats which were dosed with vitamin A.

Liver tissue which was available from a previous study (5) in which vitamin A acetate was fed to chicks at levels which ranged from zero to 0.5% of the diet, was analyzed for total tocopherol in order to study further the vitamin A inhibitory effect on tissue tocopherol over a wide range of vitamin A intakes. When it was observed in the analysis for tocopherol that chromatography was unsatisfactory in removing the interference, presumably due to the massive amounts of vitamin A in liver tissue at the high levels of vitamin A intake, some modifications in the method for tocopherol analysis were made and are reported in the present publication.

EXPERIMENTAL

Vitamin A-depleted chicks, 2 weeks of age, were fed a vitamin A-low diet which contained per kg: 17.6 mg of *dl*, α-tocopheryl acetate and was supplemented with zero, 0.5, 5, 50, 500, and 5,000 mg of vitamin A acetate/kg of diet. Other details of the experimental procedures, such as bleeding, randomizing of experimental groups, feed intakes, body weights, and others, were described previously (5).

Apparatus. A semi-micro hydrogenation apparatus as described by Quaife and Biehler (6) was used for the elimination of interference due to vitamin A with the Emmerie-Engel reaction.

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Procedure. Details of the procedure for the determination of tocopherol in tissue were described previously (7). The procedure involves a hot ethanol extraction of the tissue, molecular distillation, and chromatography through Florex XXS (recently designated as Florex AA RVM). The Emmerie-Engel colorimetric reaction was used to determine the tocopherol content of the effluent. Vitamin A was determined by use of the Carr-Price colorimetric reagent which was prepared as described by the AOAC (8).

RESULTS AND DISCUSSION

Table 1 shows the apparent tocopherol values when the routine procedure is used for liver tocopherol analysis. The determination of all liver samples for apparent tocopherol was not made at this time because of an obvious interference shown by rapidly drifting readings of the Emmerie-Engel color. Another more obvious reason for suspecting interference was that the small band of blue color normally observed on the top of Florex columns from liver extracts where adequate amounts of vitamin A were fed, had spread over the entire length of the column when liver extracts from chicks fed high levels of vitamin A were chromatographed. The capacity of these columns to adsorb vitamin A had been surpassed. Because vitamin A was passing through the column and being read as increased Emmerie-Engel color, it would be interpreted incorrectly, from the values listed in table 1, that high vitamin A intakes resulted in an increase of liver tocopherol. When the eluate from the first adsorption column was concentrated and passed through a second column, a decrease in the amount of color developed by the Emmerie-Engel reaction resulted. It was thought, therefore, that a more detailed study of the analytical procedure was desirable. An aliquot of the molecular distillate was chromatographed through a series of Florex columns until no vitamin A could be detected. The Carr-Price and Emmerie-Engel reactions were used to determine the amount of vitamin A and tocopherol in the eluates from each column. Another aliquot of the molecular distillate was hydrogenated in absolute ethanol at a pressure of 1 kg/cm^2 (15 psi) for one minute, using 5% palladium on calcium carbonate as a catalyst (6). Vitamin A was determined immediately after hydrogenation; tocopherol was determined after an additional purification step through Florex.

Table 2 shows the results from 3 samples of liver tissue containing 1195, 4795, and 4896 μg of vitamin A/g which were used to compare hydrogenation with chromatography as techniques for the removal of interference due to excessive amounts of vitamin A when analyzing for total tocopherol. The 2 techniques have often been used to eliminate the interference due to small amounts of vitamin A. Sample 1, which had an initial apparent tocopherol of 11.1 μg/g decreased to 5.7 μg/g of tissue after successively chromatographing aliquots of the molecular distillate through 4 columns of adsorbent. Hydrogenation alone, although eliminating all Carr-Price reactants, required a further chromatography step to remove extraneous Emmerie-Engel reacting material. The value of 6.8 μg/g after hydrogenation compares reasonably well with 5.7 μg/g obtained after the sample was chromatographed 4 times. Samples 2 and 3 contained massive amounts of vitamin A in the tissue. Sample 3 was a liver sample taken from chicks fed a diet which contained 500 mg of vitamin A acetate per kg. This diet contained 17.6 mg of added *dl*, α-tocopheryl acetate/kg. Sample 2 represents chicks fed a simi-

TABLE 1

Apparent tocopherol in liver tissue from chicks fed at increasing levels of dietary vitamin A

Days fed supplement	Milligrams vitamin A acetate/kg diet					
	0	0.5	5	50	500	5,000
	μg/g	μg/g	μg/g	μg/g	μg/g	μg/g
5	13.5	11.6	13.4	12.0	15.0	27.0
10	9.0	9.7	9.8	9.4	52.3	22.6
20	Not determined due to obvious interference					

TABLE 2

Comparison between Florex chromatography and hydrogenation in removing excessive vitamin A and other reducing substances which react with the Emmerie-Engel reagents

No. of Florex XXS columns	Vitamin A in liver samples, µg/g [1]		
	Sample no. 1	Sample no. 2	Sample no. 3
	1195	4795	4896
	µg apparent tocopherol/g		
1 [2]	—	—	52.3
1	11.1	58.6	—
2	9.8	36.0	21.3
3	8.2	10.5	6.4
4	5.7	10.1	3.8
5	—	9.7	—
Hydrogenation	13.0	14.9	—
Hydrogenation + 1 Florex column	6.8	9.5	3.9

[1] Chicks of samples no. 1 and 3 received 17.6 mg of *dl*,*α*-tocopheryl acetate and of sample no. 2, 216 mg/kg of diet.
[2] Contained approximately 8 g of Florex XXS. All other columns contained approximately 4 g.

lar diet, but one to which 216 mg of *dl*, *α*-tocopheryl acetate were added. The apparent tocopherol of sample 2 appears to have stabilized at approximately 10 µg/g of tissue after passage through 4 columns of adsorbent, a figure which agrees well with the 9.5 µg/g obtained after hydrogenation and chromatography. Excellent agreement is also achieved for sample 3 when similar comparisons are made. Even though the diet representing sample 2 had approximately 12 times the tocopherol of sample 3, the liver tocopherol was not appreciably increased. Experience had indicated that the liver tocopherol should have been about 6 times that shown. It was observed that chromatography through one column which contained 8 g of the adsorbent was not as efficient in removing vitamin A as were two 4-g columns. Eluates from the first column of each of the 3 samples showed the typical blue color with the Carr-Price reaction, confirming that the capacity of the column to adsorb vitamin A was surpassed. The quality of the blue color formed from the eluates of the second column was questionable, whereas eluates from the 3 subsequent columns produced a drifting yellowish-brown color and not the typical Carr-Price blue. This muddy color was *increasing* in absorbance instead of *decreasing* as does the typical Carr-Price blue.

Results of the effect of extraction, molecular distillation, chromatography, and hydrogenation upon the effectiveness of removing vitamin A are presented in table 3. Approximately 40% of the vitamin A was extracted from the tissue. The remaining 60% was either not extracted during the 20-hour extraction with hot absolute ethanol or was possibly altered in structure so as not to give a Carr-Price reaction. Approximately 67% of the Carr-Price reacting materials, which were extracted from liver tissue, were distilled by molecular distillation along with the tocopherol, which is known to distill quantitatively. Of the Carr-Price reacting materials that were placed on the first column of Florex, approximately 90% were adsorbed. It was believed that the second column should have adsorbed the remainder of the vitamin A. However, the high apparent vitamin A which eluted from the second and subsequent columns might have been due to breakdown products of vitamin A that occurred either in vivo or during the extraction or purification procedures. It was observed that the absorbance at 620 mµ of the Carr-Price color of the yellow-brown eluates from columns 3 and 4 increased but then decreased again with the eluates from column 5. Also, during routine analysis of liver for tocopherol the top centimeter of the Florex adsorbent is blue and is known to be produced by vitamin A, carotene, xanthophylls, and N, N′-diphenyl-*p*-phenylenediamine (DPPD). In the series of columns which had the high vitamin A extracts, column 1 was all blue, column 2 was partially blue, occasionally the top

TABLE 3

Effect of extraction, molecular distillation, and chromatography on the vitamin A extracted from liver tissue

Vitamin A (Carr-Price)	Sample 1	Sample 2
In tissue (µg/g)	1195	4795
Extracted (%) (20 hr, hot ethanol)	36.9	42.9
Molecularly distilled (%)	65.9	68.7
Placed on column 1 (µg)	1095	8070
Eluted from column 1 (µg)	61	865
Adsorbed on column 1 (%)	94	89
Placed on column 2 (µg)	24	346
Eluted from column 2 (µg)	9	152
To be hydrogenated (µg)	711	3497
After hydrogenation (µg)	0	0

portion of column 3 and usually the top portion of column 4 were first a rusty red. After several minutes of elution, it turned brown and, finally but not always, blue. At times it was observed that only a reddish-brown color would form and disappear with no blue in evidence. No explanation is offered for these observations without further study. However, from the overall observations the indication is that either bands of breakdown products of vitamin A due to the effect of the adsorbent or the other extraction procedures, or metabolic products due to the excessive vitamin A intake are eluting from the columns and are causing the nontypical Carr-Price reaction.

An attempt was made to correct for the vitamin A remaining in the final extract by first determining the tocopherol equivalents of the vitamin A present and subtracting this value from the total apparent tocopherol in the liver. By reacting pure vitamin A alcohol with the Emmerie-Engel reagents, it was found that 1 μg of vitamin A alcohol was equivalent to 0.01 μg of α-tocopherol. The correction to the apparent liver tocopherol values shown in table 1 was found to be so insignificant that the presence of other reducing substances was obvious.

It was found necessary to determine whether a standard sample of tocopherol could be recovered after passing it through 6 successive Florex columns. To make this test, 120 μg of pure *d,α*-tocopherol was treated as the other extracts, aliquots taken, and recoveries calculated after elution of the standard through each column. The percentage of recoveries from the 6 columns ranged from 98 to 102. To test the effect of the solvent, an amount of benzene equivalent to the amount concentrated after passage through 6 columns of Florex was evaporated to dryness. The Emmerie-Engel color determined was negligible.

Table 4 lists the results of tocopherol determinations made on liver tissue where the molecular distillate was hydrogenated and then passed through a column of Florex adsorbent. The actual (Carr-Price) vitamin A content of the livers is also included. The table shows that tocopherol values decrease when the vitamin A in the diet exceeds 5 mg/kg. The value of 1.6 μg/g at the 5,000 mg/kg level in the diet represents a tissue essentially devoid of tocopherol. The value is within the range of liver tocopherol levels obtained in chicks fed a vitamin E-low basal diet. In view of a recent observation (9) that interference with absorption was found to be mutual between cholesterol and vitamin A, the data presented in this report indicate that high intakes of vitamin A either directly or indirectly interfere with the absorption of tocopherol or destroy it in the intestine. Balance studies would help resolve this point. The decreasing liver tocopherol values with *time* at all levels of vitamin A intake and at a *dl,α*-tocopheryl acetate level of 17.6 mg/kg of diet, may be indicative of a gradually increasing tocopherol requirement of the growing bird.

Plasma tocopherol values are presented in table 5. After only 5 days of supplementation with vitamin A, a sharp decrease

TABLE 4

Actual tocopherol and vitamin A in liver tissue from chicks fed at increasing levels of dietary vitamin A

Days fed supplement	Milligrams vitamin A acetate/kg diet					
	0	0.5	5	50	500	5,000 [1]
	μg/g	μg/g	μg/g	μg/g	μg/g	μg/g
5	11.4(0) [2]	12.0(0.6)	12.5(33)	8.7(486)	6.0(2064)	— [3](3006)
10	8.8(0)	9.4(0.8)	8.8(59)	5.2(861)	3.9(4896)	3.3 (3104)
20	— [4]	8.0(1.5)	7.6(89)	4.4(853)	3.0(5908)	1.6 (2437)

[1] Feed consumption at the 5,000 mg/kg level at 5, 10, and 20 days of supplementation was 27, 48, and 85%, respectively, of the other 4 supplemented levels where feed was restricted.
[2] Values in parentheses are micrograms of vitamin A per gram of tissue as reported previously (5).
[3] Insufficient sample remaining for duplicate determinations.
[4] Mortality due to vitamin A deficiency.

TABLE 5

Apparent plasma tocopherol in chicks that had been fed at increasing levels of vitamin A [1]

Days fed supplement	Milligrams vitamin A/kg diet					
	0	0.5	5	50	500	5,000
	μg apparent tocopherol/100 ml plasma					
5	1162	1044	1200	486	420	478
10	853	1046	823	451	164	135
20	—	784	784	309	109	68

[1] Values shown are the average of duplicate determinations.

in the apparent tocopherol (1,200 to 486 μg/ml) occurred when the vitamin A content of the diets increased from 5 to 50 mg/kg, respectively. The overall decrease in apparent plasma tocopherol was from about 1,200 to 68 μg/100 ml.

In conclusion, it has been shown that vitamin A acetate in the diet at a level of 50 mg/kg, or more, resulted in lowered liver and plasma tocopherol values. There was sufficient vitamin A in liver samples at these higher levels of vitamin A intake, even after 5 days of supplementation, to overload a column of Florex adsorbent used in the analytical procedure and to give erroneous tocopherol values. Hydrogenation of the tocopherol extract followed by chromatography was shown to successfully remove this interference, which was presumably due to vitamin A or its metabolic breakdown products, or both.

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In vivo Intestinal Transport of Calcium and Water from Solutions Recycled through Healed Gut Loops in Dogs [1]

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ABSTRACT An *in vivo* study was made of the dynamic changes of Ca, and water concentrations in solutions recycled through healed jejunal gut loops of healthy adult dogs. When the solution contained no Ca, Ca moved into the gut lumen regardless of the osmolarity of the initial solution, until the calcium concentration in the lumen reached 2 to 3 mEq/liter. Above this concentration Ca was absorbed. The rate of Ca absorption increased with increasing Ca concentrations in the lumen. Net Ca absorption was inversely proportional to the average rate of water absorption. Because water was absorbed more rapidly than Ca, the concentration of the latter always rose with an increasing rate throughout the course of the experiment. Hypotonicity caused slight, if any, change in Ca or water absorption rates. Hypertonicity altered water absorption markedly; there was a movement of water into the lumen until the osmolarity of the contents approached 385 ± 12 milliosmoles/liter. During this interval of 2.2 ± 0.2 hours Ca absorption was significantly slowed. In all instances, when osmotic equilibrium had been obtained, Ca and water absorption proceeded at the usual rates. These studies suggested that under physiological conditions, net Ca transfer is in one direction — from gut lumen to blood.

It has become evident that calcium absorption is more complex than previously suspected. In vitro studies such as those of Schachter (1) and in vivo studies of Cramer and Dueck (2) have suggested that physiological factors control Ca absorption. However, little is known about physico-chemical interactions in the gut lumen, which also must be important. While some study of the interactions of substances in the lumen has been made, the usual short measurement periods with either in vitro or in vivo techniques have not permitted study of progressive changes in conditions which may occur in the contents of the lumen, over the normal 4- to 6-hour period of Ca absorption (3). A previous study investigated the effect of osmolarity on absorption during short intervals. It was found that changes in osmolarity did not alter the Ca concentration above which calcium absorption was limited (2). Nevertheless it appeared evident that under some conditions, rates of Ca absorption or excretion might be sensitive to water concentration (osmolarity). The purpose of this investigation was to study net Ca and water transport between the gut lumen and blood while the contents were allowed to approach equilibrium under physiological conditions. It was possible to do this by recycling solutions through healed jejunal gut loops of dogs until water absorption was nearly complete.

METHODS

The terminology to be followed considers influx or absorption to mean toward the body cell from the gut lumen, whereas "efflux" refers to movement in the opposite direction.

Healed Thiry-Vella fistulas of 120 cm were prepared in 10 dogs as described previously (2). The perfusion technique was similar in most respects to that of the previous study, but modified as follows: A single rubber acorn held against the inlet by gentle pressure replaced the double balloon of the tube into the gut loop and yet eliminated all leakage. No outlet tube was used. After all 250 or 500 ml of the initial solution were pumped through the gut loop, the residual fluid was washed out with air. The difference of volume of

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fluid pumped in, and that recovered, was taken as net water absorption. A sample was removed for osmotic pressure and Ca analysis. The solution was then filtered through a pad of glass wool, warmed to body temperature and repumped through the dog gut loop as before. The amount of water and Ca lost in filtering and sampling was noted when the volume was remeasured. The pressure and temperature of perfusate were maintained to those of the body. These cycles of pumping were continued until the volume was quite low. This required 3 to 5 hours depending on the rapidity of water absorption of a given dog on the day of experiment. Calcium was analyzed by the technique of Copp (4). This was modified in the case of very low Ca concentrations in perfusate by using a 1-ml sample and a Ca standard of 1 mg/100 ml. Osmotic pressure was estimated by measuring the freezing point depression of a drop of the solution in a glass capillary tube, by the method of Ramsey (5). Various solutions designated "isotonic" were initially adjusted to isotonicity by omitting an equivalent amount of NaCl when $CaCl_2$ was added. The hypotonic solutions were 0–154 milliosmoles/liter (0–0.45 g/100 ml) as NaCl initially. Hypertonic solutions were 513 milliosmolar NaCl, except in a few experiments in which 1340 milliosmoles were used. An osmole is an equivalent weight per liter, if one assumes complete dissociation.

RESULTS [2]

Examples of changes of water and Ca in the isotonic contents of gut loops are illustrated in figures 1 and 2. Solutions

[2] Significance of differences mentioned in the text are taken to indicate a P value of 0.01 or less as derived by Student's t test.

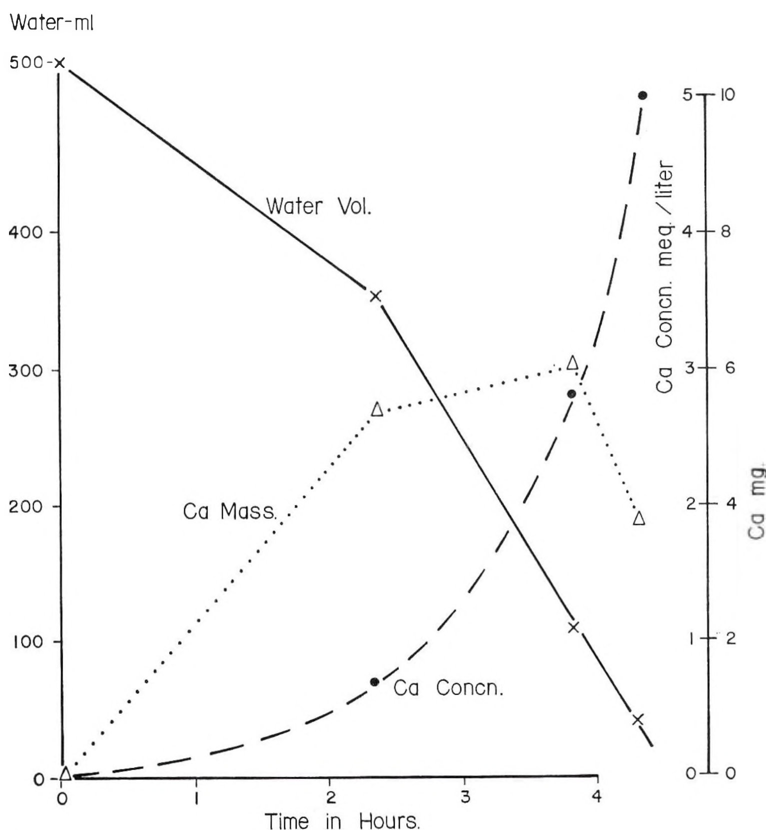

Fig. 1 Net efflux and subsequent absorption of Ca in the gut loop contents when no Ca was added initially. Net water absorption is also illustrated. Gut lumen contents were isotonic.

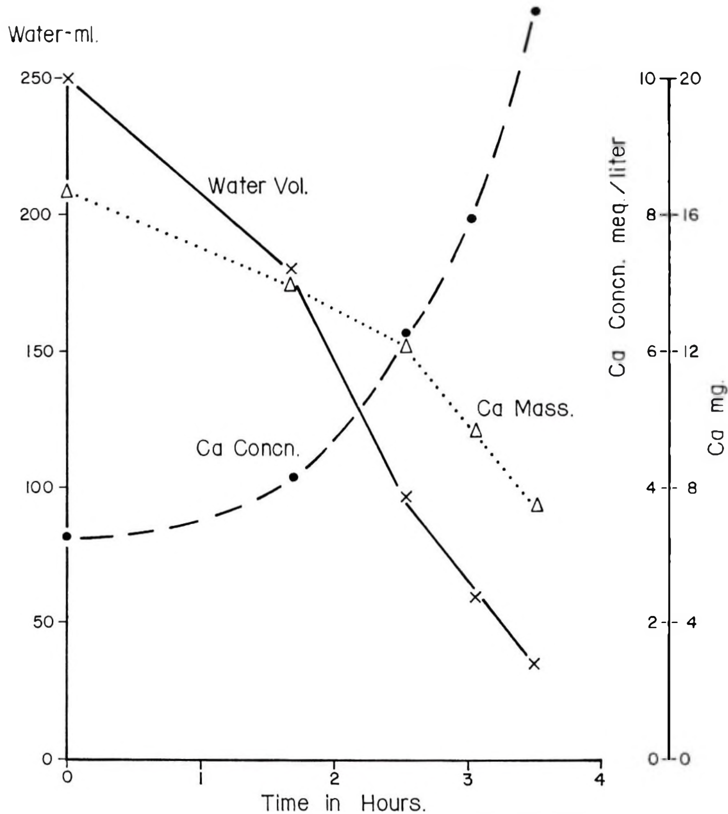

Fig. 2 Net absorption of Ca and water when some Ca was added initially. Gut lumen contents were istonic.

of various Ca concentrations were recirculated through the fistula for several cycles. Table 1 gives additional data, showing averages of all experiments. As shown in figure 1, when no Ca was added initially, Ca accumulated in the contents of the isotonic gut for 2.5 hours, after which net movement of Ca out of the gut lumen occurred. Regardless of the original Ca concentration, the Ca concentration increased at what appears to be an exponential rate, doubling every hour at low Ca concentrations and every 2.5 hours at higher initial Ca concentrations. It appears that water is absorbed more rapidly than is Ca so that the relative Ca concentration increased. At Ca concentrations of 2 to 3 mEq/liter or above, Ca was usually absorbed from the gut lumen. At higher Ca concentration the absolute Ca absorption rate (milligrams absorbed per hour) increased, but the fraction of Ca absorbed decreased. Below 2 mEq/liter there w usually movement of Ca into the lumen the gut.

Water absorption was found to be a proximately linear in 4 out of 5 expe ments. This is in contrast with the c servation of Code (6). Code found a sen log plot of the percentage of H_2O remai ing to give a straight line, whereas t present study showed the H_2O remaini to give a straight line with linear gra paper. This may reflect the fact that flui flowed slowly through the loop with t present technique. This work also ma use of jejunal fistulas, whereas Code us duodenal and ileal fistulas. Water and (absorption is shown in 2 phases, for co parison at various osmolarities. Water a sorption continued at an undiminish rate until the gut was almost dry. Wat absorption was about twice as efficie (percentage absorbed per hour) as C

TABLE 1

Changes of Ca and water in solutions recycled through gut loops of dogs

No. experiments	Initial Ca conc	Final Ca conc	Net Ca flux		Net water flux	
			0–2.5 hours	3–5 hours	0–1.5 hours	2–5 hours
	mEq/liter	*mEq/liter*	*mg/hour*	*mg/hour*	*ml/hour*	*ml/hour*
Isotonic solutions						
10	0	2.4 ± 0.5 [1]	(−) [2] 1.6 ± 0.2	5.1 ± 0.7	110 ± 12	117 ± 14
3	2.0	7.0	(−) 0.9	4.2	74	66
3	3.0	9.3	4.5	4.3	67	110
5	5.0	11.5	4.8	6.2	78 ± 6	65 ± 5.1
3	12.5	39.5	14.0	15.0	79	58
3	25.0	45.5	14.5	16.0	68	86
Avg % absorbed/hour			22.0 ± 2.3		40.0 ± 4.0	
Hypotonic solutions, 0–160 milliosmoles/liter						
10	0	2.3 ± 0.5	(−) 1.5 ± 0.1	1.3 ± 0.2	104 ± 0.2	101 ± 15
3	0.8	6.5	(−) 1.0	3.5	90	78
3	2.0	6.0	(−) 0.5	2.3	68	76
3	3.2	8.8	4.2	4.5	80	88
5	5.0	23.0	8.0	8.5	63 ± 9	63 ± 8
Avg % absorbed/hour			20.0 ± 2.1		42.9 ± 3.8	
Hypertonic solutions, 510 milliosmoles/liter						
10	0	1.6 ± 0.6	(−) 2.7 ± 0.6	1.0 ± 0.3	17 ± 3.0	100
3	0.7	2.6	(−) 2.0	0.6	(−) 8.0	45
3	2.0	3.1	(−) 0.2	3.5	15	74
3	3.0	5.6	2.2	5.1	(−) 13	40
5	5.0	7.5	6.0	10.0	10	86
Avg % absorbed/hour			15.2 ± 2.1		10.7 ± 1.4	
Extremely hypertonic solution, 1350 milliosmoles/liter						
3	0	10.7	(−) 2.6	7.6	37	92

[1] SE is shown for some data.
[2] Negative values indicate plasma to gut lumen net flux.

absorption. In similar experiments where water volume was kept small and constant but allowing the Ca to concentrate as shown here, 80 to 85% of Ca could be absorbed. The tendency of the gut to concentrate Ca suggests that Ca is important in maintaining isotonicity in the lumen by balancing rapid monovalent cation absorption.

Hypotonicity

Table 1 shows the results when hypotonic solutions of zero to 154 milliosmoles/liter containing various amounts of $CaCl_2$ were recycled through the gut loop. Net flux from the plasma into the gut, or "efflux" is designated (—), whereas net lumen-to-plasma flux (influx or absorption) is positive. These symbols relate to direction of flux toward the body cell. When no Ca initially was placed in the gut, net transport of Ca from plasma into lumen occurred. This efflux period was not significantly different from that found in isotonic controls. At the point of change from Ca efflux to influx, the average value of the gut Ca concentration was 1.5 mEq/liter. Above this concentration the Ca absorption rate was approximately proportional to the initial Ca concentration. On the other hand, water was absorbed at approximately the same rate and same efficiency regardless of Ca concentration. Hypotonic solutions delayed Ca absorption slightly after the first hour. Calcium efflux, which again occurred when the initial Ca concentration was less than 2.25 mEq/liter, was not altered significantly by hypotonicity. Hypotonicity resulted in only slightly greater water absorption, although some workers have reported greater increases (7,8). Blickenstaff actually measured filtration at 15-cm water pressure (7), whereas Visscher et al. (8) used hypertonic controls only. This may support those studies which suggest that water

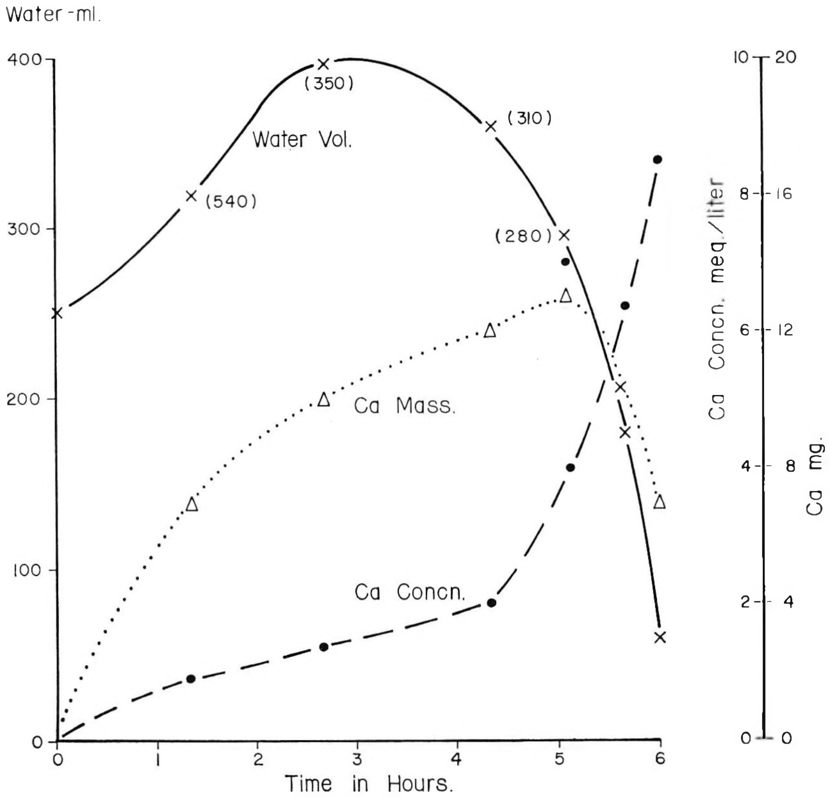

Fig. 3 Net Ca and water efflux and subsequent absorption, when contents of lumen were initially hypertonic and contained no calcium. Osmolarity in milliosmoles per liter is shown in parentheses on volume curve.

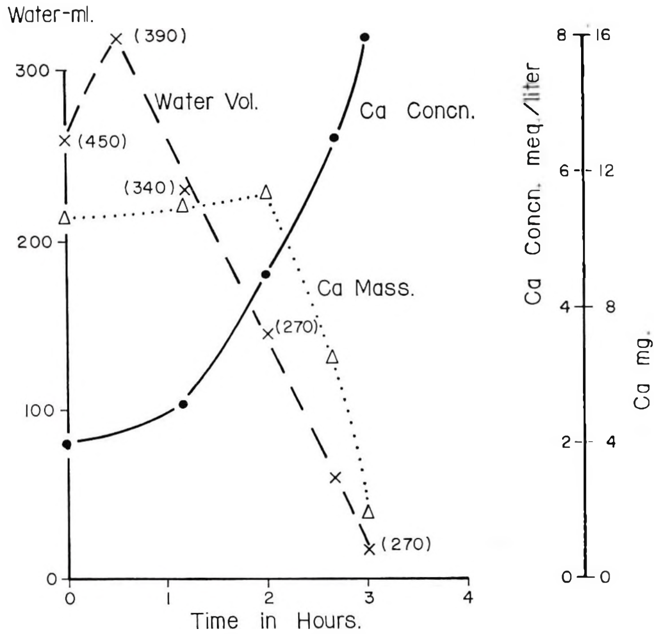

Fig. 4 Efflux and subsequent absorption of water when contents of lumen were hypertonic. Ca absorption was delayed until Ca became more concentrated and the contents

is absorbed actively (9). The fundamental independence of Ca and water absorption pathways is shown by the fact that in the first 2.5 hours water left the hypertonic gut lumen, whereas Ca entered. Indirect dependence of Ca absorption on water is shown in that rapid water absorption led to higher Ca absorption by increasing the Ca concentration (2). No graphs are shown since they reveal no differences from isotonic controls.

Hypertonicity

Figures 3 and 4 show examples of the effect of hypertonicity on net Ca and water absorption during recycling. Water initially entered the lumen and subsequently was absorbed. Calcium was absorbed only after its concentration exceeded 4 to 6 mEq/liter.

As shown in table 1, when no Ca was added to gut perfusate solutions of either 513 or 1350 milliosmoles/liter, Ca again entered the gut, but the rate of Ca efflux into the lumen was not significantly different from that in the isotonic or hypotonic gut.

Calcium absorption usually was significantly slower, whereas lumen contents were quite hypertonic. This resulted in a lower avearge Ca absorption rate. Ca absorption rates were normal after 2 to 4 hours, by which time the contents were only slightly hypertonic. As before, when no Ca was added to the contents of the lumen initially, Ca accumulated in the lumen as rapidly as it had in the isotonic gut. Water absorption was diminished markedly, but recovered within an average period of 2.5 hours. The average rate of water absorption was one-fourth that of the controls. Marked variability was noted. As table 1 shows, in those cases where net water flux reversed, this reversal occurred while the lumen was still somewhat hypertonic, at about 385 milliosmoles/liter (range, 360 to 400). This confirms a previous report by Vaughan (9). No damage to the gut loops was apparent by endoscopic examination of the mucosa. Hence only the initial wild swings of water flux, and the delayed Ca and water absorption appear to characterize the effect of hypertonicity. In extreme hypertonicity (1350 milliosmoles/liter), the time to attain isotonicity, and for Ca absorption to commence, was extended beyond 3 hours.

DISCUSSION

The design of the experiment should be considered when interpreting the results. In some Thiry-Vella studies, short gut loops and rapid flow insure that the original properties of the perfusate are maintained throughout, with the hope that turbulent flow will insure mixing and contact with mucosa. With the present method, long gut loops and slowly recirculated perfusion allow the gut itself to mix and bring solutions into contact with all the mucosa and it allows homeostatic mechanisms to adjust many conditions such as pH, osmolarity and others to those of the body. Only the factors which are adjusted by slow, homeostatic mechanisms can be studied. The important parameter which this technique was designed to measure, was net transport in both directions across the gut.

Previously, workers seemed to think of Ca transport in terms of a simple two-way transport across a single path. For many years this was pictured as a diffusion which would permit Ca through the membrane in either direction, depending on the Ca gradient. More recently evidence was advanced which suggested that the mechanism for in vivo net influx was not passive, and was either an active or facilitated transport (2). As a result of the present work, Ca transport must be pictured as having at least 2 plasma-to-gut pathways, and at least one gut-to-plasma pathway. One of the pathways from plasma to gut, via the digestive juices, has been studied extensively in man (10) and in rats (11). The other pathway, a direct one through or from the gut wall, was described only recently (12) and has been studied further in the present work. The path of this Ca entering an in vivo gut loop from the blood has not been established. It seems evident that it represents only part of the total Ca transported into the lumen, since it is only one-seventh the amount that would balance absorption from the loop. Normal flow of gastric and duodenal juices are bypassed in this preparation. The two possible sources are as part of succus entericus or as transport

through or from mucosal cells. Of these, the least likely appears to be the succus entericus, since stimulants to secretion (food or alcohol) placed in the gut loops failed to modify Ca transport. Apparently after some Ca is secreted into the lumen (see fig. 1), the independent absorption of water causes an increase in Ca concentration above 3 mEq/liter. The net Ca transport then reverses and Ca is absorbed as the Ca concentration increases. This supports the concept that the main route of plasma to gut transport is by the digestive juices, and suggests a minor second route, the direct route through the gut wall. It also leads to the conclusion that endogenous Ca excretion results largely from the excess of Ca secreted in the digestive juices over that absorbed in the gut. This work supports conclusions of Gran (11) drawn from less direct measurements.

As a result of these dynamic changes, a high Ca concentration is maintained in the intestinal tract which not only promotes Ca absorption, but protects the endogenous pool of exchangeable bone Ca. The Ca concentration in the gut lumen would always exceed 3 mEq/liter, because all body fluids approach this. Hence this efflux would not normally exist, and we can conclude that Ca is usually absorbed, not secreted, except in digestive juices.

The low solubility of some Ca compounds, together with the rapid rate of water absorption relative to the Ca absorption rate may combine to form an important factor in limiting the amount of Ca absorbed. Progressive dehydration and the characteristic slight alkalinity of the jejunum and ileum may favor formation of insoluble Ca complexes. These solids would be moved into the colon by intestinal motility where conditions of further dehydration are not conducive to further absorption. This was suggested also by previous work which showed that Sr^{85} or Ca^{45} entering the colon through the food chain was poorly absorbed, whereas a liquid dose of these isotopes introduced directly into the rat colon were well absorbed (13).

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Nutrition of Salmonoid Fishes

XIII. THE α-TOCOPHEROL REQUIREMENT OF CHINOOK SALMON

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ABSTRACT Tocopherol-deficient diets containing 1 or 5% of stripped herring oil were supplemented with zero, 10, 20, 40 and 80 mg of α-tocopherol/100 g of dry diet and were fed to duplicate lots of chinook salmon (*Oncorhynchus tshawytscha*) fingerlings for 24 weeks. Separate lots of fish were fed diets containing 1% of trilinolein and supplemented with 10, 20, 40 and 80 mg of tocopherol/100 g of dry diet. In a second feeding trial the diet containing 5% of herring oil was supplemented with 2.5, 5.0, 10, 20 and 40 mg of tocopherol/100 g of dry diet and was fed to duplicate lots of fish. The deficiency syndrome included: poor growth; exophthalmia; ascites; erythrocyte fragility; anemia; clubbed gills; epicarditis and ceroid deposition in the spleen. The symptoms were more severe in the fish fed the unsupplemented diet containing 5% of herring oil than they were in the fish fed the 1% herring oil diet and were not observed in fish fed diets supplemented with tocopherol. Under the experimental conditions used, a requirement of less than 3 mg of α-tocopherol/100 g dry diet was indicated. The herring oil used in diet preparation contributed 0.5 mg of tocopherol/100 g of dry diet (0.1 mg/g oil).

Although vitamin E has long been recognized as an essential dietary component for many animals, the quantitative requirements are related to other dietary factors such as level and type of lipid (1, 2), selenium compounds (3), sulfur-containing amino acids (4) and other biologically active antioxidants, and therefore still need investigation. Wolf (5) reported that rainbow trout (*Salmo gairdneri*) did not require this vitamin for growth. However, the tocopherol-deficient diet which he used included 9% of lard which might have contained an appreciable amount of tocopherol. Under his experimental conditions, the failure to demonstrate a positive requirement did not furnish conclusive evidence that the requirement did not exist.

For several reasons salmonoid fishes are excellent experimental animals with which to study tocopherol requirements and metabolism. The high degree of unsaturation (6) of their lipids suggests a requirement for in vivo protection against peroxidation. Typical diets for hatchery-reared salmon contain high levels of fat largely derived from marine fish normally used as a diet ingredient (7). A high level of polyunsaturated dietary fat also suggests not only an accentuated requirement for tocopherol but also its loss during oxidation of these labile fats during diet preparation and storage.

Ceroid, a yellow-brown, acid-fast pigment which has been related to the autoxidation of polyunsaturated fatty acids and to vitamin E deficiency (8) has been described in fishes suffering from various pathological conditions including suspected nutritional deficiencies and hepatoma (9). A condition known as liver lipoid degeneration in which ceroid occurs in the liver is frequently found associated with the hepatoma prevalent in some European rainbow trout (10). Although the etiology of liver lipoid degeneration has not been defined, it may result from the occasionally rancid marine-fish components of the diet. Although ceroid has not been directly implicated in rainbow trout hepatoma in the United States (11), abnormal lipid metabolism is being inves-

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tigated as one vector in this disease.[3] The requirement of α-tocopherol for the prevention of ceroid and for the protection of unsaturated body fat during intake of polyenoic dietary fats has not been thoroughly studied in salmon.

The present experiments reported were undertaken to determine whether α-tocopherol is essential in the diet of chinook salmon (*Oncorhynchus tshawytscha*), to record the deficiency syndrome, to determine whether the level of dietary fat affects the requirement, and to establish minimal needs in one set of experimental conditions. Feeding trials were conducted during the spring and summer of 1961 and 1962 and were preceded by preliminary investigations during 1959 and 1960.[4]

EXPERIMENTAL

1961 Feeding trials. Table 1 lists the various dietary parameters investigated in 1961. The herring oil used in most of the diets was purified by molecular distillation and was found to contain 96 μg of α-tocopherol/g of oil. It thus contributed approximately 0.1 or 0.5 mg of tocopherol to each 100 g of dry diet containing 1 and 5% of herring oil, respectively. Composition of the diets is listed in table 2. All diets were isonitrogenous and those containing the different fat levels were considered isocaloric, assuming 4 g of fat to be the caloric equivalent of 9 g of sucrose. Sufficient dry ingredients for approximately 3 kg of diet were weighed, thoroughly blended in a twin-shell blender and stored at 2° in sealed glass containers until needed. Oil mixes for each dietary regimen were prepared in sufficient quantity for approximately one-half of the feeding trial and were stored at 2° in nitrogen-filled serum bottles fitted with sleeve-type stoppers. For diet preparation, sufficient dry mix for 1 to 3 days of feeding was weighed into wide-mouth, screw-cap jars after which the correct amount of oil mixture was transferred from the oil mixture storage bottle by syringe and added to the dry mixture. The appropriate amount of water was then added and the diet was thoroughly blended with a spatula. The diet hardened to a stiff dough-like consistency, and was then ready to be fed. Between feedings the diet jars were tightly capped and stored at 2°.

In a preliminary experiment a study was made to determine the stability of the diets. They were freshly prepared and stored as above and samples were removed at time intervals for indication of peroxidation by the thiobarbituric acid method (12). Thiobarbituric acid values remained low for only 24 hours in diets having no added tocopherol and for periods longer than a week for diets containing tocoph-

TABLE 1

Type and level of fats and levels of α-tocopherol fed to chinook salmon (1961)

	Fat	α-Tocopherol added				
	g/100 g dry food	*mg/100 g dry food*				
Trilinolein [1]	1		10	20	40	80
Herring oil [2]	1	0	10	20	40	80
Herring oil [2]	5	0	10	20	40	80
Corn oil	1				40	
Corn oil	5				40	

[1] High purity trilinolein obtained from Hormel Institute, Rochester, Minnesota.
[2] Purified by molecular distillation and found to contain 96 μg tocopherol/g. A single lot of oil was used for both 1961 and 1962 experiments. Oil was kindly supplied by Fishery Technological Laboratory, Bureau of Commercial Fisheries, Seattle, Washington.

TABLE 2

Diet composition for α-tocopherol studies on salmon [1]

	1% Fat diet	5% Fat diet
	g	*g*
Vitamin-free casein	48	48
Sucrose	34	25
Mineral mixture [2]	4	4
Vitamin, amino acid supplement [3]	10	10
β-Carotene [4]	1	1
α-Cellulose	0	5
CMC [5]	2	2
Oil mixture [6]	1	5
Water	100	100

[1] Ingredients unless otherwise noted were obtained from Nutritional Biochemicals Corporation, Cleveland.
[2] Same as Nicolaides and Woodall (13).
[3] Vitamin supplement same as Nicolaides and Woodall (13) plus L-arginine, 1.5 g and L-cystine, 0.5 g, total weight adjusted to 10 g with α-cellulose.
[4] Two milligrams β-carotene mixed with 1 g α-cellulose.
[5] Carboxymethyl-cellulose, Hi-Vis grade (Hercules Powder Company, Wilmington, Delaware).
[6] Trilinolein, corn oil or herring oil triglycerides containing 5 μg vitamin D_3 and the appropriate amount of *dl*-α-tocopherol (free alcohol) for individual diets (table 1).

[3] Ashley, L. M., and J. E. Halver 1961 Hepatomagenesis in rainbow trout. Federation Proc., *20*: 241 (a) (abstract).
[4] Preliminary experiments of N. Nicolaides (present address, University of Oregon Medical School) and A. N. Woodall in this laboratory 1958–1960.

erol. Accordingly, diets having no tocopherol supplement were prepared fresh each morning and those containing tocopherol were prepared approximately every 3 days. Thiobarbituric acid values of diets as fed were checked during the feeding trial. At no time was a significant level detected.

The fish used were hatched from eggs of a single day's take at the Spring Creek National Fish Hatchery. After the yolk material had been absorbed the fish were fed a fat- and tocopherol-free basal ration (the 1% fat diet, table 2, with fat and tocopherol deleted) for a 2-week period when all were actively feeding. Fish were selected at random and were hand counted into the experimental troughs for the start of the feeding trial.

The diets containing herring oil triglyceride or corn oil (corn oil diets were included to serve as controls for the untested herring oil triglycerides) were fed to duplicate lots of 300 chinook salmon fingerlings, and the diets containing trilinolein were fed to single groups of 200 fingerlings. Each group was raised in individual hatchery troughs supplied with 10 liters per minute of well water at 10°. Other details of fish care, feeding and weighing are essentially the same as reported previously (13). The fish were observed for abnormal behavior to define grossly apparent symptoms of deficiency.

At the end of the 24-week feeding trial the samples were prepared and examined for the identification of various facets of the deficiency syndrome as follows.

Five fish from each lot were preserved in Bouin's fixative. Subsequently, tissues including eye, brain, heart, gills, both hemapoietic and excretory kidney, liver, spleen, pancreas, esophagus, intestine, gonad and body wall (muscle, dermis, epidermis and scales) were dissected, embedded in paraffin and sectioned at 5 μ. Histopathological examination was made of tissue sections stained with hematoxylin and eosin. Confirmation of ceroid was made with periodic acid Schiff (PAS) stained sections.

Hematological determinations including erythrocyte counts, micro-hematocrits, and hemoglobin were made by methods previously described (14). Blood smears were stained with Wright's stain to accentuate morphological changes. Erythrocyte size was determined by measuring lengths and widths of typical cells with the aid of a micrometer eye piece at a magnification of 800 ×. Since micro-hematocrit values and cell counts had indicated that a difference in cell size existed only between the groups fed the 2 tocopherol-free herring oil diets and their supplemented counterparts, cell sizes were determined for these groups only. The percentage of polychromatocytes was determined in a typical 500-cell field on 4 to 5 separate slides from each group fed the herring oil diets. Additional samples of blood were obtained from fish fed the 5% herring oil diets for erythrocyte fragility tests (15).

Approximately 100 g of fish were taken for proximate analysis (moisture, protein, lipid and ash) by methods previously described (16). Additional 100-g samples were removed from selected groups for determination of stored tocopherol and fatty acid distribution. To prepare these samples for analysis the lipids were extracted from the fish by the method of Folch et al. (17). The solvent was removed under reduced pressure in a rotary evaporator and the lipids were transferred to tared vials. To insure the absence of oxygen, the vials were filled with nitrogen and evacuated 3 times. After coming to constant weight, the evacuated vials were quickly flame-sealed and stored for analysis. During all stages of extraction, purification and concentration, the lipids were protected from air oxidation by a nitrogen atmosphere.

Tocopherols were measured by the Eggitt and Ward (18) method, modified by 2 changes as follows: the saponification time was increased from 3 to 5 minutes and the sterols were removed on the Floridin-earth column without prior crystallization from methanol. Reducing materials to the Emmerie-Engel color reaction were determined by paper chromatography. If any artifacts were present the determination was rerun. Only α-tocopherol was present in the fish oils. The completeness of the method was confirmed by determining the recovery of 90 μg of standard tocopherol added to a separate aliquot of the extracted fish oils.

Fatty acids were methylated by the dimethoxy-propane method of Radin et al. (19). The methyl esters were analyzed by gas chromatography with a column of diethyl glycol succinate on siliconized fire brick (30/60 mesh) (Aerograph Model A-110-C). Column temperature was 205°. Hydrogenation by the method of Farquhar et al. (20) was used to help identify fatty acids. The peaks were measured by the method of Carroll (21).

1962 Experiments. In the 1962 feeding trials, the α-tocopherol requirement of chinook salmon fed a diet containing 5% of herring oil triglycerides was further investigated. Levels of added tocopherol were: 2.5, 5.0, 10, 20 and 40 mg/100 g of dry diet. Duplicate lots of 300 chinook salmon fry were fed the diets for 24 weeks. Other details of the feeding trial were identical with the previous year's study with the minor exceptions that the β-carotene supplement was dispersed in the oil mixture rather than in the cellulose, and the oil mixtures were stored in brown, screw-cap bottles rather than in serum bottles. The oil mixtures showed no increase in thiobarbituric acid value during the 24-week period.

At the termination of the feeding trial, samples of fish from all groups were prepared for histopathological examination

TABLE 3

Response of chinook salmon to graded levels of α-tocopherol in diets containing 1% of trilinolein, 1 and 5% of herring oil triglycerides and 1 and 5% of corn oil (1961)

Diet fed [1]	Avg weight			Mortality	Body composition [2]			
	Start	24 weeks	Gain		H_2O	Protein	Fat	Ash
	g	*g*	*g*	%	%	%	%	%
1–L–10	0.45	2.59	2.14	13	78.2	78.1	8.2	13.0
1–L–20	0.46	2.76	2.30	8	80.9	77.1	12.2	12.5
1–L–40	0.45	2.82	2.37	12	81.7	76.6	13.6	12.8
1–L–80	0.46	2.84	2.38	8	80.9	77.4	15.2	11.9
1–H–0	0.45	2.96	2.51	19	76.0	81.4	7.4	12.9
1–H–10	0.45	3.22	2.77	20	76.7	76.3	12.1	12.3
1–H–20	0.47	3.41	2.97	15	80.0	74.4	17.4	11.6
1–H–40	0.45	3.23	2.78	14	81.0	74.9	15.2	12.0
1–H–80	0.46	3.42	2.96	14	76.0	78.0	9.9	12.4
5–H–0	0.45	4.28	3.83	44	81.8	72.9	17.7	12.2
5–H–10	0.45	6.49	6.04	31	78.5	70.2	21.1	10.4
5–H–20	0.46	6.95	6.49	30	77.5	69.8	22.3	10.3
5–H–40 [3]	0.46	7.00	6.54	24	—	—	—	—
5–H–80	0.45	6.52	6.07	24	78.3	70.0	21.1	10.4
1–C–40	0.44	2.54	2.10	25	80.9	75.6	15.5	11.9
5–C–40	0.44	3.47	3.03	24	81.9	76.1	16.4	11.6

[1] Diet code as follows: 1st number = % fat; letter = kind of fat; L = trilinolein, H = herring oil triglycerides; C = corn oil; last number = level of tocopherol added (mg/100 g dry diet).
[2] Protein, fat and ash as percentage of moisture-free fish.
[3] Body composition values were invalid, lack of sample prevented repeat.

TABLE 4

Response of chinook salmon to graded levels of α-tocopherol in diets containing 5% of herring oil (1962)

Tocopherol added	Avg weight			Feed [1] efficiency	Mortality	Body composition [2]			
	Start	24 weeks	Gain			H_2O	Protein	Fat	Ash
mg/100 g	*g*	*g*	*g*		%	%	%	%	%
2.5	0.48	7.69	7.21	0.54	29	78.7	71.4	19.5	11.3
5.0	0.48	7.97	7.49	0.55	27	79.3	70.6	19.0	11.1
10	0.48	7.46	6.98	0.52	38	79.3	68.5	20.0	11.7
20	0.48	7.79	7.31	0.58	24	79.3	70.1	20.5	11.5
40	0.48	7.96	7.48	0.65	23	79.0	70.7	19.7	11.5

[1] Gain per gram dry food fed.
[2] Protein, fat and ash as percentage of moisture-free fish.

and were analyzed for stored tocopherol, fatty acid distribution and body composition.

RESULTS

Growth. The growth performance of the fish fed the various diets in the 1961 and 1962 feeding trials is summarized in tables 3 and 4, respectively. Weight differences as a response to level of α-tocopherol fed were statistically evaluated by analysis of variance (22). The group fed 5% herring oil triglycerides without tocopherol supplement (5-H-O) gained significantly less ($P < 0.01$) than the fish receiving the same diet with the supplement. Also, the gain of fish fed the 1% herring oil diet without supplement (1-H-O)

TABLE 5

Composition of salmon lipids, 1961 feeding trial

Diet fed [1]	Tocopherol [2]	Non-saponifiable matter	Principal fatty acids			
			Palmitoleic [3]	Oleic	Linoleic	Linolenic
	µg/g lipid	%	%	%	%	%
1–L–10	155 ± 33	11	13	39	19	tr.
1–L–40	294 ± 46	10	15	35	16	1.5
1–H–0	279 ± 0	12	16	40	3	2
1–H–40	293 ± 22	10	16	44	3	1.5
5–H–0	104 ± 16	13	13	38	2.5	6
5–H–40	389 ± 21	27	12	39	2	8
1–C–40	306 ± 38	11	17	41	10	2
5–C–40	561 ± 8	11	5	40	21	1.5

[1] See footnote 1, table 3 for diet code.
[2] Two determinations per sample.
[3] Palmitoleic; palmitic acid was present to the extent of 13 to 20% in oils.

TABLE 6

Percentage fatty acid composition of salmon lipids (1962)

Fatty acid [1]	Dietary tocopherol supplement, mg/100 g dry diet				
	2.5	5.0	10.0	20.0	40.0
	%	%	%	%	%
8:0	tr	1	tr	tr	tr
10:0	tr	—	tr	tr	tr
12:0	tr	tr	tr	tr	tr
14:0	5	5	5	5	5
15:0	0.4	0.6	0.5	0.5	0.5
16:0	12	13	15	14	14
16:1	9	10	11	10	10
16:2	1	1	1	1	1
18:0	4	3	4	3	4
18:1	26	26	30	27	29
18:2	2	3	tr	3	3
18:3	8	9	8	8	8
20:4	7	7	7	6	7
20:5	6	6	6	5	5
21:0	3	3	3	4	3
22:5	1	1	1	1	1
22:6	12	10	10	13	10
24:0	2	1	2	1	1
Total	98.4	98.6	103.5	101.5	101.5
Tocopherol, µg/g lipid	23	24	78	121	248
Recovery of added tocopherol,[2] %	93	90	93	94	106

[1] Represents number of carbon atoms in chain; the number after the colon represents the number of double bonds.
[2] Recovery of the 90 µg of standard α-tocopherol added to a separate aliquot of the fish oils.

was less than the remainder of this group ($P < 0.05$). Differences in growth responses of the fish fed diets containing the trilinolein could not be tested statistically as the diets were fed to single lots of fish, and individual fish weights were not determined. There was no significant difference in weight gain in the fish fed the diets containing 5% of herring oil triglycerides with added increments of tocopherol in the 1962 experiment (table 4).

A comparison of body composition of the fish fed the various diets showed that a greater amount of lipid was accumulated by the fish fed diets containing a larger amount of fat. It also indicates that the decreased growth due to tocopherol deficiency was accomapnied by a lower lipid content of the fish.

Analyses of fish lipids. Levels of stored tocopherol in the fat of the salmon from selected groups in the 1961 feeding trial and all of the groups in the 1962 feeding trial are presented in tables 5 and 6, respectively. These tables also show the fatty acid distribution in the extracted fat of these fish.

Although the tocopherol stores generally reflected the level at which it was fed, there are a number of inconsistencies for which there is no ready explanation. The tocopherol content of the fish fed diet 1-H-O (table 5) was inexplicably high. The tocopherol content of the fish fed diets containing 2.5 and 5.0 mg/100 g tocopherol in 1962 (table 6) was much lower than that from the tocopherol-deficient fish the preceding year. A possible explanation of the latter is that oxidation of the extracted oil had taken place in 1962 with resultant loss of tocopherol. Although this may have happened, oxidation could not have been very great since there was no indication of significant change in the distribution of the more labile, highly unsaturated fatty acids (table 6).

The influence of dietary fat on the fatty acid distribution in the fish lipids is demonstrated clearly in tables 5 and 6 and although this has only partial bearing on tocopherol deficiency, it merits separate mention. The levels of oleic and palmitoleic acids remained the same regardless of type or level of fat fed or of the amount of fat deposited in the fish (table 3). The increased linoleic acid content in the fish fed trilinolein or corn oil was dramatic. There was a similar increase in the linolenic acid content of the fish fed the 5% herring oil diets (5-H-O and 5-H-40). The smaller percentage of linoleic and linolenic acids in the fish fed diets containing 1% of corn oil or herring oil, respectively, as compared with the fish fed 5% of these fats is probably due to the fact that more of these fatty

TABLE 7

Average hematological values for salmon following 1961 feeding trial

Diet fed [1]	Avg erythrocyte count	Hematocrit	Hemoglobin	Erythrocyte fragility	Polychromatocytes
	per mm³ x 10⁻⁶	*% packed cells*	*g/100 cm³*	*% NaCl* [2]	*%*
1–L–10	1.21	30.6	8.5		
20	1.14	29.7	—		
40	1.21	28.3	6.2		
80	0.91	25.1	7.7		
1–H–0	0.98	24.0	5.4		11.8 ± 1.6 [3]
10	1.03	31.2	9.3		12.9 ± 0.8
20	1.06	32.9	9.5		14.4 ± 1.9
40	1.16	34.9	9.1		12.0 ± 0.9
80	1.01	33.2	8.2		13.7 ± 2.5
5–H–0	0.48	13.7	4.0	0.50	22.4 ± 4.5
10	1.18	35.2	10.1	0.42	13.1 ± 0.3
20	1.13	36.0	10.3	0.42	12.8 ± 0.2
40	1.14	33.3	8.7	0.46	14.7 ± 0.8
80	1.18	34.0	9.1	0.44	14.0 ± 1.9
5–C–40	0.95	27.3	6.9	0.44	

[1] See footnote 1, table 3 for diet code.
[2] Per cent NaCl at which hemolysis starts (in 5–H–0 group hemolysis was usually complete at this level).
[3] SE.

acids were used for energy by the fish fed the diets containing less fat.

Hematology. Hematological determinations for the 1961 feeding trial are summarized in table 7. Erythrocyte counts, micro-hematocrits and hemoglobin values show that the fish fed the diet containing 5% of herring oil with no tocopherol supplement (5-H-O) became severely anemic. There was a significantly ($P < 0.05$) increased percentage of polychromatocytes (juvenile erythrocytes) in this group compared with that of the groups fed this diet with tocopherol supplementation. There was no significant difference in percentage of polychromatocytes in the groups of fish fed diets containing 1% of herring oil. A comparison of erythrocyte size in the fish fed the unsupplementad herring oil diets with that of the fish fed diets containing the same level of herring oil with tocopherol supplement showed that the mean size of the erythrocytes from the unsupplemented fish was significantly smaller ($P < 0.05$) than from the supplemented ones. Mean values for these measurements were (in microns ± SE): $13.9 \pm 0.4 \times 7.8 \pm 0.2$ and $15.7 \pm 0.2 \times 8.9 \pm 0.1$ for the fish fed diets containing 1% of herring oil (unsupplemented and supplemented, respectively) and $14.3 \pm 0.3 \times 7.8 \pm 0.1$ and $15.8 \pm 0.3 \times 9.2 \pm 0.1$ for the respective groups fed the 5% of herring oil diets. Figure 1 illustrates some of the morphological changes described.

Histopathology. In view of the pronounced anemia produced, remarkably little tissue pathology was observed. The

Fig. 1 A. Erythrocytes from chinook salmon fed a diet containing 5% of herring oil with no tocopherol supplement. Illustrates erythroblast (round cell with scant cytoplasm). The cell at upper center and the 2 cells adjacent to the erythroblast which show slightly rounded granular nuclei are considered polychromatocytes and the remaining cells would be considered normal. Wright's stain. × 1500. B. Normal-appearing erythrocytes from a chinook salmon fed a complete test diet. A few late polychromatocytes (having slightly rounded granular appearing nuclei) can be seen. Wright's stain. × 1500.

most pertinent pathology included clubbed gill lamellae, epicarditis and moderate ceroid deposition in the spleen. These abnormalities were most pronounced in fish fed the diet containing 5% of herring oil without supplementation, were somewhat less in incidence and severity in the fish fed unsupplemented diets containing 1% of herring oil and were not observed in any of the fish fed tocopherol-supplemented diets. These changes are illustrated in figure 2.

Other tissue pathology was observed which did not appear to be correlated to tocopherol deficiency since it was common to all groups; pathology noted included moderate-to-severe vacuolization of liver cells, unidentified blue-gray casts in renal tubules and fatty degeneration in hemopoietic kidney tissue.

Tissues from fish in the 1962 feeding trial seldom showed any of the specific histopathological changes noted above for tocopherol deficiency. The non-specific pathology was again noted.

Grossly visible symptoms which might be attributable to tocopherol deficiency and which were most frequently observed were exophthalmia and ascites. Although these conditions are occasionally observed in salmonid fingerlings and were noted to some extent in all groups in the present study, they were most pronounced in those groups fed diets having no tocopherol supplement. The eyes of affected fish projected well beyond the contour of the head, and the body cavity, being filled with fluid, gave the fish a "pot-bellied" look quite unlike their normal appearance. Affected fish frequently were lethargic.

DISCUSSION

We have attempted to measure the effect on chinook salmon of an α-tocopherol deficiency, uncomplicated by other factors such as the deficiency of sulfur-containing amino acids or selenium factor-3. The addition of 0.5% of L-cystine to the basal ration which included 48% of vitamin-free casein (reported (23) to contain selenium factor-3), should have kept these factors from being limiting. We have not attempted to measure directly the protective effect which α-tocopherol affords the animal to products of peroxidative rancidity,[5] indeed we used measures to prevent the occurrence of oxidative changes. A consideration of total tocopherol requirements must take this factor into account, but the first problem was to describe an uncomplicated deficiency.

Most of the deficiency symptoms which were observed — ceroid, edema, anemia and erythrocyte fragility are similar to those noted for other animals (24).

Neither clubbed gill lamellae of fish, nor involvement of lung tissue in other animals, has to our knowledge been noted previously in tocopherol deficiency. Unlike lung tissue, the gill has functions in addition to respiratory exchange, serving alse as a site of both osmoregulation and excretion of nitrogenous waste (25). In view of the latter and the known relationship between α-tocopherol and the development of creatinuria in the rat (2, 26), the involvement of gill tissue merits additional inquiry. Clubbing of gill lamellae has also been noted in a pantothenic acid deficiency syndrome in the chinook salmon (27) and in the various species of trout (28). Wood and Yasutake (29) summarized many of the causes of clubbed gills which were then known, and illustrated the typical morphological changes of the gill induced

[5] Interim reports on experiments on the protective effect of tocopherol to chinook salmon fed diets containing oxidized oils were presented at N. W. Fish Cultural Conference by Tom McKee, Oregon Fish Commission, and Duncan Law, Oregon State University in December 1959 and by others in 1960. Private Communication with Mr. Law indicates that a manuscript is in preparation.

Fig. 2 A. Gill filaments from chinook salmon fed diet containing 5% of herring oil with no tocopherol supplement, illustrating clubbed gill lamellae. H & E. × 100. B. Gill filaments from chinook salmon fed a diet containing 5% of herring oil supplemented with 10 mg α-tocopherol/100 g dry diet. C. Heart tissue from chinook salmon fed a diet containing 5% of herring oil with no tocopherol supplement illustrating endematous epicardium. H & E. × 250. D. Heart tissue from chinook salmon fed a diet containing 5% of herring oil supplemented with 10 mg of α-tocopherol/100 g dry diet. H & E. × 250. E. Spleen tissue from chinook salmon fed a diet containing 5% of herring oil with no tocopherol supplements. Sharply defined black areas are melanin granules, and black-to-grey areas with blurred edges are ceroid globules. In color the latter are dark red against the pale red background, and the former are black and hence more easily differentiated than illustrated here. PAS. × 600.

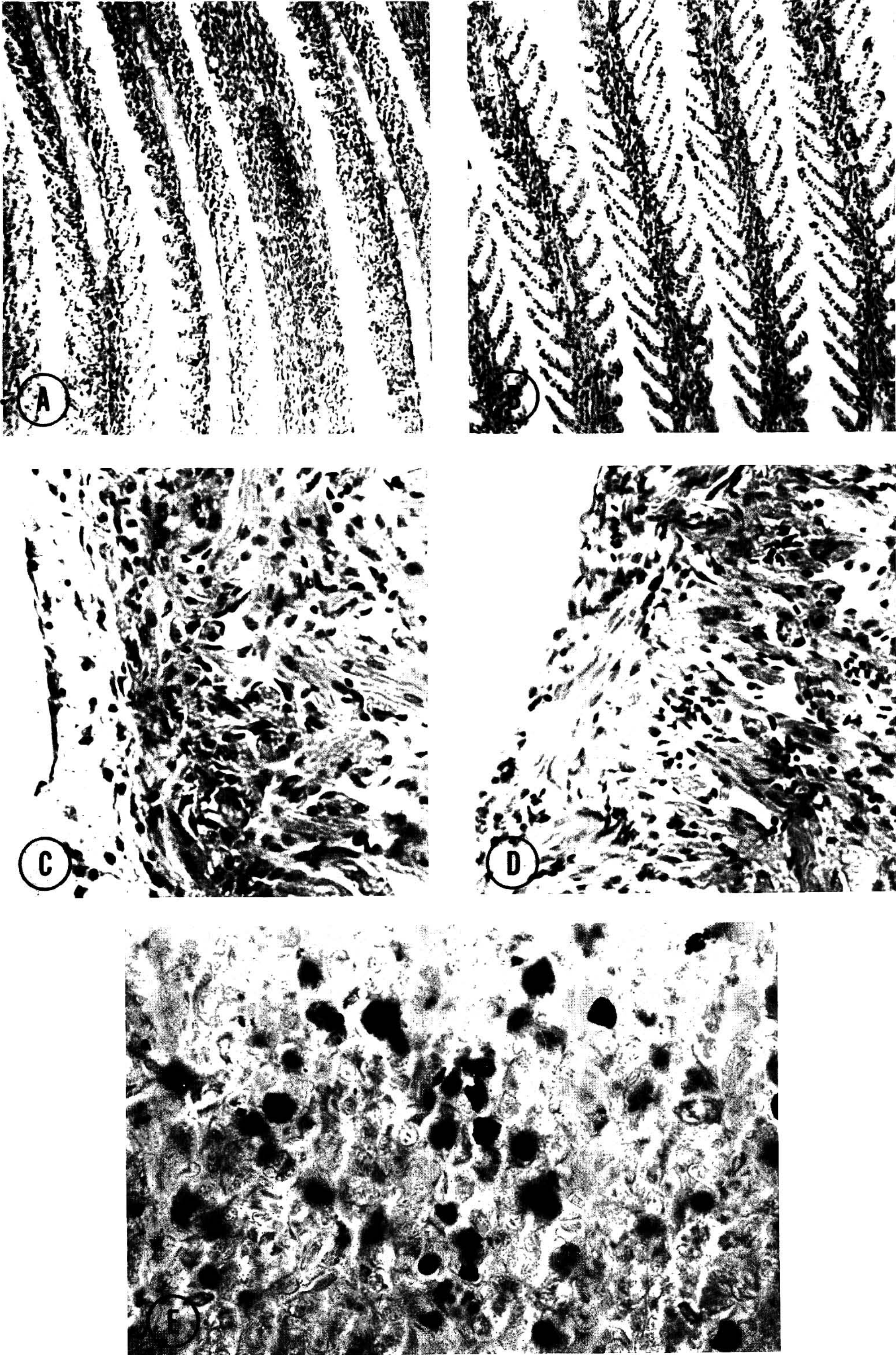

Figure 2

by these various factors. These authors pointed out the differences in the gill lesion produced by nutritional (pantothenic acid) deficiency and lesions produced by external contaminants. From these descriptions and illustrations we conclude that the gill lesion observed in the tocopherol-deficient fish was not of external origin.

Some of the erythrocyte changes noted in the tocopherol-deficient fish, such as decreased size, increased percentage of juvenile forms and increased fragility, suggest that the anemia results from premature erythrocyte breakdown rather than from a deficiency of blood-forming elements or enzyme co-factors normally associated with the formation of the heme moiety. It is possible to postulate that in tocopherol deficiency the more fragile erythrocytes have a shortened life span causing increased hemopoiesis. Where deficiency is less pronounced as with the unsupplemented 1% herring oil diet, the fish maintained a near normal red cell count, but the newly-released cells being smaller, resulted in reduced hematocrit and hemoglobin. In fish fed the unsupplemented diet containing 5% of herring oil the destruction of cells was more rapid and pronounced anemia resulted.

The 1961 experiment clearly demonstrated that α-tocopherol is required by chinook salmon; that the requirement was accentuated by a diet containing 5% of herring oil compared with one containing only 1%; and that 10 mg of α-tocopherol/100 g of dry diet were sufficient to prevent the occurrence of recognizable aspects of the deficiency syndrome. Since the evidence of positive requirement appeared unequivocal, an unsupplemented control group was not included in the 1962 experiment. Although it is attractive to base an evaluation of the tocopherol requirement on the failure of the diet supplemented with 2.5 or 5.0 mg of α-tocopherol to result in significant storage of this vitamin (that is, to consider the lack of storage as an indication of potential biochemical lesion, since no measurements of any actual biochemical lesion such as creatinuria (26) or the hemolysis test proposed for the definition of the tocopherol requirement of the rat (30, 31) were made), such a projection seems premature. In addition, no growth differences were noted nor were any specific tissue pathologies observed. We thus conclude that the α-tocopherol requirement of chinook salmon fed a diet containing 5% of herring oil triglycerides under the experimental conditions described is between 0.5 (contributed by the herring oil) and 3.0 mg/100 g of dry diet.

ACKNOWLEDGMENTS

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Effect of the Level of Dietary Protein on the Toxicity of Dieldrin for the Laboratory Rat [1]

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ABSTRACT Male Long-Evans rats were fed low (10%) and high (25%) protein diets containing zero, 100, 150, and 200 ppm of dieldrin. At all levels of dieldrin, with high and low protein diets, there was an initial slight suppression of growth for 2 to 6 days. From day 6 to 31 there was a continued slight suppression of growth among all animals ingesting 150 or 200 ppm of dieldrin. Marked mortality occurred among animals ingesting 200 ppm of dieldrin with either low or high protein but also among animals ingesting 150 ppm of dieldrin with the low protein diet. Neither kidney nor heart weight were altered in any meaningful pattern with either protein diet. Liver weight was significantly increased at all levels of dieldrin intake with high protein but not low protein diets. Glucose-6-phosphate dehydrogenase and transaminase activities were not altered by dieldrin ingestion with low or high protein. Stress response, as measured by swimming time, was not affected in any consistent manner and body fat, as a percentage of total body weight, was the same in all groups. With low protein diets, but not high protein diets, at all levels of dieldrin ingestion, there was a significant increase in liver lipids. Vitamin A per gram of liver was decreased in all dieldrin groups ingesting either low or high protein diets. However, total liver vitamin A was decreased in the low protein group at 150 and 200 ppm of dieldrin but in the high protein group only at 200 ppm of dieldrin. Kidney and cardiac muscle exhibited no alterations that could be ascribed to dieldrin ingestion. Livers of rats ingesting dieldrin exhibited cellular edema and fatty infiltration. These changes were more marked in animals fed low protein diets than in animals fed high protein diets.

Compounds of the chlorinated hydrocarbon group of pesticides (DDT, dieldrin, aldrin, endrin, and related substances) exhibit a marked toxicity for mammals. The most striking feature of intoxication with these agents is central nervous system excitation, leading to epileptiform convulsions, and terminating in respiratory failure and death (1–6). However, at sufficiently high levels of ingestion there may also be a suppression of food intake, with consequent growth inhibition or weight loss (7–8).

There are indications of degenerative changes in hepatic tissue and of enlargement of the liver relative to body weight (9–11). Sarrett and Jandorf (8) reported that rats receiving DDT had, in addition to increased liver weight, a significant increase in liver lipids (expressed as percentage of liver weight), and a significant decrease in liver phospholipid and cholesterol (expressed as percentage of liver total lipid.)

Recently Phillips (12) reported that DDT ingestion by rats, even at relatively low concentrations, results in decreased vitamin A storage in the liver, expressed either as vitamin A per gram of liver or total vitamin content of the whole liver.

In addition to these effects, a large number of metabolic alterations have been reported, such as increases in plasma potassium, lactate, and glucose, but not in calcium, sodium, magnesium, or sulfate (13), as well as slight changes in brain glutamic acid and glutamine (14), but not in cholinesterase (15), phosphatase, or arginase (6). A large number of glycolytic enzymes were demonstrated to be unaffected by these pesticides (13).

Khairy (16) has stated that ingestion of dieldrin by rats does not impair learning ability but that it does lead to deterioration of muscular efficiency.

Little or no attention has been directed toward the effect of diet composition on the toxicity of these pesticides. The

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hepatic alterations (fatty infiltration, hydropic degeneration, focal necrosis) observed following their ingestion suggest that such investigation would be fruitful, particularly with respect to variations in the protein content of the diet. Furthermore, the general question of dietary influences on toxicity has received some degree of attention. For example, Packman et al. (17) demonstrated that diet composition affects arsenic trioxide toxicity, and Meyer (18, 19) showed that diet composition exerts a modifying influence on sodium cyanide toxicity and on resistance to diphtheria toxin. According to Ambrose (20) the acute toxicity of rotenone is reduced, but the chronic toxicity is enhanced, by feeding a diet high in fat.

In the investigations reported here we have examined the effect of dietary protein intake on several parameters of dieldrin toxicity. These include growth and body composition, mortality, organ weights, enzyme activities, vitamin A metabolism, liver histology, and resistance to stress.

METHODS

Male Long-Evans rats were used for all experiments. They were obtained from an established commercial dealer and when received weighed 125 to 145 g. After assignment to groups of approximately equal weight distribution, the animals were housed in individual screen-bottom cages. Water was given ad libitum.

The low protein diet had the following composition: (in grams per 100 g) vitamin-free casein, 10; cottonseed oil, 8; USP XIV salt mixture, 4; vitamin mix,[2] 2.2; polyvinylpyrrolidone, 4; cellulose, 3; and powdered sugar, 68.8. The high protein diet differed only in that the vitamin-free casein was increased to 25 g per 100 g, at the expense of the powdered sugar. Both diets were prepared as coarse powders or granules. Dieldrin [3] was added to portions of each diet to achieve concentrations of zero, 100, 150, and 200 ppm. All diets were fed ad libitum, and the amount of food was determined by weighing the uneaten portion. Animals were weighed every fourth day. A few animals, including some of the controls, refused to eat from the first day and soon died. These were omitted from the recorded results.

All animals were observed at least twice daily and dead animals noted and discarded. At the end of either two or four weeks the surviving rats were killed according to the following procedure.

Animals in each group were anaesthetized with pentobarbital sodium.[4] Heart, kidneys, and liver were removed and rapidly weighed to the nearest milligram. One portion of liver was homogenized in twice its volume of 0.25 M sucrose and the glucose-6-phosphate dehydrogenase activity was determined by the method of Fitch et al. (21). A second portion of liver was homogenized in 0.1 M citrate buffer, pH 7.0 and used for the estimation of transaminase activity (α-ketoglutarate - phenylalanine, α-ketoglutarate - aspartate, and pyruvic - phenylalanine) according to the procedure described in Colowick and Kaplan (22). At the same time a portion of liver was homogenized in 95% ethanol and total lipids were extracted and measured by a micro - modification of the method of Bloor (23). Another portion of liver was dehydrated by grinding with anhydrous sodium sulfate and then extracted with diethyl ether, as described by Ames et al. (24). The vitamin A content of the extract was determined by the antimony trichloride reaction (25).

Portions of liver, heart, and kidney were fixed in 10% neutral formalin and histological sections were prepared and stained with hematoxylin and eosin. Sections of liver, but not of the other tissues, were also sectioned and stained with Oil Red O.

A separate group of animals, drawn from the treatment groups described above, was used for swimming stress tests. For this test a 5-g weight was attached to the tail and the animal was immersed in a cylinder of water that had been chilled to 5 to 10°. The length of time the animal was able to swim or remain afloat was measured with a stop watch. When it was no longer able to remain at the top of the

[2] The vitamin mix supplied the following amounts of vitamins: (in mg/kg of diet) α-tocopherol, 440; ascorbic acid, 990; inositol, 110; menadione, 500; p-aminobenzoic acid, 110; niacin, 99; riboflavin, 22; pyridoxine, 22; thiamine, 22; Ca pantothenate, 6.6; biotin, 0.44; folic acid, 2.0; vitamin B_{12}, 0.3; (in g/kg) choline chloride, 1.65; and vitamins A and D, 22,000 units/kg and 2200 units/kg, respectively.

[3] Hexachloro-epoxy-octahydro-dimethanonaphthalene, Shell Chemical Company, Emeryville, California.

[4] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.

water it was removed and killed for the measurement of total body fat.

The determination of total body fat was carried out by a modification of the method of Hartsook and Herschberger (26). The carcasses were autoclaved at 120° for about 2 hours and then placed in a high speed blender (3.8-liter capacity, 0.5 hp motor) together with approximately 500 ml of hot water and blended for 10 minutes. The dispersate was strained and the mat of hair was extracted two more times in the blender, with smaller quantities of hot water. The mat was then discarded and the suspension was made to volume. Total lipid was determined on an aliquot of the suspension by a modification of the method of Bloor (23). It had previously been ascertained that less than 0.4 g of lipid was lost in the discarded mat of hair.

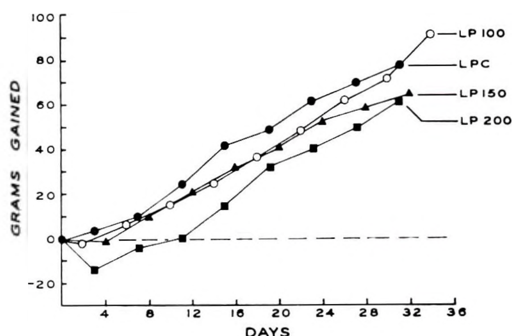
The data obtained were subjected to statistical analysis, using the *t* test (27) to determine significant differences between the treatment groups.

RESULTS

Figure 1 shows the weight gain of animals fed zero, 100, 150, and 200 ppm of dieldrin in low protein (10%) and high protein (25%) diets for 31 days. Only data on animals surviving for the entire period were used for the graphs. In the low protein groups (fig. 1, left) there was slight weight loss during the first 2 to 4 days the diet was fed, at all levels of dieldrin. From day 6 to day 31, the average weight gain in grams per day for each of the 4 groups was: zero ppm (controls), 2.76; 100 ppm, 2.80; 150 ppm, 2.36; and 200 ppm, 2.48. Thus, correcting for the immediate effects of the diet, there still appears to be a slight depression of growth at the higher levels of dieldrin. The initial weight loss or growth depression is not as marked in the case of the animals ingesting dieldrin in the high protein diet (fig. 1, right), but the grams gained per day from day 6 to day 31 are: zero ppm (controls), 4.88; 100 ppm, 4.88; 150 ppm, 4.68; and 200 ppm, 4.40, thus exhibiting a similar slight depression of growth at higher dieldrin levels.

In tables 1 and 2 are presented mean values and standard deviations for the various parameters measured and table 3 shows the *t* values for the statistical comparisons. Only differences significant at a 1% level are shown. Data from animals killed after consuming the diets for 2 weeks were not markedly different from those obtained with animals killed after 4 weeks. Therefore, only 4-week data are presented.

There is a significant decrease in weight gain among animals ingesting 200 ppm of dieldrin, with the low protein, but not the high protein diet. There is a decrease in the average number of days survived by animals fed both high and low protein diets as the level of dieldrin is increased, but the variability is so large that only in the case of high protein, 200 ppm dieldrin,



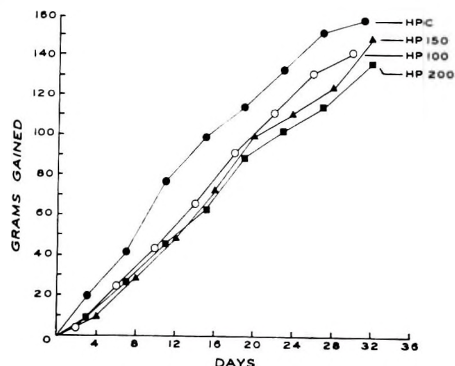


Fig. 1 (*left*) Weight gains of rats fed low protein diet with zero, 100, 150, and 200 ppm of dieldrin. LPC indicates low protein controls, and LP, low protein group. (*right*) Weight gains of rats fed high protein diet with zero, 100, 150, and 200 ppm of dieldrin. HPC indicates high protein controls, and HP, high protein group.

is there a statistically significant decrease in survival time. However, among animals ingesting 200 ppm of dieldrin there is a marked mortality which is similar in animals fed high and low protein diets. There is also an increased proportion of deaths among animals ingesting a low protein, 150 ppm dieldrin diet but not among animals ingesting a high protein, 150 ppm dieldrin diet (10/20 as opposed to 3/20, respectively). The grams of body weight gained per gram of food eaten or per gram of protein eaten (protein efficiency ratio) did not differ significantly when dieldrin-fed animals were compared with their own controls.

Kidney weight, expressed as a percentage of body weight, did not change significantly in response to diet but heart weight, as the percentage of body weight, appears to be slightly but significantly decreased with low protein diets containing dieldrin, but not with high protein-dieldrin diets. Animals ingesting a high protein diet with 100, 150, or 200 ppm dieldrin had significantly larger livers, expressed as percentage of body weight, than did their controls, whereas low protein animals at the same dieldrin levels did not.

There was no consistent change in the activities of the enzymes measured (glucose-6-phosphate dehydrogenase, α-ketoglutarate-phenylalanine transaminase, α-ketoglutarate-aspartate transaminase, and pyruvate-phenylalanine transaminase). The few scattered statistically significant changes in enzyme activity do not, in our opinion, have biological meaning, but simply represent chance occurrence of significant differences in a large series of t tests.

The length of time animals in the various groups were able to remain afloat in the stress test was, to a large extent, proportional to their body weight. This is shown in figure 2, where swimming time is plotted against body weight for all animals tested. Therefore, the data in table 1 have been expressed as swimming time (seconds) per gram of body weight. The only significant change is in the comparison of the low protein controls with the low protein 150-ppm dieldrin group. This is a result of the short swimming time of the low protein diet (150 ppm dieldrin) group.

TABLE 1

Effect of the level of dietary protein on growth, survival and organ weights of rats fed several concentrations of dieldrin in the diet

Group	Initial weight	Weight gain	Survival	Mortality	Kidney weight	Heart weight	Liver weight
	g	*g*	*days*		*% body wt*	*% body wt*	*% body wt*
Low protein, 0 ppm	134.5 ± 7.1 [1](20)[2]	79.4 ± 13.7(16)	28.8 ± 5.6(20)	4/20	0.77 ± 0.13(5)	0.43 ± 0.05(5)	4.47 ± 0.83(16)
Low protein, 100 ppm	145.4 ± 15.6 (20)	78.3 ± 13.6	31.0 ± 0.0	0/20	0.82 ± 0.08(10)	0.31 ± 0.02(10)	4.78 ± 0.66(10)
Low protein, 150 ppm	131.4 ± 8.2 (20)	62.2 ± 22.2	23.2 ± 10.3(20)	10/20	0.70 ± 0.30(5)	0.37 ± 0.05(5)	5.46 ± 0.65(5)
Low protein, 200 ppm	133.6 ± 13.6 (20)	56.2 ± 8.4(4)	14.0 ± 11.1(20)	16/20	0.91 ± 0.11(4)	0.34 ± 0.02(4)	4.72 ± 0.27(4)
High protein, 0 ppm	135.1 ± 5.1 (20)	158.1 ± 31.2(19)	31.0 ± 0.0(20)	1/20	0.90 ± 0.11(3)	0.32 ± 0.02(3)	3.90 ± 0.31(9)
High protein, 100 ppm	137.6 ± 22.3 (20)	141.2 ± 30.8(16)	26.4 ± 9.9(20)	4/20	0.79 ± 0.04(7)	0.31 ± 0.02(7)	4.91 ± 0.43(7)
High protein, 150 ppm	133.3 ± 6.7 (20)	140.9 ± 21.7(17)	29.7 ± 7.1(20)	3/20	0.91 ± 0.08(9)	0.32 ± 0.03(9)	5.24 ± 0.45(9)
High protein, 200 ppm	130.9 ± 12.0 (20)	135.2 ± 39.9(4)	15.1 ± 12.4(20)	16/20	0.91 ± 0.09(6)	0.31 ± 0.03(6)	5.17 ± 0.22(6)

[1] Mean ± SD.
[2] Number of animals contributing to each value is shown in parentheses.

TABLE 2

Effect of level of dietary protein on body and liver lipids, liver vitamin A, and swimming time of rats fed several concentrations of dieldrin in the diet

Group	Body fat	Liver lipid	Vitamin A	Vitamin A	Swimming time
	% body wt	*% liver wt*	*μg/g liver*	*μg/liver*	*sec/g body wt*
Low protein, 0 ppm	12.1 ± 1.0 [1](5)[2]	4.1 ± 0.8 (8)	208 ± 16(7)	1960 ± 149(7)	1.8 ± 0.2(8)
Low protein, 100 ppm	10.9 ± 2.5 (8)	5.9 ± 1.3(10)	152 ± 30(8)	1652 ± 553(8)	1.6 ± 0.4(10)
Low protein, 150 ppm	10.8 ± 3.4 (5)	7.8 ± 1.9(3)	151 ± 35(5)	1570 ± 238(5)	1.1 ± 0.3(5)
Low protein, 200 ppm	—	6.2 ± 0.5(4)	150 ± 11(4)	1407 ± 80 (4)	1.5 ± 0.2(4)
High protein, 0 ppm	13.1 ± 2.7 (5)	4.8 ± 0.9(9)	188 ± 24(9)	2179 ± 239(9)	1.8 ± 0.2(10)
High protein, 100 ppm	12.5 ± 1.4 (6)	5.9 ± 0.9(8)	147 ± 19(7)	2054 ± 390(7)	2.0 ± 0.4(8)
High protein, 150 ppm	13.2 ± 1.2 (6)	5.0 ± 1.0(8)	139 ± 32(7)	1994 ± 493(7)	1.8 ± 0.3(9)
High protein, 200 ppm	—	6.1 ± 0.6(6)	127 ± 8 (5)	1871 ± 300(5)	2.1 ± 0.6(6)

[1] Mean ± SD.
[2] Numbers in parentheses represent number of animals contributing to each value.

TABLE 3

Statistical comparisons of dieldrin-fed animals with their own controls

Groups [1,2]	Weight gain	Survival	Kidney weight	Heart weight	Liver weight	Body fat	Liver vitamin A/g	Liver vitamin A, total	Swimming time
Low protein									
0 ppm vs. 100 ppm	ns	ns	ns	(7.63)	ns	ns	(4.43)	ns	ns
0 ppm vs. 150 ppm	ns	ns	ns	ns	ns	ns	(3.90)	(3.68)	(5.55)
0 ppm vs. 200 ppm	(3.19)	(5.29)	ns	(3.72)	ns	ns	(6.39)	(7.09)	ns
High protein									
0 ppm vs. 100 ppm	ns	ns	ns	ns	(5.45)	ns	(3.67)	ns	ns
0 ppm vs. 150 ppm	ns	ns	ns	ns	(7.30)	ns	(3.51)	ns	ns
0 ppm vs. 200 ppm	ns	(5.75)	ns	ns	(8.48)	ns	(5.40)	(2.11)	ns

[1] Dieldrin-fed (100, 150, or 200 ppm) animals ingesting low protein diets are compared with low protein controls (0 ppm); dieldrin-fed animals ingesting high protein diets are compared with high protein controls. Only differences significant at a 1% level of confidence are shown.
[2] Numbers in parentheses are the *t* values; "ns" signifies no statistically significant difference. These represent 4-week values only.

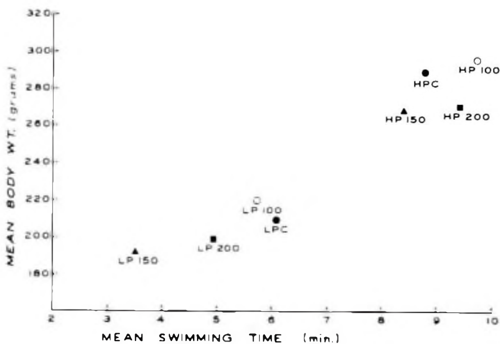


Fig. 2 Mean swimming time of rats fed low or high protein diets with zero, 100, 150, or 200 parts per million of dieldrin, plotted against mean body weight.

Statistical analyses of these data show that, when compared with their own low or high protein controls, at no level of dieldrin is there a significant alteration in total body fat. Furthermore, comparison of low protein and high protein groups ingesting the same level of dieldrin (low protein 100 ppm vs. high protein 100 ppm, etc.) reveal no statistically significant differences in body fat, as a percentage of total body weight.

With low protein diets, dieldrin ingestion at all levels tested results in a statistically significant increase in liver lipids, expressed as a percentage of liver weight. However, with the high protein diets there was no significant change in liver lipids.

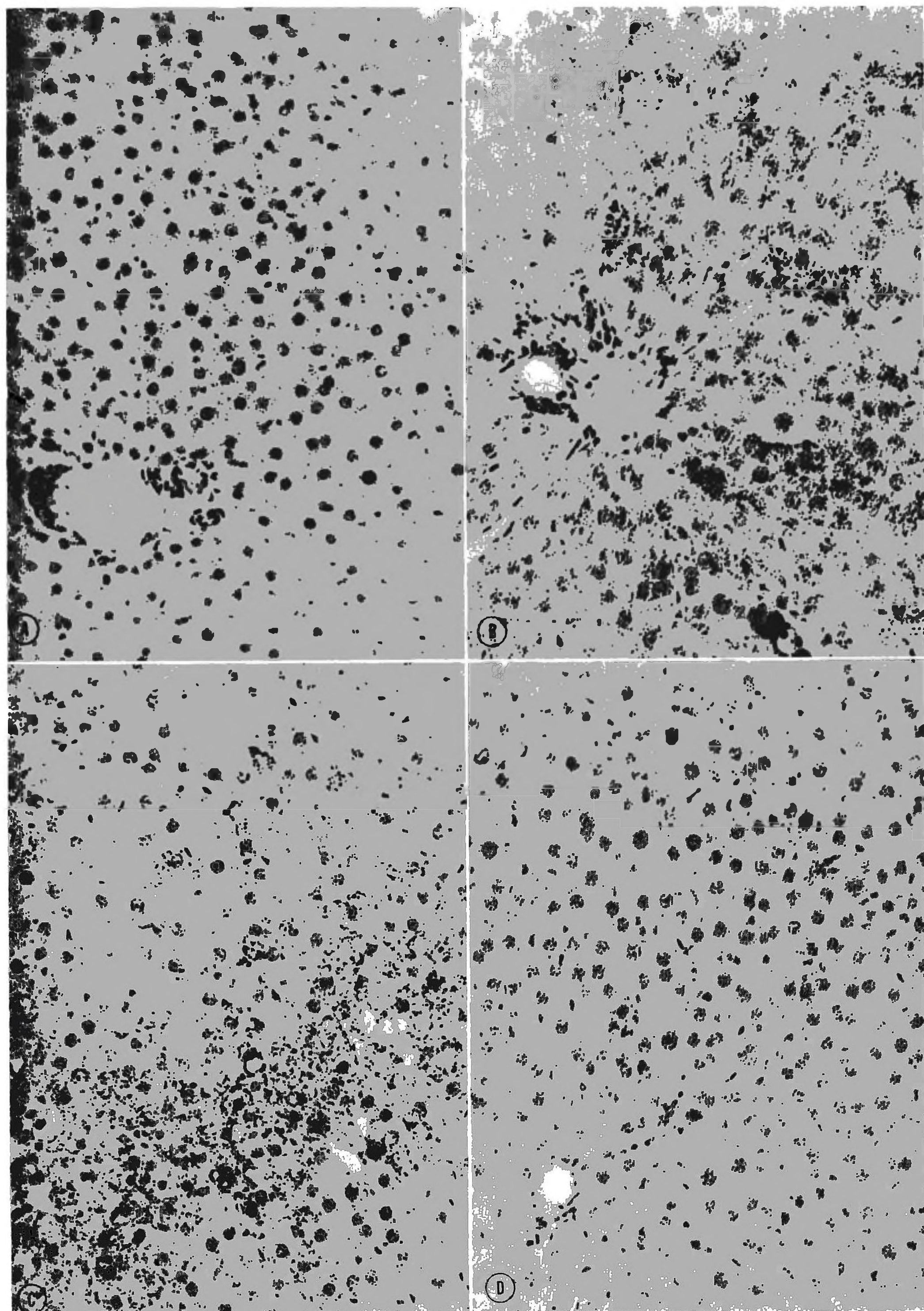


Figure 3

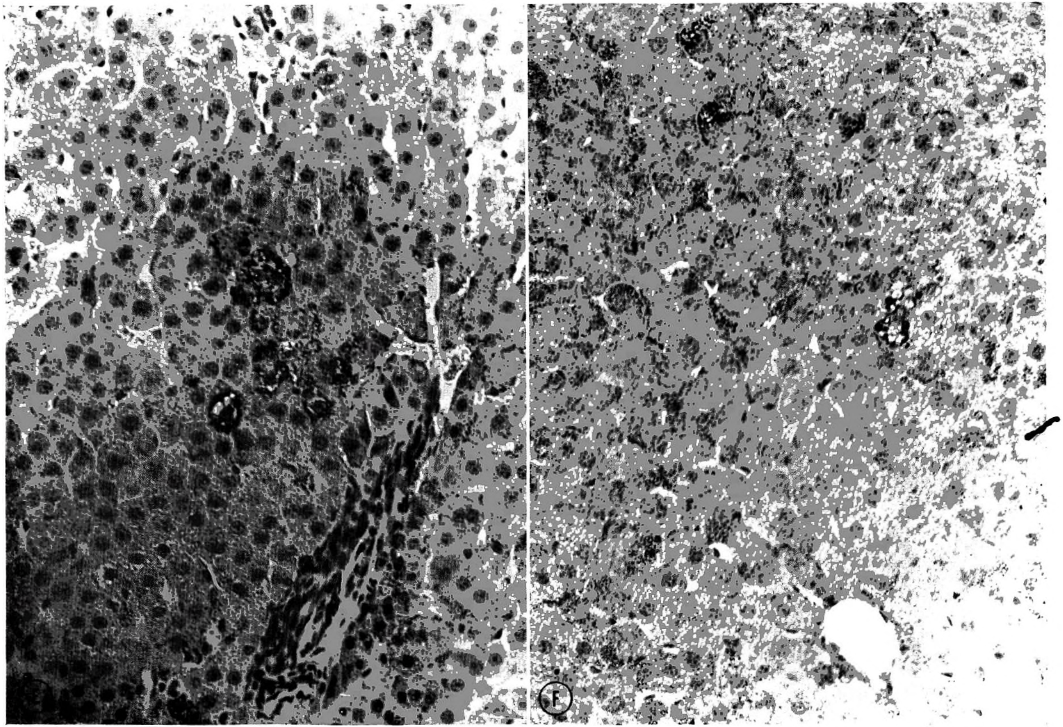


Fig. 3 Photomicrographs of rat liver sections stained with Oil Red O. (A) Low protein control; (B) low protein, 100 ppm dieldrin; (C) low protein, 200 ppm dieldrin; (D) high protein control; (E) high protein, 100 ppm dieldrin; (F) high protein, 200 ppm dieldrin.

Vitamin A per gram of liver was significantly decreased at all levels of dieldrin with both low and high protein diets, when compared with controls. Total liver vitamin A was significantly decreased at 150 and 200 ppm of dieldrin in animals ingesting low protein diets, but only in those animals ingesting 200 ppm of dieldrin with the high protein diet.

Sections of kidney and myocardium, stained with hematoxylin and eosin, revealed no significant differences when tissues from dieldrin-fed rats (at any level) were compared with their own controls. At the same level of dieldrin, high and low protein groups did not appear to differ from one another.

Pathological changes observed in the livers of dieldrin-treated animals consisted primarily of cellular edema and fatty degeneration of hepatic parenchymal cells. In liver sections stained with Oil Red O, stainable fat was localized chiefly in the peripheral zone of the hepatic lobules, although a diffuse distribution of fat was noted in some animals. These pathological changes were not observed in control animals with either diet. In general, more stainable fat was observed in the livers of dieldrin-treated animals fed low protein diets than in livers of those fed high protein diets. Figure 3 shows liver sections from animals ingesting low and high protein diets containing zero, 100, or 200 ppm stained with Oil Red O.

DISCUSSION

There is some question as to whether all of the chlorinated hydrocarbon pesticides can be grouped together with respect to their physiological and biochemical effects. Srivastava et al. (28) working with chickens, and Cline and Pearce (29), using houseflies, presented evidence suggesting that these compounds exert similar effects and, in mammals, the chlorinated hydrocarbons all appear to disturb the action of the central nervous system. However,

there in some evidence (30) that even such structurally similar compounds as aldrin and dieldrin may have different physiological effects. Most of the other parameters of toxicity have not been investigated in a sufficiently orderly manner to permit critical comparisons of the various pesticides at this time. In the absence of convincing evidence to the contrary, we have chosen to group information on all of the chlorinated hydrocarbons together for comparison with our results.

According to Barnes (1, 2) dieldrin causes some interference with growth at 25 ppm and produces death when fed at a level above 75 ppm. However, Treon and Cleveland (10) claimed that when dieldrin was fed at 75 ppm or less there was no increased mortality rate and when fed at 25 ppm for 2 years there was no growth depression. Our data indicate that with either low or high protein diets the ingestion of as little as 100 ppm of dieldrin causes an initial, short supression of growth and that ingestion of 150 or 200 ppm produces a slight but persistent inhibition of growth for at least 4 weeks, when compared with the controls. In addition, a decreased survival was evident in both low and high protein groups ingesting 200 ppm of dieldrin, but at 150 ppm only the low protein group exhibited a markedly increased mortality. This suggests that the high protein level conferred some protection on the animals. It is probable that some of the variability in the results of toxicity studies may be due to the modifying behavior of the diet. This deserves a more thorough examination.

The lack of change in total body fat and in weight gain per gram of food, in response to dieldrin ingestion, indicates that there was no gross alteration in food utilization and metabolism with either a low or high protein diet.

With respect to total liver weight (expressed as a percentage of body weight) Sarrett and Jandorf (8) have demonstrated that administration of DDT (50 mg/kg/day for 30 days) results in a significant increase in liver weight but does not alter the dry weight of the liver (expressed as a percentage of fresh weight of the liver). Part of the liver increase was certainly a reflection of the increased total lipid content (expressed as a percentage of total liver weight). Our data indicate that a high protein diet is necessary for the occurrence of liver hypertrophy.

It appears that none of the enzymes studied (glucose-6-phosphate dehydrogenase, α-ketoglutarate-phenylalanine transaminase, α-ketoglutarate-aspartate transaminase, and pyruvic-phenylalanine transaminase) are influenced by the chlorinated hydrocarbons or by the hepatic alterations which follow ingestion of these compounds.

Dieldrin ingestion does not have any clear effect on the stress test used here (swimming time), although it might be expected that swimming time measures the same kind of muscular efficiency as Khairy (16) measured with weight pulling.

In agreement with the data of Phillips (12) on DDT, dieldrin significantly depresses liver vitamin A storage. This is most clearly seen when vitamin A is expressed as micrograms per gram of liver. However, the protein content of the diet does appear to modify this effect, as the total vitamin A is significantly depressed only at a level of 200 ppm of dieldrin in the high protein diet, but was depressed at 150 and 200 ppm with the low protein diet. Whether this bears any relationship to the difference in total liver lipids of animals ingesting high and low protein diets containing dieldrin cannot be determined from these data.

The histological alterations are generally in keeping with the chemical determinations of fat, in that animals ingesting low protein diets containing dieldrin exhibited a more pronounced fatty infiltration and a higher total lipid content in the liver than did those ingesting high protein diets containing dieldrin. None of these animals exhibited severe fatty livers.

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Effect of Phytic Acid on the Availability of Zinc in Amino Acid and Casein Diets Fed to Chicks

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ABSTRACT Twenty-four 11-day-old New Hampshire × Columbian cross male chicks were used in a 12-day growth and mineral balance randomized block experiment to compare the effect, on the availability of dietary zinc, of adding phytic acid to an amino acid diet with that of adding phytic acid to a casein diet. Phytic acid decreased the availability of zinc in the absence of protein, with amino acids serving as the nitrogen source, to as great an extent as when casein was present in the diet.

It is well established that zinc is less available from plant protein than from animal protein diets (1–5).[3] It is further suggested that the natural inhibitor in plant (soybean) protein diets is phytic acid and that phytic acid must be in combination with protein to make zinc unavailable (6). McCall et al. (7) further suggest that a structural feature of the protein is responsible for zinc binding. In the present investigation we employed an amino acid diet for chicks, developed by Dean and Scott (8), to determine whether phytic acid would decrease the availability of dietary zinc in the absence of protein, with amino acids serving as the nitrogen source, to as great an extent as when a protein such as casein is present in the diet. The criteria used for measuring zinc availability were physical appearance, daily gain in weight, grams of diet required per gram gain in weight and micrograms of zinc per gram femur ash. Earlier work with pigs[4] (9), chicks,[5] and rats (10) has shown increased levels of dietary zinc to result in an increased concentration of zinc in bone tissue. The percentage ash in the dry lipid-free femur was also determined to learn whether any difference in this value existed between zinc-deficient chicks and those receiving an adequate level of dietary zinc. In an earlier study O'Dell et al. (11) noted that the percentage ash in tibiae of severely zinc-deficient chicks was lower (the difference approached significance at the 5% level) than the percentage ash in tibiae of controls.

METHODS

Twenty-four 11-day-old New Hampshire × Columbian cross male chicks, that had been fed the casein basal diet described in table 1 from seven days of age and which averaged 93 g in body weight, were alloted at random in a randomized block experiment to individual cages in a stainless steel battery for a period of 12 days. Feed and demineralized water were supplied ad libitum in glass feeders and waterers. Feed consumption and weight gains were recorded. Room temperature, which was not controlled, varied from 27.8° to 31.7°. At the end of the experiment femurs and diets were analyzed for zinc by the method of Butts et al. (12). Diets were further analyzed for phytic acid content by the method of Holt (13), adapted by developing color in a 50% ethanol medium; total phosphorus was determined by the method of Taussky and Shorr (14) and calcium by the chelometric method of Malmstadt and Hadjiioannou (15).

Analysis of variance, accompanied by the *t* test, as given in Goulden (16), was used to test the data statistically. Differ-

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[2] The data presented are taken from a dissertation in partial fulfillment of the requirements for the Ph.D. degree, University of Illinois, Urbana.

[3] O'Dell, B. L., and J. E. Savage 1957 Symptoms of zinc deficiency in the chick. Federation Proc., *16:* 394.

[4] Beardsley, D. W. 1958 Growth and chemical studies of zinc deficiency in the baby pig. Doctoral Thesis, University of Illinois, Urbana.

[5] Moeller, M. W. A. 1959 Mineral requirement of the chick with special reference to zinc and magnesium. Doctoral Thesis, University of Illinois, Urbana.

TABLE 1

Composition of amino acid and casein basal chick diets

	Amino acid	Casein
	%	%
Cornstarch	49.97	—
Cornstarch or glucose monohydrate [1]	—	48.83
Amino acid mix [2]	24.28	—
Casein [3] + amino acids [4]	—	25.42
Corn oil	15.00	15.00
Cellulose [5]	3.00	3.00
Mineral mixture [6]	5.00	5.00
Antacid [7]	1.00	1.00
$NaHCO_3$	1.00	1.00
Choline chloride	0.20	0.20
Vitamin mixture [8]	0.20	0.20
α-Tocopheryl acetate [9]	0.002	0.002

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

[2] Dean and Scott (8). The diet contained: (in per cent) L-arginine·HCl, 1.33; L-histidine·HCl, 0.62; L-lysine·HCl, 1.40; L-tyrosine, 0.63; L-tryptophan, 0.23; L-phenylalanine, 0.68; L-cystine, 0.35; DL-methionine, 0.55; L-threonine, 0.85; L-leucine, 1.20; L-isoleucine, 0.80; L-valine, 1.04; L-glutamic acid, 12.00; glycine, 1.60; L-proline, 1.00.

[3] Vitamin-Free Test Casein, General Biochemicals Inc., Chagrin Falls, Ohio.

[4] The diet contained: (in per cent) casein, 22.29; glycine, 2.14; L-arginine·HCl, 0.61; DL-methionine, 0.34; and L-histidine·HCl, 0.04.

[5] Solka Floc, Brown Company, Chicago.

[6] The diet contained: (in per cent) $CaCO_3$, 0.3000; $Ca_3(PO_4)_2$, 2.8000; K_2HPO_4, 0.9000; $MgSO_4 \cdot 7H_2O$, 0.3500; ferric citrate, 0.0500; KI, 0.0040; $CuSO_4 \cdot 5H_2O$, 0.0020; H_3BO_3, 0.0009; $CoSO_4 \cdot 7H_2O$, 0.0001; $MnSO_4 \cdot H_2O$, 0.0650; NaCl, 0.8800; and $Na_2MoO_4 \cdot 2H_2O$, 0.0009.

[7] Antacid absorbent (an aluminum hydroxide-magnesium trisilicate preparation), Warner-Chilcott Laboratories, Morris Plains, New Jersey.

[8] The diet contained: (in mg/kg of diet) thiamine·HCl, 100; niacin, 100; riboflavin, 16; Ca pantothenate, 20; vitamin B_{12}, 0.02; pyridoxine·HCl, 6; biotin, 0.60; folic acid, 4; inositol, 100; p-aminobenzoic acid, 2; menadione, 5; ascorbic acid, 250; vitamin A acetate (250,000 IU/g), 10,000 IU; vitamin D_3 (200,000 ICU/g), 600 ICU.

[9] Added to the diet dissolved in petroleum ether.

ences referred to in the text possess a probability value of less than 0.05.

Diets fed to chicks in this experiment were amino acid (AA), AA + phytic acid (PA); AA + PA + disodium ethylenediaminetetraacetic acid (EDTA); casein; casein + PA; and casein + PA + EDTA. Ingredients of the basal amino acid and casein diets are shown in table 1. The amino acid basal diet contained approximately 4 ppm Zn, and the casein basal diet contained approximately 9 ppm Zn. Extra zinc was added to the basal diets as $ZnCO_3$. Phytic acid was added directly to the zinc-supplemented basal diets. By chemical analysis the diets contained from 16 to 21 ppm Zn and 1.8% phytic acid (table 2). Higher levels of zinc in phytic acid diets when compared with those of the control diets may be accounted for as zinc contamination which resulted while mixing phytic acid into these diets. EDTA, when added, was added to phytic acid diets at a level of 230 ppm of the disodium dihydrate salt. Kratzer et al. (17) have shown that a similar level of EDTA increased the biological availability of zinc in soybean protein diets fed to turkey poults.

RESULTS AND DISCUSSION

Results of this experiment (tables 3 and 4) show that with either casein or amino acids serving as the source of nitrogen, the addition of 1.8% phytic acid to the diet decreased daily gain in weight, increased the amount of diet required per gram gain in weight and decreased the concentration of zinc in femur ash. From this it may be concluded that phytic acid decreases the availability of dietary zinc in the absence of protein, with amino acids serving as the nitrogen source, to as great an extent

TABLE 2

Chemical analyses of chick diets

Diet	Zinc	Calcium	Phytic acid-phosphorus [1]	Total phosphorus
	ppm	%	%	%
Amino acid	17.3	1.2	0.00	0.81
Amino acid + phytic acid	20.5	1.2	0.51	1.36
Amino acid + phytic acid + EDTA [2]	20.6	1.2	0.51	1.36
Casein	16.4	1.2	0.00	0.93
Casein + phytic acid	19.8	1.2	0.51	1.46
Casein + phytic acid + EDTA	19.1	1.2	0.51	1.46

[1] Per cent phytic acid = 3.57 × % phytic acid-phosphorus.

[2] EDTA indicates ethylenediaminetetraacetic acid.

TABLE 3

Average daily gain in weight of chicks from 11 to 23 days of age and average grams of diet required per gram gain in weight

Diet	No. chicks	Daily gain in wt	Feed/gain ratio
		g	
Amino acid	4	11.9 ± 0.91 [1]	1.6 ± 0.4
Amino acid + phytic acid	4	5.9 ± 0.48	2.3 ± 0.11
Amino acid + phytic acid + EDTA	4	10.1 ± 0.66	1.8 ± 0.03
Casein	4	13.5 ± 0.78	1.4 ± 0.02
Casein + phytic acid	4	7.9 ± 0.69	2.0 ± 0.03
Casein + phytic acid + EDTA	4	12.5 ± 0.50	1.5 ± 0.04

[1] SE.

TABLE 4

Average percentage ash in the dry, lipid-free chick femur and average micrograms of zinc per gram of femur ash

Diet	No. chicks	Femur ash	μg Zn/g ash
		%	
Amino acid	4	52 ± 0.4 [1]	286 ± 9.1
Amino acid + phytic acid	4	51 ± 0.3	127 ± 2.7
Amino acid + phytic acid + EDTA	4	52 ± 0.4	172 ± 9.7
Casein	4	52 ± 0.9	305 ± 10.2
Casein + phytic acid	4	52 ± 0.4	126 ± 12.5
Casein + phytic acid + EDTA	4	52 ± 1.0	233 ± 16.8

[1] SE.

as when a protein such as casein is present in the diet, in contrast with the earlier report by O'Dell and Savage (6), who suggested that phytic acid must be in combination with dietary protein to make zinc unavailable. Thus it appears that protein, if required, acts in the intestinal tract and may be of metabolic origin. Specific amino acids may chelate zinc as discussed by Gurd and Wilcox (18).

Chicks fed both the casein and the amino acid phytic acid diets were obviously zinc-deficient. However, except for being smaller, their physical appearance was not noticeably different from that of the controls. Since feathers of both control and zinc-deficient birds were damaged by the cages, valid comparisons of this nature could not be made. Other clinical symptoms characteristic of zinc deficiency, such as keratosis and dermatitis of the feet, might have develeoped if the chicks had been kept on trial for a longer period of time. The percentage ash in the dry, lipid-free femur of zinc-deficient birds fed phytic acid diets was not significantly lower than that of controls.

Chicks receiving phytic acid + EDTA in their diet gained more weight per day, required less diet per gram gain in weight and had a higher concentration of zinc in their femur ash than chicks fed phytic acid diets without EDTA. When compared with chicks fed the amino acid control diet, chicks fed the amino acid diet to which phytic acid + EDTA had been added gained less weight per day, had a higher feed-to-gain ratio and a lower concentration of zinc in their femur ash. Differences in weight gain and feed-to-gain ratio were not apparent between chicks receiving the phytic acid + EDTA diet and the control group when casein served as the source of nitrogen. However, chicks receiving the casein phytic acid + EDTA diet had lower levels of zinc in their femur ash than chicks fed the casein control diet.

Thus, addition of EDTA increased the availability of zinc in phytic acid diets. Similar effects of EDTA when plant protein diets were fed have been noted with turkey poults (17), chicks (4) and rats (10). The fact that daily gain in weight and feed-to-gain ratios of chicks fed phytic acid + EDTA diets differed from the control in the case of the amino acid diet, but not when casein served as the nitrogen source, suggests that although

chicks fed the casein phytic acid + EDTA diet received adequate amounts of zinc, birds fed the amino acid phytic acid + EDTA diet did not receive quite enough of this trace mineral for optimal growth and feed efficiency. Since the amino acid diets contained slightly higher levels of zinc than casein diets (table 2), this observation supports further the statement made earlier that phytic acid decreases the availability of dietary zinc in the absence of protein, with amino acids serving as the nitrogen source, to as great an extent as when casein is present in the diet.

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Studies of the Metabolism of Polyunsaturated Acids by Short-term Experiments [1]

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ABSTRACT Short-term feeding experiments were examined as a method for studying the metabolism of fatty acids. Rats which had been maintained with fat-free diets for 60 days were administered supplements of individual polyunsaturated fatty acids at levels ranging from 50 to 1000 mg/day. Characteristic changes in the fatty acid composition of the liver lipids, which were investigated previously after feeding 40 mg of polyunsaturated fatty acids daily for 60 days to weanling rats, were observed when as little as 50 mg of the same acids were fed for 5 days to adult EFA-deficient rats. An optimal dietary amount of either linoleic acid or linolenic acid, judged by maximal response in the metabolites of these in the liver, was 200 to 400 mg/day/animal. High dietary levels of either linoleic acid or linolenic acid resulted in an apparent decrease in the levels of the metabolites of these in the liver lipids. Nor-linoleic acid (17:2ω6) was converted to 19:2ω6 in the tissues but was not dehydrogenated. Dietary linolenic acid was shown to exert an inhibitory effect upon the conversion of arachidonic acid (20:4ω6) to 22:5ω6.

Conventionally, in our laboratory, nutritional experiments to study the in vivo interconversions of polyunsaturated fatty acids have been based upon the change in the fatty acid composition of tissue lipids after feeding small amounts of fatty acids to weanling rats for periods of 60 to 100 days. The duration of these experiments was dictated by the time studies, in which the biochemical symptoms of essential fatty acid deficiency had reached a maximum, established in previous studies (1). Recently, Schlenk et al. (2) have shown that polyunsaturated fatty acids, when fed to adult fat-deficient rats in relatively large doses for 5 days, produce marked changes in the fatty acid composition of tissuc lipids. The utility of short-term feeding experiments has been confirmed in our laboratory in a study of fatty acid interrelationships (3). The obvious advantages of economy of time and substance prompted a more detailed study of the short-term curative response. Therefore, the effect of dose level and of the type of polyunsaturated fatty acid supplement were studied.

The present study consists of 3 experiments. In the preliminary experiment, linoleate and linolenate were administered singly to fat-deficient male rats in graded dose levels for 5 days to establish the minimal dietary levels necessary to produce measurable changes in the fatty acid composition of tissue lipids. This experiment was also conducted to determine whether there were optimal dietary levels of linoleic and linolenic acids above which an increase in fatty acid intake did not produce an increase in metabolites of these acids in the tissues. In the second experiment 6 fatty acids having widely different structures were fed to fat-deficient rats at one level for 5 days. This experiment was conducted to determine whether the dose levels indicated to be optimum for linoleate and linolenate in the first experiment are applicable to a variety of naturally occurring polyunsaturated fatty acids. A seventh acid, 8,11-heptadecadienoic (nor-linoleic) acid, was included in the second experiment to study the convertibility of a fatty acid which, according to its terminal structure, belongs to the linoleic acid family, but which contains an odd number of carbon atoms. The third experiment in this series is an application of the short-term experiment to a current metabolic problem. A competitive relationship exists in liver lipids between the linoleic and linolenic families of acids (3, 4). The purpose of the third experiment was to determine where linolenate was exerting

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TABLE 1

Fatty acid composition of liver lipids (% of total methyl esters)

Group	Dietary fatty acid	Purity	Amount	16:1	17:2 ω 6	18:2 ω 6	18:3 ω 6	18:3 ω 3	19:2 ω 6	20:3 ω 9
		%	mg/day/animal							
Experiment 1										
1	0		0	12.7		1.6				7.7
2	18:2 ω 6		50	9.9		6.4				6.0
3	18:2 ω 6		200	5.2		7.8				2.5
4	18:2 ω 6		400	4.9		13.9				1.2
5	18:2 ω 6		1000	2.3		26.3				1.0
6	18:3 ω 3		100	6.5		3.1		1.3		3.0
7	18:3 ω 3		400	4.4		4.2		7.0		2.2
8	18:3 ω 3		1000	2.9		2.9		14.3		3.6
Experiment 2										
9	0		0	13.9 ± 2.5 [2]		2.3 ± 0.3				8.8 ± 2.1
10	18:2 ω 6	98.4	100	9.2 ± 1.3		6.3 ± 1.1				4.9 ± 0.5
11	18:3 ω 3	98.4	100	10.1 ± 1.5		1.2 ± 0.3		1.6 ± 0.3		4.5 ± 0.5
12	18:3 ω 6	93.2	100	9.5 ± 1.8		1.2 ± 0.4	1.3 ± 0.3			3.0 ± 0.8
13	17:2 ω 6	93.7	170	8.1 ± 1.0	7.60 ± 2.96	0.9 ± 0.1			2.4 ± 0.3	7.2 ± 1.6
14	20:4 ω 6	90.7	100	7.4 ± 2.4		0.7 ± 0.2				1.9 ± 0.3
15	20:5 ω 3	79.3	100	7.2 ± 0.5		0.7 ± 0.2				2.7 ± 0.7
16	22:6 ω 6	93.3	100	6.7 ± 0.7		0.8 ± 0.2				2.6 ± 0.5
Experiment 3										
17	18:3 ω 3 20:4 ω 6		0 50	9.7 ± 4.2		1.2 ± 0.2		0.4 ± 0.2		3.2 ± 1.8
18	18:3 ω 3 20:4 ω 6		20 50	6.3 ± 0.2		1.0 ± 0.1		1.1 ± 0.4		3.0 ± 1.0
19	18:3 ω 3 20:4 ω 6		40 50	6.9 ± 1.4		0.8 ± 0.2		1.5 ± 0.2		1.9 ± 0.4
20	18:3 ω 3 20:4 ω 6		100 50	6.6 ± 0.4		0.8 ± 0.2		1.9 ± 0.0		2.3 ± 0.1
21	18:3 ω 3 20:4 ω 6		150 50	6.3 ± 1.7		0.8 ± 0.1		2.4 ± 0.8		2.0 ± 0.1
22	18:3 ω 3 20:4 ω 6		200 50	6.6 ± 0.6		0.7 ± 0.1		3.7 ± 0.7		2.4 ± 0.4

Group	Dietary fatty acid	Purity	Amount	20:3 ω 6	20:4 ω 6	20:5 ω 3	22:4 ω 6 [1]	22:5 ω 6	22:5 ω 3 [1]	22:6 ω 3
		%	mg/day/animal							
Experiment 1										
1	0		0	1.5	4.3	0.2		0.7		0.7
2	18:2 ω 6		50	1.1	12.6	0.4		1.6		0.9
3	18:2 ω 6		200	0.9	21.0	0.3		5.9		0.8
4	18:2 ω 6		400	1.0	15.2	0.2		4.2		0.7
5	18:2 ω 6		1000		11.9	0.1		1.9		0.8
6	18:3 ω 3		100		2.7	4.0		0.2	0.9	4.7
7	18:3 ω 3		400		1.8	6.9		0.2	3.0	5.0
8	18:3 ω 3		1000		3.8	4.2		0.3	1.9	2.4
Experiment 2										
9	0		0		4.3 ± 1.1	0.4 ± 0.2	0.2 ± 0.1	1.0 ± 0.3		1.0 ± 0.2
10	18:2 ω 6	98.4	100	1.6 ± 0.2	10.8 ± 1.7	0.5 ± 0.2	0.5 ± 0.1	2.4 ± 0.7	0.1 ± 0.1	1.9 ± 0.5
11	18:3 ω 3	98.4	100		3.5 ± 0.9	4.5 ± 1.0	0.3 ± 0.2	0.6 ± 0.1	2.3 ± 0.5	6.4 ± 1.5
12	18:3 ω 6	93.2	100	3.0 ± 0.8	19.0 ± 3.1	0.2 ± 0.1	0.9 ± 0.3	3.9 ± 1.6	0.3 ± 0.1	2.4 ± 0.7
13	17:2 ω 6	93.7	170		6.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	1.9 ± 0.1	0.1 ± 0.1	1.8 ± 0.1
14	20:4 ω 6	90.7	100	0.7 ± 0.2	18.6 ± 3.2	0.3 ± 0.2	2.2 ± 0.7	4.5 ± 1.4	0.7 ± 0.1	2.2 ± 0.6
15	20:5 ω 3	79.3	100	0.4 ± 0.1	3.7 ± 1.0	5.9 ± 1.2	0.2 ± 0.1	0.4 ± 0.2	4.1 ± 1.2	6.4 ± 1.3
16	22:6 ω 6	93.3	100	0.4 ± 0.2	2.8 ± 0.4	2.2 ± 0.5	0.2 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	14.4 ± 1.4
Experiment 3										
17	18:3 ω 3 20:4 ω 6		0 50	1.2 ± 0.1	16.2 ± 2.1	0.2 ± 0.1	1.2 ± 0.4	3.1 ± 0.1	0.4 ± 0.1	2.2 ± 0.5
18	18:3 ω 3 20:4 ω 6		20 50	0.7 ± 0.2	15.5 ± 0.6	0.6 ± 0.4	1.0 ± 0.1	2.0 ± 0.5	1.4 ± 0.1	5.3 ± 0.0
19	18:3 ω 3 20:4 ω 6		40 50	0.8 ± 0.3	15.3 ± 3.3	0.7 ± 0.2	0.9 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	6.0 ± 2.0
20	18:3 ω 3 20:4 ω 6		100 50	0.7 ± 0.1	16.6 ± 1.3	1.3 ± 0.5	0.8 ± 0.3	1.0 ± 0.1	2.1 ± 0.4	6.7 ± 1.1
21	18:3 ω 3 20:4 ω 6		150 50	0.6 ± 0.2	15.6 ± 2.5	2.5 ± 0.4	0.8 ± 0.2	0.9 ± 0.3	2.4 ± 0.1	7.8 ± 1.3
22	18:3 ω 3 20:4 ω 6		200 50	0.8 ± 0.1	14.9 ± 0.1	3.1 ± 1.1	0.6 ± 0.1	0.5 ± 0.1	2.6 ± 0.5	7.3 ± 0.4

[1] Blanks in these columns indicate these values were not calculated in experiment 1. Blanks in all other columns indicate values below experimental error.

[2] Mean ± SD.

its inhibitory effects in the sequential conversion of linoleate to 22:5.

EXPERIMENTAL

Weanling male rats (Sprague-Dawley strain) were conditioned prior to administration of the fatty acid supplements by maintaining them for 60 days with semipurified fat-free diet (5).[2] The fatty acids as methyl esters were then administered by mouth for 5 days. The purities of the fatty acid esters were determined by gas-liquid chromatography (GLC) and are reported in table 1. The γ-linolenic acid was prepared from the seed oil of *Oenothera lamarckiana* (6). Nor-linoleic acid was received from Professor S. Bergström (7).[3] All the other supplements were purchased from the Hormel Institute. To distinguish structural isomers and to denote metabolic relationships, the fatty acids are referred to according to their terminal structures. Thus, the number following the ω in the abbreviated notations indicates the position of the last double bond, counting from the terminal methyl group.

Experiment 1. In the preliminary experiment, individual rats were fed daily supplements of either 50, 200, 400 or 1000 mg of methyl linoleate (18:2ω6), or 100, 400 or 1000 mg of methyl linolenate (18:3ω3). The daily doses were divided and fed so that the animals did not receive more than 200 mg of supplement at one feeding. Three rats that received only the basic fat-free diet served as control animals.

Experiment 2. Thirty-six rats were divided into groups of six, and the animals in each group received one of the following supplements daily: 100 mg of either methyl linoleate (18:2ω6), methyl linolenate (18:3ω3), methyl γ-linolenate (18:3ω6), methyl arachidonate (20:4ω6), methyl, 5,8,11,14,17-eicosapentaenoate (20:5ω3) or methyl 4,7,10,13,16,19-docosahexaenoate (22:6ω3). The amount of fatty acid supplement to be fed daily was determined from the results of experiment 1, and was intended to be the minimal amount of supplement to produce highly significant changes in the fatty acid composition of the liver lipids. A seventh group of 2 rats received a daily supplement of 170 mg of methyl 8,11-heptadecadienoate (17:2ω6), dictated by the amount of sample available. An eighth group of 6 rats served as control animals, not given any supplements.

Experiment 3. Twelve rats were divided according to diet into 6 groups containing 2 animals in each group. All of the animals received daily 50 mg of methyl arachidonate (20:4ω6) and either zero, 20, 40, 100, 150 or 200 mg of 18:3ω3.

In all 3 experiments, after 5 days of diet supplementation, the animals were killed by ether anesthesia and their livers were removed and kept in saline solution at −20° until analysis was performed. The liver tissues were homogenized with chloroform:methanol (2:1) in a mechanical blender and the lipids were isolated. They were transesterified by refluxing with 30 volumes of 5% HCl in methanol. The resulting methyl esters were analyzed by GLC as described in detail previously (4). The ester peaks on the GLC charts were identified by comparison with authentic standards whose structures had been determined by ozonolysis and reduction. Quantification was carried out by triangulation, and the results are reported as area percentage. The results reported for 16:1 and 18:2ω6 may represent mixtures of structural isomers since the isomers of these acids are not resolved under our conditions of GLC analysis. The levels of 14:0, 16:0, 18:0 and 18:1 in the tissue lipids did not vary markedly with the dietary treatment and, therefore, they are not reported. The fatty acid composition of the lipids was determined for each animal and, where feasible, reported as the mean per diet group with standard deviations.

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[2] The composition of the semipurified fat-free diet was as follows: (in per cent) vitamin-test casein, 18.0; sucrose, 74.0; salt mixture, 4.0; α-cellulose, 4.0; and vitamin mixture. The vitamin mixture contained: (in mg/kg of diet) vitamin A, 4.00; thiamine, 30.00; riboflavin, 30.00; pyridoxine, 8.00; vitamin B_{12}, 0.05; vitamin D_2, 4.00; vitamin E, 230.00; vitamin K_5, 2.00; DL-Ca pantothenate, 100.00; niacin, 100.00; *i*-inositol, 220.00; *p*-aminobenzoic acid, 75.00; biotin, 0.20; folic acid, 1.00; and choline chloride, 1000.00. Salt mixture: Wesson, L. G., Science, 75: 339, 1932. All components of the basic diet except sucrose were obtained from Nutritional Biochemicals Corporation, Cleveland.

[3] We are grateful to Prof. S. Bergström of the Karolinska Institute, Stockholm, for the nor-linoleate, and to Dr. H. J. Thomasson of Unilever Research Laboratory, Vlaardingen for the methyl esters of the fatty acids of *Oenothera lamarckiana*.

RESULTS AND DISCUSSION

Experiment 1. The fatty acid compositions of the liver total lipids are shown in table 1. Even the lowest levels of supplementation, 50 mg of 18:2ω6 or 100 mg of 18:3ω3, fed as a daily supplement to fat-deficient rats for 5 days, produced considerable change in the fatty acid composition of liver lipids. The characteristic inverse relationships between the dietary level of 18:2ω6 and the levels of 16:1 and 20:3ω9 in the tissues are indicated by the fatty acid compositions of the animals that received different dose levels of dietary 18:2ω6. The levels of 18:2ω6 and 18:3ω3 in the liver lipids increased consistently with increases in their content in the diet. However, with the exception of 20:3ω6, the content of their metabolites in the liver lipids, ω6 and ω3 acids, respectively, appeared to decrease after reaching maximal values at intermediate levels of dietary 18:2ω6 and 18:3ω3.

The limited results of this preliminary experiment indicate that 200 to 400 mg/day is a useful dietary range for a short-term feeding study using fat-deficient rats. The lowest limit of supplementation to produce a measurable response appears to lie below 50 mg/day. The possible self-inhibition indicated that high levels of supplementation in this exploratory experiment should be verified with larger numbers of animals.

Experiment 2. The naturally occurring fatty acids fed in experiment 2 produced changes in the fatty acid composition of the tissue lipids that are, with one exception, characteristic of those produced in long-term feeding studies (5,8). When equal amounts of 18:2ω6 and 18:3ω3 had been fed singly to weanling rats for at least 60 days, the levels of 20:3ω9 in the tissue lipids were significantly lower in the animals fed 18:3ω3 (5, 8). However, feeding equal amounts of 18:2ω6 and 18:3ω3 in the present study resulted in low but equal levels of 20:3ω9 in the tissue lipids. Perhaps the inconsistency may be the result of a limiting rate of removal of 20:3ω9, so that suppression of its level by 18:2ω6 or by 18:3ω3 is not significantly different in 5 days.

Dietary 18:3ω6 is efficiently converted to 20:4ω6 in the tissues, because feeding equal amounts of 20:4ω6 or its precursor, 18:3ω6, resulted in equal levels of 20:4ω6 in the tissue lipids. However, the level of 20:4ω6 in the tissue lipids of the rats that received 18:2ω6 was only one-half that observed in the rats that received 20:4ω6. This observation is consistent with the results of a radioactive tracer study by Mead et al. (9) which shows that very little degradation of 18:3ω6 occurs before incorporation of the intact molecule into 20:4ω6.

Nor-linoleic acid (17:2ω6) was found at a level of 7.6% of total liver fatty acids in the rats fed 17:2ω6. The only other unusual fatty acid component noted was an unknown acid having an equivalent chain length on GLC columns of ethylene glycol succinate as would be expected for 19:2ω6.[4] It was found in the liver lipids at a level of 2.4% of total fatty acids. The 19:4 metabolite, comparable to arachidonate, was not detected by GLC. From these limited data it appears that 17:2ω6 differs from linoleate in its metabolism in that it may undergo chain lengthening, but that it probably does not undergo subsequent dehydrogenation to a significant degree. Therefore, the ω6 terminal structure, which appears to be necessary for full essential fatty acid activity, does not confer upon the molecule the ability to be dehydrogenated. Conversely, the proximal structure, the spacing between carboxyl and the first double bond, must be an important factor in metabolizing ability. In linoleate the proximal structure is Δ9, but it is Δ8 in 17:2ω6. The importance of this spacing is suggested by the results of Schlenk et al. (10) which show that 17:2ω5 and 17:3ω3, both having the Δ9 structure, were fully convertible to more highly unsaturated acids.

The results of experiment 2 show that the metabolism of a polyunsaturated fatty acid can be studied with less than one gram of the acid per rat.

Experiment 3. A competitive relationship exists between the linoleate (ω6) and linolenate (ω3) families of polyunsaturated fatty acids (3, 4). Since this relationship was demonstrated by feeding varying amounts of 18:2ω6 and 18:3ω3,

[4] Unpublished data, H. Hofstetter, and R. T. Holman, 1964.

the precursor fatty acids of each family, it was not determined where in each conversion sequence the inhibitory effects were exerted. The total inhibitory effect of 18:3ω3 upon the metabolism of 18:2ω6 that was illustrated in previous studies, could have resulted from an inhibition of the conversion of 18:2ω6 to 20:4ω6 as well as in subsequent steps. The results of experiment 3 show that although the level of 20:4ω6 in the tissues remained constant when dietary 18:3ω3 was increased from zero to 200 mg/day, the levels of 22:4ω6 and 22:5ω6, both metabolites of 20:4ω6 (3, 5, 8), were reduced 51 and 84%, respectively. Therefore, according to steady state concentrations, 18:3ω3 appears to exert an inhibitory effect upon the conversion of 20:4ω6 to 22:5ω6, as well as upon the conversion of 18:2ω6 to 20:4ω6 (4).

ACKNOWLEDGMENT

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Effects of Coprophagy Prevention on Intestinal Microflora in Rats

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ABSTRACT Prevention of coprophagy by the use of tail cups resulted in reduced weight gains and major changes in the number of fecal and cecal lactobacilli, enterococci and coliform bacteria in rats receiving 3 different diets containing 22% casein. The number of lactobacilli decreased, whereas the enterococci and coliforms increased in relation to the counts in non-cupped animals. These results were generally similar for the 3 diets used (fat-free, 20% corn oil or 20% hydrogenated coconut oil) although relatively minor differences in the bacterial counts due to the type of diet tended to be accentuated by the prevention of coprophagy.

The role of coprophagy in the nutrition of the rat has been studied extensively by Barnes and his co-workers and has been the subject of a recent review (1). These workers have established that prevention of coprophagy in rats by the use of tail cups resulted in a decreased growth rate which was reversible only when the animals were allowed direct access to the feces as they emerged from the anus.

Coprophagy prevention has also been reported to reduce the number of lactobacilli in the cecum and feces of rats (2). This suggested that coprophagy might be an important factor in maintaining the normal ecological balance of the intestinal microflora of rodents through the process of continual reinfection. To test this hypothesis the present study was initiated to determine the effects of coprophagy prevention on several representative groups of intestinal microorganisms in the rat.

The microorganisms chosen for study, in addition to the lactobacilli were the coliforms and the enterococci because these organisms collectively form an appreciable segment of the facultative anerobic intestinal microflora of rats (3), and adequate selective culture media for their enumeration were available.

It has been well established that dietary alterations, especially in the carbohydrate portion, may influence the composition of the intestinal microflora of animals (4), and a similar effect has been postulated for various fats and fatty acids (5). In the present study a high carbohydrate, low fat diet was compared with a diet containing 20% corn oil and also a diet containing 20% hydrogenated coconut oil.

EXPERIMENTAL METHODS

Weanling male rats of the NIH Sprague-Dawley stock were distributed at random into 6 groups of 8 animals each and housed individually in screen-bottom cages. Two groups of animals were fed each of the experimental diets, the composition of which is shown in table 1. Diet and distilled water were available ad libitum.

After the animals had received their respective diets for one week, those in one of the two groups fed each diet were fitted with modified plastic tail cups similar to those described by Barnes et al. (1,6). The animals were inspected daily to insure that the cupping device remained in position.

Fecal lactobacillus counts were determined for all animals at weaning according to the methods previously described (2). After the animals had been fed the experimental diets for one week, and be-

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TABLE 1

Composition of experimental diets

	Diet A	Diet B	Diet C
	g/kg of diet	*g/kg of diet*	*g/kg of diet*
Vitamin-free casein	220	220	220
Salt mixture (Wesson) [1]	40	40	40
Corn oil	—	200	—
Coconut oil (hydrogenated)	—	—	200
Cornstarch	740	540	540
Vitamin mixture [2]			

[1] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.

[2] To each kilogram of diet the following vitamins were added at the indicated level: vitamin A acetate, 7.0 mg; calciferol, 0.1 mg; α-tocopheryl acetate, 0.5 g; menadione, 10 mg; thiamine·HCl, 10 mg; pyridoxine·HCl, 10 mg; riboflavin, 20 mg; Ca pantothenate, 20 mg; niacinamide, 100 mg; choline chloride, 2.0 g; vitamin B_{12}, 100 μg; biotin, 50 μg; p-aminobenzoic acid, 30 mg; folic acid, 12.5 mg; and ascorbic acid, 100 mg.

fore the tail cups were affixed, determinations were made of the fecal lactobacillus, enterococcus and coliform counts of the feces. For the enterococcus counts Difco Mitis-Salivarius agar medium was used and the plates were incubated at 37° for 48 hours in an atmosphere of 95% nitrogen-5% CO_2. Coliform counts were obtained on Difco Violet Red bile agar after 18 hours at 37°. Only those colonies typical of coliform bacteria on this medium were enumerated. The microbiological counts were repeated periodically during the course of the experiment which ran for a total of 8 weeks from the time the animals were weaned.

Before the experiment was started, certain studies were performed to determine factors which might influence the reliability of the microbiological counts. It was determined for example, that the lactobacillus, enterococcus and coliform counts of fecal pellets decreased gradually if the feces samples were allowed to remain in air at room temperature. After 24 hours the counts had decreased by approximately 90% when compared with freshly voided feces. However, the counts remained essentially unchanged when the feces were kept for the same period of time in closed plastic vials of the type used for the tail cups. For this reason only freshly voided feces were sampled from the non-cupped animals, whereas the entire accumulation of feces in the tail cups for a 24-hour period was pooled in making the bacterial counts from the cupped animals.

In summarizing the data the counts were expressed as logs to the base 10 and averaged. Standard errors of the mean did not exceed 0.2 log units for any of the groups at any of the sampling periods.

At the termination of the experiment the animals were killed by ether inhalation and the cecum was removed and weighed together with its contents. An aliquot of the cecum contents was then taken for microbiological determinations for each animal.

RESULTS

At the termination of the experiment all of the non-cupped animals except one were still alive and in excellent condition. One of the animals fed the corn oil diet died of unknown causes during the first week. The animals with the tail cups did not gain weight as rapidly as the non-cupped controls and they were much more nervous and irritable. One animal died in each of the cupped groups. In the fat-free diet group one rat died during the first week, before the tail cups were applied. One rat fed the corn oil diet died during the fifth week and one animal fed the coconut oil diet died in the fourth week after the application of the cups. The marked disparity in body weight of the animals with and without tail cups is shown in table 2. These results are consistent with the observations of Barnes et al. (1).

Following the application of the tail cups there was an immediate and marked decrease in the fecal lactobacillus counts in all groups of animals. The greatest differences were observed for the 2 fat-containing diets (fig. 1). These results are consistent with previous observations in which basal diets of similar composition, but deficient in pyridoxine or vitamin K, were

TABLE 2

Final body weight of rats after consuming various diets for 8 weeks, with and without tail cups

Diet	Controls		Cupped animals	
	No.	Avg wt	No.	Avg wt
		g		*g*
Fat-free	8	289 ± 14.8 [1]	7	179 ± 5.3
Corn oil	7	277 ± 26.0	7	200 ± 7.3
Coconut oil	8	297 ± 12.2	7	192 ± 8.9

[1] SE of mean.

used (2). The differences in the fecal lactobacillus counts between the cupped and non-cupped animals persisted in varying degrees throughout the duration of the experiment despite a gradual decrease in the control animals with time. In sharp contrast with the results with the lactobacilli, the coliform counts in the cupped animals increased markedly and progressively during the period of observation. At the same time the coliform counts in the non-cupped animals showed a gradual decline (fig. 2).

The pattern observed with the enterococcus counts was similar to that of the coliform counts in that there was an initial increase in the cupped rats; however, there was a tendency for the number of enterococci to decrease during the later stages of the experimental period. In the uncupped rats receiving the fat-free diet the enterococcus counts remained relatively constant, whereas in the corresponding animals fed the fat-containing diets there was a moderate decrease in the number of these organisms cultivable from feces (fig. 3).

At the termination of the experiment determinations were also made of the numbers of lactobacilli, coliforms and enterococci in the cecum contents of the animals. For the most part the counts of the respective microrganisms in the cecum contents were of the same order of magnitude as those for the feces sampled at the same time. However, the number of lactobacilli in the cecum of the animals fed the hydrogenated coconut oil diet was about a log less than in the feces (fig. 1). This was apparently related to the diet since the increment of difference was similar in the presence or absence of the tail cups. In contrast, cecal coiliform counts (fig. 2) for the cupped animals were about 2 logs less than fecal counts with the fat-free diet and the corn oil diet and one log lower with the coconut oil diet. In the non-cupped animals the

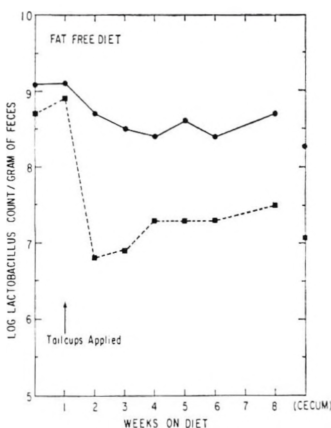

Figure 1A

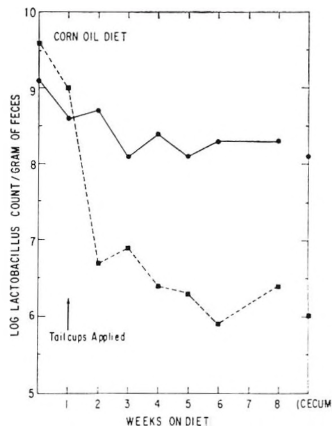

Figure 1B

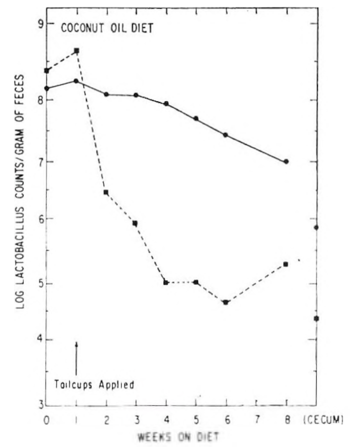

Figure 1C

Fig. 1 Effect of coprophagy prevention on lactobacillus counts. A. Fat-free diet; B. corn oil diet; C. coconut oil diet. ●——● indicates non-cupped; ■ - - - - ■ indicates cupped.

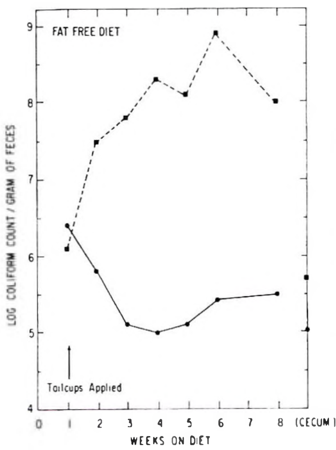

Figure 2A

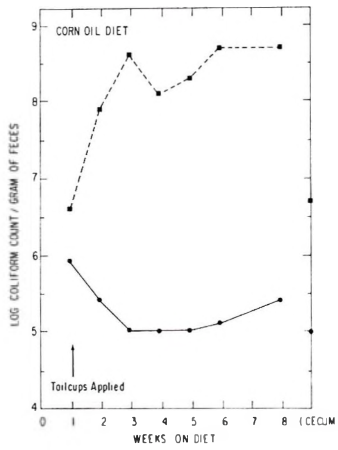

Figure 2B

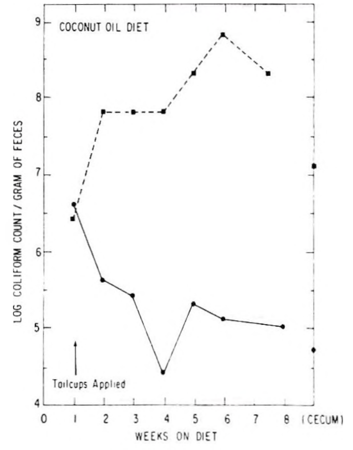

Figure 2C

Fig. 2 Effect of coprophagy prevention on coliform counts. A. Fat-free diet; B. corn oil diet; C. coconut oil diet. ●——● indicates non-cupped; ■ - - - - ■ indicates cupped.

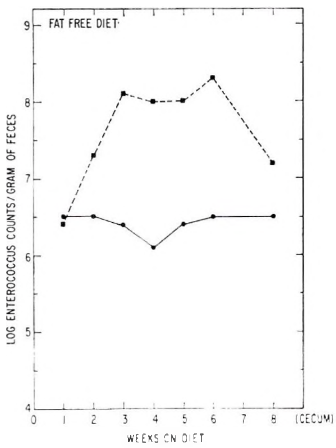

Figure 3A

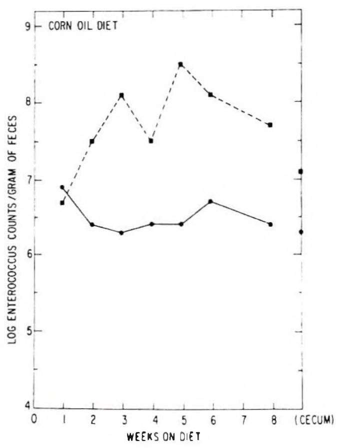

Figure 3B

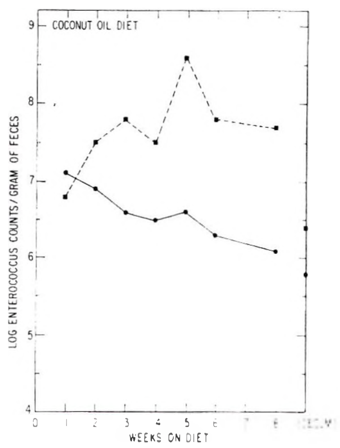

Figure 3C

Fig. 3 Effect of coprophagy prevention on enterococcus counts. A. Fat-free diet; B. corn oil diet; C. coconut oil diet. ●——● indicates non-cupped; ■ - - - - ■ indicates cupped.

cecal coliform counts were not significantly lower than the fecal counts. The relationship between cecal and fecal enterococcus counts appeared to be more uniform with the possible exception of the cupped animals fed the corn oil diet (fig. 3).

In no instance did the cecum counts exceed those of the feces, a fact which might have been anticipated because of the higher water content of the cecum content.

Compared with the effects of the tail cups, the type of diet used did not appear to have as strong an effect on the number of the organisms studied. In the non-cupped animals the lactobacilli showed a downward trend with time that was most pronounced in the case of the coconut oil diet. The number of enterococci was essentially unchanged with the fat-free and corn oil diets and only slightly reduced with the coconut oil diet. The coliform counts also showed a general downward trend with

time, but this did not appear to be especially affected by the diets in the noncupped animals.

In the animals in which coprophagy was prevented, the reduction in lactobacillus counts was greatest in the coconut oil group and least in the high starch, fat-free group. For the coliform counts the net increases were similar with all the diets but the rate of increase was greatest with the corn oil and least with the coconut oil diet. The enterococci showed a diphasic response in the cupped animals, an increase which reached a peak at 5 or 6 weeks after cupping, followed by a downward trend. At the end of the experiment the enterococcus counts in the cupped animals were similar in the coconut oil and the corn oil groups and only slightly lower in the fat-free group.

DISCUSSION

Prevention of coprophagy can lead to enhanced requirements for several micronutrients and even in the presence of all known required nutrients, animals in which coprophagy is prevented fail to grow as well as controls wearing sham tail cups (1). It has also been reported that voluntary food consumption is reduced in animals in which coprophagy is prevented but that the efficiency of food utilization is unchanged (1).

Observations of this type have usually been interpreted to mean that the animal derives some nutrient from the feces which is synthesized by the intestinal microflora at a site in the intestinal tract where it cannot be directly absorbed by the host. However, feeding of feces or cecum contents of normal rats does not necessarily reverse the growth depression observed in animals in which cophophagy is prevented. The possibility that prevention of coprophagy may influence the ecological balance of the intestinal microflora was suggested by observation (2) that the number of lactobacilli in the feces or cecum contents is greatly reduced in animals fitted with tail cups to prevent coprophagy. These observations were confirmed in the present study in which it has also been shown that the number of enterococci and coliform organisms increases markedly under the same conditions. It thus appears that coprophagy is an important factor in establishing and maintaining the composition of the intestinal microflora. In the absence of coprophagy some microorganisms, namely, the lactobacilli, may be unable to sustain themselves at "normal" levels in the intestinal tract, whereas other species may increase in number.

It has long been recognized that variations in the composition of the diet may also influence the number and type of microorganisms in the intestinal tract of experimental animals. In the present investigation the basal fat-free diet containing 22% casein and 74% starch and the high fat diets, containing 20% of corn oil or hydrogenated coconut oil, had generally similar effects on the number of lactobacilli, enterococci and coliform organisms of the lower gut of the control, uncupped, animals. The principal difference observed between the diets was a greater reduction in lactobacillus counts in animals receiving the coconut oil diet. The presence of the tail cups increased the difference between the lactobacillus counts of animals receiving the fat-free and coconut oil diets and caused a reduction in the number of lactobacilli in those receiving the corn oil diet. The observations in the present study clearly indicate 1) that coprophagy prevention was a far greater determinant in altering the balance of intestinal microflora than any of the diets used, and 2) that coprophagy prevention served to accentuate dietary-induced differences in the intestinal flora presumably because the factor of reinoculation was eliminated.

At the end of the experiment the animals were 12 weeks of age. Final body weights of the cupped animals were between 28 and 38% lower than those of the uncupped animals fed the same diet. These results are consistent with the observations of Barnes et al. (1) using a diet containing 25% casein. These authors have also shown that rats in which coprophagy was prevented for up to 16 hours daily still gained weight at a normal rate with no apparent difference between the time of day or night during which they had access to the feces. Rats allowed access to their

feces for only 2 or 4 hours daily did not gain weight at a normal rate.

It is thus apparent that complete prevention of coprophagy in rats has at least 2 consequences: 1) the animals do not exhibit normal weight gains, and 2) their intestinal microflora can undergo major alterations. Although it is tempting to assume that the 2 phenomena are interrelated, there is at present no definitive evidence to justify such an assumption. If a microbially produced growth-promoting agent is contained in feces, it apparently is not available to the animal unless the feces may pass directly from the anus to the mouth, for it has been reported (1) that opening the tail cups at their distal end to permit coprophagy only after the fecal pellets had passed through the tail cups did not restore the normal growth rate. Further studies will be required to determine the role of intestinal microorganisms individually and collectively in the nutrition of the host.

ACKNOWLEDGMENTS

The authors are grateful for the technical assistance of Richard Kogoc, William Mills and William Poole.

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Effect of Dietary α-Tocopherol on Protein Metabolism in Vitamin A-deficient Rats [1]

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ABSTRACT Rats maintained with a vitamin A-deficient diet were fed at 2 levels of α-tocopherol. Control animals were pair-fed. Higher dietary α-tocopherol caused an increase in the liver stores of vitamin A in the control animals. Vitamin A deficiency increased liver concentration of α-tocopherol and lowered serum albumin but increased globulins. In vitro incorporation of C^{14}-amino acids into diaphragm protein was significantly higher in the tissue of vitamin A-deficient rats fed at the lower α-tocopherol intake than in pair-fed controls. In contrast, the incorporation of C^{14}-amino acids into protein from diaphragm of vitamin A-deficient rats receiving the higher α-tocopherol intake was significantly lower than that of pair-fed controls. These changes in protein metabolism may be explained through the effect of vitamin A and E on membrane properties.

Previous studies by Vakil and Roels (1) have indicated that vitamin A deficiency markedly lowered serum albumin in rats and increased globulin levels. Dusheiko (2) reported an increased S^{35}-methionine incorporation into liver, kidney and serum proteins in rats fed a low protein and vitamin A-deficient diet. Chagovets and Dusheiko (3) also reported a decrease in the total serum protein in vitamin A deficiency. Harmon et al. (4) noted increased serum γ-globulins and decreased albumins without significant change in serum total proteins in swine fed a vitamin A-deficient diet. Edwin et al. (5) reported an increased liver α-tocopherol level in vitamin A-deficient rats. Diehl and Sanders (6) observed that vitamin E deficiency increased the incorporation of glycine-1-C^{14} into muscle protein of rabbits.

Since vitamin A deficiency influences the α-tocopherol status of the animal, and since both vitamins appear to affect protein metabolism, we have investigated the influence of different levels of dietary α-tocopherol on protein metabolism in vitamin A deficiency. This was studied by determining total and individual serum proteins in vitamin A-deficient rats fed at lower and higher levels of dietary α-tocopherol. The in vitro incorporation of C^{14}-amino acids into the proteins of diaphragm from the same rats was determined. The effect of different dietary levels of α-tocopherol on the liver storage of vitamins A and E in vitamin A-deficient rats and pair-fed controls is also reported.

EXPERIMENTAL PROCEDURE

Animals and diets. Twenty-one-day-old, male albino rats of the Sherman strain weighing between 40 and 44 g were housed in individual cages and maintained with the experimental diets. Littermates matched for weight formed the "pairs": the rat fed the vitamin A-deficient diet ate ad libitum and its littermate fed the control diet was pair-fed.

The major ingredients of the basal diet were (% weight): vitamin-free casein,[4] 18; glucose, 68; cellulose, 5; peanut oil, 5; salt mixture USP XIV, 4. The following vitamins were added to the basal diet (mg/kg): thiamine·HCl, 2; riboflavin, 4; pyridoxine, 4; choline, 1,000; inositol, 1,000; *p*-aminobenzoic acid, 300; nicotinamide, 100; folic acid, 2.5; vitamin B_{12}, 0.05; biotin, 0.1; ergocalciferol, 0.042; vitamin K, 10; Ca pantothenate, 10. The dietary levels of vitamin A and α-tocopherol

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[3] Williams-Waterman Fellow.

[4] Nutritional Biochemicals Corporation, Cleveland.

used in the 4 experimental groups are shown in table 1.

Each pair of animals was killed under light ether anesthesia after 24 hours' fasting, when the deficient rat had lost weight for 2 or 3 consecutive days. This usually occurred when the animals had consumed the diet for about 40 days.

Chemical Methods. Incorporation of C^{14}-amino acids into protein: To measure the incorporation of C^{14}-amino acids into muscle proteins, the method described by Wool and Krahl (7) was used, with the following modifications: one hemi-diaphragm from each rat was incubated with UL-C^{14}-phenylalanine. The concentration of UL-C^{14}- phenylalanine (140 mc/mg) in the incubation mixture was 1.2×10^{-5} M. The other hemi-diaphragm was incubated with 1×10^{-4} M methyl-C^{14}-methionine (9.3 mc/mg). These different concentrations were used to have one microcurie of radioactivity present in each test.

The dry protein sample obtained after incubation and washings, was dissolved in performic acid and an aliquot representing about 10 mg protein was plated on a planchet along with 10 mg of sucrose. This yielded a uniform layer on drying. The samples were counted in a windowless gas-flow counter. All counts were corrected for self-absorption.

Blood collected from the heart of the animals under light anesthesia was centrifuged and the serum was separated. Total serum protein concentration was determined by the biuret method described by Gornall et al. (8) and the serum proteins were fractionated by paper electrophoresis.[5]

Twenty per cent (w/v) liver homogenates were prepared in distilled water and aliquots were used for vitamin A, α-tocopherol and nitrogen determinations. Total nitrogen was determined according to the method described by Scales and Harrison (9).

The method of Roels and Trout (10) was used for the vitamin A determination, and that of Bieri et al. (11) for the α-tocopherol.

RESULTS

Growth and food intake. Table 2 shows the growth and the food efficiency ratio (weight gain g/food consumption g) of the different groups of rats.

Vitamin A and α-tocopherol

The liver stores of vitamin A and α-tocopherol are shown in table 3. No vitamin A was detectable in the livers of the deficient animals. Liver stores of vitamin A were significantly higher ($P < 0.001$) in the control rats receiving the high α-tocopherol intake (diet group AE$^+$, 172 µg vitamin A/g dry tissue) than in the control group receiving the lower level of dietary α-tocopherol (diet group AE, 123 µg vitamin A/g dry tissue). When the vitamin A levels were calculated per gram of fresh liver tissue or on a nitrogen basis, the same significant difference between vitamin A storage in the AE group and in the AE$^+$ group was observed. Table 3 also shows that the rats receiving 500 mg α-tocopherol/kg diet stored only twice as much α-tocopherol in their livers as those fed the 50 mg α-tocopherol/kg diet. The AE group stored 22.2% of its total dietary vitamin A in the liver against 33.3% in the AE$^+$ group ($P < 0.001$).

[5] Spinco Instruction Manual (Procedure B), Beckman Instrument Company, Spinco Division, Palo Alto, California.

TABLE 1

Dietary vitamin A and α-tocopherol in the various experimental groups of rats

	Pair-fed		Pair-fed	
	Deficient	Control	Deficient	Control
Diet group	– AE	AE	– AE$^+$	AE$^+$
	mg/kg of diet		*mg/kg of diet*	
Vitamin A [1]	0	1.72	0	1.72
α-Tocopherol [2]	50	50	500	500

[1] Vitamin A obtained from United States Pharmacopeia Reference Standards was used.
[2] Pure *dl*-α-tocopherol (Merck, U.S.) was used.

TABLE 2

Growth and food efficiency ratio of rats fed a complete or vitamin A-deficient diet with lower and higher level of α-tocopherol [1]

Diet group	Initial body wt	Total food intake	Final body wt		Food efficiency	
	g	*g*	*g*			
–AE	44.3	359.2	116.1		0.200	
				$P < 0.001$		$P < 0.001$
AE	41.8	359.6	136.7		0.264	
–AE+	43.9	350.1	118.5		0.213	
				$P < 0.01$		$P < 0.001$
AE+	41.0	342.7	133.5		0.270	

[1] Seven rats/group.

TABLE 3

Liver stores of vitamin A and α-tocopherol in the different experimental groups

Diet group	α-Tocopherol		Dietary α-tocopherol stored in liver	Vitamin A		Dietary vitamin A stored in liver
	No. of rats			No. of rats		
		μg/g dry liver tissue	%		*μg/g dry liver tissue*	%
–AE	7	135	0.62	27	—	—
		$P < 0.2$				
AE	7	107	0.60	27	123	22.2
–AE+	7	273	0.16	17	—	—
		$P < 0.05$				
AE+	7	189	0.13	17	172	33.3

Serum proteins. Table 4 illustrates the effect of vitamin A deficiency and different levels of dietary α-tocopherol on serum proteins. Vitamin A deficiency and different dietary levels of α-tocopherol did not influence serum total proteins. The percentages of α-, β- and γ-globulin fractions were significantly greater in the vitamin A-deficient animals fed higher dietary α-tocopherol, whereas a significant increase in the β- and γ-globulin fractions was noted in vitamin A-deficient rats receiving the lower level of α-tocopherol, without any significant change in the α-globulin fraction. The percentage albumin was significantly lower in both groups of vitamin A-deficient animals receiving low and high levels of α-tocopherol in their diet.

C^{14}-Amino acid incorporation in muscle protein. Table 5 summarizes the results of the incorporation of the C^{14}-labeled amino acids into diaphragm protein. The specific activity of the proteins of the "control" diaphragm was taken to be 100% and the specific activity of the "deficient" tissue was expressed as a percentage of its control.

Significantly higher C^{14}-amino acid incorporation into diaphragm protein was noted with both methyl-C^{14}-methionine and UL-C^{14}-phenylalanine in viamin A-deficient rats fed the lower dietary α-tocopherol. Significantly lower incorporation was observed with both the C^{14}-amino acids in vitamin A-deficient animals fed at the higher dietary α-tocopherol level.

The average incorporation of UL-C^{14}-phenylalanine in vitamin A-deficient rats fed at the lower level of dietary α-tocopherol was 386 count/min/mg protein, whereas that of the control animals was 288 count/min/mg protein. For methyl-C^{14}-methionine the incorporation in the deficient rat diaphragm was 152 count/min/mg protein compared with 107 count/min/mg protein in the controls. In contrast, the average incorporation of UL-C^{14}-phenylalanine in vitamin A-deficient rats fed at the higher level of dietary α-tocopherol was 345 count/min/mg protein and

TABLE 4

Serum proteins of vitamin A-deficient rats and pair-fed controls fed at different dietary levels of α-tocopherol

Diet	No. of pairs of rats	Total protein	Albumin	Globulin α-	Globulin β-	Globulin γ-
		g/100 ml	%	%	%	%
AE		6.1	69.1	11.5	11.1	8.0
	19	$P < 0.9$	$P < 0.001$	$P < 0.5$	$P < 0.001$	$P < 0.001$
−AE		5.9	61.0	11.1	13.3	14.3
AE+		6.3	69.7	8.9	11.4	9.9
	10	$P < 0.9$	$P < 0.001$	$P < 0.05$	$P < 0.01$	$P < 0.001$
−AE+		6.2	58.4	10.4	13.6	17.5
		Serum proteins of vitamin A-deficient rats as % of control				
		%	%	%	%	%
Lower vitamin E level	19	97 $P < 0.9$	88 $P < 0.001$	96 $P < 0.5$	118 $P < 0.001$	179 $P < 0.001$
Higher vitamin E level	10	98 $P < 0.9$	84 $P < 0.001$	117 $P < 0.05$	119 $P < 0.01$	177 $P < 0.001$

TABLE 5

Influence of vitamin A deficiency and dietary α-tocopherol on the in vitro C^{14}-amino acid incorporation into protein of rat diaphragm

Diet	No. of pairs of rats	C^{14}-amino acid used	Vitamin A-deficient as % of control	Significance of difference between control and deficient tissue
			%	
Lower vitamin E level	14	UL-C^{14}-phenylalanine	134 ± 11	$P < 0.01$
	18	methyl-C^{14}-methionine	142 ± 17	$P < 0.02$
Higher vitamin E level	20	UL-C^{14}-phenylalanine	74 ± 5	$P < 0.001$
	13	methyl-C^{14}-methionine	84 ± 11	$P < 0.05$

that of the control animals 473 count/min/mg protein. Methyl-C^{14}-methionine incorporation in the vitamin A-deficient rats fed the higher α-tocopherol was 417 count/min/mg protein compared with 497 count/min/mg protein for their pair-fed controls.

DISCUSSION

As expected, the food efficiency ratio was lower in vitamin A-deficient animals than in pair-fed controls. Although the vitamin A-deficient animal had the same food intake, the lower final body weight clearly reflected the lower food efficiency ratio due to vitamin A-deficiency. Different levels of α-tocopherol intake did not change this.

Many authors, Moore (12), Guggenheim (13) and others, have reported that dietary α-tocopherol increases tissue levels of vitamin A. This has been interpreted largely as a protective action (antioxidant effect) of α-tocopherol. We have confirmed that higher levels of dietary α-tocopherol increase liver vitamin A stores. Vitamin A deficiency significantly increased the liver concentration of α-tocopherol in the rats fed the high α-tocopherol diet compared with that of the controls. Vitamin A-deficient rats receiving the lower levels of dietary α-tocopherol also tended to store more α-tocopherol in their livers, although the difference was not statistically significant. Edwin et al. (5) fed a 100-mg α-tocopherol/kg diet to vitamin A-deficient rats and noted a 60% increase in the liver concentrations of α-tocopherol in the deficient animals compared with controls, but

these workers did not report the statistical significance of the differences they observed.

Since high levels of dietary α-tocopherol do not lead to proportionately higher tissue stores of this compound (a 10 times higher dietary intake led to a 2 times higher liver concentration) it appears that saturation levels were reached with the higher α-tocopherol intake, whereas this was not the case with the lower dietary level. The percentage of dietary α-tocopherol stored in the liver of the rats in the AE and − AE groups was 4 times higher than that of the animals receiving 10 times as much dietary α-tocopherol (AE^+ and $-AE^+$).

The dietary α-tocopherol level influenced serum protein metabolism both in the control and in the vitamin A-deficient groups: the AE group had a much higher α-globulin level than the AE^+ group, although there was no difference in serum total proteins between these 2 groups. The α-globulin level of the $-AE^+$ group was also higher than that of the AE^+ group, whereas there was no change in α-globulins due to vitamin A deficiency in the rats receiving the lower levels of dietary α-tocopherol (AE and − AE).

In an attempt to elucidate this interaction of vitamins A and E in protein metabolism, we studied the in vitro incorporation of C^{14}-amino acids into muscle protein of vitamin A-deficient rats and pair-fed controls fed at different dietary levels of α-tocopherol. In the tissues from the animals fed at the lower α-tocopherol level, the amino acid incorporation into diaphragm protein was significantly increased in vitamin A deficiency.

In contrast, the diaphragm of vitamin A-deficient rats fed at the high α-tocopherol levels incorporated significantly less C^{14}-amino acid into protein than that of pair-fed controls.

This observation might be explained through an increased permeability of the cell membrane in vitamin A deficiency: this increased permeability of the membrane might in turn change the intracellular free amino acid pool available for protein synthesis.

Dingle (14) has shown that vitamin A influences the stability of biological membranes, and Roxas et al.[6] observed an increased permeability or fragility of membranes of subcellular particles of liver tissue from vitamin A-deficient rats. Glauert et al. (15) have shown that α-tocopherol reverses the effect of vitamin A on cell membrane stability; this would tend to reduce the loss of amino acids from the intracellular pool. Brown and Morgan (16) reported negative nitrogen balance in vitamin A-deficient rats, indicating increased protein catabolism and Malathi et al. (17) observed increased free amino acid levels in tissues of vitamin A-deficient rats.

In all our experiments, the diaphragms were equilibrated in amino acid-free Krebs-Henseleit medium prior to the incubation in fresh medium in the presence of C^{14}-labeled amino acids. If the vitamin A-deficient rats receiving the "normal" tocopherol intake had increased cell membrane permeability, then this in vitro equilibration of the diaphragms in amino acid-free Krebs-Henseleit buffer might have depleted the in vivo existing intracellular free amino acid pool. When the thus depleted "equilibrated" diaphragm was then transferred to fresh Krebs-Henseleit medium containing the labeled amino acid, the relative concentration of the labeled amino acid would be considerably higher in the tissue from the deficient animals than in the controls.

In contrast, the decreased incorporation of the C^{14}-amino acid in the diaphragm of the rats receiving the higher α-tocopherol intake could be explained through a lesser penetration of the labeled amino acid into the cell and a greater dilution of the radioactive amino acid inside the cell, resulting from an increased intracellular free amino acid pool in the tissue of the vitamin A-deficient animal. If the higher dietary α-tocopherol had reversed the increased cell membrane permeability due to vitamin A deficiency, then the "equilibration" of the diaphragm in amino acid-free Krebs-Henseleit medium would not deplete the intracellular free amino acid pool markedly. This, combined with a higher intracellular free amino acid pool in the vitamin A-deficient animal compared with that of the control would result in the observed

[6] Roxas, B., M. Trout, G. L. Sessa, A. Guha and O. A. Roels 1964 The release of ribonuclease from a particulate fraction of vitamin A deficient rat liver. Federation Proc., 23: 293 (abstract).

lower specific activity in the diaphragms from the deficient animals.

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Microbiological and Enzymatic Assays of Riboflavin Analogues [1]

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ABSTRACT A number of riboflavin analogues, most of them recently synthesized, were examined with respect to their abilities to replace or antagonize the utilization of riboflavin by *Lactobacillus casei* and with respect to their substrate activities with flavokinase which catalyzes phosphorylation of riboflavin. Diethyl-, 6-methyl-, and 2′-deoxyriboflavin were shown to serve as sole sources of flavin for the growing microbe and were found to be phosphorylated in the flavokinase system. D-Erythroflavin, 6,7-dihaloriboflavins, and other flavins substituted only in position 6 or 7 are poor replacers of riboflavin for growth. Certain of these latter analogues, for example, D-erythroflavin which is poorly phosphorylated and the dihaloriboflavins which are phosphorylated moderately well, act as antagonists at high concentrations in the presence of riboflavin. Other analogues which are inactive as either vitamins or substrates for flavokinase, but antagonize the utilization of riboflavin by inhibiting its conversion to flavin mononucleotide, include 2′,3′,4′-trideoxyriboflavin and 6-methyl-7-aminolumiflavin.

Within a few years following the elaboration of the structure of riboflavin, a sizeable number of analogues were synthesized in the laboratories of Kuhn and Karrer for chemical and biological tests intended to circumscribe the behavior of this vitamin. The syntheses and testing of riboflavin analogues has continued and the basic structural requirements for biological activity as delineated by such investigations has been reviewed (1, 2). Interest has been renewed in the further syntheses and biochemical behavior of flavins with current attention focused on the mechanisms of formation of flavin coenzymes and the reactions of such coenzyme forms in flavoprotein catalyses. In particular, studies on the substrate specificity of flavokinase, which catalyzes the formation of flavin mononucleotide (FMN) from riboflavin and adenosine triphosphate (ATP), have led to the recent syntheses of several new riboflavin analogues (3, 4).

The present study describes the microbiological activities of these riboflavin analogues and their reactivities in the flavokinase system. The current observations extend considerably our understanding of the relation of the structure of riboflavin to its biological activity, particularly when interpreted together with results from contiguous investigations which have been made recently. These investigations include studies on the FMN specificity of flavin adenine dinucleotide (FAD) pyrophosphorylase, which catalyzes the conversion of FMN to FAD (5), and the coenzymatic functions of analogues of both FMN with reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome c reductase (6) and FAD with D-amino acid oxidase (7).

EXPERIMENTAL

Materials. Dichlororiboflavin (6,7-dichloro-9-(1′-D-ribityl)isoalloxazine) was supplied commercially.[2] 6-Methyl-7-aminolumiflavin (7-amino-6,9-dimethylisoalloxazine) was synthesized from 2-nitro-4-methylaminotoluene and violuric acid according to Hemmerich et al. (8). Similarly 7-aminoriboflavin (7-amino-9-(1′-D-ribityl)isoalloxazine) was prepared from *m*-nitroaniline and violuric acid. The 6-methoxy- and 6-ethoxyriboflavins (6-alkoxy-9-(1′-D-ribityl)isoalloxazines) were made as described previously (4) by condensation of barbituric acid with the

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[2] Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

corresponding 2-phenylazo-D-ribaminobenzenes by the procedure of Tishler et al. (9). A series or flavins prepared from the appropriately substituted *o*-nitroanilines and aldoses by the method of Kuhn et al. (10) include: D-erythroflavin (6,7-dimethyl-9-(1′-D-erythrityl)isoalloxazine), 2′-deoxyriboflavin (6,7-dimethyl-9-(1′-D-deoxyribityl)isoalloxazine), 6-methylriboflavin (6-methyl-9-(1′-D-ribityl)isoalloxazine), and diiodoriboflavin (6,7-diiodo-9-(1′-D-ribityl) isoalloxazine. Diethylriboflavin (6,7-diethyl-9-(1′-D-ribityl)isoalloxazine was similarly prepared as described by Lambooy (11). Dibromoriboflavin (6,7-dibromo-9-(1′-D-ribityl)isoalloxazine) and 2′,3′,4′-trideoxyriboflavin (6,7-dimethyl-9-(5′-hydroxypentyl)isoalloxazine) were synthesized via displacement reactions with the appropriate aminoalcohol and corresponding dinitrobenzes as described by Weygand et al. (12). 6-Aminoriboflavin (6-amino-9-(1′-D-ribityl)isoalloxazine) was prepared from 2,4-dinitrofluorobenzene as described previously (13) by displacement of HF with D-ribamine (14). The 6-methylpyridinoriboflavin (6-methyl-8(N)-pyrido-9-(1′-D-ribityl)isoalloxazine) was similarly synthesized via displacement of HCl from 2-chloro-3-nitro-5-methylpyridine with D-ribamine. As we are unaware of prior synthesis of this analogue, the following percentage composition is given for the monoacetate salt.

$C_{17}H_{21}O_8N_5$

Calculated:	C 48.2, H 5.0, N 16.5
Found:	C 48.1, H 5.1, N 16.1

The absorption spectrum which is observed with this compound exhibits maximal absorbancies near 265, 330 and 445 mμ which typify flavins. Both absorption and infrared spectra were obtained to characterize and establish adequate purities of all flavins used.

Flavokinase preparations from rat livers were prepared as reported previously (15). Specific activities of the preparations were from 30 to 35 mμmoles of FMN formed per milligram of protein per hour at 37°.

Methods. Microbiological assays were performed essentially as described by Snell and Strong (16) using *Lactobacillus casei e* 7469 ATCC and the Difco riboflavin assay medium. Titrametric determinations of lactic acid production in 10 ml of media were made after 72 hours of incubation at 37°.

Mixtures for assaying flavokinase activity contained, unless otherwise noted, 0.1 mM flavin, 1 mM ATP, 0.1 mM Zn^{++}, 75 mM potassium phosphate buffer (pH 8), and 1 mg of protein in 5 ml of total volume. Incubation was carried out in the dark, generally for 90 minutes. The reaction was terminated by the addition of 2 ml of 17.5% trichloroacetic acid. The flavin phosphate formed was determined by the differential extraction method of Burch et al. (17), as modified (18). In some instances, the products were applied to Whatman no. 1 paper, and the spots were observed under ultraviolet light after development of the chromatograms in ascending solvents. Secondary confirmation of phosphorylation, or lack of phosphorylation, and of purity of the flavins and flavin products was obtained in this manner (19).

RESULTS

L. casei assays. The microbiological assays of riboflavin analogues which can substitute moderately well for riboflavin are shown by the data in figure 1. For comparative purposes, diethylriboflavin and 6-methylriboflavin were included under the same assay conditions, since both of these analogues have previously been shown to serve as a sole source of flavin in the growth of *L. casei* (1). With all of

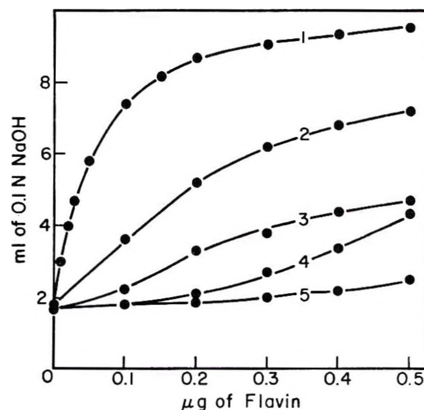

Fig. 1 Biological activities of riboflavin and analogues which support moderate growth of *L. casei*. Notations are: 1, riboflavin; 2, diethylriboflavin; 3, 6-methylriboflavin; 4, 2′-deoxyriboflavin; 5, D-erythroflavin.

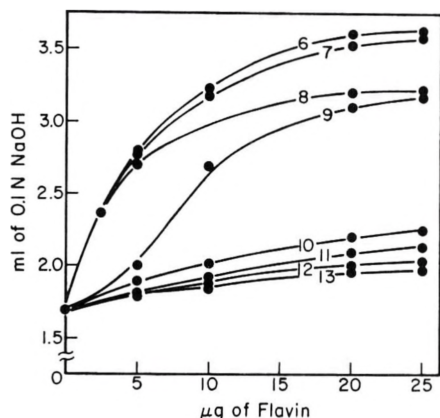

Fig. 2 Biological activities of riboflavin analogues which support poor growth of *L. casei*. Notations are: 6, dichlororiboflavin; 7, dibromoriboflavin; 8, 6-methylpyridinoriboflavin; 9, 6-methoxyriboflavin; 10, 6-iodoriboflavin; 11, 6-ethoxyriboflavin; 12, 6-aminoriboflavin; 13, 7-aminoriboflavin.

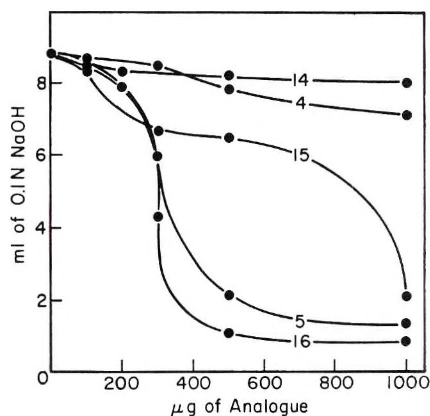

Fig. 3 Biological activities of riboflavin analogues which antagonize riboflavin in supporting growth of *L. casei*. Notations are: 14, diiodoriboflavin; 4, 2′-deoxyriboflavin; 15, 6-methyl-7-aminolumiflavin; 5, D-erythroflavin; 16, 2′,3′,4′-trideoxyriboflavin.

the analogues shown, growth of the microorganism is obtained in a reasonably low range of flavin addition. The relative order of riboflavin-replacing activity is thus: diethylriboflavin > 6-methylriboflavin > 2′-deoxyriboflavin > D-erythroflavin.

Those riboflavin analogues which exhibit only weak vitamin activity when added at high levels to the culture medium are shown by the data in figure 2. Again for comparative purposes, dichloroflavin, a well-known antagonist of riboflavin under conditions where both are supplied (1, 2) is included. Dichlororiboflavin, dibromoriboflavin, 6-methylpyridinoriboflavin, and 6-methoxyriboflavin exhibit poor riboflavin-replacing activities. The 6-iodo-, 6-ethoxy-, and 6-aminoriboflavins are nearly inactive, as is 7-aminoriboflavin.

The data in figure 3 illustrate the inhibition of growth elicited by several riboflavin analogues when added to medium containing riboflavin which is more than adequate for maximal growth when added alone. The 2′,3′,4′-trideoxyriboflavin is most potent as an antagonist, followed in decreasing potency by D-erythroflavin. D-Erythroflavin, which acts in moderate amounts as a partial replacer of riboflavin (cf. fig. 1), is a reasonably effective antagonist of riboflavin when the former is added in large excess over the natural vitamin. 6-Methyl-7-aminolumiflavin cannot be converted into functional coenzyme forms and acts only as a moderate antagonist to riboflavin. 2′-Deoxyriboflavin, which has greater vitamin activity than D-erythroflavin (cf. fig. 1), is a weak antagonist. Diiodoriboflavin is inactive as a vitamin and is very weakly antagonistic to riboflavin.

Flavokinase assays. The rates of conversion in vitro of riboflavin analogues as catalyzed by flavokinase to their corresponding FMN analogues are shown by the data in figure 4. Those other flavins

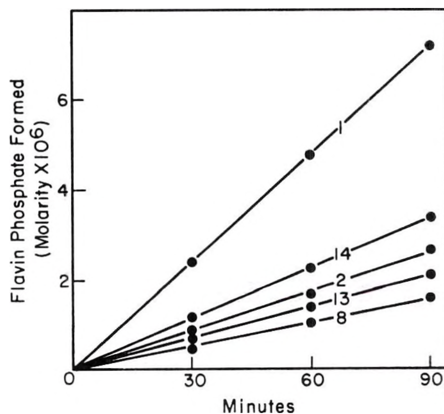

Fig. 4 Flavokinase-catalyzed phosphorylation of riboflavin and analogues as a function of time. Notations are: 1, riboflavin; 14, diiodoriboflavin; 2, diethylriboflavin; 13, 7-amino-riboflavin; 8, 6-methylpyridinoriboflavin.

which can elicit some growth of *L. casei* in the absence of riboflavin and therefore must necessarily be converted to functional coenzyme forms, have already been shown to serve as substrates for flavokinase (3, 4, 15).

The relations of the concentrations of flavin substrates to the rates of formation of flavin phosphates with flavokinase are shown by the Lineweaver-Burk plots presented in figure 5.

Comparative values calculated from data in figure 4 for the rates of the enzymatic phosphorylation of riboflavin and the riboflavin analogues and Michaelis constants (K_m values) calculated from the data in figure 5 are shown in table 1. Although diiodoriboflavin is phosphorylated slightly more rapidly than diethylriboflavin, the K_m value for the latter may suggest greater affinity of this flavin for the enzyme. A similar relationship also

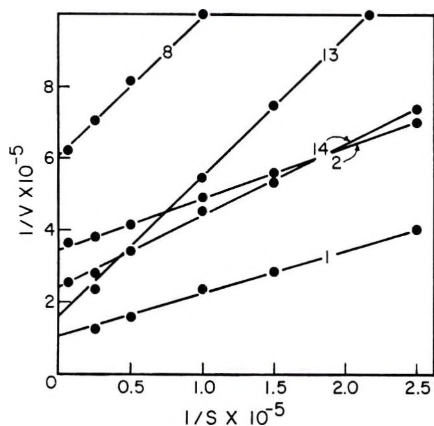

Fig. 5 Flavokinase-catalyzed phosphorylation of riboflavin and analogues as a function of flavin concentration. Notations are: 8, 6-methylpyridinoriboflavin; 13, 7-aminoriboflavin; 14, diiodoriboflavin; 2, diethylriboflavin; 1, riboflavin.

TABLE 1

Comparative phosphorylation and K_m values for riboflavin and analogues

Flavin	Flavin phosphate formed	K_m value
500 mμmoles/5 ml	*mμmoles/5 ml*	$M \times 10^5$
Riboflavin	45	1.2
6,7-Diiodoriboflavin	21	8.3
6,7-Diethylriboflavin	18	0.4
7-Aminoriboflavin	14	2.6
6-Methylpyridinoriboflavin	10	0.6

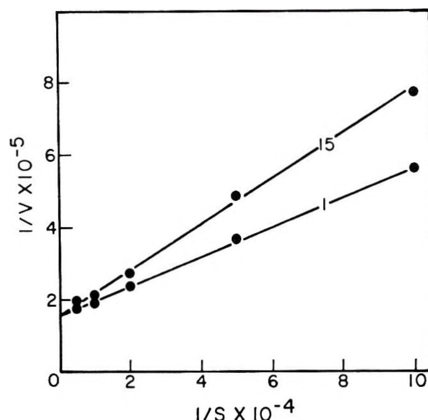

Fig. 6 Completive inhibition of flavokinase-catalyzed phosphorylation of riboflavin by 6-methyl-7-aminolumiflavin. Notations are: 15, 10^{-4} M 6-methyl-7-aminoriboflavin plus riboflavin; 1, riboflavin.

appears with 7-aminoriboflavin and 6-methylpyridinoriboflavin, both of which are relatively poor substrates for flavokinase.

6-Methyl-7-aminolumiflavin behaves as a competitive inhibitor of the phosphorylation of riboflavin as shown by the plots in figure 6. The K_i value calculated for this analogue is 4.0×10^{-5}M.

DISCUSSION

Sufficient information is now available to correlate the properties of most of the riboflavin analogues, both in vivo and in vitro. The particular biological behavior of any such analogue is a reflection of the composite of several factors which include permeability to the cell, reactivities with flavokinase and FAD pyrophosphorylase, and coenzymatic functions. In the light of the results described herein, together with previous investigations, the nutritional activities of those riboflavin analogues tested in this work can be reasonably well understood on a biochemical basis. Those few analogues which partially serve as a sole replacer of riboflavin in a growing organism must necessarily structurally resemble riboflavin to a close degree, e.g., diethylriboflavin, 6-methylriboflavin, and 2′-deoxyriboflavin. Those analogues which antagonize may act at one or more sites in the transformations of riboflavin to coenzymes and in the function of these coenzymes.

D-Erythroflavin is poorly phosphorylated in the flavokinase system, and the FMN analogue so formed is not readily converted to an FAD analogue with FAD pyrophosphorylase (5). However the redox potential of its coenzyme forms are approximately the same as the coenzymes of riboflavin, and D-erythroflavin-4′-phosphate has been found to be active with TPNH cytochrome *c* reductase from yeast (6). Overall, then, the most limiting reactions of D-erythroflavin are in the conversions to coenzyme forms, so that the microbiological activity of the flavin is more that of an antagonist at high concentrations than that of a vitamin at low concentrations.

Dichlororiboflavin is readily converted to coenzyme forms (5), but the more positive redox potential of these forms (4) does not permit adequate coupling of oxidation-reduction reactions normally served by FMN and FAD. The net result is again biological antagonism, but at the stage of flavin coenzyme function rather than formation. Similar behavior is noted with dibromoriboflavin which is slightly less potent, both as a replacer of and as an antagonist of riboflavin. The dibromo analogue is not phosphorylated quite so readily as dichlororiboflavin and has a slightly more unfavorable redox potential. Thus its coenzyme forms are even more poorly functional, but smaller quantities are formed. Diiodoriboflavin, another member in this series, is essentially inactive for the same reasons, except that conversions to coenzymes and their functions are even more limiting.

Lack of a 7-methyl substituent markedly decreases the vitamin activity of flavins mainly because of lessened conversion to coenzyme forms (3. 4). A comparison of 6-methylriboflavin with 6-methylpyridinoriboflavin additionally suggests impaired coenzymatic functions of the latter as accounting for its much weaker vitamin activity, as both analogues are converted to FMN analogues about equally well.

Both 6-methyl-7-aminolumiflavin and especially 2′,3′,4′-trideoxyriboflavin are antagonists to the biological utilization of riboflavin. Neither of these analogues is converted to coenzyme forms, but they do inhibit the conversion of riboflavin to FMN.

Finally, some of the complications should be noted. Results on the assays of flavins with different analogues do not appear to agree. For instance, Lambooy and Aposhian have shown that diethylriboflavin has an antiriboflavin activity in the growing rat (20). Beinert has pointed out some of the difficulties involved (2). Some compounds are active only in the presence of suboptimal amounts of riboflavin, whereas others show stimulation at low and inhibitions at high levels. In certain instances, any complete attempt to correlate biological activity of a flavin must await further examination of those other systems involved.

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Activity of Amino Acid-activating Enzymes in Tissues from Protein-depleted Rats [1]

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ABSTRACT The activity of amino acid-activating enzymes in tissues from protein-depleted rats was tested by the $PP_i - P^{32} \rightleftharpoons ATP$ exchange reaction. Previously we had found that following protein depletion and in concomitance with a greater incorporation of amino acids, the activity of the liver amino acid-activating enzymes significantly increased. Presently the aim was to test the possible generalization of the above by investigating whether the activity of the amino acid-activating enzymes in various tissues of protein-depleted rats would vary in a manner analogous to the differences reported for amino acid incorporation in these tissues. The results, besides confirming that in protein depletion the liver amino acid-activating enzymes increase when tested in the presence of the complete mixture of amino acids or in the presence of individual amino acids, have also shown that in heart and gastrocnemius muscle the level of these enzymes is less than normal, in agreement with the decreased amino acid incorporation in these organs. A general hypothesis was thus set up that, in tissues from protein-depleted animals, the amount of protein synthesis is regulated through modifications of the level of amino acid-activating enzymes.

The level of amino acid-activating enzymes, as measured by their activity in the presence of 4 added amino acids, increases in the liver of protein-depleted rats (1).

In previous studies it was also noted that protein-depleted animals incorporate into the proteins of liver relatively more lysine-C^{14} (2) and methionine-S^{35} (4–6) than control animals.

A possible interpretation of these observations might be that the amount of protein synthesis in the liver of protein-depleted animals is regulated through modifications of the level of the amino acid-activating enzymes, which catalyze the first step of protein synthesis.

The present research was planned to verify the validity and possible generalization of the above, extending the studies made on liver to other tissues, such as heart and skeletal muscle, where, in a condition of protein depletion, less incorporation of the label, as compared with control animals, had been observed (2–4). Correspondingly, a lesser activity of the amino acid-activating enzymes was to be expected in those tissues if the above hypothesis were correct.

In addition, the liver amino acid-activating enzymes were further investigated and tested, in the presence of the complete mixture of amino acids and in the presence of individual amino acids, to determine whether they would increase uniformly following protein depletion. Assays were also made of liver mitochondrial-activating enzymes.

The results showed that a positive correlation between the amount of protein synthesis, measured as amino acid incorporation in vivo (2–6), and the activity of the amino acid-activating enzymes exists in all tissues examined.

EXPERIMENTAL

Liver, heart and gastrocnemius muscle from adult male rats, of Wistar strain, were used. The rats were fed ad libitum a protein-free diet, or a 20% casein diet, supplemented with 0.3% DL-methionine. The rats were maintained with the experimental diet for 30 to 60 days, as the amino acid-activating enzymes have been shown to exhibit the highest activity at this stage of protein depletion (1).

Reagents. All chemicals were of analytical reagent grade. Solutions were adjusted to pH 7.4. Radioactive inorganic pyrophosphate-P^{32}, adenosine triphosphoric acid disodium salt (ATP-Na_2 salt) and

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L-amino acids were obtained commercially.[2] None of the amino acids, when checked for purity by 2-dimensional paper chromatography, showed any additional spots. Activated charcoal [3] was washed with 5% trichloroacetic acid, then with distilled water until the washings were neutral, and dried in an oven.

Enzyme preparations: liver, heart and gastrocnemius muscle supernatant fraction. The animals were decapitated. Liver, heart, and gastrocnemius muscle from the hind legs, were quickly removed and placed in an ice-cold solution of 0.05 M KCl and 0.02 M Tris (2-amino-2-hydroxymethylpropane-1:3-diol, adjusted to pH 7.4 with HCl). Heart and gastrocnemius muscle were finely chopped with scissors. Homogenation was performed in a motor-driven Potter homogenizer with a Teflon pestle, in Tris-KCl buffer (pH 7.4). Suitable aliquots of the homogenate were taken for DNA analysis. The homogenates were then centrifuged in an M.S.E. "superspeed 40" centrifuge at 105,000 × *g* for one hour. Six milliliters of the liver supernatant fraction were passed through a column, 2 × 25 cm, of Sephadex G–25 (medium size, previously equilibrated with Tris-KCl buffer) [4] to decrease the content of endogeneous amino acids, as suggested by Bucovaz and Davis (7). The head and the tail of the eluate were discarded and a central portion of about 4 ml, which had the highest exchange activity and protein concentration, was used for the enzyme assay. Following treatment of liver supernatant with Sephadex, an exchange of inorganic pyrophosphate-P^{32} labeled (PP_i-P^{32}), with ATP in the absence of added amino acids occurred to the extent of about 0.38 μmoles/hr/mg protein.

Gastrocnemius and heart supernatant were treated with Carbowax,[5] as suggested by Pennington (6), since preliminary testing had shown that by this method a greater amino acid-dependent exchange activity was obtained (threefold that of the endogenous exchange), than with Sephadex treatment (twofold that of the endogenous exchange). The amino acid-dependent exchange in liver (fourteen- and sixfold that of the endogenous exchange for protein-depleted and normal rats, respectively) was not significantly improved by Carbowax treatment; therefore the more rapid Sephadex treatment was adopted. Suitable aliquots of the treated supernatant fractions were used for assay of the amino acid-activating enzymes and for protein determination.

Liver mitochondrial extract fraction. This fraction was prepared from a 10% homogenate in 0.25 M sucrose, adjusted to pH 7.4. Suitable aliquots of the homogenate were used for DNA analysis. After removal of nuclei and cell debris, the mitochondria were sedimented, washed 3 times and the final pellet was taken up in a very small volume of ice-cold 0.25 M sucrose. The mitochondria were disrupted according to the method of Truman and Korner (9). The resulting material was centrifuged at 1,500 × *g* for 10 minutes and the supernatant passed through a small column of Sephadex G-25. Suitable aliquots were then used for assay of the amino acid-activating enzymes and for protein determination.

Assay method for amino acid-activating enzymes. The enzyme activity was determined by measuring the rate of exchange between PP_i-P^{32} and ATP, according to DeMoss and Novelli (10), in the absence of added amino acids (endogenous exchange) and in the presence of the complete mixture of amino acids, or in the presence of the individual amino acids. The incubation mixture of 1 ml final volume contained: (in μmoles) Tris-KCl buffer (pH 7.4), 100; ATP-disodium salt, 10; KF, 50; $MgCl_2$, 10; pyrophosphate-P^{32} (about 100,000 count/min), 10; amino acid(s), 4 of each; and appropriate amounts of enzyme preparations. The mixture was incubated at 37° for 15 minutes and the reaction was stopped by the addition of 2 ml of 5% trichloroacetic acid. Each incubation was made in duplicate or in triplicate. The ATP was separated by adsorption on charcoal according to a procedure described by DeMoss and Novelli (10). The adsorbed ATP was hydrolized with 4 ml of

[2] Inorganic pyrophosphate-P^{32} was purchased from the Radiochemical Centre, Amersham, Bucks, England; ATP-Na_2 salt and L-amino acids were purchased from the Nutritional Biochemicals Corporation, Cleveland.

[3] Carbo Activatus, Merck, Darmstadt.

[4] Sephadex-G25, medium size, Pharmacia, Uppsala, Sweden.

[5] Carbowax 20 M (polyethylene glycol), G. T. Gurr Ltd., 136 New Kings Road, London S.W.6, England.

N HCl for 15 minutes, in a boiling water bath. The mixture was filtered and the phosphorus-P^{32} activity of 0.1-ml aliquots of the filtrate were measured in a gas-flow, end-window counter.

Analysis. Inorganic phosphate was determined according to Fiske and Subba-Row (11). Supernatant and mitochondrial proteins were determined by the biuret method, as modified by Cleland and Slater (12). The DNA was determined by the method of Webb and Levy (13).

RESULTS

Liver supernatant fraction. The activity of the amino acid-activating enzymes, tested in the presence of the complete mixture of 20 amino acids, is shown in table 1. The results, calculated according to DeMoss and Novelli (10), are expressed in terms of micromoles of exchange per hour per milligram of protein supernatant and, also, in terms of micromoles of exchange per hour per microgram of deoxyribonucleic acid-phosphorus (DNA-P). Since in mature rats the concentration of DNA per average cell has been found to be constant during protein depletion, the results so expressed can be more conveniently used to compare the enzymic activity between normal and protein depleted animals.

If enzyme activity is taken as a measure of enzyme concentration, table 1 shows that protein depletion causes a marked increase of the amino acid-activating enzyme concentration in the liver. The percentage increase of exchange is highly significant, in spite of variability between experiments, both when the rate of PP_i–$P^{32} \rightleftharpoons$ ATP exchange is expressed in terms of supernatant protein ($P < 0.001$) or when it is expressed in terms of DNA-P content ($P < 0.05$). This indicates that the observed increase is real, and not secondary to a preferential loss of nonspecific proteins in the soluble fraction of liver cells.

In figure 1 are shown the results obtained when the amino acids were tested individually. In all cases an increased $PP_i \rightleftharpoons$ ATP exchange rate, as a consequence of protein depletion, was observed. The amino acids did not stimulate the increase uniformly; however, in consideration of the extreme lability of some of the amino acid-activating enzymes, it is difficult to ascribe a biological meaning to this lack of uniformity.

Liver mitochondrial extract fraction. The observed increase of amino acid-activating enzymes in the liver supernatant of protein-depleted rats may be explained as a *de novo* synthesis of enzyme molecules, or as an activation of preexisting enzymes. However, the possibility cannot be excluded that protein depletion might damage the organizational structure of mitochondria, where the presence of amino acid-activating enzymes has been demonstrated (14), with consequent release of such enzymes in the soluble fraction. To obtain some indication as to the content of amino acid-activating enzymes in mitochondria from protein-depleted rats, extracts of mitochondria were tested for amino acid-activating enzyme activity.

Table 2 shows that the amino acid dependent PP_i–$PP^{32} \rightleftharpoons$ ATP exchange catalyzed by mitochondrial extracts from pro-

TABLE 1

Effect of protein depletion on the activity of rat liver amino acid-activating enzymes [1]

Diet	PP_i – $P^{32} \rightleftharpoons$ ATP exchange [2]	Increase	PP_i – $P^{32} \rightleftharpoons$ ATP exchange	Increase
	μmoles/hr/mg protein	%	*μmoles/hr/μg DNA-P*	%
Control	2.20 ± 0.35 [3] (5)[4]		0.76 ± 0.10 (5)	
Protein-free	5.32 ± 0.43 (6)	142	1.48 ± 0.29 (6)	95

[1] Conditions: 0.2 ml enzyme preparation (between 2.1 and 4.3 mg supernatant protein in each incubation mixture); 100 μmoles Tris-KCl buffer (pH 7.4); 50 μmoles KF; 10 μmoles ATP, disodium salt, (adjusted to pH 7.4); 10 μmoles pyrophosphate-P^{32}; 10 μmoles $MgCl_2$; complete amino acid mixture (adjusted to pH 7.4), 4 μmoles of each of the following amino acids; alanine, arginine, aspartic acid, cysteine, cystine, glycine, glutamic acid, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine. Cystine and tyrosine did not dissolve completely. Total volume 1 ml. Incubated at 37° for 15 minutes.

[2] Inorganic pyrophosphate-P^{32} labeled; net exchange (exchange in presence of added amino acids minus endogenous exchange) calculated for each experiment and here arithmetically averaged.

[3] SE of the mean.

[4] Numerals in parentheses indicate the number of experiments, each carried out with tissue pooled from 4 rats.

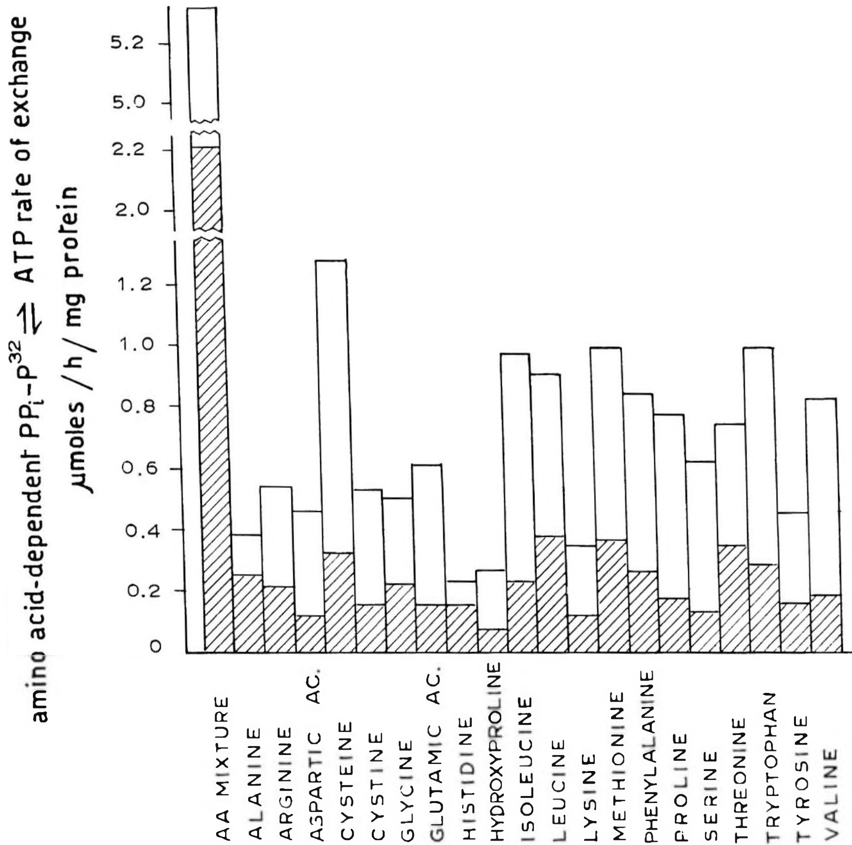

Fig. 1 Activity of individually tested amino acid-activating enzymes in normal and protein-depleted rat liver. Assay conditions as in table 1. Between 2.1 and 4.3 mg supernatant protein and 4 μmoles of each individual amino acid were present in each incubation mixture. The shaded bars represent the amino acid-dependent rate of exchange in normal rats, the open bars the increase over normal observed in protein-depleted rats. Each bar represents the average of 4 experiments, each carried out with tissue pooled from 4 rats. Net exchange (exchange in the presence of added amino acids minus endogenous exchange) calculated for each experiment and here arithmetically averaged.

TABLE 2

Effect of protein depletion on the activity of rat liver mitochondrial amino acid-activating enzymes [1]

Diet	$PP_i - P^{32} \rightleftharpoons$ ATP exchange		$PP_i - P^{32} \rightleftharpoons$ ATP exchange	
	Exp. 1 [2]	Exp. 2	Exp. 1	Exp. 2
	μmoles/hr/mg protein [3]		*μmoles/hr/μg DNA-P* [3]	
	2	2	2	2
Control	0.47	0.29	1.21	1.21
Protein-free	1.80	0.61	2.79	3.43

[1] Assay conditions as in table 1. Between 0.6 and 2.0 mg protein were present in each incubation mixture.
[2] Each numbered experiment was carried out with tissue pooled from 4 rats.
[3] Net exchange (exchange in presence of added amino acids minus endogenous exchange) calculated for each experiment and here arithmetically averaged.

tein-depleted rats is more than twofold that of control rats. However these results do not establish whether the observed increase is real, or is simply due to a greater ease of extraction of these enzymes from the mitochondria of protein-depleted rats. The concentration of amino acid-activating enzymes in mitochondria is so low, if expressed in terms of the DNA-P content, that even in the case of passage of these enzymes from mitochondria to the soluble fraction they would contribute only negligible amounts to the total high quantity observed in the liver supernatant.

Heart and gastrocnemius muscle supernatant fraction. In table 3 are reported data relative to the activity of the amino acid-activating enzymes in heart and gastrocnemius muscle supernatant.

When the results are expressed in terms of supernatant protein content, no significant difference is observed between protein-depleted and control animals after feeding the protein-free diet for either 30 or 60 days. When the results are expressed in terms of DNA-P content, therefore in terms of average cell, it is possible to show a significant decrease of enzyme concentration in the supernatant from protein-depleted rats (heart, $P < 0.05$; gastrocnemius, $P < 0.001$).

The decrease is of the same order of magnitude after feeding the protein-free diet for either 30 or 60 days. These results indicate that protein depletion, in contrast with what happens in the liver, causes in the heart and gastrocnemius muscle a parallel loss of amino acid-activating enzymes and of nonspecific soluble proteins.

DISCUSSION

The results obtained have clearly shown the existence, in a condition of protein depletion, of a positive correlation between the amount of protein synthesis, measured as amino acid incorporation into living animals (2–6), and activity of amino acid-activating enzymes in liver, heart and gastrocnemius muscle.

In fact, where a higher incorporation of the label, relative to control animals, had been observed, as for liver (2, 4–6), the level of amino acid-activating enzymes has been observed to increase markedly. Where the incorporation of the label was less than normal, as for heart and gastrocnemius muscle (2–4), the level of amino acid-activating enzymes was observed to be less than normal.

The demonstration of the dynamic way in which the level of amino acid-activating enzymes varies, correlated with the previously measured quantity of protein synthesis in organs with diversified metabolic characteristics and needs, appears to support the validity and postulated generalization of the hypothesis that the amount of protein synthesis in protein-depleted animals is controlled by the level of amino acid-activating enzymes.

As for the nature of the specific stimulus which induces the observed modifica-

TABLE 3

Effect of protein depletion on the activity of rat heart and gastrocnemius muscle amino acid-activating enzymes [1]

Diet	Heart		Gastrocnemius muscle	
	$PP_i - P^{32} \rightleftharpoons ATP$ exchange [2]		$PP_i - P^{32} \rightleftharpoons ATP$ exchange [2]	
	µmoles/hr/mg protein	*µmoles/hr/µg DNA-P*	*µmoles/hr/mg protein*	*µmoles/hr/µg DNA-P*
Fed for 30 days:				
Control	1.01 ± 0.089 [3] (4)[4]	0.31 ± 0.032 (4)	0.51 ± 0.065 (4)	0.64 ± 0.048 (4)
Protein-free	0.91 ± 0.027 (4)	0.24 ± 0.010 (4)	0.50 ± 0.064 (4)	0.41 ± 0.025 (4)
Fed for 60 days:				
Control	0.96 ± 0.086 (4)	0.31 ± 0.031 (4)	0.45 ± 0.036 (6)	0.64 ± 0.048 (6)
Protein-free	1.09 ± 0.038 (4)	0.25 ± 0.011 (4)	0.54 ± 0.052 (6)	0.51 ± 0.058 (6)

[1] Assay conditions as in table 1. Between 1.6 and 1.9 mg supernatant protein were present in each incubation mixture.
[2] Net exchange (exchange in presence of added amino acids minus endogenous exchange) calculated for each experiment and here arithmetically averaged.
[3] SE of the mean.
[4] Numerals in parentheses indicate the number of experiments, each carried out with tissue pooled from 4 rats.

tions of the level of amino acid-activating enzymes, the most direct effect of protein depletion is a decrease of the total pool of free amino acids. The crucial regulatory factor governing the activity or the synthesis or both, of the amino acid-activating enzymes could thus be represented by the size of the free amino acid pool. Assuming that the increased activity of the amino acid-activating enzymes reflects an increase of enzyme population, it is possible to postulate that when the total pool of free amino acid decreases, the enzyme-forming system reacts by increasing the size of amino acid-activating enzyme population.

This tentative hypothesis may eventually apply only to liver, because heart and gastrocnemius muscle have been shown to react in an opposite manner to the decrease of the free amino acid pool. A unique behavior of liver might be expected, considering the peculiar metabolic and physiological characteristics which markedly differentiate liver from other body tissues. Thus liver might have retained, or acquired, some mechanism that provides for emergency needs for protein synthesis in a manner similar to that reported for regenerating liver (15, 16).

A possible solution to the problem would be to propose that, in protein depletion, the postulated mechanism of regulation of the amino acid-activating enzyme population, which we assume to be controlled by the fluctuations of the free amino acid pool, is operating only in liver, and not in heart and gastrocnemius muscle. In the latter organs the amino acid-activating enzymes would behave in a nonspecific fashion, as shown by the fact that they decrease together with other soluble proteins.

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Overcoming the Inhibition of Intestinal Proteolytic Activity Caused by Raw Soybean in Chicks of Different Ages [1,2]

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ABSTRACT The response of the chicks to transfer from a heated soybean diet (HSD) to a raw soybean diet (RSD) was studied at different ages from one to 6 weeks. The proteolytic activity in the small intestine, nitrogen content of intestinal chyme and pancreas hypertrophy were investigated. An initial inhibition of proteolytic activity was observed at all ages studied. The ability to overcome the inhibition when examined between 1 to 6 weeks generally appears to increase with age. Increase in pancreas weight was noticed after the raw soybean diet had been fed for even one day. Nitrogen content of the chyme was higher in chicks with the RSD than with the HSD indicating an interference of the RSD with normal protein digestion. The study with underprocessed soybean diet showed intermediate results between the HSD and RSD. The probable factors responsible for the rate and length of inhibited proteolytic activity in the small intestine are discussed.

Growth depression occurring in chicks fed a raw soybean diet has been studied extensively and the reports reviewed (1). The relationship between the presence of antitrypsin in raw soybean and the inhibited growth is still to be established.

It was shown in previous work (2) that when raw soybean was fed to chicks from the first days of life, it inhibited the proteolytic activity in the small intestine. The inhibition disappeared when the chicks reached the age of 6 weeks.

Hypertrophy of pancreas was observed from the earliest days of life and persisted in the chicks fed the raw soybean diet also at the age when normal proteolytic activity was almost completely restored. Chernick et al. (3) and Lyman and Lepkovsky (4) observed enlarged pancreas containing excessive amounts of proteolytic enzymes in chicks and rats fed a raw soybean diet. The increased pancreatic secretion could be a means of overcoming the inhibition of proteolytic enzymes in the intestine.

Both the inhibited proteolytic activity and the hypertrophy of the pancreas appear to be involved in the growth depression of chicks fed a raw soybean diet.

In a recent study Bornstein and Lipstein (5) reported that the growth depression accompanies the transfer to a raw soybean diet at any age of the chicks, but the older chicks appear to overcome the inhibiting action of raw soybean more rapidly.

Saxena et al. (6) reported that the growth-inhibiting effect of a raw soybean diet decreased as the age at which the chicks were transferred to the raw soybean diet increased, and ceased at 6 weeks of age. Pancreatic hypertrophy was evident up to 12 weeks of age.

It was the purpose of this study to establish (a) whether the inhibition of proteolytic activity in the small intestine occurs only in very young chicks, or whether it is related to the transfer to a raw soybean diet at any age, and (b) whether the length of the period required for overcoming the inhibition of proteolytic activity changes with the age at which chicks start to be fed the raw soybean diet.

EXPERIMENTAL

The diets used for control and experimental chicks consisted of an all-vegetable ration (21% protein) that contained 30%

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heated, raw or underprocessed soybean meal, respectively. Two trials were carried out with raw soybean meal and one with underprocessed soybean meal (commercial meal passed through a horizontal desolventizer for about 40 minutes at approximately 98° without addition of moisture). The processing of the raw and heated soybean meals used and the diet composition are described in a previous paper (2).

One hundred white Leghorn male chicks were raised with a heated soybean diet up to one week of age. At that time, and at 2, 3, 4 and 6 weeks of age, 12 chicks were transferred each time to a separate compartment where they were fed the raw soybean diet. The food was removed for the night and returned in the morning to insure intensive food consumption of all chicks at the same time, so that the intestines would contain approximately the same amounts of chyme (e.g., the weights of the intestinal contents at 4 weeks of age were: (g/chick) 3.7 in control chicks and 4.5, 3.6, 3.8 and 4.1 after 1, 2, 4 and 8 days, respectively, with the raw soybean diet). Chicks fed the raw soybean diet consumed 80 to 90% of the feed consumed by control chicks. In the first 2 hours 40% of the daily intake was consumed by both groups.

At one, 2, 4 and 8 days after the transfer to the raw soybean diet, 3 chicks were killed 2 hours after they had started eating in the morning. Three control chicks fed the heated soybean diet were killed at each age, on the first day of each experimental period.

The following measurements were performed on experimental and control chicks: proteolytic activity and nitrogen content of the chyme of the small intestine, pancreas weight, its dry matter and nitrogen content. The proteolytic activity was determined as follows. The intestinal chyme from the posterior end of the duodenum up to the juncture with the cecum was squeezed out and the mucus separated. The chyme was homogenized with 10 volumes of distilled water in an Ultra-Turrax apparatus. The proteolytic activity of the homogenate was determined by the Kunitz method (7), the extent of casein digestion being expressed as the increase of the optical density at 280 mμ against a blank made at zero time. Dry matter was determined by drying at 105° overnight. Total nitrogen was determined by the Kjeldahl method.

The degree of toasting of the soybeans was established by Fröhlich's cresol-red absorption test (8). The raw soybean meal absorbed 2.7 mg cresol-red/g, the underprocessed 3.0 mg/g, and the heated meal, 3.8 mg/g.

RESULTS

The proteolytic activity in the small intestine of chicks that were transferred to the raw soybean diet at different ages, at various times after starting the new diet, is shown in figure 1.

At all ages at which the chicks were examined immediately after transferring to the raw soybean diet, there was a marked inhibition of the proteolytic activity in the small intestine.

In one-week-old chicks, the inhibition lasted practically throughout the 8 days of the experiment. Even though some beginning of proteolytic activity could be observed at the eighth day, the curve was low in comparison with that of the controls.

When the chicks' feed was changed to the raw soybean diet at the age of 2 weeks, they were able to overcome the inhibitory factors more rapidly and on the eighth day of the experiment the enzyme curve was normal. In the following week, overcoming of the inhibitory factors occurred even more quickly, and normal proteolytic activity, such as that shown by the control chicks, was reached on the fourth day.

Four-week-old chicks showed slower adaptation to the raw soybean diet. The enzyme curves, even though normal in shape, had not reached control levels by the eighth day.

At the age of 6 weeks, normal proteolytic activity was restored between the second and the fourth day after starting the raw soybean diet.

The proteolytic activity in the small intestine of chicks that were transferred to the underprocessed soybean diet at various ages, is shown in figure 2.

When the chicks were transferred to underprocessed soybean diet they over-

Fig. 1 Proteolytic activity in the small intestine of chicks fed the heated soybean diet (HSD) and chicks transferred to the raw soybean diet (RSD) at various ages. The numbers at the curves indicate the days after transfer to the raw soybean diet. The curves represent averages of 6 chicks.

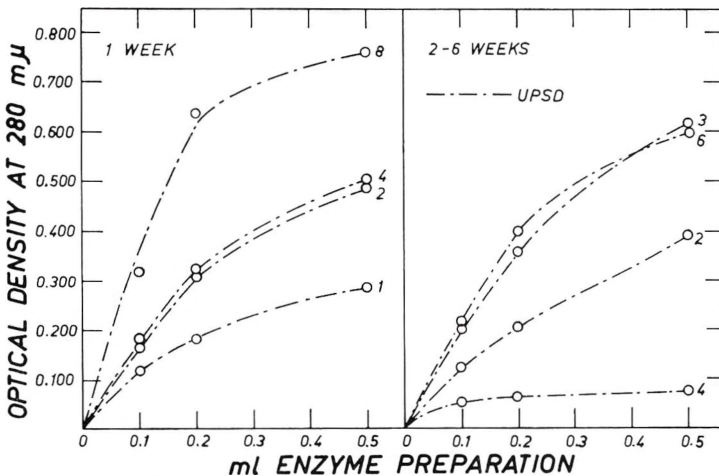

Fig. 2 Left, proteolytic activity in the small intestine of one-week-old chicks fed the underprocessed soybean diet (UPSD). The numbers at the curves indicate the days after transfer to this diet. Right, proteolytic activity in the small intestine of chicks 2 to 6 weeks old on the first day after transfer to the underprocessed soybean diet. The numbers at the curves indicate the age in weeks at which the chicks were transferred to this diet.

TABLE 1

Pancreas weight-to-body weight ratio in chicks fed the heated soybean diet compared with that for chicks transferred to the raw soybean and underprocessed soybean diets at various ages

Age in weeks	Pancreas weight-to-body weight ratio [1]					L.S.D. (at $P = 0.05$)
	Heated soybean diet	Days following transfer from heated soybean diet				
		1	2	4	8	
	Raw soybean diet					
1	0.42 [1]	—	0.52	0.60	—	0.10
2	0.43	0.66	0.55	0.76	—	0.20
3	0.48	0.67	0.60	0.53	0.69	0.16
4	0.38	0.44	0.47	0.54	0.63	0.15
6	0.35	0.42	0.40	0.46	0.36	0.05
	Underprocessed soybean diet					
3	0.57 [2]	0.63	0.75	0.78	0.59	0.06
4	0.43	—	0.45	0.56	0.63	0.11

[1] Pancreas weight(g)/100 g body weight. The numbers are averages of 6 chicks from the 2 trials.
[2] Averages of 3 chicks.

came the inhibited proteolytic activity in the intestine much more rapidly than did those fed raw soybean diet. At the ages of 2 to 6 weeks the chicks reached a level of proteolytic activity similar to that of control chicks 2 days after they had been transferred to the underprocessed soybean diet. Also in this experiment the 3-week-old chicks showed greater ability to overcome the inhibitory factors than did the 4-week-old ones.

The raw and underprocessed soybean diets, even when fed for such brief periods as one to 8 days, caused pancreas hypertrophy. The pancreas weights, calculated on the basis of 100 g body weight, are shown in table 1. The transfer of the chicks to the raw soybean diet brought about an immediate response in the pancreas. The pancreas-to-body weight ratios were consistently higher in chicks fed the raw soybean diet than in those receiving the heated soybean diet, although some variation was observed on the different experimental days.

The average dry matter of the pancreas of chicks fed both the heated and the raw soybean diets was 24.6%. Average nitrogen content was 30 mg/g fresh tissue (from 26 to 32 mg/g). No consistent differences between groups were observed.

The nitrogen content of the chyme of the small intestine is shown in table 2.

The nitrogen content of the intestinal chyme was higher at all ages in chicks fed the raw soybean diet than in those receiving the heated soybean diet. The difference was significant after two days' consumption of the raw soybean diet, but not after that.

TABLE 2

Nitrogen content of intestinal chyme of chicks fed heated soybean and raw soybean diets [1]

Age in weeks	Heated soybean diet	Days following transfer to raw soybean diet	
		2	4
	mg nitrogen/g chyme		
1	9.52	11.63	8.81
2	6.81	9.04	8.95
3	7.81	10.51	8.13
4	7.39	13.66	9.59
6	7.91	9.59	8.04
Average	7.89	10.89	8.70
L.S.D. at $P = 0.05$		1.48	

[1] Each number is the average of 3 chicks (exp. 1).

DISCUSSION

The results presented show that the ability of chicks to overcome the inhibition of proteolytic activity caused by the raw soybean diet is influenced both by the age of the chick and by the length of time the chicks were fed the raw soybean diet.

Young chicks, of one and two weeks of age, suffer from inhibition of proteolytic activity longer than older chicks. The length of inhibition is related to the length of the period fed the heated soybean diet preceding the one with the raw soybean

diet, e.g., chicks maintained with the raw soybean diet from the first days of life had depressed proteolytic activity at 3 weeks of age (2), whereas in the present experiment, chicks which were transferred from heated soybean to the raw soybean diet at 2 weeks of age reached normal activity at the eighth day (i.e., at 3 weeks of age) when tested under the same conditions.

It appears that overcoming the inhibition can be a result of several interacting factors: 1) the ability of the pancreas to grow rapidly and to secrete enzymes, particularly at the rising part of the curve of the pancreas-to-body ratio (2); and 2) the ratio between the maximal amount of enzymes that can be secreted at a given age and the amount of the inhibitor consumed with the feed.

The different behavior of chicks at various ages may be a result of domination of one of the above factors. For instance, at 3 weeks of age the first reason may predominate: the pancreas is probably at its highest ability for growth and secretion. At this age the curve of the pancreas-to-body ratio reaches a peak (2). On the other hand, in adult hens that, according to Saxena et al. (6), do not develop hypertrophic pancreas, the amount of enzymes secreted by mature pancreas may be sufficient to counteract the antitrypsin present in the feed.

The differences in behavior of 4- and 6-week-old chicks can be based on the different rates at which feed consumption is increased. From 3 to 4 weeks there is usually an increase of 33% in feed intake, whereas from 5 to 6 weeks the increase may be only 20% (9). Since during this period the pancreas-to-body weight ratio is on the descending part of the curve (2), it is easier to overcome the inhibition at 6 weeks than at 4 weeks.

In a separate trial, run parallel to trial 3 of Bornstein and Lipstein (5), 8-week-old chicks overcame the inhibition only after the eighth day of the experiment. The relatively long inhibition for this age may be related to the fact that the trial was carried out with heavy crossbred chicks (Cornish × White Rock). Also these chicks developed pronounced pancreas hypertrophy.

The higher nitrogen content in the small intestine of chicks fed the raw soybean diet compared with that of chicks receiving the heated soybean diet (table 2) is in accord with disturbed proteolytic activity observed at all ages immediately after transfer to the raw soybean diet. Carroll et al. (10, 11) reported that digestibility coefficients when calculated from the level of nitrogen in the small intestine were much lower in rats fed raw soybean than in those receiving a heated soybean diet. These coefficients were also lower than those calculated on the basis of nitrogen in the feces. These authors showed that part of the nitrogen of the raw soybean diet is absorbed in the cecum and colon. The latter fact was observed also in a previous study (12) in which chicks fed a raw soybean diet digested and absorbed part of the nitrogen in their ceca; hence chicks with ceca retained more nitrogen than chicks from which the ceca had been removed.

Therefore it appears that, although part of the higher level of nitrogen in the small intestine of chicks fed the raw soybean diet might be from a pancreatic source, the largest part is from the food and is digested later as it reaches the cecum.

The results of the experiment with the underprocessed soybean diet agree well with those of other workers (5, 13), who reported much less growth depression of chicks fed the underprocessed soybean diet than of those fed the raw soybean diet. In the underprocessed soybean diet, part of the inhibitors are destroyed (14, 15), and therefore the chicks reach normal levels of proteolysis in a shorter time than when fed the raw soybean diet (fig. 1).

Several authors (5, 16, 17) have reported that a raw soybean diet causes growth depression not only in young chicks but also in older ones when transferred to a raw soybean diet. The results of this study point to the possibility that transferring chicks from a heated soybean to a raw soybean diet at any age is accompanied by inhibition of intestinal proteolysis and pancreas hypertrophy resulting in growth depression.

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Phosphorus, Calcium and Magnesium Relationships in Ovine Urolithiasis [1]

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ABSTRACT Lambs were maintained with a natural calculogenic diet containing 45% sorghum grain or corn; the incidence of gross calculi was 56 and 39%, respectively. Stones were predominantly magnesium phosphate. The physiological response to both diets was similar with a significant increase ($P < 0.01$) in serum phosphorus level evident after only 3 weeks (from 6.7 mg/100 ml to 9.4), a slight but nonsignificant decrease in serum calcium, and a significant increase ($P < 0.01$) in serum magnesium that gradually increased to the end of the 84-day experiment (2.6 mg/100 ml to 4.1). Animals developing gross calculi had significantly higher serum phosphorus, ($P < 0.01$), excreted more phosphorus in the urine and had a higher concentration of urine phosphorus ($P < 0.01$) than animals not developing gross calculi. This was evident at the first analysis at 3 weeks and throughout the experiment. Average urine calcium excretion and serum calcium levels were lower in the gross calculi group, but the differences were not statistically significant. Magnesium levels in the serum and urine were high in both calculi and noncalculi animals. The data suggest that a physiological excess of phosphorus may produce elevated serum phosphorus and magnesium even though the diets meet NRC recommendations for calcium and phosphorus level and ratio.

Dietary mineral imbalance has long been suggested as an etiological factor in ovine phosphatic urolithiasis. High dietary phosphorus in particular has been associated with calculi development (1, 2). However, neither Eveleth et al. (3) nor Glenn et al. (4) could induce appreciable calculi incidence with diets having a low Ca-to-P ratio. Still, Lindley et al. (5) and Elam et al. (6) have shown that K_2HPO_4 supplemented diets are extremely calculogenic.

More recently, Packett et al. (7) and Udall (8) have induced high calculi incidence using fattening diets, balanced with respect to calcium and phosphorus content but containing a high percentage of sorghum grain or corn along with cottonseed meal. Emerick et al. (9) have observed high serum phosphorus in lambs with urolithiasis although not receiving a high phosphorus diet. Emerick and Embry (10) have observed that increasing the level of calcium in the diet appears to provide partial protection against the occurrence of urinary calculi in sheep receiving high levels of dietary phosphorus. Crookshank and Robbins (11) have also observed that supplemental dietary calcium is protective against urolithiasis. Kunkel et al.[2] have found that the incipience of calculi was related to the level of serum magnesium and that there was a significantly wider Ca-to-P ratio in the sera of lambs with incipient urolithiasis.

This investigation was conducted to further clarify the role played by phosphorus, calcium and magnesium in ovine urolithiasis produced experimentally with natural diets balanced in calcium and phosphorus.

EXPERIMENTAL

This experiment was conducted over an 84-day period during the summer months. Thirty-four Texas wether lambs were allotted to 2 groups on the basis of weight, the average weight being 31 kg. The diet was the reported calculogenic diet, or a modification, of Packett et al. (7) as given in table 1 along with the respective mineral analyses. The experiment was conducted under dry lot conditions. The sandy surface of the lots provided good

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[1] Journal Paper no. 2307 of the Purdue University Agricultural Experiment Station, Lafayette, Indiana.

[2] Kunkel, H. O., E. E. Whitaker, L. V. Packett, Jr. and H. R. Crookshank 1961 Relationship of serum magnesium, calcium and phosphorus to incidence of urinary calculi in lambs. J. Animal Sci., 20: 940 (abstract).

TABLE 1

Composition of diet [1]

	Group number	
	1	2
	%	%
Sorghum grain	45	—
Corn	—	45
Cottonseed meal	10	10
Cottonseed hulls	27	27
Alfalfa, dehydrated	8	8
Molasses	10	10
Mineral composition		
Phosphorus	0.30	0.26
Calcium	0.30	0.24
Magnesium	0.22	0.21

[1] The diet was pelleted and fed free choice in the presence of NaCl and water.

drainage and did not necessitate the use of litter.

Blood samples were taken initially and at the end of 3, 7 and 11 weeks of the experiment to follow the pattern of mineral change. Three- to five-day urine collections were made during the fourth, eighth and twelfth weeks.

Serum inorganic phosphorus and calcium were determined, respectively, by an adaptation of the method of Fiske and Subbarow (12) and by the method of Ferro and Ham (13). Serum magnesium was determined by atomic absorption spectroscopy according to the method of Griffith et al. (14).

Total urine phosphorus was determined by the procedure described by Hawk et al. (15). Urine calcium and magnesium were determined by atomic absorption spectroscopy according to the method of Willis (16). For urine calcium, the method requiring the initial precipitation of calcium oxalate was used.

At the termination of the experiment the animals were killed and the urinary bladders and kidneys were removed and examined for gross calculi incidence. No evidence of gross calculi incidence was observed in 5 lambs from the same flock, which were killed at the beginning of the experiment.

RESULTS

All animals gained well during the course of the experiment. Uniform weight gains in both groups indicate no significant variation in dietary intake between individual animals.

The calculi produced under the conditions of this experiment were predominantly magnesium phosphate. A typical stone contained 15.7 ± 0.9% P and 17.9 ± 1.0% Mg. Positive incidence of urolithiasis was taken as the occurrence of gross calculi, obstructive or otherwise, in the kidney, bladder or urethra of the experimental animal.

Calculi incidence and serum phosphorus, calcium and magnesium levels for groups 1 and 2 are presented in table 2. Both diets were calculi-provoking and elicited the same metabolic response. Serum phosphorus and magnesium levels were significantly increased ($P < 0.01$) in both groups within 3 weeks and remained at elevated levels throughout the experiment. Serum calcium levels tended to decrease slightly. These changes in serum mineral levels are essentially the same as had been previously observed (3, 4) with diets having a low Ca-to-P ratio. In these earlier investigations however, no appreciable calculi incidence was reported.

Urine phosphorus, magnesium and calcium concentrations of individual animals in groups 1 and 2 are shown in figures 1,

TABLE 2

Calculi incidence and serum phosphorus, calcium and magnesium levels as influenced by diet and length of experimental period

	Group 1	Group 2
No. of animals	16	18
Calculi incidence, %	56	39
weeks	*mg/100 ml*	*mg/100 ml*
Serum phosphorus		
0	6.83 ± 0.26 [1,2]	6.61 ± 0.21 [3]
3	9.42 ± 0.44	9.37 ± 0.40
7	9.66 ± 0.38	9.41 ± 0.41
11	10.16 ± 0.51	9.34 ± 0.60
Serum calcium		
0	8.79 ± 0.14	8.81 ± 0.09
3	8.36 ± 0.16	8.51 ± 0.21
7	8.45 ± 0.25	8.43 ± 0.19
11	8.46 ± 0.21	8.43 ± 0.18
Serum magnesium		
0	2.48 ± 0.11 [2]	2.65 ± 0.11 [3]
3	3.73 ± 0.16	3.86 ± 0.17
7	3.93 ± 0.17	4.07 ± 0.13
11	3.91 ± 0.11	4.26 ± 0.09

[1] SE.

[2] Significant difference ($P < 0.01$) from group 1 values at 3, 7 and 11 weeks.

[3] Significant difference ($P < 0.01$) from group 2 values at 3, 7 and 11 weeks.

2 and 3, respectively. Great individual variation occurred, especially so with phosphorus levels. Although there were no noticeable differences between groups, differences occurred within each group between animals that did or did not develop urolithiasis. These differences were most noticeable in the urine phosphorus concentrations (fig. 1). In general, those animals developing calculi had urine phosphorus concentrations above 20 mg/100 ml, especially during the later weeks of the experiment when a clearer distinction between animals with and without urolithiasis would be expected. Martin and Pierce (17) have reported normal urine phosphorus levels to vary between negligible and 18 mg/100 ml.

A comparison of the urine phosphorus picture of animals, divided into groups designated as gross calculi and non-calculi, is presented in table 3. The urine phosphorus concentration of the gross calculi group was significantly greater ($P < 0.01$) than that of the non-calculi group at 8 and 12 weeks. That this difference was not due simply to variations in urine volume is shown by the similar urine volumes in each group and by the fact that the total amount of phosphorus excreted in the urine of the gross calculi animals was significantly greater ($P < 0.01$) than that for the non-calculi animals at 8 and 12 weeks. No significant differences were noted between the urine magnesium or calcium concentration values of gross and non-calculi animals, although the calcium values of the latter tended to be higher.

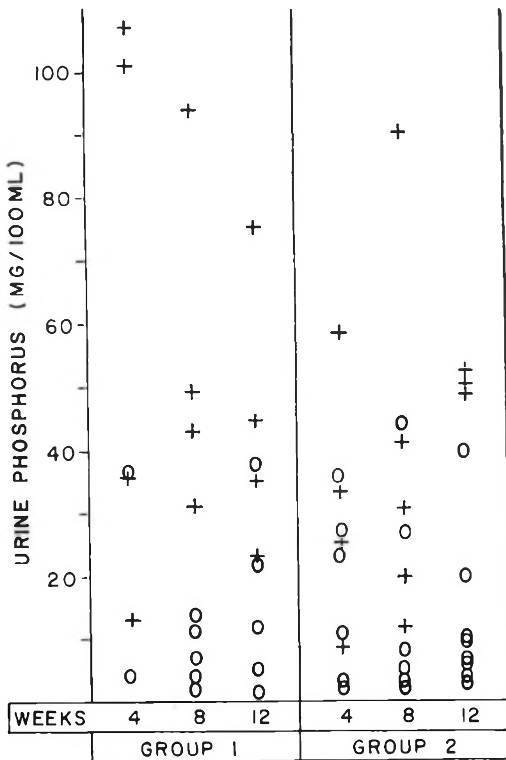

Fig. 1 Individual urine phosphorus concentration values of group 1 and 2 animals at 4, 8 and 12 weeks of the experiment; + sign indicates animals subsequently found to have gross calculi, and open circle (O) indicates those having no calculi.

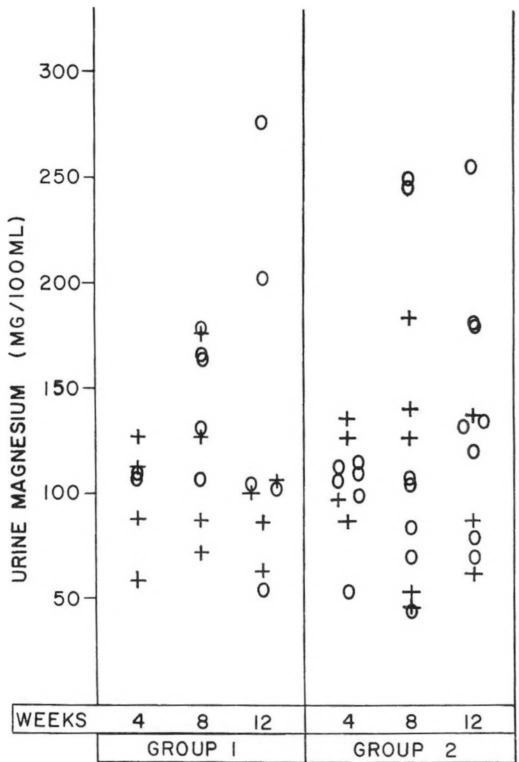

Fig. 2 Individual urine magnesium concentration values of group 1 and 2 animals at 4, 8 and 12 weeks of the experiment; + sign indicates animals subsequently found to have gross calculi, and open circle (O) indicates those having no calculi.

Although no significant differences were found between the urine magnesium levels of gross calculi and non-calculi animals, both groups had magnesium excretion val-

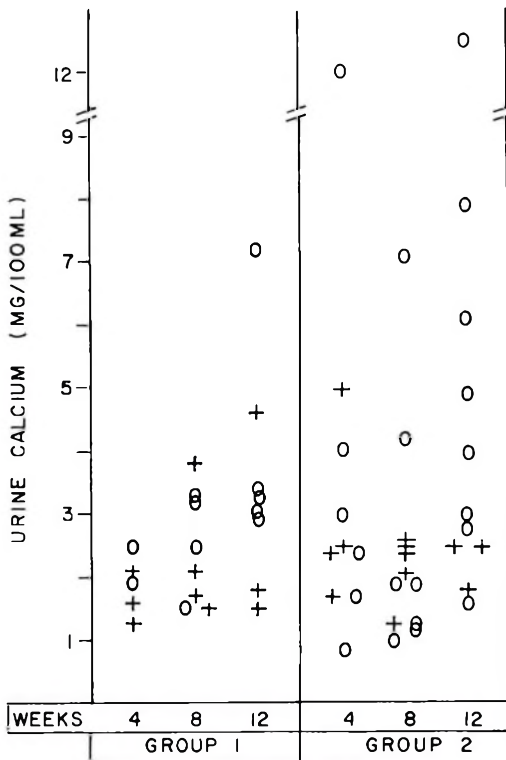

Fig. 3 Individual urine calcium concentration values of group 1 and 2 animals at 4, 8 and 12 weeks of the experiment; + sign indicates animals subsequently found to have gross calculi, and open circle (○) those having no calculi.

TABLE 3

Urine volume and phosphorus excretion at 4, 8 and 12 weeks of experiment; gross calculi vs. non-calculi animals

Period	Gross calculi	Non-calculi
weeks		
	Urine volume, ml/day	
4	849 ±129 (8) [1,2]	709 ±122 (8)
8	920 ± 84 (9)	703 ± 92 (12)
12	765 ± 65 (7)	568 ± 52 (13)
	Urine phosphorus, mg/100 ml	
4	47.9 ± 12.5 (8)	18.1 ± 4.7 (8)
8	45.8 ± 9.0 [3] (9)	11.4 ± 3.4 (12)
12	47.2 ± 5.6 [4] (7)	13.6 ± 3.4 (13)
	Urine phosphorus, mg/day	
4	481 ±167 (8)	155 ± 58 (8)
8	457 ±119 [3] (9)	109 ± 49 (12)
12	356 ± 45 [4] (7)	82 ± 22 (13)

[1] SE.
[2] Numbers in parentheses indicate number of observations.
[3] Significant difference ($P < 0.01$) from non-calculi group at 8 weeks.
[4] Significant difference ($P < 0.01$) from non-calculi group at 12 weeks.

TABLE 4

Serum phosphorus, calcium and magnesium levels; gross calculi vs. non-calculi animals

Period	Gross calculi	Non-calculi
weeks	*mg/100 ml*	*mg/100 ml*
	Serum phosphorus	
0	6.90 ± 0.21 [1,2]	6.50 ± 0.22 [2]
3	10.31 ± 0.38 [3]	8.70 ± 0.24
7	10.18 ± 0.36 [4]	8.95 ± 0.36
11	10.75 ± 0.37 [3]	8.95 ± 0.56
	Serum calcium	
0	8.70 ± 0.15	8.87 ± 0.07
3	8.00 ± 0.20 [3]	8.82 ± 0.15
7	8.18 ± 0.22	8.82 ± 0.20
11	8.18 ± 0.14	8.62 ± 0.20
	Serum magnesium	
0	2.62 ± 0.12 [2]	2.54 ± 0.10 [2]
3	3.97 ± 0.16	3.63 ± 0.14
7	4.20 ± 0.12	3.83 ± 0.09
11	4.16 ± 0.13	4.05 ± 0.10

[1] SE.
[2] Significant difference ($P < 0.01$) from respective 3, 7 and 11 weeks levels.
[3] Significant difference ($P < 0.01$) from non-calculi levels.
[4] Significant difference ($P < 0.05$) from non-calculi levels.

ues greater than those previously reported in the literature (5, 11, 18).

A comparison of serum phosphorus, calcium and magnesium values of gross calculi vs. non-calculi animals is presented in table 4. Serum phosphorus levels in both of these groups were significantly greater ($P < 0.01$) at 3, 7 and 11 weeks, than the initial values. The values for the gross calculi group at 3, 7 and 11 weeks were significantly higher than the corresponding values for the non-calculi group ($P < 0.01$, < 0.05, and < 0.01, respectively).

Although the calcium levels of the gross calculi group were consistently lower than those of the non-calculi group, the only statistically significant difference ($P < 0.01$) occurred at 3 weeks.

No significant differences were found between serum magnesium levels with respect to calculi incidence; however, significant ($P < 0.01$) increases in serum magnesium levels were observed within 3 weeks in both groups.

DISCUSSION

The significant relationship between gross calculi incidence and high serum

and urine phosphorus, implicates phosphorus as a major determining factor in the development of urinary calculi. This is consistant with the observations of Lindley et al. (5) using K_2HPO_4 supplemented diets. However, our results may not be explained on the basis of increased dietary phosphorus, since both the gross calculi and non-calculi animals received equivalent amounts of dietary phosphorus. The occurrence of urolithiasis appears to be a result of differences in mineral metabolism between individuals.

In designating animals as "non-calculi," we do not mean to imply that these animals are to be regarded as normal, since similar changes in serum and urine mineral levels occurred in all individuals. The data suggest that all animals were susceptible to urolithiasis when fed the diets used in this experiment, but that the actual formation of gross calculi was determined by the degree of change of certain metabolic variables, i.e., high phosphorus levels in the serum and urine accompanied by high magnesium levels. It appears that the increased levels of serum phosphorus and magnesium are responsible for the increased urinary excretion of these minerals.

Increases in serum phosphorus and magnesium could not be predicted from the diet components or mineral composition. Serum phosphorus levels of the order of 10 mg/100 ml were not reported by Emerick and Embry (10) until the diet contained 0.62% phosphorus. Lindley et al. (5) produced the above serum phosphorus levels using diets with 0.72% phosphorus. Thus, phosphorus intake can not be used to explain the differences in serum levels which we have observed. It is also doubtful that the dietary intake of magnesium is responsible for the increases in serum magnesium, since similar diets containing 0.2% magnesium have been fed to lambs without any increase in the serum magnesium levels (19).

The failure to explain the observed changes in phosphorus and magnesium levels on the basis of dietary intake of these minerals, gives further support to the observation of Kunkel et al.[3] that the problem of phosphatic ovine urolithiasis can be explained in metabolic as well as nutritional terms. The results of Kunkel et al. indicate that changes in magnesium metabolism are determining factors in the development of urolithiasis, whereas our results implicate abnormal phosphorus metabolism as the measurable factor in gross calculi development, although increases in serum magnesium levels did occur.

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[3] See footnote 2.

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Influence of Dietary Factors upon *Salmonella typhimurium* Infection in the Guinea Pig[1]

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ABSTRACT More than 1100 guinea pigs were used to study the effect of dietary factors upon susceptibility of the animals to salmonellosis induced by inoculation with *Salmonella typhimurium*. The chief criterion of a protective effect was reduction in mortality, but survival as measured by the time at which one-half of a group had died was also considered to be significant. Dried cooked cabbage was an effective supplement, reducing mortality from 73.5 to 50.8% among animals fed a casein basal diet. Soybean protein also exerted a protective effect but alfalfa meal was ineffective. The factor in cabbage was stable to heat, was not readily soluble in water, was not present in the ash and apparently is not identical with arginine nor the antibacterial compounds commonly occurring in cabbage. Increasing the dietary phosphorus level from about 0.4 to 1.0% caused a marked reduction in mortality regardless of the protein source, but excess phosphorus added to the soybean diet was the most protective. Although the relationship between dietary phosphorus and the factor in cabbage is not known, these compounds are clearly not identical.

The effect of nutritional status upon resistance to disease has been investigated rather extensively, but most of the experimental work has been restricted to protein and vitamin levels and to a lesser extent to unrecognized nutrients. Minerals have received little or no attention. The observation of Schneider and Webster (1) that natural products contain a dietary factor which promotes resistance against a Salmonella organism has led to a series of publications related to the unrecognized factor which has been termed the salmonellosis resistance factor (SRF) (2–4). Recently Hill et al. (5) reported that, in the presence of "normal" vitamin levels and SRF, increasing levels of protein reduced mortality in mice, but in the absence of SRF, extra protein increased mortality. When excessive levels of vitamins were fed, SRF exerted no effect, suggesting a 3-way interaction among SRF, protein and vitamins.

Although the guinea pig has not been widely used for disease-resistance studies, the consumption of cabbage by this species has been shown to prolong survival time when experimental tuberculosis was induced (6). O'Dell et al. (7) observed that an ad libitum supplement of fresh cabbage lowered the mortality rate among Salmonella-infected guinea pigs fed a diet based on casein and sucrose. The protective factor could not be identified with any of the known nutrients. Spector and Calloway (8) and Calloway et al. (9) used guinea pigs in a study of radiation protection and observed that dietary supplements of cabbage, broccoli or alfalfa reduced the mortality among x-irradiated animals fed a simple diet of bran and oats.

This paper is concerned with additional observations relative to the properties of the unidentified factor found in cabbage and with the significance of minerals in preventing mortality in guinea pigs due to salmonellosis. The protective factor in cabbage appears not to be a recognized nutrient and the evidence suggests that it is not identical to the radiation protective factor or to the SRF of Schneider. A dietary supplement of phosphorus increases resistance to salmonellosis but the effect of cabbage is not due to its phosphorus content.

METHODS AND MATERIALS

The experimental animals were female guinea pigs that were 5 to 11 weeks of age

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TABLE 1

Composition of basal diets

	Casein basal	Soy protein basal
	%	%
Acid-washed casein [1]	30.0	—
Isolated soy protein [2]	—	30.0
Sucrose	42.3	41.1
Cellulose [3]	15.0	15.0
Soybean oil	2.0	2.0
Salts I [4]	4.0	—
Salts II [5]	—	5.0
Potassium acetate	2.7	2.5
Magnesium oxide	0.5	0.5
Water-soluble vitamins in sucrose [6]	1.0	1.0
Fat-soluble vitamins in soybean oil [7]	2.0	2.0
Ascorbic acid	0.2	0.2
Choline chloride	0.1	0.2
DL-Methionine	0.2	0.5
Chlortetracycline·HCl	0.0025	0.0025
Mineral elements supplied by salts premixes		
Ca	0.84	0.96
P	0.20	0.60
Mg	0.039	0.06
Mn	0.025	0.02
Na	0.11	0.31
K	0.48	0.40
Fe	0.018	0.006
Cu	0.0014	0.0008
Co	0.00007	—
Al	0.00004	—
Zn	0.00056	0.0080
F	0.0018	—
I	0.0023	0.0010
Cl	0.38	0.52
S	0.030	0.092

[1] Commercial casein was dissolved in dilute NaOH, precipitated with HCl and washed free of salt with tap water. After drying at 65° in a forced-draft oven it was ground in a hammer mill.

[2] Promine R, Central Soya Company, Inc., Chicago.

[3] Wood pulp (Solka Floc), Brown Company, Berlin, New Hampshire.

[4] Salts I supplied: (g/100 g of diet) $CaCO_3$, 2.05; $MgCO_3$, 0.10; $MgSO_4$, 0.05; $MnSO_4 \cdot H_2O$, 0.08; NaCl, 0.28; KCl, 0.45; KH_2PO_4, 0.83; $FePO_4$ (soluble), 0.16; $CuSO_4 \cdot 5H_2O$, 0.0056; $ZnSO_4 \cdot 7H_2O$, 0.0025; $CoCl_2 \cdot 6H_2O$, 0.0003; $AlK(SO_4)_2 \cdot 12H_2O$, 0.0007; NaF, 0.004; KI, 0.003.

[5] Salts II supplied: (g/100 g of diet) $CaCO_3$, 0.90; $CaHPO_4$ (anhyd.), 2.04; $MgSO_4$ (anhyd.), 0.30; $MnSO_4 \cdot H_2O$, 0.06; NaCl, 0.26; Na_2HPO_4, 0.64; KCl, 0.77; Fe citrate, 0.036; $CuSO_4$ (anhyd.), 0.002; $ZnCO_3$, 0.0143; KIO_3, 0.0017.

[6] Water-soluble vitamins supplied: (mg/100 g diet) thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine·HCl, 1.0; Ca pantothenate, 3.0; niacin, 5.0; folic acid, 0.6; inositol, 100; D-biotin, 0.02; cyanocobalamin, 0.003.

[7] Fat-soluble vitamins supplied per 100 g diet: vitamin A palmitate, 2000 IU; vitamin D_3, 285 IU; α-tocopheryl acetate, 2.0 mg; menadione, 1.0 mg; antioxidant (Santoquin, Monsanto Chemical Company, St. Louis), 12.5 mg.

and weighed, on the average, about 500 g. They had been used in a growth factor assay prior to these studies and were randomly divided into the experimental groups described here. Groups of two were housed in raised wire-floor cages and kept in a continuously lighted room maintained between 23.3 and 25.5°. The mortality was recorded daily for 31 to 42 days, a period long enough to insure that the survivors were no longer in jeopardy. Weights were recorded 3 times weekly and fresh feed and water were supplied daily, ad libitum.

The composition of the basal diets for the guinea pigs is shown in table 1.[3] The casein and soybean protein rations contained different mineral mixtures, the composition of which is also shown in the table. Chlortetracycline has been used routinely in our guinea pig diets for several years because it is beneficial in the prevention of cervical lymphadenitis and certain types of infectious enteritis, but does not protect against salmonellosis.[4] Preliminary observations showed that chlortetracycline did not influence the results obtained in the salmonellosis resistance assay and its value in prevention of secondary infection justified its inclusion in the assay diet. Supplements were added at the expense of the entire ration. The rations were moistened with water at the time of feeding so as to make a dough-like consistency and thus increase their acceptability.

After 2 weeks to allow adjustment to the environment and the respective rations, the test animals were infected with *Salmonella typhimurium*. The organisms were isolated originally from a naturally infected guinea pig that had died of salmonellosis. The organisms were serologically identified, and were maintained by periodic culture in the trypticase soy broth (BBL) followed by growth on nutrient agar. The organisms from the nutrient agar slopes were suspended in trypticase,

[3] The authors gratefully acknowledge gifts from the following donors: Folacin and Chlortetracycline HCl, American Cyanamid Company, Pearl River, New York; biotin, Hoffmann-LaRoche Inc., Nutley, New Jersey; Vitamin A, Distillation Products Inc., Rochester, New York; Santoquin, Monsanto Chemical Company, St. Louis; other vitamins, Merck Sharp and Dohme, Rahway, New Jersey.

[4] O'Dell, B. L., W. O. Regan and A. G. Hogan. Proc. Soc. Exp. Biol. Med., *96*: 553, 1957.

and small aliquots were frozen on glass beads at −40° for storage until needed for inoculum preparation. To prepare the inoculum, a culture from the beads was grown in trypticase at 37° for 22 hours. The organisms were centrifuged for 30 minutes in a Spinco Model L ultracentrifuge at 7500 rpm using a SW 25.1 rotor, resuspended in sterile tap water, and diluted so as to give a transmittance of 80% in a "Spectronic 20" colorimeter at 650 mμ. One-half milliliter of a 1:500 dilution of the suspension was injected intraperitoneally into the guinea pigs with a semiautomatic hypodermic syringe. This dose was estimated to contain about 200,000 organisms.

RESULTS

Adequacy of purified diets. To assess the adequacy of the basal diets for uninfected animals, these diets were compared with a commercial guinea pig chow and were supplemented with a commonly used natural product, raw cabbage. As shown by the results presented in table 2, both the casein and soybean protein basal diets supported a rate of gain equivalent to that of diets of natural foods, and the rate of mortality was essentially nil. In fact only 11 deaths have been recorded among over 3,000 animals fed similar diets for a period of 4 weeks. Cabbage supplementation of the basal diets caused significant ($P < 0.01$) growth stimulation. Ascorbic acid administered daily by mouth did not improve the growth rate. Although these results suggest that cabbage provides a nutrient required to support the maximal growth rate, the basal diets were adequate for survival and growth as compared with a commonly used practical diet.

Studies relating to the protective factor found in cabbage. The rates of mortality and in some instances the time required for 50% of the animals to die (ST_{50}) were used as the criteria for assessing the efficacy of a supplement. Other observations, such as the average survival times, liver and spleen damage as measured by weight or inspection, and rates of gain or loss in body weight, were made, but none of these parameters was satisfactory as an index of Salmonella resistance. All comparisons presented here are based on data collected from animals that were under observation at the same time.

Fresh cabbage was used as the source of the protective factor in the first experiments (7), but it was difficult to control the quantity of the supplement consumed. To reduce this variable it was desirable to prepare a dry supplement which could be incorporated into the diet. Consequently, a dry supplement was prepared by cooking chopped cabbage with steam until tender. The cooked pulp and juice were then dried at 55° for 72 hours in a forced-draft oven and finely ground in a hammer mill. Table 3 shows the results obtained with the dried cooked cabbage supplement. Two levels, 8.5 and 10%, were fed but, since there was no difference in response, the data were combined. The average mortality rate among 132 animals fed the dried cabbage supplement was 50.8% for 10 trials, whereas the mortality among those fed the basal diet was 73.5%. The difference, 22.7%, was highly significant ($\chi^2 = 15.83$, df = 1; $P < 0.005$) as calcu-

TABLE 2

Adequacy of purified diets for growth and survival of uninfected guinea pigs

Diet	No. of animals	Avg daily gain, 4 weeks	Mortality
		g	%
Casein basal	23	5.9 ± 0.3 [1]	0
Casein basal + raw cabbage, ad libitum	31	7.4 ± 0.2	0
Soybean protein basal	42	6.4 ± 0.4	2.4
Soybean protein basal + raw cabbage, ad libitum	12	7.5 ± 0.2	0
Soybean protein [2] basal + 30 mg ascorbic acid daily	24	6.3 ± 0.14	0
Commercial chow pellets [3]	43	6.1 ± 0.2	2.3

[1] SE of mean.
[2] No ascorbic acid was added to this diet.
[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

TABLE 3

Effect of dried cooked cabbage upon mortality rate among guinea pigs infected with Salmonella typhimurium

Trial	Basal casein diet		Basal + dried cooked cabbage	
	Mortality	ST_{50}[1]	Mortality	ST_{50}
	%	*days*	*%*	*days*
1	90 (9/10)[2]	13	50 (5/10)	34
2	62 (8/13)	21	33 (4/12)	42+
3	83 (10/12)	15	73 (8/11)	19
4	100 (13/13)	13	50 (7/14)	24
5	93 (14/15)	10	42 (5/12)	42+
6	36 (5/14)	42+	19 (3/16)	42+
7	93 (14/15)	11	60 (9/15)	34
8	66 (17/26)	24	46 (5/11)	41+
9	75 (15/20)	11	89 (17/19)	9
10	46 (6/13)	31+	33 (4/12)	31+
Average	73.5(111/151)	19+	50.8(67/132)	32+

[1] ST_{50} indicates the time at which 50% of the animals had died; (+) indicates 50% had not died by end of trial.
[2] Ratio of number of animals that died to number infected.

lated by the Chi-square analysis described by Schneider and Webster (1). Analysis of the data for homogeneity was also made by the Chi-square method. If 73.5% is accepted as the true mortality rate for the 10 trials, then it can be shown that the mortality rate of animals fed the casein diet did not differ from trial to trial more than can be accounted for by chance alone ($\chi^2 = 7.93$; df = 9; $P > 0.50$). The rather high Chi-square value ($\chi^2 = 11.70$; df = 9; $P > 0.10$) for the animals fed the cabbage supplement was accounted for mainly by the very low mortality rate of animals in trial 6 and the high rate in trial 9. The ST_{50} for the animals fed the basal diet was 19+ days and for the supplemented animals, 32+ days.

The results of experiments designed to determine other properties of the cabbage factor are presented in table 4. For comparative purposes the casein basal diet was used as the negative control and the diet supplemented with 10% of dried cabbage as a positive control. A hot water extract of fresh cabbage was prepared by extracting chopped cabbage 3 times with boiling water. The extract was concentrated to a thick syrup *in vacuo* and was added to the casein diet at a level of 5% on a dry basis, an amount equivalent to 17% of dried cabbage. A second concentrate was prepared under different conditions which reduced the exposure of the extract to oxidative conditions. In this case the chopped fresh cabbage was autoclaved at 120° for 45 minutes. The juice was pressed from the pulp and concentrated *in vacuo* using 10 mg of mercaptoethanol/liter of juice as an antioxidant. The extract was offered to the animals as a 5% solution in water bottles and they consumed about 3.7 g of dry matter/day. The dry residues remaining after the 2 extractions were added to the basal diet at levels of 8.5 and 10%. The data indicate that both extracts and both residues caused a slight reduction in mortality. Although the residues, especially the autoclaved one, were more effective than the extracts, they were not as effective as the dried cabbage supplement. Both the animals fed the autoclaved extract and those fed the residue had longer ST_{50} values than the basal animals, indicating a slight protective effect.

Several antibacterial compounds or their precursors have been isolated from cabbage and related species (10–12). Of these compounds, three were chosen for testing for possible anti-salmonellosis activity. They were added to the casein ration as ethanol solutions to supply the following percentages of pure compounds: allyl isothiocyanate, 0.001; methylthiolsulfinate, 0.01; and S-methylcysteine sulfoxide, 0.2. None of these compounds lowered the mortality rate appreciably.

Ross et al. (13, 14) reported that chicks fed fresh chicken feces or those inoculated

TABLE 4

Effect of supplements related to cabbage upon rate of mortality among guinea pigs infected with Salmonella typhimurium

Description of supplement	Basal diet		Basal + dried cooked cabbage		Basal + supplement	
	Mortality	ST_{50}[1]	Mortality	ST_{50}	Mortality	ST_{50}
	%	*days*	%	*days*	%	*days*
Hot water extract; 6% dry matter added to feed	100.0(13/13)[2]	13	50.0(7/14)	24	84.6(11/13)	12
Hot water-extracted residue, 8.5%	100.0(13/13)	13	50.0(7/14)	24	76.9(10/13)	16
Juice of auto-claved fresh cabbage, offered as a 5% solution, ad libitum	93.3(14/15)	10	41.7(5/12)	42+	76.9(10/13)	21
Residue of autoclaved fresh cabbage, 10%	93.3(14/15)	10	41.7(5/12)	42+	53.3(8/15)	27
Allyl isothio-cyanate, 0.001%	90.0(9/10)	13	50.0(5/10)	34	91.7(11/12)	21
Methylcysteine sulfoxide, 0.2%	83.3(10/12)	15	72.7(8/11)	19	75.0(15/20)	13
Methylthiol-sulfinate, 0.01%	90.0(9/10)	13	50.0(5/10)	34	75.0(9/12)	13
Arginine·HCl, 0.5 or 1.25%	72.7(56/77)	20+	40.7(22/54)	40+	71.8(51/71)	26+
Alfalfa leaf meal, 10%	72.0(18/25)	18	52.2(12/23)	31+	72.7(16/22)	16

[1] ST_{50} indicates the time at which 50% of the animals had died; (+) indicates 50% had not died by end of trial.
[2] Ratio of number of animals that died to number infected.

with *S. pullorum*, exhibited prolonged survival times when they were injected intraperitoneally with arginine, citrulline or urea. Since the arginine level in the casein diet is marginal for the guinea pig (15–17) and cabbage protein is relatively high in arginine (18), the effect of arginine upon Salmonella resistance was tested in the guinea pig. L-Arginine·HCl was added to the casein ration at the 0.5 and the 1.25% levels. The data for the 2 levels were combined since no difference was observed between them. The average mortality rate among animals fed the arginine supplements was 71.8%, among those fed the casein ration, 72.7%, and among those fed the dried cooked cabbage supplement, 40.7%. Judging from these results the protection due to dried cooked cabbage could not be accounted for by its arginine content.

In view of the protective effect that cabbage, broccoli and alfalfa leaf meal have for x-irradiated guinea pigs (8, 9), alfalfa was tested in Salmonella-infected guinea pigs. Alfalfa leaf meal [5] was added at the rate of 10% to the casein diet. There was no difference between the rates of mortality of animals fed the alfalfa supplement and those fed the basal diet.

Effect of protein source and mineral supplement upon salmonellosis. This phase of the research evolved from the studies relating to arginine supplementation. A soybean protein diet was compounded to test the effect of a protein naturally high in arginine (19). This diet, which contained a somewhat simplified and presumably more nearly adequate mineral mix-

[5] This alfalfa meal was kindly supplied by Dr. Doris Calloway from a batch specially prepared for radiation protection studies carried out at the Quartermaster Food and Container Institute, Chicago.

ture, was compared with the casein basal diet with and without supplements. The results are shown in table 5. Arginine alone was ineffective, the dried cabbage was effective as usual, but the soybean protein diet afforded by far the most protection. As was shown previously, the marked reduction in mortality was not likely due to the high arginine content of the soybean protein. The comparison of casein and soybean protein diets was then extended to a total of 9 trials. The average mortality of animals fed the casein-ration was 64.1% (36 to 93%), whereas the mortality of those fed the soy bean protein ration was only 25.8% (14 to 50%). This difference, 38.3%, was highly significant ($\chi^2 = 37.0$; df = 1; $P < 0.005$). The average ST_{50} of animals fed the soybean protein diet was 35+ days compared with 23+ days for those fed the casein diet.

Besides the difference in source of protein in the casein and soybean protein basal diets, there were major differences in the composition of the mineral supplement. To test the effect of the mineral mixtures as well as of the protein source, an experiment was run using a 2 × 2 factorial design. As shown in table 6 the source of both protein and salts had a significant effect upon the rate of mortality, but an analysis of variance, using the arcsin transformation of the percentages of mortality, indicates that there was no significant protein-mineral interaction. The effect of salts was clearly independent of the effect of protein, and the simple effect of minerals was the same for each of the proteins within the limits of chance variation. Even so, the maximum protection occurred with salts II mixture in the presence of soybean protein.

Subsequent experiments were designed to determine the mineral element responsible for the marked depression in mortality. The soybean protein diet containing salts I was supplemented with specific salts to provide the same level of the mineral elements as supplied by salts mixture II. The results are presented in table 7. Neither the addition of a combination of zinc and iodine salts nor the omission of

TABLE 5

Comparison of casein and soybean protein diets with and without supplements

Diet description	Mortality	ST_{50}[1]
	%	*days*
Casein basal	65.5(36/55)[2]	26+
Casein + 10% dried cooked cabbage	40.5(17/42)	39+
Casein + 1.25% arginine·HCl	75.0(36/48)	24+
Soybean protein basal	22.7(10/44)	39+

[1] ST_{50} indicates the time at which 50% of the animals had died; (+) indicates 50% had not died by end of trial.
[2] Ratio of number of animals that died to number infected.

TABLE 6

Effect of protein source and type of mineral mixture upon the mortality of guinea pigs infected with Salmonella typhimurium

	Casein diet (salts I)	Casein diet (salts II)	Soy protein diet (salts II)	Soy protein diet (salts I)
	%	%	%	%
Mortality average	63.2(48/76)[1]	42.9(33/77)	27.6(21/76)	56.7(34/60)
Standard deviation	± 13.0	± 10.5	± 12.1	± 18.0

Significance lines printed under the table: a line under Casein diet (salts I) and Casein diet (salts II) marked * [2]; a line under Casein diet (salts II) and Soy protein diet (salts II) marked *; a line under Soy protein diet (salts II) and Soy protein diet (salts I) marked **.

[1] Ratio of number of animals that died to number infected.
[2] A line under any 2 rations indicates a statistically significant difference between them. Asterisks indicate level of significance. * = $P < 0.05$; and ** = $P < 0.01$.

TABLE 7

Effect of various minerals upon the mortality of guinea pigs infected with Salmonella typhimurium

Supplement	Mortality	
	Basal diet	Basal + supplement
	%	%
Soy protein basal diet with salts I		
0.008% Zn as $ZnCO_3$ + 0.001% I as KIO_3	69.2(9/13)[1]	69.2(9/13)
No added Fe or Cu	56.0(14/25)	55.6(15/27)
0.4% Ca as $CaHPO_4 \cdot 2H_2O$ + 0.6% P as $NaH_2PO_4 \cdot H_2O$	70.8(17/24)	28.0(7/25)
0.4% Ca as $CaCO_3$	50.0(11/22)	50.0(12/24)
0.6% P as $NaH_2PO_4 \cdot H_2O$	53.2(25/47)	22.9(11/48)
Casein basal diet with salts I		
0.6% P as $NaH_2PO_4 \cdot H_2O$	59.1(13/22)	16.0(4/25)
1.9% Cabbage ash	69.6(32/46)	60.0(18/30)

[1] Ratio of number of animals that died to number infected.

both iron and copper salts affected mortality. A combination of 0.4% calcium and 0.6% phosphorus as $CaHPO_4 \cdot 2H_2O$ and $NaH_2PO_4 \cdot H_2O$ depressed the mortality rate from 70.8 to 28.0%. Calcium alone as the carbonate had no effect, but the addition of 0.6% phosphorus as $NaH_2PO_4 \cdot H_2O$ depressed the mortality markedly. A similar protection was observed when the 0.6% of phosphorus was added to the casein diet containing salts I. In a subsequent trial, not shown here, an equivalent amount of sodium, fed as sodium acetate, did not decrease the rate of mortality; hence the protective effect appears to be linked to the phosphate moiety. Furthermore, there was an inverse relationship between rate of mortality and the phosphorus content of the soybean protein diet as shown by the following results: 0.44% P, 55.3% mortality (47 animals); 0.78% P, 33.3% mortality (48 animals); 1.04% P, 22.9% mortality (48 animals).

When the ash of dried cooked cabbage was added to the casein diet (salt I) at a level of 1.9%, the mortality was 60.0% compared with 69.9% with the basal. Since cabbage ash was not effective, the protection afforded by dried cooked cabbage was probably not due to its mineral composition.

DISCUSSION AND CONCLUSIONS

Disease resistance is complicated by many factors which are not easily controlled, especially such factors as virulence of the infecting organisms and the susceptibility of the host animals. During these studies the organisms have maintained a reasonably stable state of virulence presumably because of minimal manipulation and storage at low temperatures. Variations due to other factors such as the susceptibility of the host animal, age and weight of the animals, placement within the laboratory, and dietary history were controlled in part by randomization. Although the procedures used to control the variation were reasonably successful, there were occasional cases of unusually high or low mortality which cannot be explained.

Gross observations made of the liver and spleen damage or weight of survivors of the infection did not indicate any appreciable differences between rations. This was also true of such observations as rate of gain or loss during the infection trial, body temperature and feed consumption. The changes in these criteria appeared to be more closely related to the length of time that the animals survived after infection or to the severity of the infection than to an increased ability to survive.

The mortality observed among the Salmonella-infected guinea pigs fed the casein diets occurred in 2 peaks, the first about 10 days and the second about 20 days after infection. Cabbage supplementation caused a depression of mortality chiefly during the first peak with little or no effect during the period of the second mortality peak. The soybean protein diet protected during the first peak to about the same degree as that observed with the cabbage supplementation, but afforded

additional protection during the second mortality peak. The reason for the enhanced protection during the second peak is not clear, but it suggests a different protective mechanism for the cabbage-supplemented diet and soybean protein which contained the higher level of phosphorus.

Dried cooked cabbage appeared to be just as effective as fresh cabbage for promoting Salmonella resistance in the guinea pig. The protective factor of cabbage was not readily water-soluble and could not be extracted from the cabbage pulp. Antibacterial compounds which are associated with cabbage possessed no protective effect. The beneficial effect of arginine for chicks infected with *S. pullorum* (13, 14) was not observed in the guinea pig. The difference may be due to the greater efficiency of urea excretion in the guinea pig which prevents urea accumulation or to a difference in urea tolerance between the organisms used to infect the chicks and those used to infect the guinea pigs. However, the protection afforded the chicks was an increased survival of a few hours with no difference in the mortality at the end of 4 days. In the guinea pigs the experimental period was much longer and small differences in survival time were not considered significant.

There are few reports concerning the interrelationships of minerals and disease resistance. Edwards and Boyd (20) reported that chicks have increased requirements for phosphorus and magnesium when they are infected with either *S. gallinarum* or fresh chicken feces. Whether Salmonella infection increases the phosphorus requirement of the guinea pig is not known, but recent experiments in this laboratory [6] have shown that the addition of 0.6% phosphorus to the rations used in these studies did not improve the growth rate of normal animals. In fact it caused a slight depression of growth rate. The mechanism by which phosphorus increases resistance is not obvious, but possible effects may be suggested. The infectious organisms may precipitate a phosphorus deficiency by altering the intestinal mucosa and thus decreasing absorption. Such a mechanism would not likely be the primary cause of death, but it might increase susceptibility. Certain defense mechanisms such as the endotoxin detoxifying component (21) may require anions such as phosphate to be effective and the rapidly enlarging reticulo-endothelial tissues, such as the liver and spleen, may require greater amounts of phosphorus during the infection. It is also possible that excess phosphorus enhances the absorption of a resistance factor by reducing the availability of certain cations. Colburn et al.[7] observed that SRF had greatly enhanced activity in the absence of cations. Additional work must be done before a satisfactory mechanism can be suggested for increased resistance to salmonellosis which results from the higher levels of phosphorus.

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