Nutritional Studies with the Guinea Pig

X. DETERMINATION OF THE LINOLEIC ACID REQUIREMENT

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ABSTRACT Young male guinea pigs were reared for 6 to 14 weeks with purified diets containing varying levels of linoleic acid as corn oil, safflower oil, or methyl linoleate. A dietary level of about 1% of calories as linoleic acid was necessary for normal growth and skin condition. The fatty acid composition of erythrocytes from animals fed safflower oil was determined and the ratios of 5,8,11-eicosatrienoic-to-arachidonic (20:3/20:4) or oleic-to-linoleic (18:1/18:2) acids compared with the performance of the animals with respect to growth and dermal symptoms. One per cent of calories as linoleic acid gave a 20:3/20:4 ratio of 0.13 to 0.17, and a 18:1/18:2 ratio of 1.41 to 1.14. Homogenates of liver from linoleic acid-deficient guinea pigs had an elevated rate of oxidation of citrate and α-ketoglutarate.

Previous studies of the effects of dietary fat on the nutrition of the guinea pig have described the gross symptoms of essential fatty acid deficiency (1) and the changes in organ weights and fatty acid composition (2, 3). In the present paper, the requirement for linoleic acid is estimated from observations of performance and related to analytical values for fatty acid content of erythrocytes and also to the rate of oxidation of citrate and α-ketoglutarate by liver homogenates.

METHODS

Male guinea pigs of the Hartley strain, 2 to 5 days old and weighing 95 to 115 g, were used in all experiments. Diet no. 13 of Reid and Briggs (4) with the corn oil omitted was used as the basal, fat-free diet. The composition per 100 g was: casein (vitamin-free) 30; sucrose, 12.5; cellophane, 15; corn starch, 20; glucose, 12.9; potassium acetate, 2.5; magnesium oxide, 0.5; salt mixture, 6 (5); and 0.2 each of choline chloride, ascorbic acid and inositol, with liberal amounts of the other known vitamins. Additions of fat were in place of an equal weight of sucrose or glucose. Housing and feeding of the guinea pigs was conducted as described previously (1); room temperature was 25°C ± 1° and relative humidity 40 to 55%. Animals were examined regularly for scaliness, particularly on the abdomen, ears and feet. The ears have been found to be a more sensitive site than the feet, since even at relatively high linoleic-acid intakes the feet are often dry and scaly.

The trilaurin [2] was a specially prepared product essentially free of linoleic acid, whereas the corn oil [3] and safflower oil [4] were standard commercial products. The fatty acid composition of the oils is shown in table 1.

The linoleic acid content of the corn starch (6) was taken into consideration in all calculations. Starch was hydrolyzed

TABLE 1

Fatty acid composition of dietary fats [1]

Fatty acid [2]	Corn oil	Safflower oil	Trilaurin	Starch lipid [3]
12:0	—	—	99.0	—
14:0	—	—	1.0	—
15:0	—	—	—	—
16:0	11.4	6.8	—	38.0
16:1	—	—	—	—
17:0	—	—	—	—
18:0	1.5	3.0	—	1.6
18:1	27.0	12.2	—	9.2
18:2	59.5	77.1	—	48.9
18:3	0.8	0.8	—	2.3

[1] Expressed as percentage of total peak areas.
[2] Number carbons:number of double bonds.
[3] Extracted from corn starch after acid hydrolysis.

Received for publication November 8, 1963.

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[3] Mazola, Corn Products Company, Argo, Illinois.
[4] Pacific Vegetable Oil Corporation, San Francisco.

with acid and extracted with diethyl ether as described by Taylor and Nelson (7). After methylation of the fatty acids as described below, the fatty acid content was determined by gas chromatography. A fat content of 0.52% was found for the starch of which 49% was linoleic acid. Thus, the 20% of starch in the basal diet provided 0.05% of linoleic acid. This small amount is not sufficient to affect the rapid development of essential fatty acid deficiency symptoms, as shown in our previous work (2). The corn starch was necessary in the diet because the young guinea pig does not thrive well when fed a diet with sucrose or glucose as the only carbohydrate.

At the end of experiments the guinea pigs were fasted overnight and stunned by a blow on the head and the jugular vein severed. Blood was collected in a beaker containing heparin, transferred to a centrifuge tube and the red cells washed 3 times with 0.9% saline. Five-tenths milliliter of packed cells were added to 10 ml of hot ethanol-diethyl ether (3:1) with stirring. The extraction was repeated twice with 5 ml of solvent. After evaporation of the combined extracts the lipid was dissolved in 3 ml of benzene: methanol (1:2) and the fatty acids transesterified, using BF_3 as catalyst, by refluxing for 60 minutes. The methyl esters were extracted into hexane and freed of unsaponifiable material by batch-treating with 2.0 g of activated alumina weakened with 6% of water (8).

For the gas chromatography, a 4 × 180 mm column packed with 15% ethylene glycol succinate on 80–100 mesh Chromosorb W was operated at 180°C (Barber-Colman instrument with radium ionization detector). Peaks were identified by their retention times compared with standard methyl esters distributed by the National Institutes of Health,[5] and peak areas were calculated by triangulation.

The oxidation of citrate and α-ketoglutarate by liver was measured manometrically essentially as described by Smith and De Luca (9) using 10% homogenates. These were prepared in cold 0.25 M sucrose immediately after removal of the organ from the animal.

RESULTS

In the first experiment the performance of guinea pigs fed varying dietary levels of corn oil or safflower oil was studied (table 2). In a 6-week period, 1% of corn oil (0.65% linoleic acid providing 1.89% of the calories) permitted as good growth as did levels up to 15%, whereas the growth of guinea pigs fed the basal, fat-free diet was retarded, even after only 4 weeks, and a high incidence of dermatitis occurred. No skin lesions were noted in any of the animals ingesting corn or safflower oils. Ten per cent of the fat-deficient animals died. The feeding of 25%

[5] Lipid Distribution Program, National Heart Institute.

TABLE 2

Effect of varying levels of corn or safflower oil on growth, survival and dermatitis

Dietary fat	Linoleic acid		No. experiments [1]	Survival	Dermatitis [2]	Avg wt, 6 weeks
	wt %	*cal %*		%	%	*g*
None	0.05 [3]	0.15	7	90	81	287 ± 39 [4]
1 % Corn oil	0.65	1.89	1	100	0	331 ± 26
3 % Corn oil	1.83	5.16	2	100	0	330 ± 30
7.3% Corn oil	4.39	11.6	3	100	0	356 ± 42
15 % Corn oil	8.97	21.3	2	100	0	348 ± 29
25 % Corn oil	14.9	31.2	1	100	0	300 ± 44
1 % Safflower oil	0.82	2.28	2	100	0	339 ± 39
2 % Safflower oil	1.59	4.56	1	100	0	332 ± 35
7.3% Safflower oil	5.67	15.0	2	100	0	346 ± 44

[1] Six to 8 animals in each experiment.
[2] Percentage of animals with symptoms.
[3] Present in the 20% of corn starch.
[4] SE of mean.

of corn oil resulted in a slight growth depression.

The second experiment was conducted with pure methyl linoleate[6] in an otherwise fat-free diet (table 3). A positive effect on growth was noted with even the lowest level of linoleic acid (0.2% of calories).

Very good weight gain was produced with as little as 0.24% of calories as linoleic acid as well as with higher amounts. The incidence and severity of the dermatitis which occurred in 75% of the animals fed the fat-free diet was diminished by increasing amounts of methyl linoleate, but only at the highest level, 1.31% of calories, were all animals free of symptoms.

Several guinea pigs fed the highest amount of methyl linoleate were maintained with the diet for as long as 8 months (fig. 1), at which time their weight and general appearance were comparable to those of animals fed 7.3% of corn oil. One of the guinea pigs fed methyl linoleate was mated with a normal female from the stock colony. When the female was killed 6 weeks later there were 4 normal fetuses in the uterus.

In a third experiment varying levels of linoleic acid were fed in a basal diet containing 5% trilaurin. Safflower oil was substituted for the trilaurin to provide 0.46 to 1.42% of the calories as linoleic acid. After 14 weeks the animals were killed and the fatty acid composition of the erythrocytes determined (table 4). Due to the small number of animals in this experiment, together with the wide range in weights, the evaluation of the growth data is not clear-cut. The lowest level of linoleic acid, 0.46% of calories, did not have an apparent effect on weight until the fourteenth week. The next level, 0.88% of calories, was not as effective as higher amounts during the first 11 weeks of the experiment but by 14 weeks this group had attained almost the same weight as the groups fed higher linoleate intakes. It appears that an intake of about 1% of calories was necessary for optimal weight gain.

As criteria of linoleic acid status, the ratios of triene to tetraene (20:3/20:4) and of oleate to linoleate (18:1/18:2) in the erythrocytes have been calculated (table 4). According to Holman (11), a triene-to-tetraene ratio of less than 0.4 in the erythrocytes, plasma, or heart has been found in the rat to correspond with an adequate intake of linoleic acid (1% of calories or more). In the guinea pig, it appears that a ratio of 0.17 or less is necessary when growth is the criterion. With respect to dermatitis, the same ratio appears to hold since guinea pigs receiving 0.46% of calories as linoleate (20:3/20:4 = 0.48) had scaly ears, whereas those receiving higher linoleate intakes did not. This observation is in general agreement with the dermatitis scores recorded in the previous experiment. In terms of 18:1/18:2, a ratio of less than 1.4 would correspond to a linoleate intake of about 1% of calories or more. When these ratios are plotted (fig. 2) against the percentage of calories as linoleate, the shape of the

[6] Hormel Institute, Austin, Minnesota.

TABLE 3

Effect of varying levels of linoleic acid on growth, survival and dermatitis

Dietary fat	Calories as linoleic acid	Avg wt, 8 weeks [1]	Survival	Dermatitis	
	%	g	%	%	score [2]
None	0.15 [3]	254 ± 28 [4]	75	75	5
0.017% Linoleic acid [5]	0.20	305 ± 52	100	63	4
0.033% Linoleic acid	0.24	362 ± 25	100	50	3
0.067% Linoleic acid	0.34	378 ± 45	100	38	3
0.133% Linoleic acid	0.54	366 ± 67	100	25	1
0.40 % Linoleic acid	1.31	382 ± 15	100	0	0
1% Corn oil	1.89	418 ± 28	100	0	0

[1] Eight animals/group.
[2] Each animal scored from normal (0) to severe (5). The figure shown is the average of those guinea pigs with dermatitis.
[3] Present in the 20% of corn starch.
[4] SE of mean.
[5] Added as the urea complex of methyl linoleate (10).

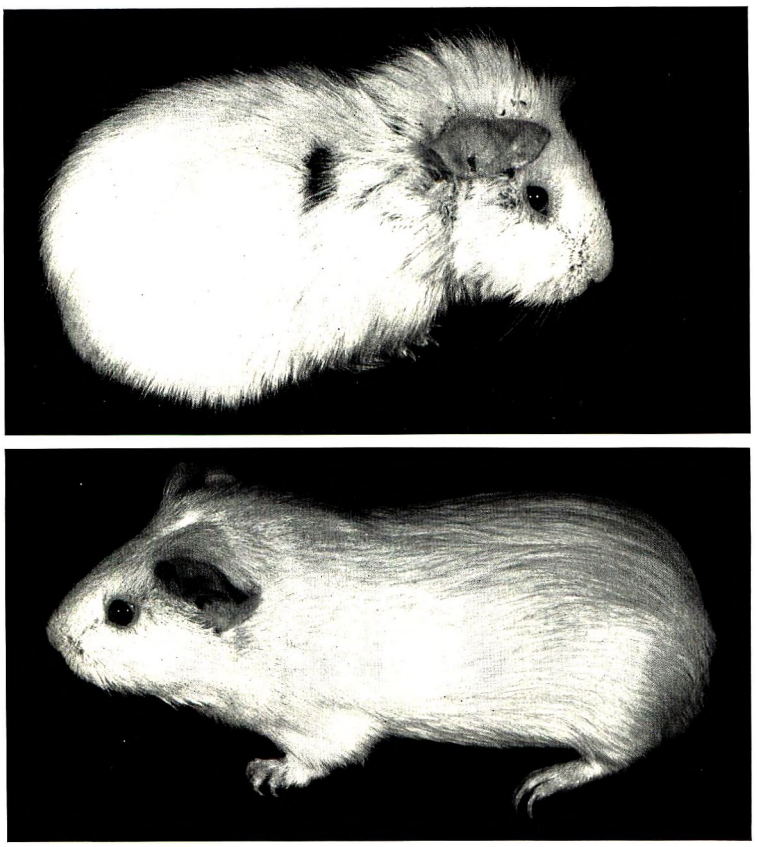

Fig. 1 Upper: animal reared with the basal fat-free diet for 8 months. Lower: animal reared with the same diet with 0.4% methyl linoleate as the urea complex, for 8 months.

curves is similar but the 18:1/18:2 ratio has a steeper slope.

While this experiment was in progress, Smith and De Luca (9) reported that liver homogenates from essential fatty acid-deficient rats showed increased rates of oxidation of several acids of the citric acid cycle. The oxidation of citrate and α-ketoglutarate by homogenates of liver from guinea pigs in the preceding experiment are shown in fig. 3. The oxidation of citrate by liver from animals ingesting 0.88 to 1.4% of calories as linoleate varied from 68 to 82 μliters of O_2/mg N (group averages) compared with a value of 124 for the EFA-deficient group. The group with the lowest linoleate supplement, 0.46% of calories, had an average value of 90 μliters O_2/mg N. There was thus a definite increase in the rate of citrate oxidation by the EFA-deficient livers, and 0.88% of calories as linoleate appeared to be necessary to reduce the rate to a minimal level.

In the case of α-ketoglutarate oxidation (fig. 3) the difference between the EFA-deficient group and the linoleate-supplemented groups was not as obvious as with citrate. Whereas the deficient group averaged 91 μliters O_2/mg N the linoleate groups ranged from 58 to 76 μliters O_2/mg N. Due to the wide range of values for the individual livers in the various groups, and the small number of samples, these data cannot be interpreted as showing a definite difference between linoleic acid-deficient and normal livers.

DISCUSSION

Comparison of the 6- or 8-week weights of linoleic acid-deficient or -sufficient animals in the 3 studies shows a considerable

TABLE 4

Effect of varying linoleic acid intakes of guinea pigs on fatty acid composition of erythrocytes, index of dermatitis, and weight gain

Safflower oil, %	0	0.22	0.42	0.50	0.60	0.69
Calories as linoleate, %	0.15 [1]	0.46	0.88	1.04	1.25	1.42
No. animals	5	4	4	5	4	3
Average wt, g						
8 weeks	394 ± 22 [2]	375 ± 8	403 ± 20	456 ± 15	457 ± 20	497 ± 16
11 weeks	465 ± 36	466 ± 12	505 ± 22	566 ± 17	561 ± 18	576 ± 37
14 weeks	509 ± 66	544 ± 16	594 ± 27	618 ± 26	640 ± 22	614 ± 39
Dermatitis [3]	4	3	0	0	0	0
RBC fatty acids,[4]						
16:0	13.0 ± 0.6 [2]	10.1 ± 1.4	12.6 ± 0.4	10.0 ± 0.2	13.1 ± 0.5	9.9 ± 0.5
16:1	3.0 ± 0.2	1.9 ± 0.3	1.4 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.2
18:0	34.6 ± 0.5	34.8 ± 2.0	35.8 ± 0.2	36.9 ± 0.3	37.1 ± 1.2	37.3 ± 0.7
18:1	23.8 ± 0.4	17.3 ± 1.9	11.8 ± 0.4	11.4 ± 1.3	8.6 ± 0.5	9.8 ± 1.0
18:2	2.8 ± 0.4	6.7 ± 0.2	8.4 ± 1.2	10.0 ± 0.4	11.2 ± 1.4	9.1 ± 1.2
20:3 [5]	6.9 ± 0.5	6.0 ± 0.8	2.8 ± 0.3	2.2 ± 0.3	1.5 ± 0.4	1.5 ± 0.2
20:4	7.1 ± 0.6	12.5 ± 1.1	16.2 ± 0.4	17.8 ± 0.4	16.2 ± 0.5	19.3 ± 1.0
20:5	2.7 ± 0.1	3.1 ± 0.5	2.4 ± 0.4	2.1 ± 0.3	2.3 ± 0.3	2.5 ± 0.2
22:5	trace	0.3 ± 0.3	1.1 ± 0.1	1.4 ± 0.2	1.6 ± 0.2	1.6 ± 0.3
22:6	trace	trace	trace	—	—	trace
18:1/18:2	8.60	2.57	1.41	1.14	0.73	1.08
20:3/20:4	0.98	0.48	0.17	0.13	0.10	0.08

[1] Present in the corn starch lipid.

[2] SE of mean.

[3] Number of animals with dermatitis.

[4] Number carbons:number of double bonds. Values are percentages of total fatty acids, with standard errors of the mean.

[5] Retention time corresponded to that for 5,8,11-eicosatrienoic acid. Traces of the 8,11,14-isomer were detectable only at the 2 highest intakes of linoleic acid.

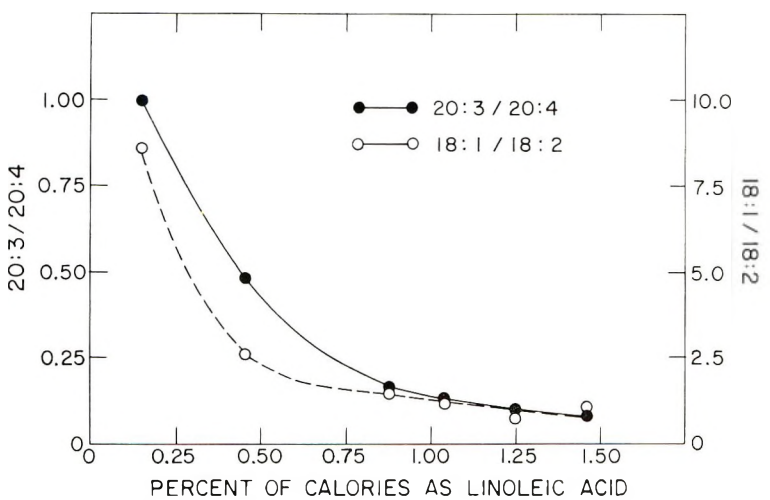

Fig. 2 Ratios of 5,8,11-eicosatrienoic:arachidonic and of oleic-to-linoleic acids in erythrocyte fatty acids of guinea pigs fed varying dietary levels of linoleic acid.

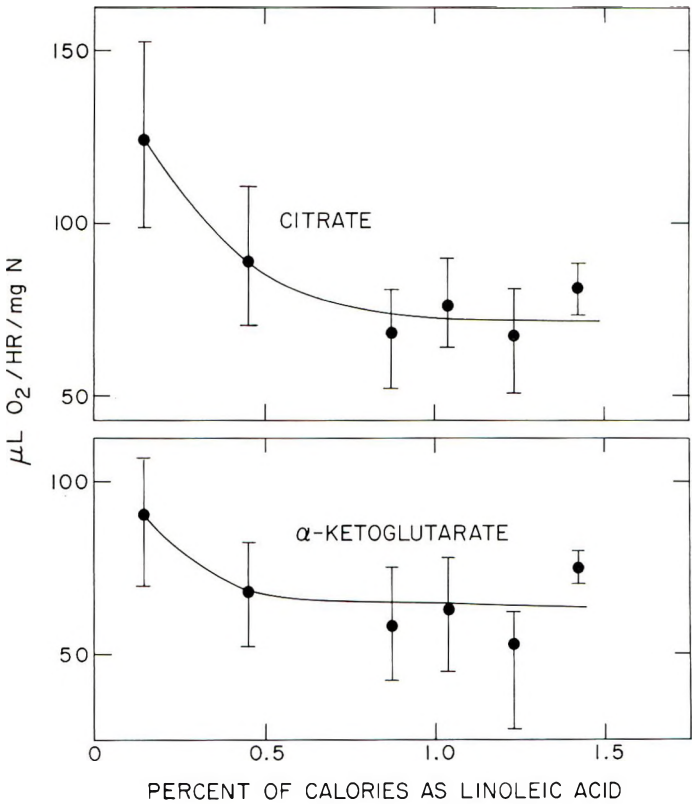

Fig. 3 Rates of oxidation of citrate and α-ketoglutarate by liver homogenates from guinea pigs fed varying dietary levels of linoleic acid. Vertical lines are standard errors.

variation in performance. The reasons for this are unknown; changes in maternal diet with seasonal variation, known to occur in our animal production section, are a possible source. As a result of this variation, comparisons of weights in the different studies are not valid.

The experiments in which corn and safflower oil were compared show that the guinea pig performs well over a wide range of fat intake, up to 15% in the case of corn oil. A higher level, 25%, depressed growth slightly but this may have been due partially to the oily consistency of the diet. Animals fed this diet were greasy and had an unkempt appearance. With respect to evaluating the requirement for linoleic acid in terms of growth and absence of skin symptoms, the lowest level of corn oil fed (1.89% of calories as linoleate or 1% of oil) was adequate.

In the experiment in which pure methyl linoleate was fed as the urea complex, only the highest level, 1.31% of calories (0.4% of linoleate) permitted both normal skin and optimal weight. The next lowest level, 0.54% of calories, was not sufficient to prevent dermatitis in all animals, although growth was satisfactory. This experiment showed that at least for an 8-week period, the linoleic acid requirement for maintenance of normal skin is considerably higher than that for optimal growth rate. Since the diet with the urea complex is very dry and powdery, with a consistency not different from the fat-free diet, the beneficial effects of increasing amounts of linoleate on the skin must be metabolic and not due to external effects of the diet on the skin and hair.

In the experiment where graded levels of safflower oil were fed in a diet contain-

ing 5% of fat for 14 weeks, on the basis of growth 0.46% of calories as linoleate was not adequate. The next level, 0.88%, produced as good weight gains at 14 weeks as did higher amounts but at 8 and 11 weeks this level may not have been sufficient. This observation suggests, as Leats (12) noted in pigs, that the linoleic acid requirement decreases as animals mature or as Wiese et al. (13) noted in puppies, the requirement increases with growth rate. In terms of dermatitis, the 0.88% level of calories was adequate. On the basis of these results the linoleate requirement of the male guinea pig is between 0.88 and 1.04% of calories. This value of about 1% agrees with the requirement estimated for human infants (14) and for rats (11). Whereas Leats (12) reported a requirement of about 1% of calories for swine, Hill et al. (15) observed a requirement of 2%.

One purpose of this study was to obtain biochemical information which could be used to assess the linoleic acid status of guinea pigs. The fatty acid content of erythrocytes was chosen as a criterion since it has been shown in the rat (11) that similar information can be obtained from erythrocytes, plasma or heart. Furthermore, for routine evaluation without killing the animal, blood is the only tissue available. The ratio of triene (5,8,11-eicosatrienoic) to tetraene (arachidonic) (table 4) shows that with a linoleic acid intake of 0.88 to 1.04% of calories, a value of 0.17 to 0.13 is obtained. This is similar to the ratio of 0.14 reported by Holman (11) for rat erythrocytes when 1.12% of the calories were linoleate.

In place of the triene-to-tetraene ratio, we would suggest that the ratio of oleate to linoleate (18:1 to 18:2) may be a more valid estimation of dietary linoleic acid adequacy. It has been shown by numerous investigators that oleate (and also palmitoleate, to a much lesser extent) increases in tissues of EFA-deficient animals, as noted in table 4. This ratio may have certain advantages over the triene-to-tetraene ratio; for example, it is now known that more than one eicosatriene can appear in tissues and one of these is a normal intermediate in the conversion of linoleate to arachidonate (16). Excellent chromatographic conditions are necessary to separate the various eicosatrienoic acid isomers and very probably many investigators have found only one peak where 2 or 3 compounds may be present (13). Data obtained by alkali isomerization also would not distinguish between the various trienes. On the basis of 18:1 to 18:2, a ratio of about 1.5 or less would indicate a sufficient intake of linoleate. Using the total amount of monoenes (16:1 + 18:1) rather than 18:1 alone did not change this ratio significantly.

Dhopeshwarkar and Mead (17) obtained normal male guinea pigs when 100 mg of linoleic acid/day were fed together with 3.65% of oleic acid. This dosage would be close to the intake of our animals receiving 0.4% of methyl linoleate in the diet (1.3% of calories, table 3) since these animals consumed about 25 g of diet/day.

In a previous study (2), we noted reddening of the muscles in fat-deficient guinea pigs, but this condition was not noted in the present experiments. Since Bender et al. (18) and also Schottelius et al. (19) had reported an increased myoglobin content in muscle of vitamin E-deficient guinea pigs, the dietary vitamin E in the current study was increased fivefold (to 100 mg/100 g). This may account for the absence of reddened muscles in the experiments.

The increase in oxidation of citrate by liver homogenates from EFA-deficient guinea pigs agrees with the results obtained by Smith and De Luca in rats (9); however, the difference between deficient and normal guinea pigs was not as great as in rat liver homogenates, even though the depletion period was similar in the two studies. In contrast with a marked difference in rate of oxidation of α-ketoglutarate by normal and EFA-deficient rat liver, very little difference was observed for this substrate in our guinea pigs. It appears that this biochemical measurement may not be as satisfactory for estimating EFA status in the guinea pig as it is for the rat.

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Response of Digestive Enzymes to Dietary Protein

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ABSTRACT Enzyme activities of the contents of small intestine were determined in rats pair-fed protein-free, 15% casein or 15% whole-egg protein diets during 1-hour intervals spaced 12 hours apart. Casein and whole-egg protein increased the total proteolytic, peptic, tryptic, and chymotryptic activities and the total endogenous nitrogen content of the digesta after 7 days of experimental feeding. Whole-egg protein was shown to increase the synthesis and secretion of trypsin, chymotrypsin and amylase when experimental diets were fed for 1, 7, 15, and 25 feedings. The presence of casein and whole-egg protein appeared to retard the rate of trypsin and chymotrypsin inactivation within the small intestine.

Previous studies have shown that both source and level of dietary protein influence the magnitude of endogenous protein secretions, including digestive enzymes, into the gastrointestinal tract. Twombly and Meyer (1) estimated that total endogenous nitrogen secretion over a digestive period of 12 hours' duration increases approximately 79% when rats are fed 15% whole-egg protein instead of a protein-free diet. Abdeljlil et al. (2) observed that casein-rich (70%) diets as opposed to starch-rich (75%) diets augment the tryptic and chymotryptic output of the rat pancreas as much as 1.7- and 3-fold, respectively. Similar observations were made by Howard and Yudkin (3) and Grossman et al. (4). The tryptic output of the dog pancreas is higher with raw meat and milk diets than with cooked meat or bread diets according to Babuskina et al. (5).

The secretory response elicited by dietary protein was investigated in the experiments reported below by fractionating and quantitating the nitrogenous components of rat intestinal contents and by determining the extent of enzyme adaptation to dietary change.

EXPERIMENTAL

Male Sprague-Dawley rats, averaging 180 g in weight and trained to eat during 1-hour periods spaced 12 hours apart, were pair-fed experimental rations composed of zero or 15% protein. Either casein or whole-egg protein [2] served as the protein source. Protein partially replaced sucrose in the diets, the remainder of which had the following percentage composition: cellulose, 5; vitamins, 1; [3] salts, 4; [4] and cottonseed oil, 5.

The rats were killed by chloroform inhalation. The contents were washed from their small intestines with cold (4°C) distilled water, ground in a tissue grinder, and centrifuged (14,000 × g) for 15 minutes to remove cellular debris and large particulate matter. The debris was analyzed for nitrogen. The supernatant was assayed for nitrogen, protein, and enzyme activity.

Nitrogen was determined by the Kjeldahl method. Protein was analyzed by Miller's modification of the Lowry method using casein as a reference (6).

Chymotryptic assays were performed using a modification of the method developed by Rhodes et al. (7). One milliliter of enzyme solution was placed in a cuvette and 2 ml of substrate-buffer-indicator solution (0.02 M ATEE, [5] 0.015 M Tris buffer, pH 8.1, 0.03 M $CaCl_2$, and 0.015% *m*-nitrophenol) were added. Readings were made at 395 mμ on a Beckman B spectrophotometer against a reagent blank set at 40% transmission. The assay was linear with respect to enzyme concentration over the range used. Tryptic assays were similarly

Received for publication December 27, 1963.

[1] Postdoctoral Fellow of the National Institutes of Health.

[2] Whole-egg powder, hexane extracted was fed at a level of 18.6% to attain a 15% level of protein.

[3] Phillips, P. H., and E. B. Hart, J. Biol. Chem., *109*: 657, 1935.

[4] Vitamin mix supplied the following vitamins: (in mg/100 g ration) thiamine, 0.3; riboflavin, 0.3; pyridoxine, 0.2; niacin, 0.4; Ca pantothenate, 4.0; folic acid, 0.5; biotin, 0.02; and vitamin B_{12}, 0.002.

[5] N-acetyl-L-tyrosine ethyl ester.

TABLE 1

Nitrogenous components of contents of small intestine

Treatment		Fraction			
Ration	Hours [1]	Insoluble protein [2]	Soluble protein [2]	Soluble protein [3]	Non-dialyzable protein [3]
		mg	*mg*	*mg*	*mg*
Protein-free	1	64	98	78	27
	2.5	15	53	39	14
	8	30	72	62	25
	12	27	89	62	25
15% Casein	1	62	149	112	32
	2.5	34	77	65	21
	8	41	116	83	29
	12	56	155	92	34
15% Egg protein	1	62	144	102	46
	2.5	61	150	112	38
	8	70	176	123	49
	12	60	164	93	44

[1] Hours after the initiation of the 1-hour feeding period.
[2] Analyzed by Kjeldahl method; protein = N × 6.25.
[3] Analyzed by Miller's method using Folin-phenol reagent (6).

performed using either BAEE [6] or TAME [7] as substrate.

Total proteolytic activity was determined by measuring the solubility of casein in 5% trichloroacetic acid after 20 minutes of incubation with the enzyme (8). The starch-iodine method described by Smith and Roe (9), was used to assay for amylase. The peptic-like protease activity of small intestinal contents, presumably of gastric origin, was assayed at pH 1.2 using a hemoglobin substrate (10).

RESULTS

Intestinal contents of rats fed their assigned ration for one week were treated as described above and then further fractionated by dialysis for 12 hours at 4°C against 0.005 M phosphate buffer, pH 7.5. The contents of groups of 3 rats each were pooled to provide sufficient sample for the fractionation studies. Results were divided by 3 to give an average value of nitrogen or enzyme activity per rat. The nitrogenous components of the small intestinal contents are shown in table 1. Approximately 20 to 40% of the nitrogen was insoluble. This insoluble protein was probably composed mainly of cell debris and mucous secretions. The Miller method gave lower values for total soluble protein, a possible reflection of the amount of non-protein nitrogen, including amino acids, in the small intestine. The color developed in the Miller analysis is a function of the number of peptide bonds and aromatic amino acid residues in the sample. When protein was not fed, the soluble protein fraction was composed chiefly of enzymes and partially digested enzymes, mucus, and cell debris. The non-dialyzable fraction contained the intact enzymes. The protein content of almost every fraction increased when protein was fed. The largest increases were observed in rats fed whole-egg protein.

The enzyme activities of the contents of the small intestine after 1 week of experimental feeding are shown in table 2. Tryptic and chymotryptic activities were measured on model substrates, but to clarify presentation, values for their activities were converted to proteolytic units. The factors for converting from esterolytic to proteolytic activity were determined in a study of purified rat trypsin and chymotrypsin. The conversion factors are the quotients of the proteolytic activity and the esterolytic activity of given quantities of purified enzymes. The results shown in table 2 are also from the study with pooled samples and therefore do not indicate the variability among animals.

When pair-fed rats received casein, tryptic activity increased slightly within intestinal contents but activities of chymotrypsin and other proteases increased at all time intervals. Greater increases in all 3 activities were produced when egg protein

[6] N-benzoyl-L-arginine ethyl ester.
[7] p-Toluene sulfonyl-L-arginine methyl ester.

TABLE 2

Sources of intestinal proteolytic enzyme activity after 7 days of experimental feeding

Ration	Hour [1]	Source of proteolytic activity		
		Trypsin [2,3]	Chymotrypsin [2,3]	Other proteolytic enzymes [2,4]
Protein-free	1	235	44	96
	2.5	269	33	83
	8	138	34	269
	12	245	46	178
15% Casein	1	314	62	250
	2.5	328	87	158
	8	293	71	451
	12	236	63	196
15% Egg protein	1	381	171	415
	2.5	583	154	345
	8	361	105	354
	12	305	73	184

[1] Hours after the initiation of the 1-hour feeding period.
[2] Milligrams of casein digested/20 minutes.
[3] Method of calculating the proteolytic activity of trypsin and chymotrypsin is presented in the text.
[4] The activity of other proteolytic enzymes was obtained by difference.

TABLE 3

Potential peptic activity of contents of small intestine

Ration [1]	Hours [2]	Potential peptic activity [3]
Protein-free	1	13.5
	5	12.3
	8	0.0
	12	14.0
15% Casein	1	39.9
	2.5	57.7
	8	24.4
	12	28.7

[1] Rations were fed ad libitum for 7 days.
[2] Hours after the initiation of the 1-hour feeding period.
[3] Milligrams hemoglobin digested/20 minutes.

was fed. Apparently, trypsin was the most active single proteolytic enzyme secreted into rat intestinal contents. A variety of proteases of pancreatic and intestinal origin were probably responsible for the additional proteolytic activity. Their activity was dependent in part upon initial digestion by trypsin and chymotrypsin.

The data presented in table 3 demonstrate that the potential peptic activity of the contents of small intestine was also increased by feeding casein. The term "potential" activity is used because pepsin is not active under the neutral or slightly basic conditions prevailing in the intestine. Pepsin is denatured above pH 6, (11) but peptic-like enzymes in intestinal contents were still able to function under acidic conditions.

To ascertain the time required to produce a response to dietary change, enzyme activities of the digesta were measured 2.5 hours after the initiation of the following feeding periods: 1, 7, 15 and 25. The digestive enzymic adaptation of rats, previously fed a casein diet, to a protein-free or whole-egg protein diet, is shown in table 4. After only one feeding, tryptic, chymotryptic, total proteolytic, and amylolytic activities were reduced with the protein-free diet and elevated with the whole-egg protein diet. Differences between rats fed egg protein and rats fed the protein-free diet were all significant at the 2% level. Proteolytic and chymotryptic activities were not appreciably altered after 25 feedings of a protein-free diet. Tryptic and amylolytic activities were reduced after 7 and 15 feedings but not after 25 feedings. The pancreatic enzyme activity of rat digesta adapted to the whole-egg protein diet, reaching a maximum by the seventh feeding period. The increases in proteolytic, chymotryptic and amylolytic activities in rats fed whole-egg protein were significant at the 1% level. Possibly the rats were no longer able to achieve a maximal response to the dietary stimulus due to the restricted food consumption.

The statistical results of the adaptation study show the variability that can be expected within groups of animals in an experiment of this nature. Generally, vari-

TABLE 4

Adaptation of intestinal enzyme activity to diet [1]

Treatment [2]		Enzyme [3]			
Ration	No. of feedings	Proteolytic	Trypsin	Chymotrypsin	Amylase
		μmoles tyrosine/ 20 min	*μmoles TAME/ min*	*μmoles ATEE/ min*	*mg starch/min*
Casein	> 15	422 ± 24 [4]	192 ± 14	180 ± 26	461 ± 113
Protein-free	1	195 ± 32	111 ± 15	56 ± 10	288 ± 43
	7	198 ± 27	69 ± 12	51 ± 3	139 ± 6
	15	182 ± 25	77 ± 10	63 ± 12	0 ± 0
	25	209 ± 7	124 ± 10	97 ± 13	256 ± 29
Whole-egg protein	1	509 ± 27	288 ± 28	267 ± 16	684 ± 124
	7	933 ± 40	402 ± 36	782 ± 58	1386 ± 250
	15	755 ± 46	389 ± 64	650 ± 92	1172 ± 66
	25	590 ± 45	326 ± 24	452 ± 19	605 ± 53

[1] TAME indicated *p*-toluene sylfonyl-L-arginine methyl ester; ATEE indicates N-acetyl-L-tyrosine ethyl ester.
[2] Groups of 4 rats each were used.
[3] Enzyme activities of intestinal contents were determined 2.5 hours after the initiation of feeding.
[4] Standard error of the mean.

TABLE 5

Inactivation of chymotrypsin and trypsin within intestinal contents

Ration	Hours [1]	Inactivation of chymotrypsin at 4°C		Inactivation of trypsin at 4°C	
		After 12 hours	After dialysis for 12 hours	After 12 hours	After dialysis for 12 hours
		%	%	%	%
Protein-free	1	14.9	66.2	12.5	30.2
	2.5	62.9	88.5	39.1	55.5
	8	42.6	59.6	+33.7	+6.2
	12	32.1	64.3	8.4	23.9
Casein	1	4.7	44.1	+12.0	+4.3
	2.5	48.5	74.4	10.5	40.5
	8	31.2	63.8	+12.8	11.2
	12	32.3	47.1	13.9	32.2
Whole-egg	1	0.0	71.9	0.0	10.0
	2.5	27.5	70.6	24.2	37.4
	8	16.7	53.6	6.8	20.6
	12	2.3	35.0	27.1	59.9

[1] Hours after the initiation of the 1-hour feeding period.

ability is fairly low as long as animals are from the same lot and are treated identically.

The dialysis experiment, summarized in table 5, was performed to test whether casein and whole-egg protein effect the increase in enzyme activity solely by augmenting enzyme synthesis and secretion. Enzyme activities of soluble intestinal contents, maintained at 4°C for 12 hours, were compared to activities observed after dialysis for 12 hours against 0.005 M phosphate buffer, pH 7.5. In all instances less tryptic and chymotryptic activity remained after dialysis than after standing. Proportionally more chymotrypsin was inactivated after standing 12 hours in solutions of intestinal contents of rats fed a protein-free diet. Least inactivation of chymotrypsin occurred after the whole-egg protein diet was fed. Thus, protein protected chymotrypsin in solution. Results with trypsin were variable. Occasionally tryptic activity increased when the solutions of intestinal contents stood for 12 hours. This phenomenon might be related to an organic inhibitor such as trypsin inhibitor, which in the rat is digested after incubation with trypsin and chymotrypsin (12). Rat trypsin inhibitor has been recovered

from pancreatic juice and hence is secreted into the intestine. Its destruction on standing in a solution of intestinal contents might explain increases in tryptic activity.

DISCUSSION

Studies of the nitrogenous components of the digesta indicate that dietary protein augments secretion of digestive enzymes as well as other endogenous proteins. Probably only small amounts of dietary protein were recovered in the insoluble fraction. Casein is fairly soluble under conditions prevailing in the small intestine. Egg protein is rendered soluble by the action of HCl and pepsin.[8] Thus, mucous secretions and cell slough-off may increase when protein is fed. The enzymic contribution to the non-dialyzable fraction of contents of rats fed the protein-free diet cannot be accurately assessed but was probably large since the other proteins will be partially digested as they become soluble. Rats consumed, on the average, 525 mg of protein. Sufficient proteolytic activity was present in the small intestine to digest all protein consumed in 20 minutes, but past experiments indicate that less than one-half of the protein entered the small intestine by 2.5 hours (1). Thus, hydrolysis must have been rapid and casein probably did not accumulate in the non-dialyzable fraction. In vitro, egg protein is hydrolyzed less rapidly than casein by trypsin and chymotrypsin.[9] The increase in the size of the non-dialyzable fraction, especially after rats were fed casein, is indicative of increased enzyme secretion.

That dietary protein increased the enzyme activity of the contents of the small intestine was shown by the results of the enzyme assays. After 7 days of experimental feeding, casein and whole-egg protein diets, in contrast with the protein-free diet, increased the proteolytic enzyme activity of rat digesta throughout the digestive period. The increases were greatest during the first 8 hours of digestion when more dietary protein was present in the gastrointestinal tract. The mechanisms regulating the enzymatic response to diet were not established in this experiment although the response to the whole-egg protein diet may have been due in part to excessive stimulation of pancreatic secretion by ovomucoid trypsin inhibitor as indicated by Lyman et al. (13).

The results of the adaptation study indicate that dietary protein directly stimulates enzyme secretion because the enzyme activity of the digesta was altered after one feeding of the test diet. Wang and Grossman (14) have demonstrated that products of protein digestion stimulate pancreatic secretion.

The adaptation study also showed that the proteolytic and amylolytic activities of intestinal contents adapted to continued feeding of whole-egg protein, a result suggestive of increased synthesis of pancreatic enzymes. The influence of dietary protein on non-proteolytic enzymes was evidenced by the fact that amylolytic activities increased when casein and whole-egg protein were fed.

Dietary protein appears to increase the enzymic potential of the digesta not only by increasing the synthesis and secretion of various digestive enzymes but by retarding the rate of breakdown of these enzymes within the intestine. Pelot and Grossman (15) noted that trypsin and chymotrypsin are inactivated in the rat intestine. Chymotrypsin is inactivated more rapidly than trypsin. The authors observed that the ratio of chymotryptic to tryptic activity decreased from 4.96 in the pancreatic juice to 0.85 in the fourth quarter of the small intestine. Similar ratios, calculated from the data shown in table 4, demonstrate that proportionally greater increases of chymotrypsin were observed when protein diets were fed. Because the activity of the less stable enzyme was more readily increased, dietary protein must protect the enzymes. This effect was also noted in the dialysis study. Enzyme inactivation occurred to a greater extent after proteolytic substrates of dietary and endogenous origin were removed by dialysis. Dietary protein may exert its protective effect by retarding the rate of proteolysis or digestion of the enzymes, themselves. The loss of activity during dialysis might have been due in part to loss of active enzyme "fragments,"

[8] In in vitro experiments whole-egg protein goes into solution after approximately 15 minutes of incubation with 10 cm^3 of 0.1 N HCl and 0.2 mg of pepsin.

[9] A solution of whole-egg protein (5 mg/cm^3), previously subjected to 1 hour of peptic digestion, is hydrolyzed by trypsin and chymotrypsin (0.02 mg/cm^3) at approximately 31% the rate of a similarly treated solution of casein.

a phenomenon noted by Bresler et al. (16). However, less than 5% of the lost activity could be recovered in the dialyzate.

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Nonspecific Role of Arginine in the Promotion of Muscular Dystrophy in the Chick[1]

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ABSTRACT Experiments were conducted with diets deficient in vitamin E and sulfur amino acids to study the specificity of arginine in the promotion of breast muscle lesions in the chick. It was observed that no muscle degeneration occurred in chicks when the cystine-deficient diets used were primarily deficient in lysine, isoleucine, tryptophan or tyrosine, whether or not supplemental arginine was provided as an additional stress. Supplementation with the limiting amino acid did not promote muscular dystrophy unless subsequent to the addition, the sulfur amino acids became first limiting for growth. When this latter condition was met, muscular dystrophy-promoting activity could be shown for lysine, isoleucine, tryptophan and tyrosine as well as arginine, demonstrating that the arginine effect was nonspecific and that development of the condition merely required that the amino acid intake was primarily deficient in the sulfur amino acids. Exaggeration of the amino acid imbalance inherent in cystine-deficient, muscular dystrophy-supporting diets, had no effect on the development of the myopathy, indicating that an excess of essential amino acids per se relative to cystine was not contributing to the disorder. Methionine protectiveness was shown to be superior at the higher (but suboptimal) sulfur amino acid intakes used, apparently due to a reduced utilization of the amino acid for growth at the higher rates of gain.

In the initial studies on muscular dystrophy in the chick, Dam et al. (1) observed that chicks fed a diet simultaneously deficient in vitamin E and sulfur amino acids developed a myopathy characterized by grossly visible white striations in the breast musculature. These workers observed that supplementation with 0.01 % α-tocopheryl acetate or 0.5% L-cystine would prevent the condition and later Machlin and others (2,3) demonstrated the effectiveness of methionine and high levels of diphenyl-*p*-phenylenediamine and ethoxyquin.

During investigations on the specificity of the sulfur amino acids in prevention of the disease, Nesheim et al. (4) observed that muscular dystrophy did not occur in chicks fed a diet deficient in vitamin E and the sulfur amino acids when the diet was also deficient in arginine. The inclusion of arginine in the basal diet resulted in a high incidence and severity of muscle degeneration before the chicks were 5 weeks of age. From the results obtained in this study, as well as in later experiments (5), it was postulated that the arginine effect specifically involved a reduction of methionine effectiveness possibly by a decreased conversion of the amino acid to cystine. Preliminary studies in the authors' laboratory (6), however, indicated that arginine has a nonspecific role in the disorder, and that arginine supplementation merely altered the sequence of limiting amino acids in the diet, causing the sulfur amino acids to become first limiting for growth. To further test this premise, in the present study 5 experiments were conducted with sulfur amino acid-deficient diets in which essential amino acids other than arginine were first limiting for weight gain, or arginine was primarily deficient but the sulfur amino acids were in a third rather than a second limiting position.

Additionally, in view of the amino acid imbalance necessarily present in the sulfur amino acid-deficient diets used for the production of muscular dystrophy in the chick, a study was conducted to investigate the relation of amino acid excesses to development of the disorder.

Received for publication November 13, 1963.

[1] This work was supported in part by a grant from the Ontario Research Foundation, Toronto, Canada. The authors are also indebted to Merck and Company, Ltd., Montreal, for the lysine and several of the vitamins employed and to Distillation Products Industries, Rochester, New York, for generous supplies of vitamin E-stripped lard.

TABLE 1

Composition of basal diets [1]

Ingredients [2]	Diet no.						
	1	2	3	4	5	6	7
	%	%	%	%	%	%	%
Casein	—	—	—	—	—	20.00	13.30
Zein	10.98	—	—	21.98	21.98	—	—
Peanut meal	20.37	—	—	—	—	—	—
Blood meal	—	16.00	6.66	—	—	—	—
Gelatin	—	5.54	16.66	—	—	—	—
Sesame meal	—	6.80	—	—	—	—	—
L-Tryptophan	0.10	—	—	0.074	0.02	—	—
DL-Threonine [3]	0.28	—	0.24	—	—	—	—
DL-Valine [3]	0.12	—	—	0.40	0.40	—	—
Glycine	0.50	—	—	1.04	1.04	0.60	0.40
DL-Methionine	—	0.10	0.34	—	—	0.10	0.067
L-Leucine	—	—	0.28	—	—	—	—
L-Lysine·HCl	—	—	—	1.45	1.45	—	—
L-Arginine·HCl	—	—	—	0.07	0.19	1.00	0.67
L-Histidine·HCl·H_2O	—	—	—	0.08	0.08	—	—
Glucose	55.43	59.34	63.60	62.69	62.62	66.08	73.34
Cellulose	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Stripped lard	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Vitamin mixture [4]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral mixture [5]	4.22	4.22	4.22	4.22	4.22	4.22	4.22

[1] Protein analyses (N × 6.25) were as follows: basals 1–5, 20%; casein, 90.6%; gelatin, 90.2%; zein, 91.0%; and peanut meal, 49.1%; blood meal, 75.0%; and sesame meal, 44.2% after extraction with ether to remove tocopherols.

[2] Zein, gelatin, cellulose (Alphacel) and amino acids purchased from Nutritional Biochemicals Corporation, Cleveland; peanut meal, General Biochemicals, Inc., Chagrin Falls, Ohio; sesame meal, Pacific Vegetable Oil Corporation, San Francisco; blood meal, Canada Packers Ltd., Toronto, Canada.

[3] Added at twice the amount of L-form required, as based on the NRC requirements (8).

[4] Supplied the following (mg/100 g diet): inositol, 25; niacin, 5; Ca pantothenate, 2; thiamine·HCl, 1; riboflavin, 1; pyridoxine·HCl, 0.45; folic acid, 0.40; menadione, 0.05; D-biotin, 0.02; vitamin B_{12}, 0.002; vitamin A palmitate, 500 IU; vitamin D_3, 38 ICU; choline chloride, 150.

[5] Supplied the following (g/100 g diet): $CaHPO_4 \cdot 2H_2O$, 1.89; $CaCO_3$, 0.65; KH_2PO_4, 0.69; $MgCO_3$, 0.192; NaCl, 0.6; and (mg/100 g diet): $FePO_4 \cdot 4H_2O$, 33.3; $MnSO_4 \cdot H_2O$, 33.3; KI, 0.26; $CuSO_4 \cdot 5H_2O$, 1.67; $ZnCl_2$, 1.0; $CoCl_2 \cdot 6H_2O$, 0.17; $Na_2MoO_4 \cdot 2H_2O$, 0.83.

EXPERIMENTAL

Female White Plymouth Rock chicks, obtained from a commerical hatchery, were used in all trials. For experiments 1, 2, and 3, the chicks were fed a 20% casein basal diet (table 1, basal no. 6) for the first week, at the end of which time they were selected by weight range for random assignment to the treatment groups. For the remaining experiments, one-day-old chicks were assigned at random to the various pens. The chicks were housed in wire-floor battery brooders in lots of 15 with feed and water provided ad libitum. At the termination of the 5-week feeding period the birds were killed and the breast muscles assessed for incidence and severity of muscular dystrophy. The composition of the vitamin E and sulfur amino acid-deficient basal diets is shown in table 1. Amino acid supplementation of the gelatin, zein, and peanut, blood, and sesame meal-containing basals was based on the calculated average analyses from the values given by Block and Bolling (7) and supplementation of the casein basal diet was based on values obtained from microbiological assays conducted in this laboratory. Where required, amino acids were included in the basal rations in amounts calculated to produce the desired sequence of limiting amino acids and in dietary treatments in amounts to approximate the NRC requirement (8).

RESULTS AND DISCUSSION

Promotion of muscular dystrophy by amino acids other than arginine. Results of the 5 experiments are presented in table 2. In the first experiment a combination of zein and peanut meal was used (basal diet 1) to provide lysine and the sulfur amino acids at 30 and 74%, respectively, of the NRC requirement (8). The arginine level used in the basal diet

TABLE 2

Evidence of a nonspecific role for arginine in the promotion of muscular dystrophy in the chick

Group no.	Amino acid additions [1]						Limiting amino acid [2]	Gain in wt [3]	Feed efficiency	Muscular dystrophy	
	L-Trypto-phan	DL-Iso-leucine	DL-Me-thionine	L-Tyro-sine	L-Argi-nine	L-Lysine				Incidence [4]	Severity [5]
	% of diet		% of diet		% of diet		% NRC requirement [6]	g 7–35 days	g gain/g feed consumed (× 100)	%	
						Experiment 1 (basal diet 1)					
1	—	—	—	—	—	—	lysine (30)	52 ± 7	16	0/15	0	0.0
2	—	—	—	—	0.83	—	lysine (30)	56 ± 8	17	0/14	0	0.0
3	—	—	0.20	—	—	—	lysine (30)	51 ± 6	15	0/10	0	0.0
4	—	—	—	—	—	0.70	M-74, C-74 (74)	219 ± 11	33	15/15	100	3.1
5	—	—	0.20	—	—	0.70	—	375 ± 9	47	0/15	0	0.0
						Experiment 2 (basal diet 2)					
1	—	—	—	—	—	—	isoleucine (60)	72 ± 8	22	0/15	0	0.0
2	—	—	—	—	0.83	—	isoleucine (60)	77 ± 9	20	0/15	0	0.0
3	—	—	0.18	—	—	—	isoleucine (60)	71 ± 7	21	0/14	0	0.0
4	—	0.40	—	—	—	—	M-82, C-74 (79)	326 ± 9	35	13/14	93	2.2
5	—	0.40	0.18	—	—	—	—	424 ± 13	45	0/14	0	0.0
						Experiment 3 (basal diet 3)					
1	—	—	—	—	—	—	tryptophan (33)	13 ± 4	6	0/15	0	0.0
2	—	—	—	—	0.83	—	tryptophan (33)	8 ± 2	4	0/14	0	0.0
3	0.14	—	—	—	—	—	isoleucine (53)	30 ± 3	16	0/15	0	0.0
4	0.14	—	—	—	0.83	—	isoleucine (53)	37 ± 5	17	0/14	0	0.0
5	0.14	0.60	—	—	—	—	P-103, T-27 (64)	166 ± 8	33	0/15	0	0.0
6	0.14	0.60	—	—	0.83	—	P-103, T-27 (64)	158 ± 10	33	0/15	0	0.0
7	0.14	0.60	0.18	—	—	—	P-103, T-27 (64)	169 ± 10	36	0/15	0	0.0
8	0.14	0.60	—	0.52	—	—	M-116, C-30 (78)	274 ± 9	41	10/15	67	2.5
9	0.14	0.60	0.18	0.52	—	—	—	291 ± 11	44	0/15	0	0.0
						Experiment 4 (basal diet 4)		g, 1–35 days			
1	—	—	—	—	—	—	arginine (35)	18 ± 1	9	0/13	0	0.0
2	0.11	—	—	—	—	—	arginine (35)	23 ± 2	9	0/15	0	0.0
3	—	—	—	—	0.83	—	tryptophan (47)	51 ± 4	18	0/15	0	0.0
4	0.11	—	—	—	0.83	—	M-102, C-57 (83)	70 ± 4	26	10/15	67	1.8
5	0.11	—	0.30	—	0.83	—	—	203 ± 9	42	0/15	0	0.0
						Experiment 5 (basal diet 5)					
1	—	—	—	—	—	—	tryptophan (20)	6 ± 1	4	0/12	0	0.0
2	—	—	—	—	0.72	—	tryptophan (20)	7 ± 2	6	0/9	0	0.0
3	0.16	—	—	—	—	—	arginine (43)	61 ± 4	20	0/15	0	0.0
4	0.16	—	—	—	0.72	—	M-102, C-57 (83)	83 ± 4	26	12/13	92	2.8
5	0.16	—	0.30	—	0.72	—	—	217 ± 7	36	0/15	0	0.0

[1] DL-Isoleucine used at twice the amount of L-form required; arginine and lysine added at proportionately higher levels as the hydrochlorides; amino acid additions replaced glucose in basal.
[2] Limiting amino acid for weight gain.
[3] Mean ± SE of mean.
[4] Incidence: fraction or percentage of surviving chicks showing visible breast muscle lesions.
[5] Severity: average score for birds having visible white striations using a scale of 1 to 5.
[6] Calculated level of limiting amino acid, as percentage of the NRC requirement (8), shown in parentheses. Individual and average values given for methionine plus cystine and for phenylalanine plus tyrosine.

(1.2% of the diet) was considered adequate to obviate an arginine deficiency but sufficiently low to avoid the possibility of an excess contributing to the development of muscular dystrophy. The data show that chicks which received the lysine-deficient basal ration either with or without supplemental arginine had no breast muscle degeneration, demonstrating that under these dietary conditions arginine had no effect in promoting muscular dystrophy. Birds that received the basal diet supplemented with lysine showed markedly improved weight gains and feed efficiency and a high incidence and severity of muscle lesions. Feeding methionine in addition to lysine resulted in a further stimulation of weight gain and complete prevention of the disorder. These results demonstrate that the muscular dystrophy-promoting activity attributed to arginine also can be shown for lysine, and suggest that development of the dystrophic condition may simply require that the amino acid intake be primarily deficient in the sulfur amino acids.

The results obtained in the first experiment prompted a second similar study on arginine specificity. For this purpose, a mixture of blood meal, gelatin and sesame meal was used (basal 2) in which isoleucine and the sulfur amino acids were the first and second limiting amino acids, respectively. As observed in the previous experiment, no muscular dystrophy occurred in chicks fed a diet containing a deficient but not growth-limiting level of the sulfur amino acids. Similarly, birds receiving additional arginine showed no evidence of muscle degeneration. Supplementation of the basal diet with isoleucine, however, prompted a marked improvement in weight gain, feed efficiency and a high incidence of breast muscle degeneration demonstrating that isoleucine, as well as lysine, could simulate the "arginine effect."

In the third experiment, a diet was formulated using gelatin, blood meal and supplemental amino acids (basal 3) to contain an increasing, stepwise deficiency of tryptophan, isoleucine, tyrosine (plus phenylalanine) and cystine. The object was to demonstrate dystrophy-supporting activity for a third amino acid (tyrosine) and to study the specificity of the isoleucine effect observed in the previous experiment. The data show that chicks that received the basal diet for the 5-week feeding period experienced only minute increases in body weight and had no breast muscle degeneration. The addition of tryptophan to the basal diet prompted a slight improvement in weight gain and feed efficiency but did not result in the development of the degeneration. Supplementation with isoleucine as well as tryptophan improved the rate of gain of the chicks but did not promote muscular dystrophy. Further supplementation with tyrosine resulted in an additional improvement of weight gain and the development of muscle lesions. Finally, feeding methionine in addition to tryptophan, isoleucine and tyrosine stimulated further growth and prevented the disorder. It is readily apparent from these data that tyrosine (as well as isoleucine and lysine) could simulate the dystrophy-promoting effect attributed to arginine, and that amino acid supplementation had no effect in the development of muscular dystrophy unless subsequent to the supplementation the sulfur amino acids became first limiting for growth — thus isoleucine would not promote the degeneration in this experiment when placed in a limiting position not immediately prior to that of cystine. The observation that chicks receiving the tryptophan, or tryptohan-plus-isoleucine supplements to the basal diet had improved weight gains but no dystrophic lesions, is evidence against the possibility that the development of muscular dystrophy in the previous experiments resulted from an improved rate of growth. This is not unexpected, however, as the sulfur amino acid-deficient basal diets used were more severely deficient in essential amino acids other than cystine and thus provided the chicks with an excess of the sulfur amino acids relative to the amounts that could be utilized for maintenance and growth. Although supplementation with the most severely deficient amino acids would have enabled the utilization of part of the excess of the sulfur amino acids for improved growth, no dystrophy would have occurred until supplementation pro-

ceeded to where cystine became the growth-limiting amino acid.

It seemed pertinent also to study the muscular dystrophy-promoting activity of arginine in a more direct manner by the use of a diet in which arginine assumed a limiting position for weight gain. Thus, in a fourth experiment a diet was formulated using zein plus supplemental amino acids (basal 4) in which the limiting amino acids were arginine, tryptophan and cystine in place of the usual deficiency sequence of arginine to cystine when casein is the dietary source of protein for chicks. The data show that arginine supplementation was ineffective in promoting the myopathy when the sulfur amino acids were not in the next limiting position. Muscular dystrophy occurred, however, in chicks that received both tryptophan and arginine, demonstrating that tryptophan as well as lysine, isoleucine and tyrosine could simulate the "arginine effect." Modification of the basal diet so that tryptophan, arginine and cystine became the sequence of limiting amino acids (basal 5) resulted in arginine rather than tryptophan becoming effective in the promotion of muscle lesions, confirming the previous observations that arginine has a nonspecific role in the muscle disorder.

In contrast to the foregoing observations, Scott and Calvert (5) reported in a recent study that arginine may be involved in the disorder by antagonizing the dystrophy-preventing action of methionine, but not cystine. These workers noted that supplementation of an arginine and sulfur amino acid-deficient diet with cystine plus arginine prevented the onset of muscular dystrophy, whereas a combination of methionine plus arginine was ineffective in preventing the disorder. However, a calculation of the levels of methionine and cystine furnished by the experimental diets used in that study reveals that the cystine level was excessively high compared with methionine. Consequently when the supplement of arginine plus cystine was fed, growth was limited by a deficiency of methionine, and cystine in excess of that which could be used for maintenance and growth was provided in a sufficient amount to prevent dystrophy. On the other hand, when arginine plus methionine in a suboptimal amount were added, growth was stimulated and neither methionine nor cystine remained in excess to protect against dystrophy. Thus the difference observed in the effectiveness of cystine and methionine in the presence of arginine can be explained by an excessively high cystine content of the diet used and does not necessarily support a *specific* role for arginine.

Amino acid imbalance in dystrophy. As the development of muscular dystrophy in the chick requires the use of sulfur amino acid-deficient diets, it is apparent that these diets contain an inherent imbalance in terms of excessive amounts of essential amino acids relative to the levels of the sulfur amino acids. To study the relation of this imbalance to the development of muscular dystrophy, diets were used containing either 13.3 or 20% of casein and an amino acid mixture equivalent to one-half the protein plus supplemental amino acids (excepting cystine and methionine) to exaggerate the imbalance. To enable evaluation of an imbalance effect a low level of methionine was incorporated into the basal diets to retard the development of the disorder.

The data (exp. 6, table 3) show that exaggeration of the amino acid imbalance had no effect on the development of muscular dystrophy at either the 13.3 or 20% casein level. Similarly, no substantial difference in the development of dystrophy was observed in chicks receiving either 20% casein (lot 1) or 13.3% casein fortified with amino acids (other than cystine and methionine) to simulate 20% casein (lot 4). As these diets were formulated so as to differ only in sulfur amino acid content (0.39% vs 0.59%), it then appears that if the more deficient diet had been supplemented with the sulfur amino acid differential no alleviation of the muscle disorder would have been provided. This indicates that methionine is relatively ineffective when added to a diet severely deficient in the sulfur amino acids. To investigate this possibility further, an additional experiment was conducted in which graded levels of methionine were added to the 13.3 and 20% casein basal diets. The data (exp. 7) reveal that at low dietary levels of sulfur amino acids methionine supplementation was ineffective in pre-

TABLE 3

Influence of amino acid imbalance on the development of muscular dystrophy and of dietary sulfur amino acid level on methionine protectivness

Group no.	Dietary variables				Avg wt gain	Feed utilization	Sulfur amino acid utilization	Muscular dystrophy	
	Casein level	Amino acid mixture	DL-Methionine added	Sulfur amino acid level				Incidence [1]	Severity [1]
	% of diet	*% of diet*	*% of diet*	*% of diet*	*g, 1–35 days*	*g gain/g feed consumed (× 100)*	*g gain/g methionine + cystine consumed*	*%*	*%*
				Experiment 6					
1	20.0 (Basal 6)	—	—	0.59	401	—	—	73	2.1
2	20.0 (Basal 6)	12.42 [2]	—	0.59	366	—	—	73	2.3
3	13.3 (Basal 7)	—	—	0.39	260	—	—	82	2.8
4	13.3 (Basal 7)	8.28 [3]	—	0.39	203	—	—	80	2.2
				Experiment 7					
1	13.3 (Basal 7)	8.28 [3]	—	0.39	181	30	70.1	81	4.1
2	13.3 (Basal 7)	8.28 [3]	0.1	0.49	238	39	79.1	91	3.8
3	13.3 (Basal 7)	8.28 [3]	0.2	0.59	308	46	78.7	80	3.5
4	13.3 (Basal 7)	8.28 [3]	0.3	0.69	346	48	68.8	64	2.8
5	20.0 (Basal 6)	—	—	0.59	382	47	78.9	73	3.8
6	20.0 (Basal 6)	—	0.1	0.69	411	51	73.2	60	2.2
7	20.0 (Basal 6)	—	0.2	0.79	454	53	67.4	36	1.1
8	20.0 (Basal 6)	—	0.3	0.89	248	45	50.4	0	0.0

[1] As explained in footnotes 4 and 5 in table 2.

[2] Represented one-half of the amino acids in basal diet 6, except cystine and methionine which were omitted: (in % of diet) L-arginine·HCl, 0.90; L-histidine·HCl·H_2O, 0.32; L-lysine·HCl (95%), 0.89; L-tyrosine, 0.51; L-tryptophan, 0.11; L-phenylalanine, 0.44; DL-threonine, 0.80; L-leucine, 0.86; DL-isoleucine, 1.04; DL-valine, 1.26; glycine, 0.47; DL-serine, 0.62; L-glutamic acid, 2.08; DL-aspartic acid, 0.63; L-alanine, 0.30; L-proline, 1.19.

[3] Represented one-half of the amino acids in basal diet 7 except cystine and methionine which were omitted. Amino acid levels were two-thirds those shown in footnote 2.

venting muscle degeneration. Thus, the addition of 0.3% DL-methionine to the 20% casein basal diet completely prevented the disorder, but an equivalent supplement to the 13.3% casein basal diet had no effect on the incidence of dystrophy. Comparison of the ratio of weight gain to sulfur amino acid ingestion for the various treatments showed that the ratio tended to decrease with higher sulfur amino acid intakes. This indicates that at the superior weight gains the proportion of ingested sulfur amino acids used for gain was decreased and in accordance with the muscular dystrophy data, a higher proportion became available for the prevention of muscular dystrophy. In apparent contradiction to the above, *reduction* of the sulfur amino acid intake from 0.49 to 0.39% also resulted in a decreased ratio of weight gain to sulfur amino acid intake. However, this observation was not unexpected as it is well known that the methionine requirement of birds for maintenance is higher than for growth (9). Thus at the lower rate of growth, the greater influence of the maintenance requirement could have depressed the amount of gain derived from methionine and cystine.

Although at low sulfur amino acid intakes the growth rate may progressively become more responsive to supplements of these amino acids, nevertheless at the requirement level the weight gain response becomes constant. As a higher rate of growth is attained there would be an inflection and reversal of the gain response to increased sulfur amino acid intake, as indicated by the data persented here.

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Nutrition and Biochemistry of Survival During Newcastle Disease Virus Infection

II. RELATION OF CLINICAL SYMPTOMS AND STARVATION TO NUCLEIC AND FREE AMINO ACIDS OF AVIAN LIVER[1]

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ABSTRACT The symptoms resulting from a Newcastle disease virus (NDV) infection of cockerel chicks, when arrayed in order of increasing virus involvement, correlated with a linear depression of body weight and increase in liver size. These phenomena were accompanied by a significant linear increase in liver DNA per gram of tissue and decrease in RNA, total protein and free amino acids, all in terms of DNA. The significant differences in these liver constituents between symptoms within treatments demonstrate the need for utilizing outcome groups to avoid masking nutritional effects at cellular levels. During the period of active virus involvement, 5 days post-inoculation, on the basis of milligrams per gram liver, starvation was more significant than disease per se in its effect on the increase in DNA and decrease in RNA/DNA and protein/DNA. However, in terms of the whole liver, DNA was affected principally by the disease which also increased RNA and protein. The free amino acids, on the other hand, although showing individuality, were significantly reduced by both disease and starvation. During the animal's recovery from NDV, DNA and RNA/DNA returned to normal levels; however, protein/DNA was significantly above normal and the free amino acids again were depressed. These phenomena were correlated with anabolic requirements related to an accelerated rate of growth in birds recovering from the infection.

Metabolic changes within the cell are rapid and influenced by many variables which complicate the interpretation of diet and disease interrelationships.

The first report of this series (1) showed that the virulence of a Newcastle disease virus (NDV) infection, the stage of the disease cycle, and the age of the animal could significantly influence liver protein and nucleic acids of the immature cockerel chick. The work also revealed that, in the evaluation of biochemical changes in the liver, consideration must be given to the effect of the infection on the animal's growth during the period of active involvement of the NDV and recovery from the disease.

The 2 experiments reported here were undertaken to define further some of the variables related to NDV infection 1) by observing the relation of the clinical status of the infected chick to liver total protein, deoxyribonucleic (DNA), ribonucleic (RNA), and free amino acids; and 2) by separating the effects on these liver components of the disease per se from those of starvation.

METHODS AND RESULTS

Procedures common to both experiments were as follows: One-day-old White Leghorn cockerels of known breeding were provided the reference diet (1) and water ad libitum. At the end of a 28-day preliminary period, birds to be infected were transferred to an air conditioned isolated animal room and inoculated with 0.1 ml of a 10^{-1} concentration of NDV, a level which produces approximately 50% mortality. Noninfected chicks were maintained as controls under identical conditions.

At the periods designated, the chicks were weighed, killed and the livers removed, weighed and immediately frozen by dry ice refrigeration. Selection of samples, preparation of tissue, and procedures

Received for publication December 16, 1963.

[1] This work was supported in part by grants from the U.S. Public Health Service (E-3645) and the Grange League Federation, Ithaca, New York.

for analysis for total protein and nucleic acids have been described (1). The same homogenates were used for the nucleic and free amino acid determinations. Free amino acids were determined according to Squibb (2) by thin-layer chromatographic separation of the amino acids and scanning of the chromatograms with a Photovolt TLC densitometer. All values were calculated on the basis of per gram of fresh tissue and analyzed statistically according to Snedecor (3).

Experiment 1. This was designed to study the relationship between clinical symptoms, or degree of NDV involvement, and biochemical observations in the liver.

At time of inoculation 300 birds were divided into 12 equal groups. All groups continued to receive the reference diet and water ad libitum until killed. The initial groups were large so that sufficient numbers of livers would be available for analysis for each of the clinical symptoms usually observed with this level of NDV. Starting 72 hours after inoculation, 10 of the groups were killed at 4-hour intervals and the remaining 2 groups at 8-hour intervals. The sampling periods thus covered the fourth, fifth and part of the sixth day post-inoculation. Body temperatures were recorded at time of killing. Noninfected controls were killed in 2 groups of 25 birds each 72 and 96 hours after the start of the experiment.

When chemical analyses were completed, values were grouped according to the bird's clinical symptoms as listed in table 1, irrespective of sampling period. Each grouping contained a reasonable representation of sampling periods. "Dying" birds were those that were completely prostrate but the heart was still beating faintly; "dead" birds were sampled within a few minutes of death.

Table 1 shows that as virus involvement progressed there was a significant ($< 1\%$) linear reduction in body weight, whereas liver size as percentage of body weight increased linearly ($< 1\%$). Body temperatures decreased critically in birds with acute paralysis and in those about to die. Per gram of tissue, DNA showed a significant linear ($< 1\%$) rise, whereas the RNA/DNA and protein/DNA ratios were reduced linearly ($< 1\%$) as the disease

TABLE 1

Relation of clinical symptoms to liver protein, nucleic and free amino acids in chicks infected with a Newcastle disease virus [1]

Clinical symptoms	Body wt	Liver as % of body wt	Body temperature	DNA	RNA	Protein	Lysine	Histidine	Arginine	Aspartic acid	Alanine	Valine	Leucine [2]
	g		*°C*	*mg/g liver*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*
Control	434	2.3	40.7	1.55	9.05	125	0.55	0.32	0.48	0.91	0.55	0.41	0.58
Respiratory	407	3.2	41.3	1.95	7.58	108	0.28	0.24	0.27	0.60	0.35	0.23	0.35
Partial paralysis	358	3.4	40.9	1.95	7.46	106	0.27	0.20	0.27	0.54	0.34	0.22	0.30
Acute paralysis	323	3.5	38.1	2.18	6.61	97	0.21	0.22	0.21	0.53	0.28	0.16	0.23
Dying	303	3.9	32.6	2.44	5.78	85	0.20	0.14	0.18	0.32	0.23	0.16	0.21
Dead	310	4.2	27.2	2.49	5.37	90	0.11	0.15	0.12	0.22	0.25	0.12	0.12

[1] Average number birds/group = 10.
[2] Leucine and isoleucine combined.

progressed. All of the free amino acids determined were reduced linearly (< 1%) as the degree of virus involvement increased.

Experiment 2. During the active involvement stage of the disease, 3 to 5 days post-inoculation, a virulent NDV infection depresses dietary intake; hence starvation is a confounding factor (4). To explore the magnitude of this effect, 200 one-day-old cockerel chicks were fed the reference diet (1) and water ad libitum for 28 days. At the end of this period the birds were divided into 3 groups: 1) noninfected controls continued with ad libitum feeding; 2) noninfected controls starved for a period equal to that of the active involvement stage of the virus, namely, the fourth, fifth and sixth days post-inoculation; and 3) birds to be inoculated with NDV and denied access to feed during the same period as group 2. Water was continually available to all groups. Chicks were killed at the same hour in each sampling period.

On the sixth day after NDV inoculation chicks were weighed, killed, and liver samples obtained from each experimental lot. As a result of the observations noted in trial 1, outcome grouping was utilized in the infected group by selecting 9 birds with respiratory symptoms and 9 with partial paralysis. The remaining infected birds were continued on trial and representative chicks were killed 9 and 19 days post-inoculation to evaluate the effect of renewed appetite which is associated with initiation of recovery of the chick from the infection.

Table 2 shows that 6 days after inoculation both feed deprivation and infection significantly (< 1%) reduced body weights from those of the ad libitum controls. Despite starvation, liver size as percentage of body weight was higher (< 1%) in the infected than in either of the noninfected groups. Per gram of tissue, DNA was significantly higher (< 5%) in the feed-restricted controls and in the birds infected with NDV (< 1%). Compared with the chicks fed ad libitum, the RNA/DNA ratios were significantly lower in both the starved controls (< 5%) and infected birds (< 1%). Protein/DNA was depressed significantly (< 5%) in the

TABLE 2

Effect of starvation and disease on liver protein, nucleic and free amino acids of chicks during the active involvement stage of a Newcastle disease virus infection [1]

Treatment	Days post-inoculation	Body wt	Liver as % of body wt	DNA	RNA	Protein	Lysine	Arginine	Histidine	Aspartic acid	Alanine	Valine	Leucine [2]
		g		*mg/g liver*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*
Control, ad libitum	6	382	2.2	1.79	8.93	117	0.60	0.26	0.51	0.69	0.76	0.46	0.69
Control, starved	6	299	2.0	2.23	6.93	112	0.53	0.18	0.40	0.52	0.61	0.36	0.52
NDV, starved	6	275	2.8	2.46	6.87	91	0.35	0.16	0.29	0.44	0.44	0.30	0.41
NDV, repleted	9	357	3.1	1.70	8.99	133	0.51	0.26	0.38	0.62	0.55	0.32	0.49
NDV, repleted	19	603	2.2	1.92	9.06	141	0.32	0.18	0.25	0.43	0.38	0.23	0.30

[1] Average number birds/group = 10.
[2] Leucine and isoleucine combined.

infected birds as were the levels of free amino acids ($< 1\%$).

Three days after the ad libitum feed was restored to the infected chicks (9 days post-inoculation) DNA values had decreased to normal and body weight, RNA/DNA and protein/DNA had increased significantly ($< 1\%$). Some, but not all, of the free amino acids increased significantly during this initial stage of recovery. At 19 days post-inoculation the infected birds had gained an average of 328 g in body weight since the active involvement stage of the NDV. RNA/DNA and protein/DNA showed only slight increases since the initial repletion period, but the free amino acids again were reduced to levels similar to those observed at 6 days post-inoculation.

DISCUSSION

The highly significant linearity of the data and the slope of the curves indicate that the clinical symptoms as arrayed in experiment 1 (table 1) fall in order with the increase in involvement of the NDV. Since intensity of virus involvement is highly variable within groups in a sampling period, failure to match clinical symptoms between treatments could easily mask a nutritional effect (s).

Body temperatures of birds with respiratory symptoms or partial paralysis, when compared with those of controls, appeared normal. However, these group averages do not show the rapidly changing *individual* metabolic picture. A previous report from these laboratories (5) has shown that temperatures of some NDV-infected chicks may reach 42.8°C during the active involvement stage of the virus. On the other hand, any significant drop in body temperatures below 39.4°C, which usually occurs in birds with acute paralysis or those classified as dying, is indicative of the extremely low metabolic rate which precedes death.

The enlargement of the liver in terms of percentage of body weight correlates with the degree of NDV involvement. Furthermore, it appears to be related to the disease per se since the effect is independent of dietary intake.

In recognition of the variables present in diet and disease interrelationships, liver DNA was used as a cellular constant and base of reference for total protein, RNA and the free amino acids. The increase in DNA indicated that parenchymal cell numbers per gram of tissue increased, whereas RNA, protein and the free amino acids decreased with the degree of involvement. These data indicate that protein metabolism is affected in relation to degree of virus involvement.

Food deprivation of noninfected and infected birds during the period of active virus involvement was used in experiment 2 as a means of separating the effect of disease per se from that of starvation on the liver constituents under study. From the results shown in table 2, the increase in DNA per gram of liver appeared to be due largely to starvation and not the infection. Histological examination of representative livers from all treatment groups showed that morphological changes were agonal rather than distinct disease effects. When total liver DNA is calculated, however, it becomes evident that on this basis disease produced the greater effect due to the infection enlarging the liver. The decrease in RNA/DNA per gram of tissue was principally the result of starvation. Food deprivation or restriction of nitrogen intake has been shown to depress RNA in the rat (6, 7) and chick (8).

The reduction in the free amino acid levels per gram of tissue was the result of a combined disease-starvation effect, an effect which was variable among the amino acids determined. For example, disease per se reduced lysine 70%; histidine 30%; and arginine, aspartic acid, alanine, valine and the leucines approximately 50%. When calculated on the basis of total liver weight the same variability was evident. A reduction in the size of the amino acid pool in the starved rat has been reported by Allison and Wannemacher (7).

With initial repletion (9 days post-inoculation) there was an increase in RNA/DNA, protein/DNA and the free amino acids per gram of tissue and on a total liver basis. However, at 19 days post-inoculation the free amino acid pool again was reduced, whereas total liver RNA and protein increased. The increase in liver RNA, protein and reduction of free amino

acids is attributed to the anabolic requirements for the rapid growth which occurred during recovery.

ACKNOWLEDGMENTS

Dr. Henry Siegel made the histological examinations. The nucleic acid determinations were made by Marie Utzinger and the free amino acids by Harry Veros. Donald Clody cared for the animals.

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Nutrition and Biochemistry of Survival During Newcastle Disease Virus Infection

III. RELATION OF DIETARY PROTEIN TO NUCLEIC AND FREE AMINO ACIDS OF AVIAN LIVER[1]

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ABSTRACT Cockerel chicks conditioned with diets containing protein ranging from deficient, balanced and imbalanced to surfeit were infected with Newcastle disease virus at 28 days of age. Liver DNA averaged higher in the surfeit protein groups. RNA, protein and free amino acids in terms of DNA were highest in the birds provided normal levels of dietary protein. Changes in RNA generally were reflected in similar trends for protein and the free amino acids. Based on the data observed, it was postulated that protein metabolism was most efficient in birds provided dietary protein in ranges normally assumed to be within requirement. Highest mortality, attributed to double jeopardy, was observed in the deficient and surfeit groups. Significantly lower hemagglutination-inhibition (HI) antibody titers correlated with extreme protein deficiency. Mortality was independent of HI antibody titers and showed no apparent correlation with liver nucleic and free amino acid levels.

There is considerable evidence in the literature that dietary crude protein may influence the course of infection in avian species. Increased susceptibility and mortality in chicks fed surfeit quantities of protein and infected with *Salmonella gallinarum* have been observed by Smith and Chubb (1), Hill and Garren (2) and Boyd and Edwards (3). Britten et al.[2] reported the same phenomenon in chicks infected with coccidiosis (*Eimeria tennella*), as did Boyd and Edwards (3) with Newcastle disease virus (NDV). However, the latter workers observed that with an *Escherichia coli* infection a low protein diet produced the greatest mortality.

In light of this evidence, work directed toward defining the pathways and mechanisms related to the apparent influence of protein on the chick's susceptibility to infection would be of value. The experiments reported here were designed to investigate further the effect of varying levels of dietary crude protein on mortality and on liver total protein, deoxyribonucleic (DNA), ribonucleic (RNA) and free amino acids of control and NDV-infected chicks.

METHODS AND RESULTS

The effect of dietary protein on the liver nucleic and free amino acids of control and NDV-infected chicks was studied in 2 experiments using diets ranging from 7.7 to 41.3% crude protein. For both trials the management of the chicks, NDV inoculation procedures, sampling techniques and analyses of tissue were similar to those previously described (4–6). The test diets (table 1), which were isocaloric within experiments, were offered to the chicks ad libitum from one day of age to the end of the trials. Mortality was recorded daily. Biochemical results were calculated in terms of milligrams per gram of fresh liver tissue and the data analyzed statistically according to Snedecor (7).

Large numbers of chicks (450 to 550) were used for each experiment so that sufficient birds would be available for pairing of symptoms between treatments (6). Using this technique, outcome groups of 10 birds each with similar respiratory symptoms were selected for the biochemical studies. The birds selected were killed and livers removed zero, 2, 5, and 14 days post-inoculation; the killing time was

Received for publication December 16, 1963.

[1] This work was supported in part by grants from the U.S. Public Health Service (E-3645) and the Grange League Federation, Ithaca, New York.

[2] Britten, W. M., C. H. Hill and C. W. Barber 1963 Dietary protein levels and resistance of chickens to coccidiosis. Federation Proc., 22: 435 (abstract).

TABLE 1
Composition of experimental diets

Crude protein, %	Experiment 1			Experiment 2		
	7.7	24.5	41.3	13.8	21.3	41.3
	%	%	%	%	%	%
Soybean oil meal (50%)	0	40	80	25	40	80
Corn meal	96	56	16	16	16	16
Sucrose	0	0	0	55	40	0
DL-Methionine [1]	0	+	+	+	+	+
Minerals [2]	4	4	4	4	4	4
Vitamins A, D, K [3]	+	+	+	+	+	+
Vitamin B complex [4]	+	+	+	+	+	+

[1] Added at 0.3% of the soybean oil meal.
[2] See (4) in Literature Cited.
[3] Supplies per kg diet: 6000 IU vitamin A; 300 ICU vitamin D and 0.125 mg menadione sodium bisulfite.
[4] See (15) in Literature Cited.

scheduled at the same hour each day. The periods corresponded to the incubation and active involvement stages of the virus and initiation of recovery of the host (5).

Experiment 1. In this trial, chicks reared with diets (table 1) containing deficient to surfeit quantities of imbalanced and balanced protein were inoculated with NDV at 28 days of age. The effects of these dietary regimens on mortality, liver protein, nucleic and free amino acids are shown in table 2.

Up to the time of inoculation there was a continuing mortality, amounting to 58%, in the group given the 7.7% protein diet. At the end of the trial total mortality was lowest in the 24.5 and highest in the 7.7% protein group. The chicks reared with rations containing either deficient or surfeit protein had slightly higher DNA and lower RNA/DNA ($< 5\%$) values. Protein/DNA was lowest ($< 1\%$) in the 7.7% protein group, with comparable ratios observed in the other 2 groups. Free amino acids averaged higher in the chicks fed 24.5% protein, whereas the lowest values were obtained from the 41.3% group.

Considering the entire disease cycle, DNA averaged higher ($< 5\%$) in the chicks fed the surfeit protein diet. In terms of DNA, RNA was highest ($< 5\%$) in the birds fed the 24.5% protein ration. Compared with the chicks receiving 24.5% protein, protein/DNA was less in the 7.7 and 41.3% ($< 1\%$, $< 5\%$) protein groups. The free amino acids were highest (levels of significance ranged from $< 5\%$ to $< 1\%$) in the birds fed the 24.5% protein, with no significant differences between the deficient and surfeit groups.

Serum antibody titers were determined by the hemagglutination-inhibition (HI) test (8) on survivors at the end of the 14-day infection period. These titers varied from an average of 6100 units for the 7.7% protein group to 22,500 for the 24.5% and 20,500 units for the 41.3% protein groups.

Experiment 2. This trial replicated the procedures of the first experiment except that in the test diets (table 1) corn was kept constant and soybean oil meal varied as the principal source of crude protein. As in experiment 1, total mortality (table 2) was lowest in the group provided 21.3% protein.

At the time of inoculation, liver DNA, which increased linearly with dietary protein, was significantly higher ($< 1\%$) in the 41.3% protein group. In terms of DNA, highest levels of RNA ($< 1\%$), protein and free amino acids ($< 5\%$) were observed in the 21.3% protein group, with no significant differences noted between the deficient or surfeit protein groups.

During incubation of the NDV there was an increase in RNA/DNA, protein/DNA ($< 5\%$) and the free amino acids in all treatment groups. During active involvement of the virus there were decreases from levels noted 2 days post-inoculation in RNA/DNA ($< 5\%$) and protein/DNA ($< 1\%$). From 2 to 14 days post-inoculation the free amino acids remained at approximately the same levels in the 13.8% protein group but were significantly reduced (< 5 to $< 1\%$) in the other groups.

TABLE 2

Effect of dietary protein on mortality, liver protein, nucleic and free amino acids of chicks infected with a Newcastle disease virus [1]

Dietary crude protein	Days post-inoculation	Total mortality	DNA	RNA	Protein	Lysine	Histidine	Arginine	Aspartic acid	Alanine	Valine	Leucine [2]
		%	*mg/g liver*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*	*mg/mg DNA*
Experiment 1												
7.7	0		1.77	6.77	96	0.39	0.24	0.36	0.39		0.24	0.33
	2		1.38	7.35	115	0.30	0.39	0.36	0.54		0.23	0.36
	5		1.39	7.80	130	0.31	0.31	0.34	0.48		0.24	0.32
	14	76	1.68	6.25	85	0.29	0.19	0.35	0.46		0.24	0.32
24.5	0		1.63	8.88	127	0.45	0.29	0.40	0.50		0.26	0.37
	2		1.52	8.99	136	0.58	0.42	0.53	0.70		0.38	0.51
	5		1.67	7.69	119	0.31	0.26	0.32	0.49		0.22	0.32
	14	28	1.40	9.91	147	0.59	0.33	0.54	0.70		0.36	0.56
41.3	0		1.75	7.48	126	0.27	0.25	0.29	0.29		0.22	0.25
	2		1.95	7.03	120	0.22	0.19	0.30	0.32		0.16	0.21
	5		2.03	6.27	108	0.23	0.19	0.23	0.34		0.22	0.30
	14	40	1.63	7.52	129	0.33	0.33	0.42	0.47		0.28	0.42
Experiment 2												
13.8	0		1.64	7.25	91	0.53	0.26	0.32	0.56	0.55	0.26	0.35
	2		1.52	8.54	117	0.66	0.35	0.38	0.78	0.68	0.34	0.48
	5		1.56	7.76	96	0.54	0.27	0.42	0.76	0.69	0.32	0.49
	14	43	1.86	7.82	99	0.69	0.32	0.35	0.63	0.65	0.34	0.48
21.3	0		1.72	9.00	109	0.77	0.34	0.47	0.83	0.68	0.41	0.54
	2		1.59	9.19	127	0.75	0.41	0.44	0.92	0.75	0.42	0.62
	5		1.75	7.98	102	0.60	0.29	0.41	0.87	0.67	0.37	0.51
	14	25	2.24	7.37	95	0.55	0.26	0.33	0.64	0.54	0.32	0.44
41.3	0		2.20	7.08	94	0.50	0.28	0.37	0.61	0.55	0.30	0.44
	2		1.78	8.45	114	0.54	0.35	0.46	0.80	0.64	0.35	0.48
	5		2.42	6.59	86	0.34	0.22	0.28	0.62	0.47	0.24	0.32
	14	50	2.60	6.17	80	0.44	0.21	0.26	0.49	0.47	0.24	0.35

[1] Each of the averages represents 10 chicks.
[2] Leucine and isoleucine combined.

DISCUSSION

These studies deal with the effect of deficient to surfeit quantities of balanced and imbalanced protein on mortality and on protein metabolism in the livers of control and NDV-infected cockerel chicks. The mortality observed in the group fed 7.7% crude protein, when evaluated in terms of noninfected controls, suggests that both dietary protein deficiency and infection contributed to the final total. When the low protein groups are not considered, due to the confounding effect of double jeopardy, then in both experiments the highest mortality was observed in the groups given 41.3% protein, which is in accord with the observations on *S. gallinarum* (2), NDV (3), and coccidiosis[3] infection of chicks fed surfeit quantities of protein.

Extreme protein deficiency may reduce antibody titers in the chick as well as in the rabbit (9). The higher mortality observed in the birds fed 41.3% protein was not due to less antibody response since HI titers of both the 24.5 and 41.3% protein groups of exeperiment 1 were similar. The free amino acid levels in the deficient and surfeit protein groups were similar yet there was a considerable magnitude of difference between these groups in HI titers. This would suggest that HI titer response is independent of the free amino acid pool or that only small quantities of these essentials were utilized for the production of antibodies.

The higher mortality experienced in the surfeit protein groups may have resulted from the stress of infection rendering toxic the excess protein intake. This would result in double jeopardy similar to that of the deficient groups. There is also the possibility that surfeit dietary protein increased virus proliferation.

The results of these replicated experiments provide an opportunity to speculate on the relationship of dietary protein to protein metabolism in the liver of the chick prior to and during NDV infection.

A severe dietary deficiency of protein depressed RNA, protein and free amino acids, in terms of DNA, in the livers of control and NDV-infected chicks. Similar diet effects have been reported for noninfected rats (10–13). The data herein show that surfeit protein also resulted in lower levels of these liver constituents in the chick. In this case, protein metabolism was reduced due to the utilization of protein as energy. Even though highest levels of RNA, protein and free amino acids were obtained when protein was provided in normal ranges during the active involvement stage of the virus, the magnitudes of difference do not account for the lower mortality observed in these groups.

With the use of outcome grouping for symptoms to equalize the effects of starvation between treatments (6), the behavior of the free amino acids prior to and during infection is of interest. First, the 2 nonessential amino acids did not show any apparent differences from the essentials in reaction to diet or infection. Second, under conditions of extreme protein deficiency the free amino acid pool remained at reasonably high levels. However, this was not unexpected since Nasset et al. (14) demonstrated in the dog that the free amino acid mixture in the small intestine was not greatly affected by the test meal ingested, a phenomenon they hypothesize as being due to hydrolysis of food proteins and to autodigestion of hydrolytic enzymes and other endogenous proteins. Third, high levels of dietary protein did not result in an increase in the free amino acid pool. Since excess protein is utilized in part as energy, the extent of catabolism of the free amino acids in this respect is of interest. Regardless of utilization as energy, dietary imbalance must also be considered an influence on the liver free amino acid pool.

Calculation of total liver DNA, RNA, protein and free amino acids showed some differences from trends based on per gram of tissue. This was predictable since such data reflect changes in size of the body and liver which result from infection (6). Total liver DNA showed a uniform linear increase with protein content of the diet, indicating a greater proliferation of cells. Changes in RNA generally were reflected in the protein and the free amino acid pool of the liver. Protein metabolism was most efficient when birds were provided dietary protein in ranges normally assumed to be within requirement.

[3] See footnote 2.

ACKNOWLEDGMENTS

The nucleic acid determinations were made by Marie Utzinger and the free amino acids by Harry Veros. Donald Clody cared for the animals.

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Liver Lipid Accumulation in Isoleucine-deficient Rats[1,2]

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ABSTRACT Rats made acutely deficient in isoleucine by force-feeding a diet completely devoid of isoleucine, had higher concentrations of liver triglycerides than control rats. When liver slices from fed, but deficient rats were incubated with acetate-2-C^{14}, total incorporation of the label into the fatty acids of triglycerides was nearly 4 times higher than in the triglycerides of control livers. Fatty acid analyses of the triglycerides of deficient rat livers revealed higher proportions of palmitic, stearic and oleic acids and lower proportions of linoleic and arachidonic acids than were in control livers. Lipoprotein synthesis appeared not to be impaired by the deficiency, since there were no differences in serum cholesterol, phospholipid, and lipoprotein concentrations in the 2 groups. Some impairment in the removal of liver triglyceride in deficient rats was suggested, however, because more linoleic acid accumulated in these animals than in the controls. The results indicated, therefore, that increased fatty acid synthesis in the liver may have been an important factor contributing to the excessive accumulation of liver triglyceride in isoleucine-deficient rats.

The importance of the liver in lipid metabolism is apparent from its role in the transformation of unesterified fatty acids into triglycerides; the oxidation of fatty acids; and the synthesis of fatty acids, triglycerides, cholesterol and phospholipids and their secretion into the blood stream as part of a lipoprotein structure. Therefore it is not unexpected that derangement of one or more of these processes results in alterations in the lipid content of the liver.

Omission of choline from the diet has been known for many years as a cause of severe liver fat infiltration in experimental animals (1). The lipid accumulation in this nutritional deficiency has been attributed to decreased transport of triglycerides from the liver (2, 3), reduced fatty acid oxidation (4), and increased liver fatty acid and triglyceride synthesis (5). Although the relative contribution of each of these factors to the over-all lipid accumulation in choline-deficient animals remains unknown, the studies of Olson et al. (3) indicate that impaired synthesis of low-density lipoproteins may be an important factor because this serum lipoprotein fraction transports mostly triglycerides and, in choline-deficient rats, the concentrations of β-lipoproteins are greatly reduced.

Imbalances or deficiencies of essential amino acids have also produced fatty livers. Dietary imbalances involving lysine, tryptophan, and threonine increased deposition of liver fat in rats (6, 7). Rats fed diets completely lacking tryptophan (8), isoleucine and phenylalanine,[3] methionine (9), and histidine or threonine (10) also develop fatty livers. However, in these instances, much less lipid accumulates and its distribution is periportal as compared with the large quantities of centrolobular distributed lipid obesrved in choline deficiency.

Although many studies have been concerned with the mechanism through which liver lipids accumulate in choline deficiency (2, 3) or in ethionine, ethanol, and carbon tetrachloride toxicities (11–14), much less attention has been directed toward the causes of the lipid infiltration into the livers of animals made deficient in essential amino acids. During studies on acute deficiency of isoleucine in rats, it was noted that deficient animals had more

Received for publication October 24, 1963.
[1] This investigation was supported in part by Grant-in-aid A-3046 from the National Institutes of Health, U.S. Public Health Service, Bethesda, Maryland.
[2] A preliminary report of part of this study was presented at the 6th International Congress of Nutrition, Edinburgh, Scotland, 1963.
[3] Samuels, L. T., H. C. Goldthorpe and T. F. Dougherty 1951 Metabolic effects of specific amino acid deficiencies. Federation Proc., *10*: 393 (abstract).

liver fat than control animals. Experiments were therefore continued to investigate factors that might be responsible for the increased lipid deposition.

EXPERIMENTAL

Female, Long-Evans rats, having an average body weight of 136 g, were housed in individual screen-bottom cages. They were fed, by stomach tube, in 2 feedings daily, 10 g of a purified diet, the composition of which is shown in table 1. A control group was fed the complete diet, and the experimental group was fed the same amount of a similar diet unsupplemented with isoleucine. Details of the force-feeding procedure have been described previously (15, 16). The animals were fed for 10 days. On the eleventh day, rats from both groups were fed 5 g of their respective diets, and then were decapitated. Blood was collected and allowed to clot. An appropriate portion of the serum was extracted with alcohol:ether (3:1) (17) and total cholesterol (18) and phosphorus (19, 20) were determined on the extracts.

Serum lipoproteins were estimated in a Spinco Model R electrophoresis apparatus by means of the staining and dye elution procedure of Jencks and Durrum (21). Duplicate 20-μliter serum samples applied to papers saturated with veronal buffer (pH 8.6) were run for 4 hours. The strips were stained with oil red O, and the developed color was eluted with 25% acetic acid in ethanol, and read at 520 mμ in order to get some estimate of the relative concentrations of the serum lipoproteins, the ratio of optical density of 20 μliters of stained serum to the optical density of the stain eluted from an equivalent size of background paper was compared in both groups.

Livers were removed and weighed. For the acetate-incorporation studies, 500-mg of slices 0.5-mm thick were prepared from the large lobe and incubated for 3 hours at 37°C in 4.5 ml of Krebs-Ringer bicarbonate buffer (pH 7.3), with 0.3 μc of sodium acetate-2-C^{14}. Following the incubation, the slices were washed and extracted with alcohol and ether as described by Okey et al. (22). The solvents were removed in a rotary evaporator, under vacuum, and re-extracted into petroleum ether. Triglycerides and phospholipids

TABLE 1

Basal diets and amino acid mix composition

	Control	Deficient	Amino acid mix [1]	
	%	%		%
Sucrose	46.25	48.65	L-lysine·HCl	1.78
Amino acid mix			L-arginine·HCl	0.91
(without isoleucine)	26.2	26.2	DL-tryptophan	0.27
Fortified oil [2]	1.0	1.0	DL-phenylalanine	0.04
USP XIV salts	4.0	4.0	DL-leucine	3.64
Cottonseed oil [3]	9.0	9.0	DL-isoleucine	(2.38)
Cellulose [4]	10.0	10.0	DL-valine	2.80
Vitamin mix [5]	1.0	1.0	L-histidine	0.68
Choline chloride	0.15	0.15	DL-methionine	0.60
DL-Isoleucine	2.4	—	DL-threonine	1.62
			DL-serine	2.26
			glycine	0.38
			DL-tyrosine	1.13
			L-cystine	0.27
			L-proline	2.27
			DL-aspartic acid	1.17
			L-glutamic acid	4.22
			DL-alanine	1.12

[1] Amino acid mix was compounded to approximate the L-amino acids in 18% casein. An additional 0.2% of L-cystine was added to the mix. Since the D-forms of leucine, isoleucine, valine and threonine are not completely utilized by the rat, the concentration of these DL-amino acids was doubled.

[2] Fortified oil provided per 100 g of diet: vitamin A, 1700 IU; vitamin D, 100 IU; α-tocopheryl acetate, 6.66 mg; menadione, 0.5 mg.

[3] Dietary cottonseed oil analyzed to contain the following percentages of fatty acids: myristic, 1.0; palmitic, 19; palmitoleic, 0.8; stearic, 2.0; oleic, 17; linoleic, 52; linolenic, 0.5.

[4] Cellu Flour, Chicago Dietetic Supply House, Chicago.

[5] Vitamin mix provided mg/100 g diet: thiamine·HCl, 0.2; riboflavin, 0.3; pyridoxine·HCl, 0.25; Ca pantothenate, 2.0; inositol, 10.0; biotin, 0.01; folic acid, 0.02; niacinamide, 1.0; vitamin B_{12}, 0.02.

were separated on silicic acid by the semi-micro procedure of Lis et al. (23). Aliquots of the appropriate fraction were plated on stainless steel planchets as described by Williams and Pertel (24), and counted to within 5% accuracy with a Nuclear-Chicago gas-flow counter (model 161A) having a counting efficiency of about 30%.

Nitrogen was determined by semi-micro Kjeldahl on a portion of the whole liver after digestion with sulfuric acid. Copper sulfate was used as the catalyst. Glycogen was precipitated from potassium hydroxide digests of liver with alcohol, and analyzed by the method described by Colowick and Kaplan (25).

The remaining portion of the liver was extracted with alcohol followed by an overnight Soxhlet extraction with diethyl ether. Total liver lipid content was determined by the oxidation procedure of Bloor (17). A portion of this extract was then separated on silicic acid, as previously described (23), into triglycerides and phospholipids, and the fatty acid composition of the fractions was determined by gas-liquid chromatography [4] following methylation of the fatty acids. Total lipids (17) were determined on the triglyceride fraction and phosphorus (19, 20) was analyzed on the phospholipid fraction so that the quantity of the fractions and their fatty acids could be calculated. Statistical analysis of results was made by *t* test (26).

RESULTS

Although rats of both groups were fed the same amount of diet (10 g/day), the results in table 2 show that whereas control animals gained, rats fed the diet lacking isoleucine lost approximately 2 g daily. However, total liver weight and liver nitrogen and cholesterol content remained similar in both groups. The major differences between the 2 groups of animals were in the total liver lipid and glycogen, both of which were significantly higher in the deficient animals than in the controls.

Table 3 shows the results obtained after incubation of liver slices with acetate-2-C^{14},

[4] Wilkin's Aerograph, Model A90C. Operating temperature was 190 to 200°C; liquid phase was diethylene glycol succinate polyester adsorbed on Chromasorb W and packed in 152-cm coiled stainless steel columns. Retention times of the sample fatty acids were related to those of a mixture of weighed amounts of known fatty acid standards obtained commercially. A set of these standards was analyzed daily. Relative sensitivity factors were computed from the standards and applied to the sample fatty acids.

TABLE 2

Body and liver weights of control and isoleucine-deficient rats, and nitrogen, lipid and glycogen content of the livers [1]

Diet	Body wt	Liver				
		Weight	Nitrogen	Total cholesterol [2]	Total lipid	Glycogen
	g	*g*	*mg/g*	*mg/g*	*mg/g*	*mg/g*
Control	159 ± 4 [3]	5.3 ± 0.3	33.9 ± 0.8	2.5 ± 0.1	36.2 ± 1.7	20.7 ± 2.4
Without isoleucine	115 ± 4	5.5 ± 0.2	34.4 ± 1.0	2.3 ± 0.1	59.9 ± 3.5 [4]	58.4 ± 3.8 [4]

[1] Values expressed in terms of wet weight of liver.
[2] Cholesterol was determined in 5 animals; all other determinations were from 6 or more rats.
[3] Mean ± SE.
[4] Significantly different from control group ($P < 0.01$).

TABLE 3

Liver lipids and in vitro acetate-2-C^{14} incorporation into liver slices [1]

Diet	Triglyceride	Phospholipid	Acetate incorporation			
			Triglyceride	Phospholipid	Triglyceride	Phospholipid
	mg/g	*mg/g*	*count/min/mg*		*count/min/liver*	
Control	8.3 ± 1.2 [2]	20.9 ± 0.5	249 ± 101	136 ± 35	1490 ± 221	2850 ± 209
Without isoleucine	29.3 ± 6.3	21.7 ± 0.9	278 ± 107	269 ± 35 [3]	5340 ± 324 [3]	5760 ± 224 [3]

[1] Six rats/group. Values expressed in terms of wet weight of liver.
[2] Mean ± SE.
[3] Significantly higher than control group ($P < 0.01$).

followed by fractionation of the extracted lipids into triglycerides and phospholipids. Only triglycerides accumulated in the liver in isoleucine deficiency. The concentration of phospholipid remained unchanged.

Acetate incorporation into liver triglycerides was similar for both groups when expressed as specific activity of the triglycerides. However, because of the greater amount of triglyceride in the deficient animals, total acetate incorporation in this group was nearly 4 times that of the controls. Acetate incorporation into the fatty acids of the phospholipids was about twice as much for the deficient animals as for the controls.

To determine whether the accumulated liver triglycerides in the isoleucine-deficient rats could have been derived primarily from the diet or from adipose tissue, fatty acid analyses of the triglycerides were made by gas-liquid chromatography. The results are shown in table 4. The percentage of linoleic acid in the liver triglycerides of the deficient animals was significantly lower, whereas that of palmitic acid was significantly higher than in control animals. Inasmuch as the oil in the diet fed these rats contained over 50% linoleic acid (table 1), a decrease in the proportion of this fatty acid, relative to the control, would not be expected if the triglycerides had accumulated because of increased mobilization from adipose tissue or from dietary fatty acids. The dilution of linoleic acid in the deficient rats by the greater accumulation of the more readily synthesized fatty acids (especially palmitic) suggests that the liver lipid was synthesized within the organ.

The fatty acid composition of the liver phospholipids of the isoleucine-deficient animals did not differ appreciably from the controls (table 5). The somewhat higher proportion of palmitic acid in this liver lipid fraction in the deficient animals may have been a reflection of the greater amount of this fatty acid in the triglycerides.

To learn whether the capacity of the liver of the isoleucine-deficient rat to form lipoproteins and secrete them into the blood was impaired, serum cholesterol, phospholipid, and relative lipoprotein concentrations were determined in control and deficient animals. The results are shown in table 6. Although there were slight decreases in the serum concentrations of cholesterol and the relative amounts of lipoprotein in the deficient animals, the values were not significantly different from those of the control animals.

DISCUSSION

Accumulation of lipid in the liver may occur under the following conditions: 1)

TABLE 4

Fatty acid composition of liver triglycerides in control and isoleucine-deficient rats [1]

Diet	Fatty acid						
	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Arachidonic
	wt % fatty acid						
Control	0.8 ±	24.6 ± 1.1 [2]	2.02 ± 0.24	3.55 ± 0.26	20.6 ± 1.0	40.3 ± 1.3	6.65 ± 0.31
Without isoleucine	1.2 ±	32.5 ± 1.1 [3]	2.41 ± 0.66	4.04 ± 0.38	19.6 ± 1.2	33.6 ± 2.2 [3]	5.40 ± 0.17
	mg fatty acid/g liver [4]						
Control	0.07 ±	2.2 ± 0.1	0.19 ± 0.03	0.32 ± 0.3	1.9 ± 0.3	3.9 ± 0.6	0.6 ± 0.1
Without isoleucine	0.35 ±	9.7 ± 1.4	0.78 ± 0.26	1.2 ± 0.2	6.0 ± 1.0	9.0 ± 1.0	1.6 ± 0.3
Deficient/ control	4.7	4.4	4.2	3.7	3.2	2.9	2.6

[1] Nine rats/group.
[2] Mean ± SE. Fatty acids below C_{14} made up less than 3 or 4% of total and have been omitted.
[3] Significantly different from control group ($P < 0.01$).
[4] Calculated from weight per cent of fatty acid × 0.95 × total lipid in triglyceride fraction. Values expressed in terms of wet weight of livers.

TABLE 5

Fatty acid composition of liver phospholipids from control and isoleucine-deficient rats [1]

Diet	Fatty acid						
	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Arachidonic
	wt % fatty acid						
Control	trace	15.9 ± 0.7 [2]	trace	28.0 ± 1.8	5.16 ± 0.70	15.4 ± 1.0	35.1 ± 0.9
Without isoleucine	trace	19.4 ± 1.1	trace	27.6 ± 1.8	5.60 ± 0.80	16.6 ± 1.0	30.2 ± 1.5

[1] Nine rats/group.
[2] Mean ± SE.

TABLE 6

Serum cholesterol, phospholipid, and lipoprotein from control and isoleucine-deficient rats [1]

Diet	Total cholesterol	Phospholipid [2]	Lipoprotein [3]	
			Fraction 1	Fraction 2
	mg/100 ml	*mg/100 ml*		
Control	95 ± 3.8 [4]	187 ± 7.1	8.8 ± 1.0	4.7 ± 0.05
Without isoleucine	81 ± 4.8	201 ± 4.8	7.5 ± 0.7	3.1 ± 0.4

[1] Five rats in control group and 9 rats in the isoleucine-deficient group.
[2] Mg/100 ml phosphorus × 24.5 = mg/100 ml phospholipid.
[3] Ratio of OD of 20 μliters stained serum to OD of stain from equivalent-size piece of background paper. Fraction 1 is a fast moving band and may include albumin as well as α-lipoprotein. Fraction 2 is a slower moving band and corresponds to β-lipoprotein.
[4] Mean ± SE.

increased mobilization of dietary or adipose tissue fatty acids to the liver; 2) increased fatty acid synthesis; 3) decreased transport of fatty acids from the liver to the plasma; and 4) decreased utilization or oxidation of liver fatty acids.

The deposition of liver triglycerides as a result of increased mobilization of dietary fat (since the animals had been fed) or adipose tissue fatty acids was investigated in this study by comparing the proportion of linoleic acid in the triglycerides of deficient rats with that in the controls. This procedure has been used to demonstrate that increased mobilization contributes to the lipid accumulation in ethanol toxicity,[5] carbon tetrachloride poisoning (27), and ethionine toxicity (12). Since the rat cannot synthesize linoleic acid, an increased proportion of this fatty acid in the accumulated liver triglyceride, compared with that in control animals, indicates that the fatty acids were derived from dietary or adipose tissue sources. However, a dilution of the linoleic acid in the liver lipids of the treated animals, as compared with controls, indicates increased fatty acid synthesis in the liver. In the present experiment, the percentage of linoleic acid in the deficient rat liver triglycerides was lower than that in the controls. Therefore, increased mobilization of fatty acids to the liver must not have been responsible for the excess triglyceride observed in the livers of the deficient rats. On the other hand, the dilution of the linoleic acid in the deficient animals by a disproportionately high level of palmitic acid, considered together with the increased in vitro acetate incorporation into the liver triglycerides suggests that an appreciable part of the accumulated liver triglyceride may have resulted from in situ fatty acid synthesis. Yoshida and Harper (5) have demonstrated, in rats made deficient in threonine, that the accumulation of neutral fat into the livers of these animals was also accompanied by increased acetate incorporation into the liver and body fat. The results were interpreted by the authors to mean that increased fatty acid synthesis occurred in these animals and that liver lipid could have accumulated as a consequence.

It is now generally conceded that no lipid circulates in the blood as free lipid,

[5] Maling, H. M., M. G. Horning, W. M. Butler, Jr., B. Highman and B. B. Brodie 1960 Triglyceride deposition in rat liver through derangement of fat transport by various chemical agents. Federation Proc., 19: 229 (abstract).

but is combined with protein in a variety of lipoprotein complexes synthesized by the liver. Any impairment in the ability of the liver to form the protein moiety of these complexes should therefore cause a decrease in the blood lipids and a concomitant increase in the lipid content of the liver. Seakins and Robinson (28) have presented evidence that such a mechanism may be a primary factor in the liver lipid accumulation in rats poisoned with carbon tetrachloride. An inability to synthesize and secrete lipoproteins into the blood would not appear to be a major factor causing triglyceride deposition in livers of isoleucine-deficient rats, since serum levels of cholesterol, phospholipids, and lipoproteins were essentially the same as in control groups. However, table 4 shows that linoleic acid did accumulate in the livers of deficient animals, suggesting that some impairment of removal mechanisms may have occurred. Thus it appears that liver triglyceride may accumulate in isoleucine-deficient rats as the result of increased fatty acid synthesis as well as from some impairment in the ability of the organ to remove the lipid. The results indicate that in situ fatty acid synthesis by the liver might contribute an appreciable proportion of the excess liver triglyceride in isoleucine-deficient rats, since the concentrations of palmitic, palmitoleic, stearic, and oleic acids increased more rapidly than the concentration of linoleic acid. Liver lipid derived mainly from synthesis, as compared with lipid that accumulates primarily as the result of impaired removal, might account for the histologic differences in distribution of the liver lipid reported in choline- and essential amino acid-deficient rats.

Samuels et al.[6] suggested that the increased liver lipid associated with essential amino acid deficiencies was due to deamination of the excess amino acids and their subsequent conversion into fat. However, Sidransky and Farber (29) were unable to demonstrate that rats force-fed diets devoid of threonine incorporated labeled amino acids into liver lipids any differently than did controls. Previous studies from this laboratory (16) have also shown that rats force-fed diets lacking isoleucine had a lower proportion of carcass fat than animals fed the complete diet. If deaminated amino acids were being converted into fat, these smaller animals, whose energy requirements were lower than those of the controls, would be expected to have deposited more fat in the carcass as well as in the liver. It appears, therefore, that some other biochemical mechanism will be necessary to explain the increased fatty acid synthesis in essential amino acid deficiencies.

An increased liver glycogen, as observed in the isoleucine-deficient rats, has been previously reported for rats force-fed diets deficient in threonine (29). Moriwaki (30) has also reported that glycogen accumulated in the livers of rats fed ad libitum diets devoid of lysine, histidine, phenylalanine, tryptophan, valine, or isoleucine. In an earlier experiment (16), rats made isoleucine-deficient under conditions similar to those reported here had significantly enlarged adrenals compared with controls. Therefore, it is possible that the higher liver glycogen in the deficient rats of the present experiment resulted from increased adrenal cortical activity. However, Sidransky and Farber (29) are of the opinion that an intact adrenal, capable of excessive steroid secretion, was not essential for the glycogen response in their rats fed threonine-free diets. Because a number of essential amino acid deficiencies have been reported to cause increased liver glycogen as well as increased liver lipid deposition, further investigations should be undertaken to see whether the 2 responses may be related through some disturbance in carbohydrate metabolism.

ACKNOWLEDGMENT

The authors wish to thank Mrs. Shirley Thenen for her technical assistance in parts of this study and for the statistical analyses.

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Effect of Potassium on Juxtaglomerular Cells and the Adrenal Zona Glomerulosa of Rats[1,2]

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ABSTRACT In a series of 4 experiments (100 rats), 2 to 9 weeks in duration, excess potassium, either dietary or added to drinking water, did not alter juxtaglomerular (JG) granulation. In the same animals, however, the zona glomerulosa hypertrophied, confirming previous observations. It was concluded that high potassium, like sodium deficiency, produces widening of the zona glomerulosa, but unlike sodium deficiency, it does not affect JG cells. These results fit in with the observations of others that stimulation of aldosterone by high potassium is by direct action and an exception to the renal control, characteristic of other forms of secondary aldosteronism. Histologic changes in the zona glomerulosa were of much greater magnitude with variations in sodium intake than with variations in potassium intake, at least at levels compatible with life in these chronic experiments.

In 1948, Deane et al. (1) showed that variations in sodium and potassium intake produced histologic changes in the zona glomerulosa without affecting the other zones of the adrenal cortex of rats. Sodium deficiency or potassium excess caused widening of the zona glomerulosa, whereas atrophy resulted from either sodium excess or potassium deficiency. Their observations were the first to emphasize the specific role of the zona glomerulosa in electrolyte-regulating activity.

Later, it was shown in our laboratories that sodium deficiency produced hypergranulation and other changes indicative of hyperactivity of juxtaglomerular (JG) cells in several species (2–5). Excess sodium suppressed activity of JG cells. Prompted by Deane's earlier observations (1), adrenal cortex was also studied in these animals with the result that changes in JG cells correlated very well with changes in the zona glomerulosa (6). The same relationship held true in the case of human JG cells in patients with varying degrees of hyponatremia (7).

It was natural that the question of potassium should be raised with respect to its effect on JG cells. Excess potassium causes widening of the zona glomerulosa (1) and increases aldosterone secretion (8). In the present study, 4 experiments using rats were carried out in an attempt to stimulate JG cells with excess potassium, but despite the fact that the zona glomerulosa was significantly hypertrophied, no evidence indicating hyperactivity of JG cells could be demonstrated.

METHODS

Four experiments were carried out using 100 male rats of the Wistar strain. Details of each experiment are as follows.

Experiment 1. Seventeen rats, weighing approximately 200 g were fed a standard chow diet ad libitum. Five rats (group 1) were given 4% KCl in drinking water; 6 rats (group 2) were given tap water to drink; 6 rats (group 3) were given 2% NaCl in drinking water. The duration of the experiment was 29 to 33 days.

Experiment 2. Ten rats, weighing an average of 58 g were fed a standard chow diet for 3 weeks. Five rats (group 1) were allowed to eat ad libitum and were given 4% KCl in drinking water (distilled); the other 5 rats (group 2) were pair-fed with

Received for publication November 12, 1963.

[1] These studies were supported by grant A-1114, U.S.P.H.S., and a grant (240) from the Nutrition Foundation.

[2] A brief summary of these experiments was included in a paper presented at a meeting of the Council for High Blood Pressure Research, American Heart Association, Cleveland, October 24, 1962 (12) and at the Federation of American Societies for Experimental Biology, Atlantic City, 1963 (Hartroft, P. M., and E. Sowa 1963 Effect of potassium on juxtaglomerular cells and adrenal zona glomerulosa. Federation Proc., 22: 548 (abstract)).

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rats in group 1 on the basis of body weight and were given distilled water to drink.

Experiment 3. Thirty-nine (39) rats, weighing an average of 98 g, were fed semi-purified diets ad libitum and given distilled water to drink. Nine rats (group 1) were fed a potassium-deficient diet (table 1) supplemented with 12% KCl; 10 rats (group 2) were fed the potassium-deficient diet supplemented with 6% KCl; 10 rats (group 3) were fed the potassium-deficient diet supplemented with 1.2% KCl to serve as controls; 10 rats (group 4) were fed the potassium-deficient diet alone. The duration of the experiment was 2 to 7 weeks.

TABLE 1

Composition of the potassium-deficient diet

	%
Casein (extracted) [1]	20
Cellulose [2]	2
Potassium-free salt mix	4
Sucrose	65
Vitamin mix [1]	2
NaCl	0.5
Corn oil	7
Composition of the potassium-free salt mix	
Calcium carbonate	20.94
Magnesium sulfate	19.70
Sodium phosphate (dib.)	14.80
Calcium phosphate (trib.)	41.00
Ferric phosphate ($2H_2O$)	3.20
Cupric sulfate ($5H_2O$)	0.15
Manganese sulfate	0.04
Cobaltous chloride ($6H_2O$)	0.02
Sodium iodide	0.014
Zinc sulfate ($7H_2O$)	0.116

[1] Obtained from Nutritional Biochemicals Corporation, Cleveland.
[2] Alphacel, Nutritional Biochemicals Corporation.

Experiment 4. Thirty-four (34) rats, weighing an average of 43 g were fed semi-purified diets and given distilled water to drink. Eight rats (group 1) were fed a sodium-deficient diet (9) supplemented with 7% KCl; 8 rats (group 2) were fed the sodium-deficient diet alone; 10 rats (group 3) were fed the sodium-deficient diet supplemented with 0.6% NaCl and 7% KCl; 8 rats (group 4) were fed the sodium-deficient diet supplemented with 0.6% NaCl to serve as controls. Rats of groups 2, 3 and 4 were pair-fed with rats of group 1. The duration of the experiment was 2 months.

Juxtaglomerular cells were studied by a counting method described previously (2,7) and, except for experiment 2, the zona glomerulosa was studied in frozen sections stained for lipid with oil red O (9). Serum levels of sodium and potassium were determined by flame photometry in experiments 2, 3 and 4. Animals were decapitated.

RESULTS

Results are tabulated separately for each experiment in tables 2 and 3. In the 3 experiments in which adrenal cortex was studied, the zona glomerulosa was significantly wider in rats ingesting excess potassium (either 4% KCl in drinking water or 7 to 12% KCl added to the diet; combined average, 71 μ) than in control rats (combined average, 50 μ, $P < 0.01$). In contrast, degree of granulation of JG cells (JGI) was not significantly different between control (combined average, 23) and high-potassium rats (combined average, 21) in any of the experiments.

In sodium-restricted rats, high intake of potassium resulted in a lower JGI than in sodium-deficient rats with a normal potassium intake (exp. 4, table 3). It should be noted that these rats failed to grow as well as rats in other groups of this

TABLE 2

Plasma sodium and potassium [1]

Group	Plasma Na	Plasma K
	mEq/liter	*mEq/liter*
Experiment 2		
1 High KCl	158 ± 2.0 *,[2]	8.7 ± 0.55 **
2 Control	148 ± 3.1	5.9 ± 0.13
Experiment 3		
1 High KCl, 12%	143 ± 1.4	6.4 ± 0.31 *
2 High KCl, 6%	144 ± 1.5	7.1 ± 0.26
3 Control	141 ± 0.7	7.4 ± 0.24
4 K-deficient	138 ± 0.5 *	3.6 ± 0.14 **
Experiment 4		
1 Na-deficient, High KCl	126 ± 1.9	9.6 ± 0.27
2 Na-deficient, normal KCl	124 ± 2.6	8.6 ± 0.53
3 High KCl	140 ± 0.5	8.3 ± 0.49
4 Control	140 ± 0.5	7.1 ± 0.53

[1] The *t*-test was used to test difference between experimental and control group within each experiment except in experiment 4 in which group 1 was tested against group 2.
[2] SE of mean.
* Significant at the 1% level.
** Significant at the 5% level.

TABLE 3

Juxtaglomerular cells and adrenal zona glomerulosa [1]

Group	JGI [2]	Zona glomerulosa width
		micra
Experiment 1		
1 High KCl	34 ± 5.3 [3]	59 ± 5.2 *,[4]
2 Control	31 ± 3.4	42 ± 1.5
3 High NaCl	9 ± 1.5 **	27 ± 1.5 **
Experiment 2		
1 High KCl	18 ± 3.0	—
2 Control	20 ± 2.7	—
Experiment 3		
1 High KCl, 12%	16 ± 2.5	76 ± 3.1 **
2 HighKCl, 6%	21 ± 3.2	58 ± 3.6
3 Control	14 ± 2.9	51 ± 3.4
4 K-deficient	17 ± 3.3	46 ± 2.4
Experiment 4		
1 Na-deficient, high KCl	54 ± 8.5 *	430 ± 47
2 Na-deficient, normal KCl	85 ± 9.3	350 ± 23
3 High KCl	17 ± 3.1	77 ± 4.2 **
4 Control	26 ± 3.0	57 ± 3.6

[1] The *t*-test was used to test difference between experimental and control group within each experiment except in experiment 4 in which group 1 was tested against group 2.
[2] JGI indicates degree of granulation of juxtaglomerular cells.
[3] SE of mean.
[4] Micra ± SE.
** Significant at the 5% level.
* Significant at the 1% level.

experiment, despite pair-feeding, but a similar depression of growth occurred with high potassium and normal sodium intake, except in experiment 2, where pair-feeding was carried out on the basis of body weight.

In experiment 3, in which varying levels of dietary potassium were used, the width of the zona glomerulosa was proportional to the amount of potassium in the diet, but the only significant difference from control rats was in the group that received 12% KCl. There were no significant differences in JGI in this experiment.

Although plasma potassium levels (table 2) tended to be higher in the groups receiving the high potassium intake, this difference was inconsistent and was statistically significant only in experiment 2; plasma potassium was in fact lower in the high potassium group of experiment 3 than in the control group.

Histologic changes in the zona glomerulosa were of much greater magnitude in sodium-deficient rats than in high-potassium rats in these experiments (table 3).

DISCUSSION

These results demonstrate that a high intake of potassium, like sodium deficiency, produces widening of the zona glomerulosa, confirming the observations of Deane et al. (1), but unlike sodium deficiency, it does not affect JG cells in a parallel way. Thus, variation in potassium intake is an exception to the relationship between activity of JG cells and of the zona glomerulosa (6).

This conclusion corroborates, but does not explain, the observation of Davis et al. (10) that stimulation of aldosterone by excess potassium is not under renal control. Previously, it had been shown by Davis' group and others, as reviewed recently (11), that hypersecretion of aldosterone by the zona glomerulosa, secondary to hemorrhage, constriction of the inferior vena cava, sodium deficiency, and others, was dependent upon intact kidneys and that the substance responsible for this control — aldosterone stimulating substance — was angiotensin, formed by the action of renin on angiotensinogen. Present evidence strongly supports JG cells as the source of renin (4, 12). Thus, JG cells control the secretory activity of the zona glomerulosa in most forms of secondary aldosteronism. The action of high potassium, on the other hand, is apparently a direct one, independent of JG cells, but the exact mechanism by which it stimulates aldosterone is unknown.

Two other observations were made in the present study. First, hypergranulation of JG cells in sodium-deficient rats was partially inhibited by high potassium intake, whereas there was no inhibitory effect on hypertrophy of the zona glomerulosa. The explanation is not obvious, at least on the basis of the parameters measured. A similar effect was recently described by Wardlaw and Pike (13) in sodium-deficient, pregnant rats. Although these rats had more severe hypertrophy of the zona glomerulosa than did comparable sodium-deficient, non-pregnant rats, JGI was significantly lower. The possible

link is that their pregnant group had higher serum levels of potassium than the non-pregnant group. Admittedly, a comparison of high intake of potassium and high serum levels with normal potassium intake may not be valid, but the coincidence deserves further study. The possibility that the lower JGI represented hypersecretion cannot be ruled out, but it is highly unlikely. In chronic conditions, especially in the rat, hyperactivity of JG cells is invariably accompanied by increased granulation (12).

The other observation was the much greater change in the zona glomerulosa with variations in sodium intake than with variations in potassium intake (exps. 1 and 4, table 3), at least at levels compatible with life. Higher supplements of potassium or more severe potassium deficiency (that is, the same diet in weanling rats) than those used in these experiments were fatal. Again the explanation is not apparent, but this observation might suggest that potassium plays only a minor role in control of aldosterone and that the renal control, by JG cells, in other forms of secondary aldosteronism is of primary importance.

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Adrenal Lipids of Cholesterol-fed Guinea Pigs and Rats[1]

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ABSTRACT Male and female guinea pigs and rats were fed diets with or without cholesterol. Adrenal lipids were separated into cholesterol ester, triglycerides and phospholipids. The fatty acid composition of each lipid class was determined. Cholesterol feeding had no effect on the total lipid level of adrenals in either species. It did increase the cholesterol content, particularly the esterified fraction, at the expense of triglycerides. Phospholipid levels were relatively unaffected. Adrenal cholesterol ester contained 75 to 80% of unsaturated fatty acids, including large amounts of a 22:4 acid. Cholesterol feeding led to a decrease of the ratio of the 20:4/20:3 acids. It had little effect on the fatty acid composition of triglycerides and phospholipids. Guinea pig adrenals had considerably higher lipid levels than those of rats. In contrast with that of rats, guinea pig adrenal cholesterol ester contained less 22:4 acid, and the predominant fatty acids of its phospholipids were palmitic and linoleic acid, whereas those of rats were stearic and arachidonic acids. Also in contrast with rats, unesterified cholesterol levels in guinea pig adrenals increased in response to dietary cholesterol. Certain sex differences were observed between adrenal lipid levels and their changes in response to dietary cholesterol. Present hypotheses of the function of adrenal lipids are discussed.

It has been quite well-established that the amount and kind of dietary lipid and of certain hormones affect lipid metabolism in man and many species of animals (1–3). Dietary cholesterol and cortisone, for instance, increase serum cholesterol levels in certain animals (4, 5), whereas some polyunsatuarted fatty acids and thyroid hormone may have the opposite effect (2, 6).

The mechanism by which these and related effects are produced is obscure. Adrenal cholesterol has been shown to be a precursor of adrenocortical hormones (7) and it is known that adrenal lipids contain a high proportion of polyunsaturated fatty acids (2). Very little, however, is known of the changes in adrenal lipid composition in response to changes in dietary lipids.

We are reporting data on the adrenal lipid composition of 2 commonly used laboratory animals, rats and guinea pigs, that had been fed a semi-purified diet with or without added cholesterol. Both male and female animals were studied.

MATERIALS AND METHODS

Guinea pigs. Male and female guinea pigs at 2 and 3 weeks of age were used. They were fed a semi-purified diet of basically the same composition as that used by Reid and Briggs (8) as modified by Richie,[2] containing 10% cottonseed oil.[3] At the end of 2 weeks the animals were divided into control and cholesterol-fed groups, the diet of the latter differing from that of the former by the addition of 1% cholesterol at the expense of sucrose. At the end of the experimental period of 50 to 60 days, 5 or 6 guinea pigs of each group were fasted for 20 to 22 hours, anesthetized with sodium pentobarbital, and killed by withdrawal of blood by open-heart puncture. Both adrenals were removed, blotted, weighed and immediately homog-

Received for publication December 9, 1963.

[1] This investigation was supported in part by Western Regional Experiment Station Project W-44 and by U. S. Public Health Service Grant-in-aid H-6480.

[2] Richie, J. 1959 Studies on semisynthetic diet for the growth of young guinea pigs. M. S. Thesis, University of California, Berkeley.

[3] The diet contained: (g/100 g diet) casein, 20; albumin, 10; cottonseed oil, 10; sucrose, 7; glucose monohydrate, 6.8; dextrin, 20; cellophane, 5; alfalfa meal, 5; agar, 5; salts, vitamins. For details of composition and preparation see (9).

enized in 95% ethanol. The lipids were extracted first with ethanol, then with ethyl ether. They were then separated into 4 fractions on silicic acid columns as described in previous reports from this laboratory (10, 11). The fractions obtained were 1) cholesterol esters, 2) triglycerides, 3) phospholipids, and 4) a mixture of unesterified cholesterol, mono- and diglycerides and free fatty acids. Because this last fraction represented a very small proportion of the total adrenal lipids of both the guinea pigs and the rats, its level and composition will not be presented. The fatty acid composition of the individual fractions was determined by gas-liquid chromatography of their methyl esters.[4]

The fatty acids were identified by comparing their retention times with those of known fatty acids plotted as number of carbon atoms versus $\log_{10}$ retention time. The less common fatty acids were tentatively identified by re-chromatography after hydrogenation (12). The percentage composition of the sample was computed from the areas under the peaks. The relative peak areas were calculated by the method of Carroll (13).

Cholesterol was determined by the method of Sperry and Webb (14), with modifications as described by Okey and Lyman (15). Total lipids were determined by the method of Bloor (16), and phosphorus by the method of Sumner (17). The analyses were performed on the adrenals of individual animals. Statistical comparisons of the results were made by *t* test as described by Snedecor (18).

Rats. Weanling male and female rats were fed ad libitum a semi-purified diet containing 10% safflower oil with or without 1% cholesterol.[5] At the end of the experimental period of 45 to 55 days the fasted animals were killed and the adrenals removed, weighed, and extracted as described above.

The separation of lipids and their analyses were performed as described above. Because of the small size of the adrenals, the extracts from adrenals of 3 or 4 animals were pooled in such a way that 2 pools for each group were analyzed separately. No standard errors or *t* tests of significance were calculated on the means of the 2 values.

RESULTS

Gross effects of feeding cholesterol. Table 1 presents data on the growth and adrenal weights of all the animals discussed in this report. Cholesterol feeding impaired the growth of guinea pigs, but had no effect on rats. In both species the females had larger adrenals than the males whether they were fed the cholesterol-containing diet or not ($P < 0.01$). Although cholesterol feeding had no effect on the size of rat adrenals, guinea pig adrenals were enlarged by such a regimen ($P < 0.1$ to $P < 0.05$).

The effects of cholesterol on the *levels of adrenal lipids* of guinea pigs and rats are presented in table 2.

[4] Wilkins Aerograph with thermal conductivity detector; 152-cm columns of coiled stainless steel were packed with diethylene glycol polyester on Chromasorb W; operating temperature was 190 to 200°C. A mixture of weighed amounts of known fatty acid standards was analyzed daily. Relative sensitivity factors were computed from the standards and applied to the sample fatty acids as corrections.

[5] The diet contained: (g/100 g diet) casein, 5; albumin, 10; safflower oil, 10; sucrose, 69; salts, vitamins. For details see (19).

TABLE 1

Growth and adrenal weights of guinea pigs and rats fed a diet with or without cholesterol [1]

Diet group [2]	Sex	Total wt gain		Wt at autopsy		Adrenals	
		Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat
		g	*g*	*g*	*g*	*mg/100 g body wt*	
Control	M	229 [3]	206	466	236	56.6 ± 5.6	15.1 ± 1.6
Control	F	165	147	378	180	78.5 ± 1.7	29.8 ± 1.2
+ 1% cholesterol	M	105	212	349	244	74.6 ± 7.7	14.1 ± 0.8
+ 1% cholesterol	F	98	138	322	168	97.5 ± 9.1	27.9 ± 1.1
Cholesterol/control [4]	M	0.5	1.0	0.7	1.0	1.32	0.94
Cholesterol/control	F	0.6	0.9	0.8	0.9	1.24	0.94

[1] Five or 6 animals/group; experimental period varied from 49 to 57 days.
[2] Control group animals were fed a diet without cholesterol.
[3] Mean for each group ± SE (where given).
[4] Cholesterol/control indicates the ratio of the means of the cholesterol-fed and of the control groups.

TABLE 2

Adrenal lipid composition of guinea pigs and rats fed a diet with or without cholesterol [1]

Diet group [2]	Sex	Total lipid [3]		Triglycerides [4]		Total cholesterol [5]		Cholesterol ester		Unesterified cholesterol		Phospholipid [6]		PL/FC [7]	
		Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat
Control	M	21.1 ± 0.8	8.91	10.9 ± 1.5	2.7	4.10 ± 0.20	1.59	3.81 ± 0.18	1.30	0.29 ± 0.016	0.28	4.29 ± 0.16	3.35	15	12
Control	F	21.9 ± 1.3	8.97	8.3 ± 1.2	0.3	5.44 ± 0.50	2.45	5.08 ± 0.48	2.16	0.36 ± 0.022	0.29	4.51 ± 0.25	4.30	12	15
+ 1% cholesterol	M	19.1 ± 1.7	8.60	5.8 ± 1.5	1.1	5.44 ± 0.46	2.46	4.97 ± 0.46	2.18	0.47 ± 0.013	0.27	4.21 ± 0.13	3.19	8.9	12
+ 1% cholesterol	F	22.7 ± 1.4	11.28	7.2 ± 1.6	0.6	6.94 ± 0.37	3.86	6.40 ± 0.65	3.56	0.54 ± 0.022	0.30	3.94 ± 0.73	3.96	7.3	13
Cholesterol/ control [8]	M	0.90	1.0	0.53	0.4	1.32	1.5	1.30	1.7	1.62	1.0	0.98	0.9	0.59	1.0
Cholesterol/ control	F	1.04	1.2	0.87	1.6	1.27	1.6	1.26	1.7	1.50	1.0	0.87	0.9	0.61	0.9

[1] All figures are means ± SE (where given) for each group in terms of percentage (g/100 g wet wt adrenals). Standard errors for means less than 5 are presented only where relevant to the discussion.

[2] Control group animals were fed a diet without cholesterol. Data for the guinea pig experiment represent means of groups of 5 or 6 animals. Data for the rat experiment are averages of 2 pools of adrenal extracts from 2 or 3 animals each.

[3] Total lipids for guinea pigs were determined on the original lipid extract, those for rats were calculated from the amount of total fatty acids, determined on the saponifiable fraction of the original lipid extract.

[4] Triglyceride content was calculated by difference. Due to very small amounts of this fraction in rat adrenals, the figures are to be taken only as approximations.

[5] Total cholesterol was calculated from the amount of cholesterol determined in the cholesterol-ester fraction and in "fraction 3" of the silicic acid column separation procedure.

[6] Phospholipid content was calculated from the phosphorus content, determined in the original lipid extract.

[7] PL/FC indicates the ratio of the means of phospholipid and of unesterified cholesterol content, respectively.

[8] Cholesterol/control indicates the ratio of the means of the cholesterol-fed and of the control groups.

Guinea pigs. The level of adrenal lipid was the same in both sexes, and was unaffected by dietary cholesterol. The total amount of lipid (mg/adrenal) was greater in cholesterol-fed females than in males ($P < 0.05$) because of their larger adrenals. The cholesterol was present predominantly as ester. Female guinea pig adrenals contained more cholesterol ester as well as more unesterified cholesterol than those of males ($P < 0.05$). Cholesterol feeding increased the cholesterol ester content ($P < 0.05$) as well as the unesterified cholesterol content of the adrenals ($P < 0.01$) but did not affect the level of phospholipids. Consequently, dietary cholesterol produced a decrease of both the phospholipid to total cholesterol ratio as well as of the phospholipid to unesterified cholesterol ratio. The triglyceride level of the adrenals decreased somewhat in response to dietary cholesterol. The effect was more pronounced in the males than in the females ($P < 0.01$).

Rats. Compared with those of guinea pigs, rat adrenals contained less total lipid with a higher proportion of phospholipids and a smaller proportion of glycerides. Cholesterol feeding tended to increase total lipids, but in females only. Observations concerning sex differences and response of adrenal total cholesterol and phospholipid levels to dietary cholesterol were similar to those in guinea pigs. The level of unesterified cholesterol and therefore the ratio of phospholipids to unesterified cholesterol in rat adrenals, however, was not influenced by dietary cholesterol.

Effects of dietary cholesterol on the fatty acid composition of lipids. The composition of the cholesterol esters of guinea pig and rat adrenals is presented in table 3.

Guinea pig adrenal cholesterol ester contained up to 75% of unsaturated fatty acids. Adrenals of females showed a tendency to contain higher proportions of linoleic, linolenic, and 20:3 acids but lower proportions of arachidonic and 22:4 acids

TABLE 3

Fatty acid composition of cholesterol ester of adrenals of guinea pigs and rats fed a diet with or without cholesterol [1]

Fatty acid, wt %	Guinea pig				Rat			
	Male		Female		Male		Female	
	Control	Cholesterol-fed	Control	Cholesterol-fed	Control	Cholesterol-fed	Control	Cholesterol-fed
14:0 [2]	2.3	3.0	2.4	3.3	5.4	4.4	3.8	3.0
16:0	13.8 ± 1.9	17.4 ± 1.5	12.9 ± 0.8	17.6 ± 1.3	10.7	10.8	11.4	10.0
16:1	3.9	3.7	3.8	4.8	7.1	6.2	4.8	3.6
18:0	3.8	3.8	2.9	3.1	2.7	2.6	2.6	2.6
18:1	12.3 ± 0.6	12.4 ± 0.6	12.6 ± 0.2	12.7 ± 1.3	10.0	10.8	8.8	8.6
18:2	11.2 ± 1.2	12.6 ± 0.8	14.2 ± 0.2	15.2 ± 0.6	9.2	11.2	10.2	9.1
18:3	4.9 ± 0.9	4.4 ± 0.5	6.8 ± 0.2	6.1 ± 0.5	—	—	—	—
20:4	24.1 ± 1.7	20.4 ± 1.1	22.3 ± 1.0	16.5 ± 0.5	24.8	22.8 [3]	28.0	23.6
20:3 [4]	0.9	1.6	0.9	1.8	trace	0.8	0.8	2.0
20:3	3.7 ± 0.1	6.0 ± 0.9	5.0 ± 0.4	7.2 ± 1.3	3.9	6.0	3.4	5.8
22:4	12.1 ± 2.0	8.3 ± 1.1	8.9 ± 0.9	4.8 ± 0.3	21.3	21.5 [3]	26.3	27.6
24:0	3.1 ± 0.5	1.3 ± 0.4	3.1 ± 0.3	1.1 ± 0.3	4.9	trace	trace	2.0
20:3/20:4	0.153	0.294	0.224	0.436	0.157	0.263	0.121	0.251
Total fatty acids, mg/g adrenal [5]	27.8	36.2	37.0	46.6	9.5	15.9	15.8	26.0

[1] All figures are percentages of total methyl esters measured. They represent means ± SE. Standard errors of means less than 5 are presented only when relevant to the discussion. Data for the guinea pig experiment represent means of groups of 5 or 6 animals. Data for the rat experiment are averages of 2 pools of adrenal extracts from 2 or 3 animals each.

[2] The first figure represents the number of carbon atoms, the second figure the number of double bonds.

[3] Represents value of one pool only.

[4] Some of the higher unsaturated fatty acids have been identified only tentatively. One of the 20:3 acids could be a 20:2 acid, and the 24:0 acid could be an isomeric 22:4 acid. The major 22:4 acid is very likely adrenic acid, 5, 8, 11, 14-docosatetraenoic acid (44). In addition to the fatty acids listed, guinea pig adrenal cholesterol ester contained traces of 12:0, 15:0, 17:0, 20:0 and 20:1 acids. Rat adrenal cholesterol ester contained 1 to 3% of a 15:0 acid.

[5] Total fatty acids were calculated from the cholesterol content of the fraction.

than those of males. Statistically, these differences were significant only in the cholesterol-fed groups ($P < 0.05$ to $P < 0.01$). Cholesterol feeding resulted in an increase in the proportions of palmitic and 20:3 acids at the expense of arachidonic, 22:4 and 24:0 acids. (These changes were statistically significant in males for the 20:3 and 24:0 acids at the 5% level, in females for palmitic, arachidonic and 24:0 acids at the 1% level.)

Rat adrenal cholesterol ester contained over 80% unsaturated fatty acids — even more than that observed in guinea pigs. A high proportion of 22:4 acid was particularly evident. Adrenals of female rats tended to contain a lower proportion of palmitoleic but more 22:4 acids than those of males.

In both guinea pig and rat adrenals, linoleic, palmitic and oleic acids made up 80 to 90% of the triglyceride fatty acids (table 4). Arachidonic acid concentration was low, and higher unsaturated fatty acids were absent. No sex differences were noted, and cholesterol feeding did not change the triglyceride composition.

The composition of the phospholipids is shown in table 5. The major fatty acid of both guinea pig and rat adrenal phospholipids was arachidonic acid. This was also the only lipid class containing large amounts of stearic acid. Neither sex of the animal nor cholesterol feeding had much influence on the fatty acid composition of this fraction. A definite species difference, however, was observed; whereas the guinea pig adrenal phospholipids contained a higher proportion of linoleic and palmitic acids than those of rats, the latter contained more arachidonic and stearic acid than did those of guinea pigs. In contrast with guinea pigs, the ratio of the proportion of stearic to palmitic acids in the adrenal phospholipids of rats appeared to be somewhat higher in female than in male animals fed the diets with or without cholesterol.

DISCUSSION

Our observations concerning the relation of adrenal weight to body weight are similar to those in the literature, namely, 0.06% for guinea pigs (20) and 0.02% for rats (7, 21, 22). The observation that the relative weight of female rat adrenals is greater than that of males has been reported (7, 21, 22). Conflicting reports of sex differences in size of guinea pig adrenals (7) might be explained by the observation that such sex differences appear only in guinea pigs weighing more than 500 g (20). Dietary cholesterol has previously been reported to have no effect on rat adrenal size (23).

TABLE 4

Fatty acid composition of triglycerides of adrenal of guinea pigs and rats fed a diet with or without cholesterol

Fatty acid,[1] wt %	Guinea pig				Rat			
	Male		Female		Male		Female	
	Control	Cholesterol-fed	Control	Cholesterol-fed	Control	Cholesterol-fed	Control	Cholesterol-fed
16:0[2]	21.6±1.3	21.7±1.3	21.2±1.0	21.3±1.4	21.8	20.6	19.8	20.2
16:1	1.5	2.2	1.7	2.1	5.8	5.0	4.4	4.2
18:0	6.2±0.1	7.0±0.8	6.2±0.3	5.5±0.3	6.6	6.6	6.5	6.3
18:1	18.1±0.4	19.8±1.4	17.7±0.3	17.6±0.5	22.7	24.4	21.8	22.8
18:2	49.7±1.5	40.9±4.9	45.3±1.8	44.3±4.0	36.6	35.6	36.4	37.5
20:4[3]	trace	5.5±2.8	trace	4.8±1.7	3.5	5.8	9.1	7.2
Total fatty acids mg/g adrenal[4]	10.4	5.5	7.9	6.8	8.5	8.2	5.0	3.9

[1] All figures are percentages of total methyl esters measured. They represent means ± SE. Standard errors of means less than 5 are presented only when relevant to the discussion. Data for the guinea pig experiment represent means of groups of 5 or 6 animals. Data for the rat experiment are averages of 2 pools of adrenal extracts from 2 or 3 animals each.

[2] The first figure represents the number of carbon atoms, the second figure the number of double bonds.

[3] In addition to the fatty acids listed, guinea pig adrenal triglycerides contained traces of 14:0, 15:0, 17:0 and 18:3 acids.

[4] Total fatty acids were calculated from values for triglycerides.

TABLE 5

Fatty acid composition of phospholipids of adrenals of guinea pigs and rats fed a diet with or without cholesterol

Fatty acid,[1] wt %	Guinea pig				Rat			
	Male		Female		Male		Female	
	Control	Cholesterol-fed	Control	Cholesterol-fed	Control	Cholesterol-fed	Control	Cholesterol-fed
16:0 [2]	14.0 ± 0.7	14.9 ± 0.1	14.0 ± 1.4	15.6 ± 0.2	6.9	6.9	5.9	5.7
18:0	15.1 ± 0.1	14.3 ± 0.5	12.7 ± 0.8	14.1 ± 0.7	29.8	28.8	30.4	30.0
18:1	18.2 ± 1.0	15.8 ± 0.3	18.4 ± 0.6	16.8 ± 0.9	6.1	7.4	5.8	5.9
18:2	14.5 ± 1.8	18.0 ± 1.0	16.2 ± 0.5	18.9 ± 1.5	6.5	7.0	6.0	5.6
20:4	30.7 ± 1.4	28.3 ± 1.2	29.0 ± 0.9	25.6 ± 1.1	49.9	49.3	51.5	52.4
18:0/16:0 [3]	1.08	0.96	0.91	0.90	4.32	4.30	5.21	5.30
Total fatty acids, mg/g adrenal [4]	30.3	29.7	31.8	27.8	29.0	27.2	30.4	28.0

[1] All figures are percentages of total methyl esters measured. They represent means ± SE. Standard errors of means less than 5 are presented only when relevant to the discussion. Data for the guinea pig experiment represent means of groups of 5 or 6 animals. Data for the rat experiment are averages of 2 pools of adrenal extracts from 2 or 3 animals each.

[2] The first figure represents the number of carbon atoms, the second figure the number of double bonds.

[3] In addition to the fatty acids listed, guinea pig adrenal phospholipids contained small amounts of 14:0, 15:0, 17:0, 18:3, 20:2 and 20:3 acids.

[4] Total fatty acids for guinea pig adrenal phospholipids were calculated from phosphorus determinations on the phospholipid fraction; those for rats were calculated from phosphorus determinations on the original lipid extract.

In addition to much histological and histochemical work on lipid levels of rat and guinea pig adrenals (7, 24), a number of quantitative chemical determinations have been reported. The total lipid content of rat adrenals has been reported to be 14 to 26% (25, 26), figures much higher than those reported here. This discrepancy may be due to differences in the procedures used for dissections and extractions. Small amounts of adhering perirenal fat would contribute additional amounts of neutral fat. Literature reports on the composition of guinea pig adrenal lipids are similar to our observations in respect to total lipids (27), total cholesterol (27, 28), cholesterol ester (28), and phospholipids (27), as are the reports on rat adrenal total cholesterol (23, 25, 26, 29), cholesterol ester (23, 25, 26, 29, 30), and phospholipids (31). The higher cholesterol content of adrenals of female rats compared with that of males (25, 29), and its increase in response to dietary cholesterol, primarily in the esterified fractions, have been reported (23, 29). We are not aware of any report on the response to dietary cholesterol of guinea pig adrenal lipids.

It has been observed previously that adrenals contain large amounts of unsaturated fatty acids (1, 2). Detailed investigations of the fatty acid composition of adrenal lipids, however, have only recently become technically feasible. Levels of arachidonic, linoleic and 22:4 acids in rat adrenal cholesterol ester similar to our observations have been reported (26, 30–33). Their glycerides are reported to contain about one-half the amount of linoleic acid noted in our animals (30, 31). This difference probably is a reflection of our diet which was high in linoleic acid. The predominance of stearic and arachidonic acids in rat adrenal phospholipids has been reported recently (31). Swell et al. (30), on the other hand, reported very low levels of arachidonic acid. These differences may well be the result of different procedures for lipid extractions and manipulations. It has been reported that different phospholipid classes and subclasses have different fatty acid compositions. Choline-containing and noncholine-containing phospholipids, for instance, appear to differ in that the former are richer in palmitic and linoleic acids, whereas the latter are richer in stearic and arachidonic acids (34). Lecithins have been separated into fractions differing in the concentrations of the same 2 pairs of fatty acids (35). Our observation of a difference in the fatty acid composition of adrenal phospholipids in

rats and guinea pigs suggests therefore that these species differ in the proportion of different phospholipid classes or subclasses in their adrenal lipids.

The major features of adrenal lipids of rats and guinea pigs are the high level of lipids, the presence of cholesterol, primarily as ester, the large proportion of unsaturated fatty acids in all lipid fractions and the occurrence of large amounts of unusual polyunsaturated fatty acids esterified with cholesterol. The function of adrenal cholesterol as a precursor of steroid hormones appears to be well established (7, 36), although additional, unknown functions have been postulated (4). The functions of cholesterol esters are still obscure. Morphologically the esters are reported to be concentrated in the "clear cells" of the adrenal cortex, whereas the "compact cells" contain a high proportion of unesterified cholesterol (37). Stimulation with ACTH leads to a replacement of the "clear" cells by "compact" cells and to a decrease of the cholesterol ester content of both the blood serum and of the adrenals (37–39). Work with canine adrenal homogenates has shown that they contain enzymes which can incorporate cholesterol-C^{14} into cholesterol ester and into cortical steroids and which can also hydrolyze cholesterol-C^{14} esters and incorporate their labeled carbon into steroids (40, 41). Recently it has been suggested that the first stage of steroid biosynthesis takes place while cholesterol is still esterified (42).

The function of adrenal polyenoic fatty acids is still obscure. Experiments with rats, guinea pigs and rabbits maintained with fat-free diets or with diets containing only saturated fat have shown that, under such conditions, the adrenals respond with changes in their size, accumulation of adrenal cholesterol, certain changes in their fatty acid composition and a decrease of adrenocortical hormone output (21, 22, 25, 26, 43). Sinclair (2) has proposed a role of essential fatty acids in steroid synthesis, and the possibility that specific cholesterol esters play an important role is supported by 2 recent observations. Dailey et al. (32) mentioned unpublished observations indicating changes in the proportions of arachidonic and 22:4 acids of adrenal cholesterol ester in response to stimulation with ACTH, and Grant (38) reported that the decrease of adrenal cholesterol ester in response to such stimulation is primarily in the cholesterol-linoleate fraction. The 22:4 acid observed in considerable amounts in adrenal cholesterol ester has been shown to belong to the same family of polyunsaturated fatty acids as linoleic and arachidonic acids (44). It might therefore reasonably be expected to function similarly to these "essential fatty acids" (EFA).

The effects of dietary cholesterol on the adrenals were different for the 2 species. Guinea pigs were more severely affected than rats, showing impaired growth, enlarged adrenals, and the accumulation not only of cholesterol ester but also of unesterified cholesterol in the adrenals. Cholesterol-fed guinea pigs, in contrast with rats, have been shown to develop enlarged livers and spleens and severe anemia. The adrenals of rabbits, a species which also reacts to dietary cholesterol with severe metabolic disturbances, have also been reported to be greatly enlarged and to contain increased amounts of cholesterol ester (45).

It has been reported that dietary EFA deficiency is accompanied by an increase of the ratio of the 20:3 to 20:4 acids in many animal tissues (46). Our observation of a similar increase in the cholesterol ester fraction of adrenal lipids in response to dietary cholesterol suggests a marginal or relative EFA deficiency. The tissue acted as though not enough arachidonic acid had been available to cope with the increased amounts of cholesterol.

The results of this study confirm previous observations on the influence of dietary cholesterol on the adrenal cortex, and extend the available data on detailed changes of its lipid composition. Measurement of adrenal function and of hormone levels in adrenal venous blood and of urine in cholesterol-fed animals might provide information concerning the possibility that the changes observed in blood and tissues in response to dietary cholesterol might be mediated in part by changes produced in circulating adrenocortical hormones.

ACKNOWLEDGMENTS

We are grateful for the assistance of Margaret Rose and Ruth Pertel in the care and handling of the animals, and of Ruth Babcock in the lipid analyses.

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Studies on the Utilization of Tempeh Protein by Weanling Rats[1,2]

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ABSTRACT The effect of allowing a specific mold of the Rhizopus species to grow on soybeans has been studied in weanling rats. The mold did not improve protein efficiency nor growth in these studies above the protein efficiency ratio for the unfermented soybeans. Either the mold or something elaborated by the mold depressed the acceptance of the diets containing the fermented soybeans (tempeh). It was found that the acceptance of the tempeh-containing diets by rats was decreased with each 12-hour increment in fermentation time. There was no marked change on our parameters (growth, feed consumption, digestibility and protein efficiency ratio) of deep-fat frying tempeh for 3 minutes or less or of steaming for 2 hours or less after the fermentation step. In all our studies the animals receiving diets containing tempeh have consumed less food than similar animals fed diets containing unfermented full-fat soybean meal. Our experiments do not show an improvement in the nutritional value and digestibility of soybeans fermented by a specific mold of the Rhizopus species, but they do show that properly prepared tempeh contains high quality protein.

Tempeh, a fermented soybean product, serves as an important source of protein in the diet of Indonesians and has been suggested by Autret and van Veen (1) as a possible source of low cost protein in developing countries. Van Veen and Schaefer (2) have reported that the fermented beans are easier to digest than the original cooked beans. The question of the nutritional value of fermented foods has importance for a wide variety of such foods consumed in the orient (for example, miso, ontjom, bongkrek and natto).

The nutritional quality of tempeh protein has not been adequately investigated, and the literature is meager with respect to its utilization (3).[3] Therefore, the work to be described was undertaken to evaluate the protein in soybeans fermented for varying lengths of time by a specific mold of the Rhizopus species.[4] Investigations were designed to indicate the beneficial or detrimental effects of allowing the mold to grow on soybeans. In addition, studies were conducted to evaluate the effect of 2 cooking procedures.

EXPERIMENTAL METHODS

In the determination of protein efficiency ratios (PER), it has been our standard practice to have young male rats of the Holtzman strain shipped at weanling age, and to maintain these rats with a basal diet containing 15% casein for 2 days prior to distributing them into groups according to weight. The groups are then assigned, at random, to the experimental diets. All of our growth studies with weanling rats are of 4-weeks' duration. Additional details of our experimental procedures used in the handling of rats for growth studies have been described by Hackler et al. (4).

To further test the utilization of tempeh protein, digestion experiments were carried out with weanling rats. Apparent digestion coefficients (ADC) were calculated from the nitrogen intake (NI) and the fecal nitrogen (FN) using the following formula:

$$\text{ADC} = \frac{\text{NI} - \text{FN}}{\text{NI}} \times 100.$$

Each collection period was of 5 days' duration, preceded by a preliminary period of

Received for publication December 7, 1963.
[1] This investigation was supported in part by a grant from the United Nations Children's Fund.
[2] Approved for publication as Journal Paper no. 1369, by the Director of the New York State Agricultural Experiment Station, Geneva, New York.
[3] Smith, A. K., J. J. Rackis and C. W. Hesseltine 1963 Processing losses and nutritive value of tempeh, a fermented soybean food. Cereal Science Today, *8* (4): 129 (abstract).
[4] The tempeh mold has been tentatively identified as *Rhizopus oligosporus* Saito. Hesseltine, C. W. 1961 Proceedings Conference Soybean Products for Protein in Human Foods. NRRL, Peoria, Illinois.

7 days. During the last 3 days of the preliminary period, the rats were supplied with a constant intake of food, and this level was maintained throughout the collection period as far as possible. The rats were fed the experimental rations at the same level in all periods. During the digestion experiments, the rats were confined in metabolism cages[5] which separated the feces and urine very efficiently. Daily collections of feces were dried at 70°C for 24 hours, then allowed to cool to room temperature and were composited before weighing and grinding prior to chemical analyses.

In the studies on the effect of length of fermentation, defatted soybean meal[6] was used as a standard for comparison. In all of the subsequent studies, casein has been routinely used as a standard for comparison. Since the soybeans used in these studies differ in protein and fat content from defatted soybean meal and casein, adjustments were made in the amounts of dextrose and corn oil to maintain a constant calorie-to-protein ratio. All other components[7] of the diets remained constant and were added as follows: (parts per 100) sucrose, 12; cellulose, 4; mineral mix,[8] 4; choline chloride, 0.4; vitamin mix, 1; and A-D-E oil, 1. The diets were mixed to contain 10% protein (N × 6.25) and 10% fat. The diets were analyzed for crude protein by the micro-Kjeldahl procedure as outlined in AOAC (5).

Preparation of tempeh samples. Clark variety soybeans were used in all studies for the preparation of tempeh. The soybeans were dehulled by heating them for 10 minutes at 104°C in an air dryer[9] to loosen the hulls, which were then separated from the cotyledons by running the beans through a burr mill[10] and over a gravity separator.[11] The dehulled soybeans were soaked overnight in tap water (1 kg of beans/3 liters of water containing 30 ml of 85% lactic acid. Following this the beans were steamed in the soak water for 90 minutes at 100°C. The soak water was then drained, and the beans were cooled in a cold room at 5°C. As soon as the temperature had fallen to approximately 36°C, the dehulled, soaked, partially cooked beans were mixed with a lyophilized culture of the tempeh mold (2 g of inoculum/kg of dry starting beans). The inoculum was grown on sterilized soybeans which were then quick frozen and lyophilized. Steinkraus et al. (6) have discussed the preparation of the inoculum in much greater detail.

The inoculated beans were spread on stainless steel pans (25.4 × 35.6 × 6.4 cm) to a depth of approximately 2.54 cm and covered with metal covers and incubated at 37°C.

For the study on the effect of length of fermentation the fermenting soybeans were removed at intervals of 12, 24, 36 and 72 hours. After the studies on the effect of length of fermentation, the preparation of tempeh was altered slightly. We switched from closed fermentation pans to open mesh trays[12] (35.6 × 79.3 × 1.27 cm). The soybeans were incubated at 37°C (same as before) but with a controlled relative humidity of 80%. This new procedure made it possible to reduce the fermentation time to 18 hours. The mold growth and pH changes were equivalent to that obtained at 36 hours by the earlier procedure. This latter procedure was used for all subsequent studies. The thinner layer of soybeans (approximately 1.27 cm) in the open mesh trays allowed better air circulation, resulting in shorter fermentation time. Therefore, the fungus grew more rapidly.

The lots of tempeh were steamed directly on the fermentation trays, which, in turn, were placed in the hot air dryer for dehydration. Tempeh samples were cut[13] into slices 0.32-cm thick before deep-fat frying in corn oil at 196°C. The hydrated soybeans (or tempeh) were cooked

[5] Stainless steel metabolism cages for rats, Acme Metal Products, Inc., Chicago.

[6] Soybean oil meal, 44% protein solvent extracted. A. E. Staley Manufacturing Company, Decatur, Illinois.

[7] The vitamin composition, as well as the source of the various components, has been described earlier. Hackler, L. R., D. B. Hand, K. H. Steinkraus and J. P. Van Buren 1963 J. Nutrition, *80:* 205.

[8] HMW Mineral Mix, Nutritional Biochemicals Corporation, Cleveland, Ohio. Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

[9] Laboratory forced-draft tray dryer, Proctor and Schwartz, Inc., Philadelphia.

[10] Burr mill, style 148, size 8, Bauer Bros. Company, Springfield, Ohio.

[11] Model 5A gravity separator, Olive Manufacturing Company, Rocky Ford, Colorado.

[12] Steinkraus, K. H., J. P. Van Buren, L. R. Hackler and D. B. Hand 1963 A pilot plant process for the production of dehydrated tempeh. Submitted for publication.

[13] Slicer, Model 411, Hobart Manufacturing Company, Troy, Ohio.

in 1200-g lots. At the end of 1-, 3-, 5- and 7-minute processes, the temperature of the oil was 147, 152, 167 and 190°C, respectively. The 3-minute process yielded a product that would be considered slightly undercooked and 5 minutes a product that was slightly overcooked. Since there was moisture still present in the samples fried for 5 minutes or less, these lots were dehydrated further in the air dryer.

The unfermented soybean control was handled exactly the same as the fermented soybeans but was dried immediately after the soaking step and prior to inoculation of the soaked soybeans for the production of tempeh. After preparation, all samples were dehydrated, as soon as possible, and ground in a comminuting mill.[14] The samples that had been deep-fat fried were mixed with dextrose to facilitate grinding. All samples were stored in metal cans at 2°C until used.

RESULTS AND DISCUSSION

As the fermentation time was increased, there was a definite decrease in the food consumption by the rats in this study. When soybeans were allowed to ferment for 12, 24, 36 and 72 hours, the food consumption by weanling rats was 13.70, 12.43, 12.20 and 11.68 g/day/rat, respectively. There was a similar decrease in the rate of growth as the fermentation time was increased. This was no doubt a reflection of the feed intake data, as there was no significant decline in the PER's of the rats with increases in the length of time the mold was allowed to grow on the soybeans. The rats receiving the diet containing the defatted soybean meal consumed the most feed (14.90 g/day/rat) and had the highest growth rate and PER (3.81 g/day/rat and 2.63 respectively). These results are summarized in table 1.

Part of the first study (table 1) was repeated, and a tempeh control (unfermented soybeans) was included. In this study, 30 rats were divided into 3 groups of 10 rats each and were fed the defatted soybean meal, unfermented soybeans and tempeh (36 hours of fermentation) diets as before. The results obtained confirm the earlier study and demonstrate conclusively a decline in food consumption due to fermentation. The rats receiving the defatted soybean meal-containing diet had the highest growth, food intake and PER value, 4.21, 16.46 and 2.48, respectively. The growth rate (3.68 g/day/rat) and food consumption (15.49 g/day/rat) for the rats receiving the diet containing the unfermented soybeans (full-fat) were slightly higher than the corresponding values (3.40 and 13.94, respectively) found for rats receiving the tempeh diet. The PER values for the rats receiving the diets containing the unfermented soybeans and tempeh were similar (2.32 and 2.31, respectively).

Effect of deep-fat frying and steaming. The tempeh fed to the rats in the previous experiments differed from tempeh that is ordinarily consumed in the Indonesian diet. Although the soybeans had been subjected to a preliminary heat treatment, the tempeh was not heated after fermentation. Thus, the mold was raw. The fermented soybean product (tempeh) is commonly French-fried in deep fat which may produce an effect on the digestibility of the protein. For this reason a series of experiments was designed to determine

[14] Model D comminuting mill, W. J. Fitzpatrick Company, Chicago.

TABLE 1

Summary of the effect of length of fermentation on subsequent utilization of tempeh protein by weanling rats when diets supplied 10% protein [1]

	Length of fermentation, hours				
	0 [2]	12	24	36	72
Average daily gain, g [3]	3.81 ± 0.17	3.46 ± 0.17	3.24 ± 0.17	3.11 ± 0.03	2.86 ± 0.13
Average feed intake, g	14.90	13.70	12.43	12.20	11.68
Protein efficiency ratio	2.63	2.47	2.56	2.49	2.44
Apparent digestion coefficient, %	86.9	88.0	86.2	85.3	85.4

[1] Each datum represents an average of 10 rats.
[2] Soybean oil meal (commercial) represents the zero-hour of fermentation.
[3] Average daily gain in grams ± SE of mean.

TABLE 2

Summary of the data on the effect of deep-fat frying of tempeh protein when fed to weanling rats in diets supplying 10% protein

	Tempeh, minutes deep-fat fried					Casein
	0 [1]	1 [1]	3 [1]	5 [1]	7 [2]	
Average daily gain, g [3]	2.18 ± 0.11	2.18 ± 0.09	2.44 ± 0.15	1.96 ± 0.08	0.46 ± 0.06	3.08 ± 0.08
Average feed intake, g	10.73	10.97	11.95	11.08	7.41	11.70
Protein efficiency ratio	2.02	1.93	1.98	1.80	0.61	2.54
Apparent digestion coefficient, % [4]	86.8	87.4	84.4	82.8	79.0	93.1

[1] Each datum represents an average of 20 rats from 2 studies of 10 each.
[2] Data represent an average of 10 rats.
[3] Average daily gain in grams ± SE of mean.
[4] Each datum represents an average of 4 rats, except for the casein group where 8 rats were used to determine the value.

the effect of deep-fat frying on the utilization of tempeh protein, and the results obtained are summarized in table 2.

The digestion coefficients obtained indicate a decrease in the amount of nitrogen absorbed as the length of deep-fat frying was increased. The values obtained for the 0-, 1-, 3-, 5- and 7-minute deep-fat fried tempeh samples when fed to weanling rats as the sole source of nitrogen in otherwise complete diets were 86.8, 87.4, 84.4, 82.8 and 79.0, respectively. The PER's show a similar decline after 3 minutes of frying in oil. The PER values for the 0-, 1-, 3-, 5- and 7-minute tempeh samples were 2.02, 1.93, 1.98, 1.80 and 0.61, respectively.

The growth rates of rats fed diets containing tempeh, which has been deep-fat fried for 0-, 1-, 3-, 5- and 7-minutes, were 2.18, 2.18, 2.44, 1.96 and 0.46 g/day/rat. The group of rats receiving the diet containing tempeh, which had been deep-fat fried for 3 minutes, grew faster than any of the other tempeh-fed rats in this study. This average value shown in the table may be misleading since 1 group of 10 rats out of the total of 20 rats, grew an average of 1 g/day faster than any of the other groups. In the second study there was no increase in the growth rate. Although growth rates varied considerably in the 2 studies for the 3-minute sample, the PER values were similar; therefore, we feel justified in averaging the 2 studies. In our studies with weanling rats, we have observed changes in growth rate and PER's with different shipments of rats; however, the magnitude of change is much less for PER's. Our data suggest that the PER may be superior to growth rates in weanling rats as a measure of protein quality when comparing data from several studies.

The results showing the effect of additional steaming beyond the standard 90 minutes the soybeans received prior to growing the mold on the beans are summarized in table 3. Additional steaming, up to 2 hours at 100°C, had no effect on the utilization of tempeh protein as measured by growth, feed intake, PER and digestion in this study.

The PER values for the steamed tempeh were slightly higher than the values shown in table 2 for tempeh deep-fat fried in oil.

TABLE 3

Summary of data showing the effect of additional steaming on the utilization of tempeh protein by weanling rats

	Tempeh, minutes additional steaming at 100°C				Casein
	15	30	60	120	
Average daily gain, g [1,2]	2.55 ± 0.13	2.38 ± 0.09	2.46 ± 0.13	2.43 ± 0.10	3.20 ± 0.14
Average feed intake, g [1]	11.51	11.33	11.21	11.51	11.55
Protein efficiency ratio [1]	2.21	2.10	2.19	2.10	2.76
Apparent digestion coefficient, % [3]	88.7	88.6	87.0	86.1	93.1

[1] Each datum represents the average value obtained with 10 rats.
[2] Average daily gain in grams ± SE of mean.
[3] Each datum represents the average value obtained with 4 rats, except for the casein group where 8 rats were used to determine the value.

Therefore, a new batch of tempeh was prepared and divided into 2 equal parts with one sample deep-fat fried for 3 minutes and the other steamed for 15 minutes. The PER's for these 2 supplements were identical (2.24). The PER value for the casein control was 3.02.

In our studies with rats we have observed some variation in the PER's for tempeh protein. We have found a similar variation in PER values with weanling rats fed casein. The starting weight of the weanling rats is apparently responsible for most of the variation between studies in our results.

Our data with rats do not support the observations that have been made by van Veen and Schaefer (2) that the fermented soybeans are easier to digest by humans, although our tempeh may not be identical to the one studied by van Veen and Schaefer (2). The variety of soybeans used in our studies was undoubtedly different, and our tempeh was produced with a pure culture. Tempeh is made in Indonesia with mixed cultures that appear to be largely *Rhizopus oligosporus* of which NRRL 2710 (used in our studies) is a typical representative (7).

ACKNOWLEDGMENTS

The authors are indebted to Ihsan El Rawi, Bwee Hwa Yap, Judy Dunn and Marvin Gage for their technical assistance during these studies.

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Effect of Thyroxine, Thiouracil and Ambient Temperature on the Utilization of Vitamin A by Vitamin A-deficient Rats [1, 2]

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ABSTRACT Vitamin A-deficient rats supplemented with vitamin A, exposed to 35°C for 3 weeks and restricted to 10 g of feed daily gained in body weight more efficiently than similarly treated rats at 21°C. The rats at 35° had significantly heavier livers, stored less hepatic vitamin A (μg/g), but total hepatic vitamin A storage was essentially the same at either temperature. Daily treatment with 7.5 μg L-thyroxine sodium resulted in a significantly decreased liver weight, whereas feeding thiouracil at 0.5% of the diet resulted in a significant increase in liver weight. Hepatic vitamin A storage (μg/g) in the thyroxine-treated rats was significantly greater than in the thiouracil-treated rats but total vitamin A storage was unaffected by hormone treatment. Vitamin A supplementation levels above 50 IU were followed by decreases in protein-bound iodine (PBI) with the highest level of supplementation resulting in a decrease in both PBI and thyroid weight. A significant quadratic response of thyroid weight to level of supplemental vitamin A was observed.

Research by Johnson and Baumann (1, 2) and more recent studies by Serif and Brevik (3) have demonstrated that the level of thyroid activity influences the conversion of carotene to vitamin A. Carotene conversion in calves subjected to the stress of high ambient temperature has been reported by Page et al. (4) to be less efficient than in calves maintained in a cooler environment, probably as a result of lowered thyroid activity at the higher temperature.

The effect of thyroid activity and high ambient temperature on the utilization of pre-formed vitamin A is not as well established. Logaras and Drummond (5) observed that thyroxine-treated rats supplemented with 3000 IU of vitamin A daily stored 15 to 20% more hepatic vitamin A than untreated animals. However, Johnson and Baumann (1) and Morgan and White (6) reported little difference in hepatic vitamin A storage by normal, hyper- or hypothyroidal rats supplemented at levels of 40 to 100 IU daily.

Early work conducted by Rappai and Rosenfeld (7) indicated that the increased basal metabolic rate observed in thyroxine-injected rats could be partially counteracted by the simultaneous administration of vitamin A or carotene. This vitamin A thyroid antagonism has also been studied by Smith and Perman (8).

There is little information in the literature on the effect of high ambient temperatures on the utilization of vitamin A or on the possible interrelationship between ambient temperature, thyroid activity and level of vitamin A supplementation. The study reported here was designed to compare the influence of constant 21°C ambient temperature with 35°C daytime (8:00 AM to 5:00 PM) and 30°C nighttime (5:00 PM to 8:00 AM) temperature on vitamin A utilization by vitamin A-deficient rats. An additional objective of the study was to determine the influence of thyroxine and thiouracil on the utilization of various levels of supplemental vitamin A as related to ambient temperature. Body weight gain, hepatic vitamin A storage, thyroid weight and protein-bound iodine were chosen as the response measurements.

Received for publication November 30, 1963.

[1] Arizona Agricultural Experiment Station Technical Paper no. 783.

[2] This study was supported in part by Public Health Service Grants EF-228 and A-1799.

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TABLE 1

Design of experiment with number of rats per treatment [1]

Temperature	Hormone treatment	Vitamin A (IU)			
		5	50	100	150
21°C	Control	4	5	5	5
	Thyroxine	5	5	5	5
	Thiouracil	5	5	5	5
35°C	Control	5	5	5	5
	Thyroxine	4	5	5	5
	Thiouracil	4	5	5	5

[1] Allotment on basis of 5 rats/treatment; table shows surviving rats within each treatment.

MATERIALS AND METHODS

One hundred twenty-five male weanling rats (Sprague-Dawley) were fed the vitamin A test diet, USP,[6] for a 3-week period to deplete hepatic vitamin A reserves. At the end of the 3-week depletion period, 120 individually caged rats were randomly allotted to the 24 treatment combinations shown in table 1.

During the 3-week experimental period, the vitamin A test diet was supplemented to provide a daily intake of stabilized vitamin A palmitate ranging from 5 to 150 IU. Thiouracil was fed at 0.5% of the diet and L-thyroxine sodium salt was mixed into the diet to provide 7.5 μg/10 g of diet. All rats were restricted to a feed intake of 10 g/day. The feed was mixed fresh each week, stored at 3°C and the vitamin A content of the diet measured at the beginning and end of each 7-day period to verify that each treatment group received the intended level of supplementation.

Body weights were obtained initially and at 7-day intervals during the 3-week supplementation period. At the end of the experiment, all rats were anesthetized, bled by heart puncture and the livers and thyroids removed and weighed. Hepatic vitamin A was determined according to the method of Gallup and Hoefer (9) and protein-bound iodine as described by Barker (10).

Table 1 shows the number of rats that survived to provide complete data. The treatment means for each group of 4 or 5 rats are presented in tables 2 and 3. A review of table 1 will suggest how the results of these small groups might be combined. Such calculations provide data to consider the influence of each major factor studied in this experiment as well as the interrelationships among these major factors. Although the basic plan (11) anticipates such pooling of data and the discussion will report some observations in this light, the raw data are provided by means from groups of 4 or 5 rats. The main effect treatment means of the factorial design (that is, temperature, hormone treatment or level of vitamin A supplementation) are presented in the columns or rows labeled "average."

RESULTS AND DISCUSSION

Rats exposed to 35° for 3 weeks were significantly heavier ($P < 0.01$) than those animals maintained at 21° for the same period (table 2). Both the control rats and those receiving thiouracil were significantly heavier at 35° than similarly treated rats maintained at 21°; however, a significant ($P < 0.05$) hormone treatment × temperature interaction was observed as a consequence of the similar responses in body weight gain shown by the thyroxine-treated rats at both ambient temperatures.

Expressed either in grams or as percentage of body weight, the livers of the thyroxine-treated rats weighed significantly less than the livers of the thiouracil-treated rats ($P < 0.05$). The observed effect of thiouracil on liver weight is in agreement with the published work of Leatham and Seeley (12) and May et al. (13) but the depressing effect of thyroxine on liver weight is not as clearly established by the literature. Beaver and Pemberton (14) state that liver atrophy with weight reduction occurs in hyperthyroidism but Papper

[6] Diet composition: USP salt mixture no. 2, 4%; irradiated yeast, 8%; starch, 65%; vegetable oil, 5%; vitamin-free casein, 18%.

TABLE 2

Relationship between thyroid activity and ambient temperature in vitamin A-deficient rats supplemented with vitamin A

Temperature	Hormone treatment			Average
	Control	Thyroxine	Thiouracil	
Body weight, g				
21°C	149 ± 3.1 [1]	154 ± 3.1	147 ± 3.1 [2]	150 ± 1.8 **
35°C	169 ± 3.1	154 ± 3.1	167 ± 3.1	163 ± 1.8
Average	159 ± 2.2	154 ± 2.2	157 ± 2.2	
Liver weight, g				
21°C	5.42 ± 0.51	4.94 ± 0.51	6.53 ± 0.51	5.63 ± 0.29 *
35°C	6.47 ± 0.51	5.40 ± 0.51	7.70 ± 0.51	6.53 ± 0.29
Average	5.94 ± 0.36	5.17 ± 0.36	7.12 ± 0.36 *	
Hepatic vitamin A, μg/g				
21°C	41.36 ± 2.32	44.54 ± 2.32	38.48 ± 2.32	41.46 ± 1.34 **
35°C	33.71 ± 2.32	34.69 ± 2.32	27.30 ± 2.32	31.90 ± 1.34
Average	37.54 ± 1.64	39.62 ± 1.64	32.89 ± 1.64	
Total hepatic vitamin A, μg				
21°C	229.58 ± 14.60	229.02 ± 14.60	225.62 ± 14.60	228.07 ± 8.44
35°C	237.24 ± 14.60	194.05 ± 14.60	232.57 ± 14.60	221.29 ± 8.44
Average	233.41 ± 10.33	211.54 ± 10.33	229.10 ± 10.33	
Serum protein-bound iodine, μg/100 ml				
21°C	3.27 ± 0.33	7.14 ± 0.33	0.76 ± 0.33	3.72 ± 0.19
35°C	2.36 ± 0.33	7.90 ± 0.33	0.82 ± 0.33	3.69 ± 0.19
Average	2.82 ± 0.23	7.52 ± 0.23	0.79 ± 0.23 **	
Thyroid weight, mg/100 g				
21°C	13.5 ± 2.08	8.6 ± 2.08	54.2 ± 2.08 [2]	24.8 ± 1.20
35°C	13.2 ± 2.08	10.1 ± 2.08	41.1 ± 2.08	20.5 ± 1.20
Average	13.4 ± 1.47	9.4 ± 1.47	46.7 ± 1.47 **	

[1] Mean with SE.
[2] Significant interaction, $P < 0.05$.
* $P < 0.05$.
** $P < 0.01$.

and Schaffner (15) note that in thyroid-fed rats, the liver is larger than in normal rats. All authors are in agreement, nevertheless, that the administration of thyroxine results in a marked decrease in liver glycogen and it is on this evidence that the decrease in liver weight observed in the thyroxine-treated rats in this study is explained.

The liver weights of rats maintained at 21° were significantly less than those of animals exposed to 35°, but these differences were due largely to the heavier body weights of the rats maintained at 35°. However, liver weights of the thyroxine-fed rats were heavier by almost 0.5 g at 35° even though body weights of the rats treated with thyroxine were identical at both temperatures.

Hepatic vitamin A storage, measured in micrograms per gram, was significantly greater ($P < 0.01$) in the rats maintained at 21°. Since both the control and thiouracil-treated rats at 21° were significantly smaller than similarly treated rats at 35°, the difference in hepatic vitamin A concentration per gram of liver, at first, might be assumed to be the result of a decreased need for the vitamin and therefore greater storage by the smaller rats. The thyroxine-treated rats, which weighed the same at both temperatures, also stored significantly more vitamin A (μg/g) at 21° than at 35°; hence the increased storage in micrograms per gram at 21° cannot be explained by differences in body weight alone. However, when the heavier livers of the rats exposed to 35° were considered and vitamin A storage expressed as total vitamin A, there were no differences in the amounts of vitamin A stored at the 2 temperatures.

TABLE 3

Relationship of level of vitamin A supplementation to ambient temperature in vitamin A-deficient rats

Temperature	Level of supplementation				Average
	5 IU	50 IU	100 IU	150 IU	
Body weight, g					
21°C	129 ± 3.5 [1]	158 ± 3.5	156 ± 3.5	156 ± 3.5	150 ± 1.8 **
35°C	148 ± 3.5	168 ± 3.5	169 ± 3.5	169 ± 3.5	163 ± 1.8
Average	138 ± 2.5	163 ± 2.5	163 ± 2.5	162 ± 2.5 **	
Liver weight, g					
21°C	4.23 ± 0.58	7.05 ± 0.58	6.18 ± 0.58	5.05 ± 0.58	5.63 ± 0.29 *
35°C	4.34 ± 0.58	7.48 ± 0.58	6.89 ± 0.58	7.41 ± 0.58	6.53 ± 0.29
Average	4.29 ± 0.41	7.26 ± 0.41	6.54 ± 0.41	6.23 ± 0.41 **	
Hepatic vitamin A, μg/g					
21°C	1.60 ± 2.68	16.05 ± 2.68	51.15 ± 2.68	97.04 ± 2.68 [2]	41.46 ± 1.34
35°C	1.36 ± 2.68	12.94 ± 2.68	42.59 ± 2.68	70.71 ± 2.68	31.90 ± 1.34
Average	1.48 ± 1.90	14.49 ± 1.90	46.87 ± 1.90	83.88 ± 1.90 **	
Total hepatic vitamin A, μg					
21°C	5.92 ± 16.85	113.53 ± 16.85	308.88 ± 16.85	483.97 ± 16.85	228.07 ± 8.44
35°C	5.58 ± 16.85	96.65 ± 16.85	283.25 ± 16.85	500.68 ± 16.85	221.29 ± 8.44
Average	5.75 ± 11.94	104.59 ± 11.94	296.07 ± 11.94	492.33 ± 11.94 **	
Serum protein-bound iodine, μg/100 ml					
21°C	4.37 ± 0.38	4.52 ± 0.38	3.34 ± 0.38	2.67 ± 0.38	3.72 ± 0.19
35°C	3.42 ± 0.38	4.12 ± 0.38	3.69 ± 0.38	3.56 ± 0.38	3.69 ± 0.19
Average	3.89 ± 0.27	4.32 ± 0.27	3.51 ± 0.27	3.12 ± 0.27	
Thyroid weight, mg/100 g					
21°C	22.3 ± 2.40	25.5 ± 2.40	28.4 ± 2.40	22.7 ± 2.40	24.8 ± 1.20
35°C	13.6 ± 2.40	27.0 ± 2.40	27.3 ± 2.40	17.9 ± 2.40	20.5 ± 1.20
Average	18.1 ± 1.69	26.3 ± 1.69	27.8 ± 1.69	20.3 ± 1.69 *	

[1] Mean with SE.
[2] Significant interaction, $P < 0.05$.
* $P < 0.05$.
** $P < 0.01$.

The thyroxine-fed rats at both 21° and 35° stored significantly more hepatic vitamin A, measured in micrograms per gram, than the thiouracil-fed rats, but due to the larger livers of the rats receiving thiouracil in the diet, the total vitamin A stored by each group was essentially the same.

Logaras and Drummond (5) observed that thyroxine-treated rats (68 μg/day) fed 3000 IU of vitamin A daily stored more of the vitamin than untreated controls. These workers reported that storage of vitamin A, expressed as IU/100 g liver, was proportionally greater in male rats treated with thyroxine, whereas the reverse was true of female rats. It is not possible to ascertain from their data whether these differences from control values were the result of smaller livers or decreased body size in the thyroxine-treated male rats.

Johnson and Baumann (1) did not report the influence of thyroxine or thiouracil upon the liver weight of their rats. They concluded that total hepatic and kidney vitamin A storage was essentially unaffected by treatment with thyroxine or thiouracil, although both of the treated groups retained slightly more vitamin A than the control group. This observation was explained as possibly due to the smaller size of the treated rats with vitamin A doses being concentrated in a smaller amount of tissue.

In a study designed to determine the utilization of vitamin A by hyperthyroid rats, Morgan and White (6) observed that liver hypertrophy resulted from daily doses of 60 to 80 μg thyroxine (analogue not mentioned). Compared with control animals, slightly greater total hepatic vitamin A storage was noted at the 80-μg

dosage level and a slight but not significant decrease in storage observed at the 60-μg level, measured either as total hepatic vitamin A or in terms of micrograms per gram of liver.

The serum protein-bound iodine (PBI) values presented in table 2 verified the effect of the thyroxine and thiouracil treatments. The highly significant elevation ($P < 0.01$) of PBI level in the thyroxine-fed rats and the highly significant depression ($P < 0.01$) of PBI in the thiouracil-fed rats, compared with the controls, established conclusively that the desired alteration in thyroid activity had been achieved. As would be expected, thyroid weights of the thiouracil-fed rats were significantly heavier ($P < 0.01$) and thyroid weights of the thyroxine-fed rats significantly lighter ($P < 0.01$) than those of the control animals.

Control rats maintained at 21° had higher PBI levels than the control rats maintained at 35°. The significant interaction ($P < 0.05$) between thyroid weight and temperature was due to a depression in the size of the thyroids in the thiouracil-fed rats at 35°, compared with similarly treated rats maintained at 21°. Temperature had no appreciable effect on the thyroid weights of the control rats, although the thyroxine-fed rats exposed to 35° had slightly heavier thyroids than similarly treated rats maintained at 21°. Dempsey and Astwood (16) have observed that the rate of thyroid enlargement in response to thiouracil administration was lower in rats exposed to 35° than in controls maintained at 26°. This difference in response was thought to be due to the lowered thyroid activity at the higher temperature.

The means presented in table 3 show the relationship between level of vitamin A supplementation and ambient temperature. In terms of body weight gain the critical level of supplementation appeared to be 50 IU daily. Since the rats supplemented with more than 50 IU of vitamin A/10 g of feed did not show a response in additional weight gains, it may be concluded that the 50-IU level allowed for optimal growth at both temperatures.

A sharp peak in liver weight was noted at the 50-IU level of vitamin A, with liver weights expressed as percentage of body weight heaviest at this level of supplementation. At the 150-IU level, the liver weight of the rats maintained at 21° was significantly reduced ($P < 0.05$) over that at the 50-IU level, whereas the liver weights in this same treatment group at 35° were similar to those at the 50-IU level of supplementation. Measured either as concentration in micrograms per gram or as total vitamin A, hepatic vitamin A storage showed a linear response to increased levels of vitamin A supplementation above 50-IU/10 g of feed.

A consistent effect of vitamin A supplementation on thyroid activity is apparent when the influence of level of supplementation on thyroid weight and PBI is examined. Both PBI and thyroid weight are reduced at the highest level of vitamin A supplementation in each of the treatment combinations measured. Sadhu (17) observed decreased thyroid size in rats fed large daily doses of vitamin A (30,000 IU), but the reduction in thyroid size noted in table 3 resulted from less than 1/200th of the daily level of supplementation used by Sadhu. The observed quadratic response of thyroid weight and PBI levels to increasing levels of vitamin A supplementation supports the observations of Frape et al. (18) in their work with young pigs.

The influence of level of vitamin A supplementation on protein-bound iodine and thyroid weight cited here would appear to point toward a definite antagonism between vitamin A and thyroid activity. That increasing levels of vitamin A appear to lower the basal metabolic rate and at the same time cause a reduction in thyroid size suggests that this action is mediated through the pituitary by a decreased production of thyrotrophin. If the vitamin acted upon the thyroid itself in a manner similar to thiouracil, which prevents the iodination of tyrosine, thyroid hypertrophy would result. The evidence cited in this study supports one of the theories advanced by Smith and Perman (8), that vitamin A influences thyroid activity by acting on the hypophysis to modify the release of thyrotrophic hormone.

This study has shown that ambient temperature and level of thyroid activity have an effect upon liver weight and upon

hepatic vitamin A storage expressed in micrograms per gram. However, the alterations in liver weight due to the treatments imposed offset any differences in hepatic vitamin A concentration (μg/g) so that total hepatic vitamin A storage was unaltered by ambient temperature or thyroid status. Hepatic vitamin A storage expressed in micrograms per gram would not appear to be as meaningful as the total hepatic vitamin A available to the animal. This places a limitation on the inferences that can be drawn from a liver biopsy sample taken to estimate the hepatic vitamin A status of the animal since it is not possible to calculate total hepatic vitamin A stores.

A comparison of the results of this study with those of similar published research suggests that the level of vitamin A supplementation and the degree to which thyroid activity is altered by the hypo- or hyperthyroidal agents administered must be considered before valid inferences can be drawn about the relationship of vitamin A and the thyroid. Interpretation of the results is further complicated by the alterations in liver size which result from treatment with thyroxine or thiouracil. The work of Logaras and Drummond (5) also suggests that the sex of the animal may be an additional factor to be considered in assessing the thyroid-vitamin A interrelationship. Each study cited here has used different concentrations and analogues of thyroxine and thiouracil, different levels and periods of vitamin A supplementation, different sexes and species of experimental animals and has reported hepatic vitamin A storage in different terms. A change in any one of these variables may have a great influence on the interpretation of the experimental results.

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Influence of Protein and Energy on Growth and Protein Utilization in the Growing Chicken

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ABSTRACT A factorially arranged experiment involving 4 levels of metabolizable energy (2.50, 2.78, 3.05 and 3.33 kcal/g), and 5 levels of protein (10, 14, 18, 22 and 26%) was conducted with chickens to study the effects of energy and protein on growth and protein utilization. To achieve the same balance of amino acids at all levels of protein a single protein source was used. Increasing the protein level to 26% gave an increase in weight gain and feed efficiency even with the lowest energy diet. Net protein utilization (NPU) values increased slightly at the 2 lowest protein levels as the energy level of the diet was increased. There was a much greater increase in NPU values with increasing levels of energy at the higher than at the lower levels of protein. The results indicate that both protein and energy levels should be taken into consideration when evaluating protein supplements. It is suggested that higher than maintenance levels of protein, along with a calorie-to-protein ratio in the range 55 to 65 be used for the determination of NPU values for application to the growing chicken. Nitrogen retention values followed the same trend as the NPU data except at the low protein levels where the lack of a correction for nitrogen for maintenance resulted in misleading retention values at the low levels of protein.

The influence of dietary protein level on the utilization of protein has long been established, both in growth (1) and in nitrogen balance studies (2). More recent work in this field using the chicken (3), as well as the rat (4–6), has substantiated the results of the earlier investigators namely, that the utilization of protein decreases as its level in the diet is increased.

As pointed out by Platt and Miller (7), many factors other than level of protein in the diet must be considered in the evaluation of protein supplements. The following factors have all been shown to influence the nutritive value of a protein; strain (6), age (7, 8), sex (7, 9) and environmental temperature (7). Differences in amino acid requirements between species can also affect the utilization of protein (10). Platt and Miller (7), Morrison and Campbell (9) and Morrison and Sabry (11) have emphasized the importance of considering not only the type of protein in question but also the availability of amino acids in net protein utilization (NPU) studies. The levels of minerals and vitamins in a diet can also affect NPU (12–14). Energy is probably one of the most important factors to consider when investigating the nutritive value of protein because of its influence on feed intake. The importance of considering not only the level of energy but also the calorie-to-protein ratio of a diet has been stressed in studies with the rat (5, 15, 16, 18–20).

The above review indicates that many factors must be considered in the evaluation of protein supplements if the results obtained are to be reproducible not only from laboratory to laboratory but also within laboratories. Since little or no work has been carried out with the chicken showing the influence of energy level on NPU, the present study was initiated to investigate this problem.

MATERIALS AND METHODS

A factorially arranged experiment involving 4 levels of energy (2.50, 2.78, 3.05 and 3.33 kcal of metabolizable energy (ME)/g) and 5 levels of protein (10, 14, 18, 22, and 26%) was conducted to study the effects of energy and protein levels on growth and net protein utilization (NPU). Soybean meal (50% protein) supplemented with 0.5% DL-methionine was used as the sole source of protein to achieve the same balance of amino acids in all diets. An equal parts mixture of cornstarch and

Received for publication November 6, 1963.

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dextrose served as the main carbohydrate source for all rations.

Soybean meal, the carbohydrate mixture, corn oil, fiber [2] and kaolin were varied in such a manner as to achieve the levels of energy and protein desired and at the same time maintain a relatively constant feed density. The water displacement method as outlined by Sibbald et al. (21) was used to determine feed density ratios. Vitamin and mineral supplements were added to all diets in such a manner as to maintain constant ratios between these nutrients and energy. The basal ration and the vitamin-mineral mix are shown in table 1.

TABLE 1

Composition of basal ration [1]

	%
DL-Methionine	0.11
Corn oil	2.00
Soybean oil meal (50%)	19.80
Fiber [2]	20.00
Carbohydrate mix [3]	53.71
Kaolin	0.36
Vitamin-mineral premix [4]	4.02

[1] Ration shown is the 10% protein, low energy diet. All ingredients were altered in such a manner as to give the desired levels of energy, vitamins and minerals.

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

[3] An equal parts mixture of cornstarch and dextrose.

[4] Vitamin-mineral mix provided: (in mg/1000 kcal ME) vitamin A (10,000 IU/g) 164; vitamin D_3 (1650 IU/g) 164; riboflavin (52.8 g/kg), 28; Ca D-pantothenate (70.4 g/kg), 56; niacin, 12; vitamin B_{12} (19.8 mg/kg), 220; choline chloride (25%), 40; ascorbic acid, 80; thiamine·HCl, 4; pyridoxine·HCl, 2.0; biotin, 0.08; folic acid, 0.4; ethoxyquin (50%), 80; manganous oxide (56% Mn), 36; ZnO, 22; $CuSO_4$, 4; $Fe(SO_4)\cdot 3H_2O$, 32; KI, 0.8; KCl, 656; $MgSO_4$, 812; iodized salt (0.015% KI), 1644; dicalcium phosphate, 8220; limestone, 2468; Cr_2O_3, 988.

Male, one-day-old, White Leghorn chicks were fed a commercial starting ration until one week of age. At this time the birds were divided into weight groups and distributed equally between 86 pens until each pen contained 10 chicks. Four randomly selected pens were then allotted to each of the 20 treatment groups and an equal number to the nitrogen-free control group. The 2 remaining pens of 10 chicks each were killed at the beginning of the experiment for carcass nitrogen analysis in order that the percentage nitrogen retention could be determined. During the last 6 days of the 2-week experimental period 3 excreta samples were collected from each pen pooled and, together with feed samples, analyzed to permit determination of ME values by the method of Sibbald and Slinger (22). Feed consumption and final body weights were recorded at the conclusion of the experiment and the chicks were killed by chloroform inhalation. Carcass nitrogen analyses were conducted in triplicate on the pooled samples of the 10 chicks for each pen after the birds had been frozen, ground, freeze-dried and then reground. Nitrogen retention data represent the carcass nitrogen gain as a percentage of nitrogen consumed. The NPU values were calculated using the formula described by Summers and Fisher (3).

RESULTS AND DISCUSSION

The determined ME values agreed very closely with the calculated values and are not presented. Also, water displacement density ratios for the experimental diets exhibited only small differences and these values are not shown.

In table 2 are shown the final body weights at 3 weeks of age together with the feed-to-gain ratio, feed intake and energy intake data. Increasing the protein to the highest level, at all levels of energy, resulted in increased body weights; this suggests that the protein required for maximal gain was at least as high as 26% even with an energy level as low as 2.50 kcal of ME/g of feed.

Shutze et al. (23) and Sunde (24) have shown a reduction in growth and feed efficiency by using high-protein, low-energy practical-type diets. Hill and Dansky (25) showed that the detrimental effect of a high-protein, low-energy ration could be alleviated by improving the balance of amino acids in the diet. Similarly, Harper (26) and Fisher (27) have shown that an amino acid imbalance manifests itself in a reduced feed intake. The good tolerance to the highest levels of protein in the present work may well be explained on the basis of the good balance of amino acids in the protein and the fact that the amino acid balance remained constant as the protein level was increased. Caution should be taken in using protein requirement figures obtained in studies such

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

TABLE 2

Effect of protein and energy levels on body weight, feed-to-gain ratio, feed consumption and energy intake

Dietary treatment		Mean body wt	Feed-to-gain ratio	Mean feed intake	Mean energy intake
Energy	Crude protein				
kcal ME/g	%	*g*		*g*	*kcal/W*$^{0.75}$
2.50	10	132	3.74	220	14.8
2.78	10	135	3.60	220	16.2
3.05	10	129	3.43	191	16.0
3.33	10	120	3.42	170	16.4
2.50	14	179	2.66	284	15.3
2.78	14	175	2.56	280	17.0
3.05	14	178	2.38	257	16.9
3.33	14	169	2.42	225	16.8
2.50	18	197	2.31	283	14.2
2.78	18	205	2.21	290	15.7
3.05	18	201	2.03	261	15.7
3.33	18	202	1.91	264	17.3
2.50	22	210	2.12	294	14.1
2.78	22	222	1.94	292	14.9
3.05	22	222	1.78	262	14.7
3.33	22	224	1.69	256	15.5
2.50	26	220	2.08	303	14.0
2.78	26	228	1.87	290	14.5
3.05	26	235	1.67	264	14.2
3.33	26	235	1.54	238	14.0

[1] Average initial starting weight equals 70 g.

as this for application in practical diets in which case the amino acid balance changes as the protein level is raised.

At the 2 lowest levels of protein, increasing the ME level tended to result in decreased weight gains; as the level of protein was increased this trend gradually reversed so that at the high-protein levels, increasing ME resulted in an improvement in weight gains. Statistical analysis of the data showed this interaction between protein and energy to be significant ($P < 0.01$).

In general, increasing the levels of protein or ME, or both, resulted in improvements in feed efficiency. Statistical analysis of the data indicated that as the level of protein was increased there was a curvilinear response ($P < 0.01$); increasing the energy level resulted in a significant linear response ($P < 0.01$). An ME level of 3.05 kcal/g appeared to be at least sufficient for optimal utilization of feed at levels of 10 and 14% protein, whereas this energy level was not adequate for the diets containing 18% or more of protein.

At all levels of protein, increasing the energy level resulted in decreased feed consumption and thus lower protein intake; although this resulted in growth depressions at the lower levels of protein, growth increases were obtained at the higher levels of protein. The decreased growth at the lower levels of protein, as the level of energy was increased, can be explained by the lower protein intake as feed consumption decreased. At the higher levels of protein, energy was apparently limiting and protein was being burned for energy purposes in the body; as the level of energy was increased more protein became available for tissue production and hence the increase in weight gain even though feed consumption decreased. Such an explanation is supported by the report of Platt et al. (28). Statistical analysis of the feed consumption data showed the protein × energy interaction to be significant ($P < 0.01$).

In general, ME intake per unit of metabolic body size (W 0.75) was constant, thus supporting the conclusions of Hill

and Dansky (29) with the chicken, and Sibbald et al. (19) with the rat, that rate of feed consumption is determined largely by the energy level of the diet. There was however, an indication of increased energy consumption as the protein level was increased from 10 to 14%, followed by a gradual decrease as the level of protein was increased from 14 to 26%. As suggested by Donaldson et al. (30) the overconsumption of energy with diets low in protein may represent an attempt by the birds to satisfy their protein requirements. The rather marked differences in weight between groups receiving diets containing 14 and 10% protein suggest that this method of compensation is not effective when the protein level drops to a low level. That a moderate deficiency of protein may be overcome by increased consumption of feed is well illustrated by the many experiments with chicks showing that the correction of a slight methionine deficiency will lower feed intake and thus improve efficiency of feed utilization (31, 32).

Net protein utilization and nitrogen retention values together with the calorie-to-protein ratios for the various treatments are shown in table 3. At the 10 and 14% levels of protein, NPU increased slightly as the energy level of the diets was raised even though food intake was reduced. Forbes and Yohe (15) reported that with a reduction in food intake the biological value of a protein remained constant until a point was reached where protein was burned for energy purposes in the body. Because it has been demonstrated that NPU increases as the level of protein in a diet decreases, i.e., as the protein intake decreases (3) it was to be anticipated that NPU would increase as feed intake was reduced providing that energy did not become a limiting factor. Thus the small increases observed in NPU at the low levels of protein, as the energy level was raised, are probably real affects. There were much greater differences in NPU values with increasing energy levels at the high levels of protein than at the lower levels. With the low-energy, high-protein diets, protein was probably being burned for energy purposes in the body which would tend to lower the NPU values. However, at the higher energy levels, the increased energy and the decreased feed consumption would tend to complement one another in increasing protein utilization. The calorie-to-protein ratios of all the 10 and 14% protein diets were abnormally high for chicks of this age and it is very improbable that any appreciable amount of the protein in these diets was being used for energy purposes. Statistical analysis of the NPU data indicated that there were linear responses ($P < 0.01$) to increasing both the level of protein and of energy; there was no statistically significant interaction between energy and protein.

TABLE 3

Effect of protein and energy levels on net protein utilization and nitrogen retention

Dietary treatment		Calorie-to-protein ratio	NPU [1]	Nitrogen retention
Energy	Crude protein			
kcal ME/g	%		%	%
2.50	10	114	66	54
2.78	10	126	65	54
3.05	10	139	68	54
3.33	10	151	68	53
2.50	14	81	62	55
2.78	14	90	63	56
3.05	14	99	64	57
3.33	14	108	65	58
2.50	18	63	51	46
2.78	18	70	54	49
3.05	18	77	58	52
3.33	18	84	61	55
2.50	22	52	48	44
2.78	22	57	52	48
3.05	22	63	55	49
3.33	22	69	55	51
2.50	26	44	39	36
2.78	26	48	45	41
3.05	26	53	50	47
3.33	26	58	51	47

[1] Indicates net protein utilization.

Nitrogen retention exhibited a similar picture to that of the NPU data with the exception that retention increased as the protein was increased from 10 to 14%. The difference in the 2 sets of data is due to the fact that the nitrogen used for maintenance is not considered in the nitrogen retention values but is taken into account in the NPU data.

The results presented, indicate clearly that the energy level of a diet as well as

the protein level, should be taken into account when measuring NPU or nitrogen retention with growing chickens. It was observed that at the usually recommended calorie-to-protein ratios for starting chickens (55 to 65) the NPU values were fairly constant (table 3). It thus appears that in evaluating a protein supplement, where one is attempting to relate the values to practical nutrition, it would be advisable to work at calorie-to-protein ratios that are within the normal range for the type of bird studied. Following such a procedure, higher levels of protein than recommended presently could be used and thus eliminate the criticism that evaluation of proteins at the maintenance level does not give a satisfactory assessment for application in the growing bird.

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Effect of Water Intake on Nitrogen Metabolism in Dogs[1,2]

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ABSTRACT Nitrogen balance studies in dogs were carried out to determine the effect of variable water intake on nitrogen metabolism. The results showed that increased water intake resulted in lower retention of nitrogen at low or high levels of nitrogen intake. In most cases urinary urea decreased when water intake increased. Apparent nitrogen absorption was not influenced by water intake. The urinary sulfur did not change with changes in water intake. Total serum protein and albumin concentration, albumin-to-globulin ratio and urea levels did not change significantly when higher volumes of water were given. It is concluded that in nitrogen balance work, water intake volume should remain as constant as possible to reduce the variability in nitrogen retention. Also, with a constant water intake, when protein intake is decreased, it is possible to learn when the nitrogen balance of the experimental subject becomes stable by measuring urine volume.

In studies of nitrogen retention in dogs, it was observed that the volume of urine decreased with amino acid imbalances.[3] In theory, the feeding of such diets should also have given a lower or even a negative nitrogen balance (1, 2), but this was not always the case. In these studies[3] the amino acid imbalances were induced by adding essential amino acids other than the most limiting amino acid, to a casein diet, and it was thought that variable water intake might cause the differences in excreted urine volume, which in turn, might explain the lack of effect observed. Studies by other investigators have shown that changes in water intake caused changes in nitrogen excretion in the urine (3–6). The recent studies of Konishi and McCay (7) in dogs showed that a decrease in water intake resulted in lower nitrogen excretion and that an increase in water intake had the reverse effect. The problem of water intake was, therefore, studied further.

MATERIALS AND METHODS

In experiments to be described, groups of 2 or 3 dogs of about 5 months of age, of the same sex, and from the same litter were used in each study. The animals were placed in individual metabolism cages and fed a diet consisting of gelatin, 15.0; casein (vitamin-free), 8.0; DL-methionine, 0.30; DL-tryptophan, 0.20; mineral mixture (8), 2.0; hydrogenated vegetable fat, 10.0; cod liver oil, 1.0; cellulose,[4] 2.7; sucrose, 15.0; dextrin, 22.8; dextrose, 23.0% and 5 ml of a complete B-vitamin solution (9) per 100 g of diet. The diet contained 3.36% nitrogen and 435 kcal/100 g. Depending on the experiment, the dogs were fed from 0.4 to 1.0 g of nitrogen and 100 to 150 kcal/kg of body weight/day. When necessary, the intake of calories was adjusted by feeding a protein-free diet of the following composition: (in grams) cornstarch, 20; dextrose, 40; sucrose, 24; cellulose, 3; hydrogenated vegetable fat, 10; mineral mixture (8), 2; cod liver oil, 1, and 5 ml of a complete B-vitamin solution (9) per 100 g of diet. The animals were weighed and fed daily and their nitrogen intake adjusted every 4 days. The specified amount of water was mixed with the food; in all experiments the food was consumed in less than 30 minutes. Therefore, corrections were not made for loss of drinking water by evaporation. The temperature of the metabolism room varied from 23 to 24°C and the relative humidity from 77 to 84%.

The experimental periods lasted 4 days and feces and urine were collected twice

Received for publication August 9, 1963.
[1] This investigation was supported by grant A-981 from the National Institutes of Health.
[2] INCAP Publication I-299.
[3] Bressani, R., unpublished data.
[4] Cellu Flour, Chicago Dietetic Supply House, Chicago.

a day. At the end of each period, the feces were homogenized, weighed and analyzed for nitrogen. The urine was collected in amber bottles containing 1 cm^3 of concentrated acetic acid and stored under refrigeration (4°C). The 4-day volume was measured and nitrogen determined in aliquots. The nitrogen content of the food, feces and urine was determined by the macro-Kjeldahl method. Urinary urea nitrogen and ammonia nitrogen were determined by nesslerization (10). Urinary sulfur was determined following the method of Folin (6) for total sulfur. In some experiments, blood samples were taken at the end of each 4-day period. Total serum protein was determined by the method of Lowry et al. (11, 12), blood urea by the method of nesslerization (10) and total α-amino nitrogen by the method of Albanese (13) except that tetraethylenepentamine was used to form a deeper-colored complex which was read on an Evelyn colorimeter.

RESULTS

Table 1 shows the effect on nitrogen balance and on other parameters of changing water intake from 400 to 1000 ml/day and back to 400 ml/day in one group, and from 1000 to 400 ml/day to 1000 ml/day in the second. Nitrogen intake and apparent nitrogen absorption did not change. Nitrogen balance and the urea nitrogen-to-total nitrogen ratio was lower when the higher water intake was given whether between 2 low water intake periods, or having a low intake of water in between. The urine-to-water intake ratio was highest when the intake of water was higher for both groups of dogs. The urinary total sulfur excretion did not change with changes in water intake. No consistent changes in body weight gain between water treatments was observed. Table 1 also shows the results of the next experiment carried out with 3 dogs. Water intake, high (1000 ml/day) and low (400 ml/day), was kept constant for 12 days to learn of variations in nitrogen balance for a longer period of time. Nitrogen retention was again lower when larger volumes of water were given and increased with the lower water intake. In both cases nitrogen balance remained constant within water treatment, the changes observed being small. Other determinations on the urine gave results similar to those shown for previous experiments, except urea nitrogen-to-total nitrogen ratio, which was in general high irrespective of water intake.

In the second experiment, the results of which are shown in table 2, the same 2 groups of dogs were used. One was given increasing and the other decreasing water intakes. Urea nitrogen-to-total nitrogen ratio followed the same tendency

TABLE 1

Effect of changing and of constant water intake on nitrogen balance, urine sulfur and urea nitrogen excretion in dogs [1]

Water intake [2]	Nitrogen					Urine/water intake ratio	Urine sulfur	Urea N/total N ratio
	Intake	Fecal	Urine	Absorbed	Retained			
ml/day	*mg/kg/day*						*mg/kg/day*	
400	692	41	374	651	277	0.51	14.0	0.76
1000	701	57	517	644	127	0.73	14.5	0.40
400	703	51	443	652	209	0.50	14.4	0.72
1000	665	28	450	637	187	0.74	15.8	0.46
400	668	36	388	632	244	0.61	16.2	0.78
1000	673	42	464	631	167	0.78	17.0	0.50
1000	719	35	498	684	186	0.71	17.1	0.95
1000	720	38	521	682	161	0.73	16.3	0.93
1000	719	40	514	679	165	0.72	17.6	0.89
400	723	55	482	668	186	0.57	17.0	0.61
400	722	49	467	673	206	0.61	16.8	0.82
400	722	34	471	688	217	0.65	15.8	0.91

[1] Number of dogs used, and average intitial weight: upper section 3, 10.25 kg; middle section 2, 13.46 kg; and lower section, 3, 11.42 kg.
[2] Each balance period is of 4-day duration.

TABLE 2

Effect of increasing and decreasing water intake on nitrogen balance, urine, sulfur and urea nitrogen excretion and on serum proteins and urea levels in dogs [1]

Water intake [2]	Nitrogen				Urine/water intake ratio	Urine sulfur	Urea N/total N ratio	Serum		
	Intake	Fecal	Urine	Absorbed	Retained				Total protein	Albumin	Urea
ml/day		*mg/kg/day*					*mg/kg/day*		*g/100 ml*	*g/100 ml*	*mg/100 ml*
400	714	42	401	672	271	0.44	13.1	0.89	6.62	2.89	17.40
600	712	36	459	676	217	0.61	15.0	0.67	6.75	2.97	17.69
800	715	53	451	662	211	0.68	15.8	0.87	6.48	3.04	17.73
1000	717	45	485	672	187	0.72	15.8	0.92	6.97	3.05	20.10
1000	717	45	481	672	191	0.65	15.2	0.95	—	—	—
1000	678	44	469	634	165	0.77	15.2	0.63	6.58	4.60	11.85
800	680	34	447	646	199	0.73	16.0	0.75	5.79	3.98	15.48
600	683	47	435	635	201	0.70	17.7	0.86	6.06	3.66	14.71
400	687	40	434	647	213	0.62	15.7	0.87	5.90	3.82	18.25
400	687	45	440	642	202	0.59	15.3	0.88	—	—	—

[1] Number of dogs used and average initial weight: upper section, 3, 10.68 kg; lower section, 2, 13.80 kg.
[2] Each balance period is of 4-day duration.

as previously shown in only the group of animals receiving decreasing levels of water intake. The urine-to-water intake ratio increased with increases in water consumption and decreased with decreases in water intake. Urine sulfur behaved as indicated for the results of the first experiment. Table 2 also shows the effect of changing water intake on total serum proteins, albumin, and serum urea. Total serum protein and albumin concentration showed no change when higher volumes of water were given. The albumin-to-globulin ratio remained constant within the group of dogs, and the higher values observed in the animals given decreasing intakes of water are probably characteristic of the breed of dogs used. The serum urea concentration did not change consistently with changes in water intake in either group of dogs.

Table 3 shows the results of another experiment obtained by feeding a group of dogs 2 levels of protein at a constant intake of calories of 140 kcal/kg/day. Water intake was varied from 300 to 900 ml/day and back to 300 ml/day with the same sequence at each level of nitrogen intake. During the transition in nitrogen intake, water intake remained constant at 300 ml/day. Apparent nitrogen absorption was not affected by water intake at either level of protein feeding when expressed as percentage of nitrogen intake. The nitrogen retention was again lower at the higher water intake volume independent of nitrogen intake. Nitrogen balance at the lower level of intake was about one-half the nitrogen retained at the higher nitrogen intake in milligrams per kilogram per day, and similar when expressed as percentage of intake. The urea nitrogen-to-total nitrogen in the urine was lower at the higher water intake volume for both levels of protein. The changes were more marked at the lower level of protein intake. Although not shown in the table, a corresponding change in ammonia nitrogen was observed in both experiments, and creatinine in milligrams per kilogram per day decreased slightly at the higher water intake volume, the change being not significant. It was the same at both levels of protein feeding.

TABLE 3

Effect of changing water and nitrogen intake on nitrogen balance and serum proteins in 3 dogs [1,2]

Water intake [3]	Nitrogen				Urine/water intake ratio	Urine sulfur	Urea N/total N ratio	Serum		
	Intake	Fecal	Urine	Absorbed	Retained				Total protein	Albumin	Urea
ml/day	*mg/kg/day*					*mg/kg/day*			*g/100 ml*	*g/100 ml*	*mg/100 ml*
300	1036	54	675	982	307	0.67	31.7	0.92	6.46	2.94	10.6
900	1031	48	731	983	252	0.83	35.3	0.72	6.75	3.13	12.0
300	1042	47	680	995	315	0.68	29.7	0.88	6.45	3.24	11.1
300	533	41	384	492	108	0.67	22.3	0.83	—	—	—
300	548	33	338	515	177	0.63	21.3	0.97	6.47	3.13	8.9
300	570	42	368	528	160	0.64	22.7	0.96	—	—	—
300	564	54	363	510	147	0.63	30.1	0.74	5.94	2.81	12.9
900	518	42	387	476	89	0.69	26.8	0.30	5.97	2.82	15.4
300	539	42	348	497	149	0.47	23.6	0.76	6.04	2.81	17.9

[1] Average initial weight: 5.98 kg.
[2] Average urinary creatinine excretion: mg/kg/day: 28.06.
[3] Each balance period is of 4-day duration.

Table 3 also shows that total serum protein and albumin remained constant, within levels of protein, but lower at the lower protein intake. Urea concentration in the serum did not change with changes in water intake within level of protein feeding, but was higher at the lower level of protein intake.

Table 4 shows the effect of water changes on nitrogen balance when nitrogen intake is decreased from about one to around 0.5 g/kg/day and back to about 1.0 g/kg/day. The intake of calories remained constant at 140 kcal/kg/day. During changes from high to low and from low to high intake of nitrogen, water intake remained constant at 900 ml/day. Nitrogen retention was highest when water intake was lowest, for both high and low levels of nitrogen feeding. Although not shown in the table, the α-amino nitrogen values in urine were slightly higher for the higher water intakes at both levels of nitrogen feeding, and a decrease was observed when nitrogen intake decreased. As in previous studies, urinary urea nitrogen-to-total nitrogen ratio was higher for the lower intakes of water, and lower for the higher water consumption. Ammonia nitrogen changes in the opposite direction. Table 4 also shows the values for blood protein and serum urea which varied as in previous experiments.

DISCUSSION

The lower retention of nitrogen with higher water intakes or the higher retention with lower intake of water was evident at all levels of nitrogen intake. These results are in agreement with the results of Konishi and McCay (7), who reported a small increase in nitrogen retention by dogs upon water restriction. Black et al. (3) and Grande et al. (4), working with humans observed that water deprivation increased urinary nitrogen output in subjects given a low caloric diet devoid of protein. The results presented by these investigators are not comparable nor do they agree with those presented in this manuscript or published by Konishi and McCay (7).

Contrary to the present study, Konishi and McCay (7) in dogs, as well as Larsen et al. (5) in their work with ruminants,

TABLE 4

Effect of changing water and nitrogen intake on nitrogen balance, and serum proteins in 2 dogs [1]

Water intake [2]	Nitrogen					Urine/water intake ratio	Serum		
	Intake	Fecal	Urine	Absorbed	Retained		Total protein	Albumin	Urea
ml/day			*mg/kg/day*				*g/100 ml*	*g/100 ml*	*mg/100 ml*
900	1026	49	597	977	380	0.77	5.52	2.93	14.6
300	1030	47	477	983	506	0.64	5.47	3.03	16.0
900	1023	46	572	977	405	0.71	5.59	2.86	16.1
900	522	53	393	469	76	0.78	—	—	—
900	540	50	382	490	108	0.70	—	—	—
900	546	52	336	494	158	0.68	—	—	—
900	554	52	365	502	137	0.70	—	—	—
900	554	50	336	504	168	0.72	5.25	2.29	11.3
300	554	52	237	502	265	0.49	5.39	2.45	12.6
900	562	46	285	516	231	0.64	5.41	2.44	16.0
900	970	53	508	917	409	0.60	—	—	—
900	966	60	512	906	394	0.56	5.75	2.85	10.4
900	933	32	497	901	404	0.59	—	—	—
900	943	39	606	904	298	0.62	6.01	3.07	12.9
900	936	46	509	890	381	0.59	6.14	3.28	12.6
300	941	40	418	901	483	0.47	6.65	3.28	18.1
900	937	43	560	894	334	0.54	6.53	2.82	17.3

[1] Average initial weight: 4.34 kg.
[2] Each balance period is of 4-day duration.

observed that nitrogen absorption also increased with water restriction. The disagreement in apparent nitrogen absorption with respect to water intake between the present study and that of others could be accounted for by the high apparent digestibility of the protein used in this study, which would approximate 100% digestibility if corrections were made for endogenous fecal nitrogen.

Two possible mechanisms could explain the effect of high and low water intake on nitrogen balance. First, high intakes of water may have a flushing effect in the kidney and other tissues washing out nitrogen metabolites. Second, high water intakes may have some influence on the formation of urea. When the amino acids have been absorbed, part are excreted in the urine, as suggested by the slight increase in α-amino nitrogen, although the largest part is deaminated. The ammonia produced is then discarded with the urine and not utilized in urea biosynthesis. This is indicated by the lower urea nitrogen-to-total nitrogen ratio, and higher ammonia nitrogen-to-total nitrogen ratio when the higher level of water is consumed. More data are needed, however, before the above explanation can be accepted. Some adaptation by the animal may possibly occur with respect to urea formation when the higher level of water is consumed, since a lower urea nitrogen-to-total urine nitrogen ratio was not always observed. The combined statistical analysis for 22 cases available indicated nonsignificant differences in urea nitrogen excretion according to water intake. However, the 22 cases available were separated according to water intake into a high urea nitrogen excretion group (13) and a low urea nitrogen excretion group (7). A significant difference in urea excretion was detected in the group of 14 but not in the group of eight observations. The decrease in urinary excretion of nitrogen during periods of low water intake is probably also a result of less water available for concentration and solution of the end products of protein metabolism.

The interaction of water intake and amino acid imbalance is being studied to learn whether one affects the other adversely. If so, the lack of response in nitrogen balance when the imbalanced diets were fed will be explained.[5] It has been shown that animals fed amino acid

[5] See footnote 3.

imbalanced diets show a high blood urea concentration (14). If the water intake remains low the animal needs to convert all its amino nitrogen from deamination into urea to protect itself against increases in ammonia; however, if water intake is high, this will dilute the ammonia produced and as a result, nitrogen balance will decrease, since more nitrogen will be present in the urine.

It has been repeatedly reported from work with rats that one of the characteristics associated with the feeding of amino acid imbalanced diets is the almost immediate refusal of the diet (14, 15) by the experimental animal. The studies of Lepkovsky et al. (16) showed that rats fed without water ate less food than rats fed with water. When fed without water, rats regulated their food intake so that it matched the amount of water that they could mobilize from their own tissues, thereby maintaining the proper water-to-food ratio in the gastric contents. Therefore, the work of Lepkovsky et al. (16) confirmed that water intake has a decisive effect on food intake and food intake greatly influences the amount of water ingested. It would be of interest, therefore, to learn whether the decreased food intake due to amino acid imbalance diets can be corrected by increasing water consumption.

On the basis of the results, it is suggested that in nitrogen balance work, water intake volume should remain as constant as possible in order to reduce the variability in nitrogen retention within periods of the same treatment and also when comparing 2 or more treatments. Also of interest is the preliminary observation that when protein intake is decreased, it may be possible to learn when the nitrogen balance of the experimental subject becomes stable by measuring urine volume, provided water intake and environmental conditions have remained constant.

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Nutrition of Salmonoid Fishes

XI. IODIDE REQUIREMENTS OF CHINOOK SALMON

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ABSTRACT Duplicate groups of 500 chinook salmon (*Oncorhynchus tshawytscha*) fingerlings raised in water containing 0.2 μg iodine/liter, were fed a low iodide basal ration with added sodium iodide to give 0.1, 0.6, 1.1, 5.1, and 10.1 μg iodide/g dry diet for 24 weeks. A 100-fish sub-lot of each group was fed the diets for an additional 9 months. No significant difference in growth was observed in either feeding period. At the end of the 24-week feeding period the fish fed 0.1 μg iodide had stored significantly less iodine in the thyroid region than had the fish in the remaining groups. At the end of the 9 months' extended feeding the fish fed 0.1 μg iodide had stored significantly less thyroid iodine than had the fish fed 0.6 μg, whereas the latter had stored significantly less than had the fish in the remaining groups. Under the experimental conditions reported, the iodide requirement of chinook salmon fingerlings was 0.6 μg iodide/g dry diet; for advanced parr the requirement increased to 1.1 μg.

As endemic goiter was perhaps the first nutritional deficiency syndrome to be recognized in salmonids, it is somewhat paradoxical that the quantitative iodide requirements of these hatchery propagated species remain unknown. Marine and Lenhart (1, 2) and Marine (3) correctly diagnosed as simple hyperplasia what was then thought to be thyroid carcinoma in brook trout (*Salvelinus fontinalis*). They effected a complete remission of the tumor by adding an iodide-iodine solution to the water in which the fish were raised. This pioneering work with trout did much toward furthering our knowledge of the etiology of human goiter. The literature on thyroid hyperplasia in salmonids and its treatment with iodide and thyroid preparations has been reviewed by LaRoche (4), but no experiments have been reported which define the iodide requirements of salmonids.

It was the purpose of the present investigation to determine the dietary iodide requirements of pre-migrant chinook salmon (*Oncorhynchus tshawytscha*) raised in low (0.2 μg/liter) iodide water.

EXPERIMENTAL

Approximately 15,000 free-swimming chinook salmon fry, hatched from eggs obtained at the Spring Creek National Fish Hatchery, were fed a low iodide basal ration until all fish were actively feeding and the yolk material was absorbed. After approximately 3 weeks, 5 duplicate lots of 500 fish each were hand-counted, weighed and placed in the experimental hatchery troughs for the feeding trial. The 5 groups were then fed diets consisting of a basal ration (table 1) with added iodide to give the following iodide levels; 0.1 (present in the ration), 0.6, 1.1, 5.1 and 10.1 μg/g dry diet.

TABLE 1

Ingredients of basal ration

	g/200 g diet
Vitamin-free casein	48
White dextrin	28
Oil mixture [1]	9
Vitamin-amino acid supplement [2]	10
Mineral supplement [3]	4
CMC [4]	1
Water	100

[1] Containing in grams: Corn oil, 7; USP cod liver oil, 2; α-tocopherol, 0.04.

[2] Containing in mg: thiamine, 5; riboflavin, 20; pyridoxine, 5; niacin, 75; Ca pantothenate, 50; choline chloride, 500; *i*-inositol, 200; biotin, 0.5; folic acid, 1.5; ascorbic acid, 100; menadione, 4; vitamin B_{12}, 0.01; L-arginine, 1500; L-cystine, 500; plus α-cellulose flour to make 10 g.

[3] Salt mixture USP XIV no. 2 plus in mg: $CuSO_4 \cdot 5H_2O$, 3; $ZnSO_4 \cdot 7H_2O$, 12; $MnSO_4 \cdot H_2O$, 6; $CoCl_2 \cdot 6H_2O$, 0.06.

[4] Carboxymethylcellulose, HiVis grade, Hercules Powder Company, Wilmington, Delaware.

Sufficient dry ingredients for 6 kg of diet were blended together in a twin-shell blender until completely uniform and then stored in a tightly closed container at

Received for publication November 6, 1963.

2°C until needed. For final diet preparation, sufficient dry mix for 2 to 3 days' feeding was weighed into a pint plastic freezer container, an equal weight of water was added and the two mixed thoroughly with a spatula. The container was then capped and allowed to stand briefly before feeding. Between feedings the diet was stored at 2°C. Diets containing the added iodide levels were prepared by adding an appropriate volume of sodium iodide solution to the water with which the dry ingredients were mixed.

The diet, which resembled a heavy corn meal mush, was presented to the fish by forcing it through a garlic press, clipping off of the worm-like extrusions with a spatula and allowing the particles to fall directly into the trough containing the fish. Feed was presented as rapidly as the fish would consume it and feeding was terminated as soon as any rejection of diet was noted. The fish were fed in this manner 3 times daily, 5 days/week, (Monday through Friday). The troughs were partially cleaned by drawing down the water in the troughs after each feeding. Dead fish were removed as soon as noted and all groups were carefully observed for abnormalities or signs of deficiency. The fish were weighed by methods previously described by Halver (5). Weighing was done before feeding on Monday when they were relatively free of undigested food.

The feeding trial was conducted in aluminum-painted, wooden hatchery troughs 2.1-m long, 40-cm wide and 30-cm deep. The troughs were covered with plastic screen and were supplied with 10 liters/minute of particulate-free well water maintained at a temperature of 10° ± 0.5°C for the entire 24-week period. The iodide content of the water, which was checked several times during the feeding trial, ranged from 0.1 to 0.3 μg/liter.

At the termination of the feeding trial all groups were weighed as usual, again hand-counted and allowed to go without feed for an additional two days to insure that they were free of ingested food. At this time a representative sample of the fish (ca. 100 g) was removed for proximate analysis and 10 fish were preserved for histological examination. An additional 10 fish from each group were individually weighed to the nearest 0.02 g, the thyroid-containing area consisting of the floor of the mouth from the tongue to the last gill arch was removed, freed of excess peripheral tissues, weighed, wrapped in plastic film and quick-frozen for subsequent micro-iodine determination.

Proximate analyses were conducted by methods described previously (6). Micro-iodine determinations on diet samples, water and tissues were made by a semi-automated modification[1] of a colorimetric reaction originally described by Sandell and Kolthoff (7, 8).

After the termination of the 24-week feeding trial, 100 fish from each group continued to be fed their respective diets for an additional 9 months. During this extended observation period feeding frequency was reduced to twice daily for the first 16 weeks and thereafter to once daily. After 16 weeks, 25 fish from each group were weighed individually and measured and at the termination of the extended period all surviving fish were weighed, measured and examined carefully for abnormalities. At this time thyroid areas were again collected for micro-iodine analysis and for histological examination.

RESULTS

Twenty-four-week feeding trial. Growth and performance of the fish during the initial 24-week feeding trial are summarized in table 2. Although it appears that the fish in group 1, which were fed the basal ration without iodide supplement, gained somewhat less than did the fish in the other groups, statistical evaluation of the growth data by analysis of variance, indicated that the mean gains of the fish in the 5 groups did not differ significantly.

Diet efficiency and mortality in all groups would be considered normal for chinook salmon fingerlings under the experimental conditions of this station. Careful examination disclosed no observable deficiency syndromes although evidence of simple goiter was looked for both externally and histologically. Proximate analysis of ter-

[1] Isolation and estimation of serum organically-bound iodine. II. An application for the determination of protein-bound iodine (PBI) or non-anionic iodine (NAI), G. LaRoche, D. Carpenter and A. Coxworth, Semi-annual Report Biology and Medicine, U.C.R.L., Fall, 1963.

minal samples showed no differences in gross body composition (protein, fat, ash and moisture) between groups. Average composition of the fishes: protein, 70.2; fat, 22.6; ash, 10.8 (as percentage of moisture-free fish) and moisture 78.0%.

The most striking difference that occurred during the 24 weeks was the level of stored iodine in the thyroid area. Table 2 shows that the fish that received only the iodine present in the basal ration stored less than 40% as much iodine in the thyroid area as those receiving an iodide supplement. A statistical treatment of the data by analysis of variance showed this difference to be highly significant ($P < < 0.01$).

Extended feeding period. Table 3 presents the average weight of the fish in each of the 5 groups at intervals during the extended feeding period. This table also shows the cumulative mortality of each group at selected intervals and finally the thyroid iodine storage of the fish at the end of the experiment. The weight of the fish confirmed the observation of the initial feeding period, that is, there was no significant difference in the mean weight of the 5 groups of fish.

As table 3 shows, there were no mortalities in the experimental lots for nearly 6 months. Starting in the middle of January a persistent daily mortality occurred in group 1 and within 6 weeks over one-half

TABLE 2

Growth, performance and iodide storage in chinook salmon during 24-week feeding trial

Group no.	1	2	3	4	5
Iodine fed, μg/g dry diet	0.1	0.6	1.1	5.1	10.1
Avg wt, start, g	0.46	0.46	0.46	0.45	0.46
Avg wt, finish, g	8.03	8.42	8.41	8.25	8.40
Avg gain, g	7.57 ± 0.11 [1]	7.96 ± 0.07	7.95 ± 0.07	7.80 ± 0.01	7.94 ± 0.09
Diet efficiency, g gain/g dry feed fed	1.02	1.03	1.04	1.01	1.04
Mortality, %	4.7	4.7	3.5	4.1	3.3
Iodine stored [2]	3.7 ± 0.2	9.6 ± 1.0	10.5 ± 1.6	11.1 ± 0.7	11.4 ± 1.2

[1] SE.
[2] Micrograms thyroidal iodine/100 g body weight; average of 10 determinations.

TABLE 3

Average weight, mortality rate and iodide storage of chinook salmon during extended feeding period

Group no.	1	2	3	4	5
Iodide fed, μg/g dry diet	0.1	0.6	1.1	5.1	10.1
			Average weight, g		
7/23/62 (start)	8.5	8.9	8.8	8.4	8.4
9/24/62	19.4	20.2	19.5	18.7	19.1
11/13/62	28.2 ± 1.2 [1]	30.4 ± 1.7	29.2 ± 1.2	29.1 ± 1.6	29.5 ± 1.2
4/22/63	41.1 ± 4.2	47.5 ± 2.0	47.5 ± 2.1	47.2 ± 1.7	50.4 ± 2.0
			Cumulative mortality [2]		
1/15/63	0	0	1	0	0
2/25/63	49	1	1	0	0
3/25/63	65	6	5	2	0
4/24/63	82	24	22	5	4
			Thyroid iodine storage [3]		
	6.5 ± 0.6	14.2 ± 1.7	20.3 ± 2.8	23.3 ± 2.7	22.3 ± 2.6

[1] SE.
[2] Per cent of population started 7/23/63.
[3] Micrograms iodine/100 g fish; average of 10 determinations/group.

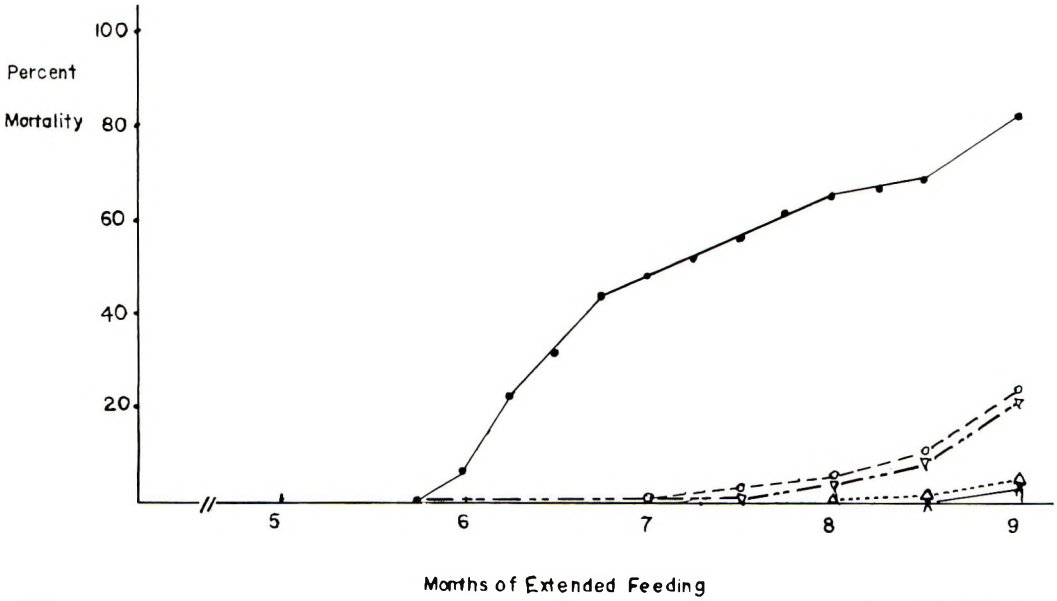

Fig. 1 Mortality rate of young chinook salmon during 9 months of extended feeding with diets containing graded levels of iodide: ·——·, 0.1; ○- - - -○, 0.6; ▽- - —▽, 1.1; △. . . .△, 5.1; ×——×, 10.1 μg iodide/g dry diet.

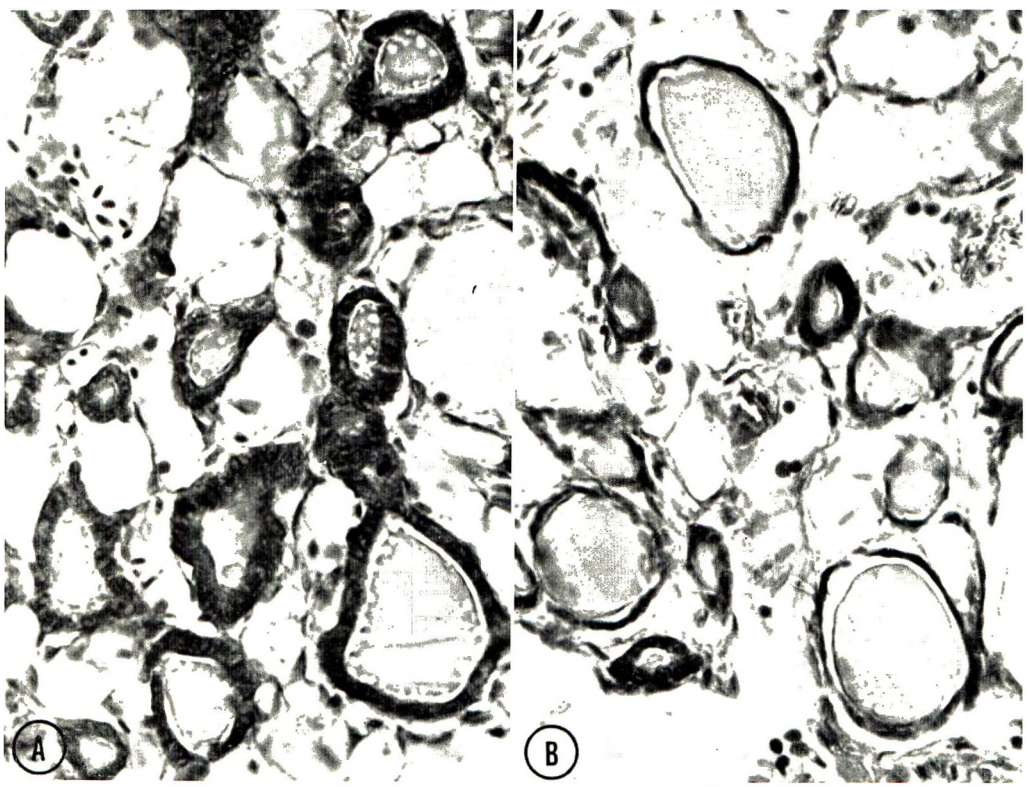

Fig. 2 Photomicrographs of typical thyroid sections from 15-month old chinook salmon advanced parr (× 250): (a) Sample from group 1, note increased epithelial cell height; (b) sample from group 5.

of the fish in this group had died. Mortalities continued until, at the termination of the experiment in late April, only 18% of the starting population remained. Figure 1 illustrates the mortality rate of the 5 groups of fish during this period. Postmortem examination disclosed that fish in all but group 5 were infected with kidney disease, a bacterial infection prevalent among salmonid fishes (9).

Although no evidence of goiter was noted on gross examination, histological examination of thyroid sections disclosed that the fish in group 1 had a somewhat increased epithelial cell height, and slightly decreased, lighter staining colloid suggesting thyroid hyperplasia. Photomicrographs of typical thyroid sections illustrating this difference are presented in figure 2.

The thyroid iodine storage in the terminal samples (table 3) again showed that the fish in group 1 contained significantly ($P << 0.01$) less iodine than the fish in all other groups. However, contrary to the observations made at 24 weeks, the iodine storage in the fish of group 2 was significantly lower ($P < 0.01$) than that of the fish in groups 3, 4, and 5.

DISCUSSION

Our results showed that iodide intakes which were insufficient to maintain adequate thyroid iodide storage and prevent thyroid hyperplasia in chinook salmon did not interfere with the animals' normal growth. This response is consistent with the results of comparable experiments reported for many other animals.

Levine et al. (10) reported no growth difference in rats fed levels of iodide from 15 to 400 μg/kg of diet but observed that 110 to 207 μg/kg of diet was essential to prevent enlargement of the thyroid and to give a thyroid iodine concentration of 0.1% (dry basis). Axelrad et al. (11) noted that mice fed diets containing 4.1 μg/100 g of diet developed goiter, whereas those fed 12.2, 32 and 100 μg/100 g of diet did not. No statements regarding difference in weight gain were made nor did their data appear to support a significant difference. Scott et al. (12) reported that pheasants or quail fed a low iodide basal ration (0.2 mg/kg of diet) showed no growth response to added iodide supplements although supplementation reduced thyroid size. Godfrey et al. (13) reported that chicks fed a basal ration containing 30 μg of iodine/kg of diet developed enlarged thyroids although they gained as well as chicks fed the same ration with added iodide to give 150, 350, 550, 750 and 1150 μg of iodine/kg of diet.

LaRoche and Leblond (14) compared the growth rate and histological appearance of the thyroid glands of Atlantic salmon (*Salmo salar*) parr[2] which were fed a presumably iodine-deficient diet (beef liver) in untreated and iodide-enriched water. It was shown that, if anything, the iodide treatment slightly reduced growth rate although the thyroid tissue of the (low iodide) controls was hyperplastic, whereas that of the fish raised in the iodide-supplemented water appeared normal.

The effect of the thyroid on the growth of salmonids is by no means clear. LaRoche and Leblond (14) compared the effects of various thyroid preparations on the growth and body transformation of Atlantic salmon parr and brook trout fingerlings and concluded that thyroid treatment had no growth-promoting effects. In a subsequent publication (15) these authors reported that the growth rate of thyroidectomized and control Atlantic salmon parr did not differ significantly for 10 months following the administration of I^{131} for thyroid destruction. Recent work,[3] however, has demonstrated that thyroidectomy in rainbow trout induces impressive morphological changes as well as reduced growth which becomes apparent 10 to 12 months after the final I^{131} treatments. In contrast with the results obtained by LaRoche and Leblond (14) Barrington et al. (16) observed that the addition of thyroid powder to a diet of liver and blowfly maggots caused increased growth in rainbow trout (*Salmo gairdnerii*). They pointed out that the growth effect might have been due to the supplementary nutrient afforded by the glandular material rather than due to added thyroid hormones. In additional experiments they

[2] Parr is a term used to designate the stage of development of a young salmon prior to the time it has undergone the morphological and physiological transformation immediately preceding seaward migration.

[3] LaRoche, G., A. N. Woodall and C. L. Johnson. Thyroidectomy in the rainbow trout (*Salmo gairdnerii*, Rich.), manuscript in preparation.

showed that maintaining trout in a solution of thyroxine also enhanced growth. However, as no evidence was presented to compare the stage of activity of the thyroid gland or iodine intake of the treated and control fish, it cannot be said with certainty that the growth response was due to the correction of a chronic thyroid insufficiency. The authors pointed out that the nature of the growth-promoting effect of thyroid hormone treatment remains unresolved.

Although our experimental results do not resolve the role of the thyroid hormones in growth regulation, interpretation of the data may help clarify this question. The observation that growth was maintained by the salmon regardless of iodide intake, even when it was clearly insufficient to prevent thyroid hyperplasia and iodine depletion, reveals 2 characteristics of the growth-regulating function of the thyroid; 1) it can be maintained by the minimal amount of iodine which was fed, and 2) it appears independent of the total thyroidal iodine present, within an appreciable range for the experimental period (15 months). The latter observation suggests a significant lowering of the thyroidal hormonal pool as indicated by thyroid hyperplasia which is often considered *prima facie* evidence of thyroid hormone insufficiency. A similar interpretation can be made from previously cited results of iodide feeding trials with other animals (10–13). An exogenous thyroxine-induced growth response such as Barrington (16) reported would not necessarily complement observations of internal growth regulation by the thyroid. The conditions simulated by their experiment could be considered growth stimulation which may require greater levels of thyroxine for its induction than these fish normally secrete. Interpreted as thyroxine-induced growth stimulation rather than normal thyroid-sponsored growth regulation, a separate mode of action such as stimulation of, or growth-promoting synergism with a given endocrine state should be considered.

The increased iodide requirement found for the advanced parr during the extended feeding period is clearly in accord with numerous observations of increased thyroid activity of salmonids prior to migration. As with other animals, fish undergo periods of thyroid stress where the requirements of the gland, and hence the requirement for iodide, is increased. Robertson and Chaney (17) reported that rainbow trout living under conditions of low iodide intake do not develop thyroid hyperplasia until confronted with the stress of sexual maturation and migration. The role of the thyroid in the smoltification[4] of anadromous salmonids has been partially reviewed by Hoar (18). Fontaine et al. (19) and Olivereau (20) have also demonstrated an increased thyroid activity during smoltification. These and previously cited references (14–16) show clearly an increased activity of the thyroid during this period. More recently Piggins (21) has confirmed these observations by demonstrating an increased production of smolts by inclusion of beef thyroid in the diet of Atlantic salmon parr.

Although the significance of increased numbers of mortalities in the fish fed the lower levels of iodide during the latter part of the extended feeding trial cannot be fully understood, sufficient corroborative evidence exists to suggest an explanation. Hoar (22) suggests that the anadromous teleost making a prolonged stay in fresh water is under added osmotic stress which demands additional thyroid hormones. It therefore appears plausible that the kidney, with its important role in osmotic regulation, may have been stressed and weakened in those fish fed insufficient iodide to meet this increased challenge to the thyroid, thus permitting the ingress of kidney disease organisms. Conversely a renal failure itself might have been the principal cause of death irrespective of the presence of kidney disease.

Baker (23), in reviewing the literature of renal and heterotropic thyroid tissue in fish, reported that heterotropic thyroid tissue develops as a compensatory device when the iodine demands of the animal exceed the available supply of this essential component of thyroid hormones. She observed extensive destruction of kidney tissues in the platyfish (*Xiphophorus maculatus*) during the development of renal thyroid tumors. Unfortunately this was

[4] The physiological and morphological changes preceding seaward migration of the juvenile fish.

not investigated in our experimental fish, but we feel that this possibility is remote as previous studies had shown that there were no radioiodide concentrating tissues in kidneys of other groups of young salmon and trout which had been maintained with a low iodide intake for a year and a half.[5]

Since a correlation between iodide intake and mortality of advanced parr obviously existed in this experiment (see fig. 1), a thyroid function linked to the maintenance of kidney integrity is suggested. An investigation of this interrelationship may necessitate modifying the iodide requirements suggested by iodine storage.

Based on the maintenance of maximal thyroid iodine storage the evidence presented indicates that, for the experimental conditions described, the minimal iodide requirement of chinook salmon fingerlings was not more than 0.6 μg/g of dry diet, whereas for advanced parr the requirement was approximately doubled to 1.1 μg/g diet. The increased iodide requirement of the advanced parr supports morphological and radioiodide observations of increased thyroid activity which many investigators have assocated with ensuing smoltification.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent care in feeding and handling of the experimental fish which Al Merritt provided. We are indebted to C. E. Smith for photomicrography and for pathological and histological examinations of the terminal samples, to Mrs. Hazel Jones and Mrs. Dana Eshleman for technical assistance and to Mrs. Marge Wagoner for diet preparation. We are indebted to Mrs. Dorothy Carpenter, University of California, for micro iodine determinations.

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[5] See footnote 3.

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Effect of Pyridoxine Deficiency upon Valine Incorporation into Tissue Proteins of the Rat[1]

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ABSTRACT Pyridoxine deficiency in the rat resulted in a consistent decrease in incorporation of L-valine-1-C^{14} into proteins of liver, spleen and serum and into subcellular fractions of liver and spleen. Rate of incorporation was increased by administration of pyridoxine shortly before injection of labeled valine. Disappearance of labeled valine from these proteins was not affected in the deficient animals.

Previous investigations have amply demonstrated the inhibitory effects of a pyridoxine deficiency upon formation of circulating antibodies in response to various antigenic stimuli (1, 2). Subsequent studies have described a similar depressant effect of a pyridoxine deficiency on delayed hypersensitivity of the tuberculin type in guinea pigs (3, 4). The relationship of nutritional factors to immunological processes has been summarized in a recent review (5). However, the precise role of pyridoxine in the sequence of events leading to fabrication of antibodies has not yet been elucidated. Since antibodies are proteins, the possibility must be considered that the inhibitory effect of pyridoxine deficiency upon antibody synthesis is a reflection of the requirement for this vitamin in the general process of protein biosynthesis. Accordingly, in the present study we have investigated the effect of a pyridoxine deficiency in the rat upon incorporation of L-valine-1-C^{14} into proteins of liver, spleen and serum.

MATERIALS AND METHODS

Animals and diets. Male, weanling, albino rats of the Holtzman strain were used. The animals were housed individually in wide-mesh screen-bottom cages and fed ad libitum unless stated otherwise. The composition of the diet used to produce a deficiency of pyridoxine and of the control diet have been described previously (6). Briefly, all animals received a basal, semi-purified diet composed of sucrose, casein, salts, lipids and the fat-soluble vitamins. In addition, each rat was fed daily a vitamin supplement in the form of a pill. For both ad libitum and inanition control groups, this pill contained adequate amounts of all B-vitamins known to be required by the rat. For the pyridoxine-deficient group only pyridoxine was omitted from the pill.

In order to control the factor of inanition which is observed in pyridoxine-deficient animals, a pair-fed inaniton control group was utilized. These animals received daily an amount of basal diet equal to that consumed by a pyridoxine-deficient partner. The food intake of pyridoxine-deficient animals receiving pyridoxine shortly before injection of tagged valine was limited similarly during the period of pyridoxine therapy. A solution of pyridoxine (5 mg/ml) was prepared in isotonic sodium chloride and adjusted to pH 7.2 for intraperitoneal injection.

Valine incorporation. L - Valine - 1 - C^{14} with a specific activity of 1.5 mc/mmole [2] was dissolved in isotonic sodium chloride for injection. After an experimental period of 9 weeks, each rat was injected intraperitoneally with 5 μc of L-valine-1-C^{14} per 100 g of body weight, bled by cardiac puncture at the desired time after injection and decapitated immediately thereafter. Blood was allowed to clot and serum separated by centrifugation. Liver and spleen were quickly excised, washed in ice-cold 0.25 M sucrose, weighed after blotting on filter paper, and a 10% homogenate prepared in ice-cold 0.25 M sucrose with a hand homogenizer. Protein content

Received for publication November 13, 1963.
[1] This study was aided by U. S. Public Health Service research grant A-727 from the National Institute of Arthritis and Metabolic Diseases, and by the office of Naval Research under Contract 1833(00), NR 101-412.
[2] Purchased from New England Nuclear Corporation, Boston.

of the tissue homogenates and serum was determined by the method of Lowry et al. (7). Subcellular liver and spleen fractions were separated by differential centrifugation of the tissue homogenates. The nuclear fraction was obtained by sedimentation at 2000 × *g* for 10 minutes, the mitochondrial fraction by sedimentation at 6590 × *g* for 20 minutes and the microsomal fraction by sedimentation at 105,000 × *g* for 80 minutes. The supernatant remaining after removal of the microsomal fraction constituted the soluble fraction.

Proteins were precipitated from aliquots of blood serum, tissue homogenates and subcellular fractions (resuspended in 0.25 M sucrose) by addition of an equal volume of 10% (w/v) trichloroacetic acid and treated according to the procedure of Rabinovitz et al. (8). A weighed amount of protein was dissolved in 1 M *p*-(diisobutyl-cresoxyethoxyethyl) dimethyl benzyl ammonium hydroxide [3] in methanol by heating at 70°C for 2 hours in a closed tube. One milliliter of this solution was employed per 5 mg of protein. Following solubilization, 1 ml of the protein solution was diluted with 9 ml of a 0.5% solution of 2,5-diphenyl-oxazole [4] in toluene and radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Pyridoxine deficiency produced a consistent decrease in the incorporation of L-valine-1-C^{14} into proteins of liver, spleen and serum (table 1). Approximately the same degree of inhibition was noted in the 3 types of protein studied. As determined by variations in the specific activity of isolated proteins, incorporation by the deficient animals ranged from 50 to 75% of that of controls. Simple inanition was without effect and the diminished rate of incorporation was restored by administering pyridoxine 19 or 24 hours before injection of labeled valine.

The inhibitory effect of pyridoxine deficiency upon incorporation of L-valine-1-C^{14} into all subcellular fractions of liver and spleen is shown in table 2. Incorporation into all fractions was affected in similar fashion by the deficiency state and could be increased uniformly by prior administration of pyridoxine.

The rate of disappearance of labeled L-valine-1-C^{14} from liver, spleen and serum proteins was obtained from measurements of specific activities of these proteins in animals killed at various intervals after injection of L-valine-1-C^{14} (figs. 1 and 2). These experiments provided additional evidence for the inhibitory effect of pyridoxine deficiency upon L-valine-1-C^{14} incorporation and indicated that the disappearance of labeled valine from these proteins was not affected in this deficiency state.

DISCUSSION

The effect of a pyridoxine deficiency upon rate of incorporation of L-valine-1-C^{14} into tissue proteins noted in this paper is considerably less than the inhibitory effect of this deficiency state upon antibody synthesis. These data do not, however, negate the possibility that the role of pyridoxine in antibody synthesis is comparable to a function in the more general process of protein biosynthesis. Variations in the effects of pyridoxine deficiency upon synthesis of specific proteins are quite likely. Although these studies suggest a similarity of action of pyridoxine in synthesis of tissue and antibody protein, further elucidation of the mechanism of antibody synthesis is essential for final comprehension of the role of pyridoxine in this process. It is of interest in this connection that Lichstein (9) has reported that pyridoxine is required for synthesis of trytophanase in a pyridoxine-requiring mutant of *Escherichia coli*. On the other hand, other workers (10–12) could find no evidence for an interference of pyridoxine deficiency with protein synthesis although an increased rate of amino acid catabolism in this deficiency was indicated (10, 13).

The observed decrease of incorporation of L-valine-1-C^{14} into tissue protein in pyridoxine deficiency may be attributable to factors other than those associated directly with protein anabolism. Increase in the amino acid pool as well as a decreased rate of intracellular concentration of amino acids may be operable. There is a considerable body of evidence to support the latter possibility (14–18).

[3] Hydroxide of Hyamine 10-X. Packard Instrument Company, La Grange, Illinois.

[4] Purchased from the Packard Instrument Company, La Grange, Illinois.

TABLE 1

Effect of pyridoxine deficiency upon incorporation of L-*valine-1-*C^{14} *into proteins of liver, spleen and serum* [1]

Animal no.	Wt	Treatment	Liver			Spleen			Serum	
			Wt	Protein/ g of tissue	Count/ min/mg protein	Wt	Protein/ g of tissue	Count/ min/mg protein	Protein/ ml	Count/ min/mg protein
	g		*g*	*mg*		*g*	*mg*		*mg*	
				Experiment 1						
1	295	Control	11.201	224	185	0.751	171	179	73.1	335
2	111	Deficient	6.028	200	134	0.310	203	131	69.3	221
3	99	Deficient	3.908	200	141	0.182	237	122	66.2	216
4	120	Deficient + vitamin B_6 [2]	5.262	201	188	0.334	190	173	68.9	342
				Experiment 2						
5	112	Deficient + vitamin B_6 [3]	4.744	218	191	0.287	221	145	69.3	292
6	112	Deficient	3.983	223	97	0.245	237	92	72.9	203
				Experiment 3						
7	120	Inanition control	6.000	213	196	0.303	212	168	70.1	351
8	108	Deficient	4.876	204	121	0.257	217	93	69.0	225
9	111	Deficient + vitamin B_6 [4]	5.103	216	179	0.295	218	161	69.3	303

[1] Bled and killed 4 hours after injection of L-valine-1-C^{14}.
[2] Received 5 mg of pyridoxine 19 hours before injection of L-valine-1-C^{14}.
[3] Received 5 mg of pyridoxine 43 and 19 hours before injection of L-valine-1-C^{14}.
[4] Received 5 mg of pyridoxine 24 hours before injection of L-valine-1-C^{14}.

TABLE 2

Effect of pyridoxine deficiency upon incorporation of L-valine-1-C^{14} into protein of subcellular fractions of liver and spleen [1]

Animal no.	Wt	Treatment	Liver					Spleen				
			Wt	N	Mito	Micro	S.F.	Wt	N	Mito	Micro	S.F.
	g		*g*	*count/min/mg protein*				*g*	*count/min/mg protein*			
			Experiment 4									
1	103	Deficient + vitamin B_6 [2]	3.930	132	92	190	149	0.269	176	116	245	177
2	112	Same	4.031					0.330				
3	96	Deficient	3.713	84	49	140	70	0.226	60	41	142	77
4	106	Same	4.019					0.315				
			Experiment 5									
5	120	Inanition control	5.931	180	131	278	182	0.308	163	121	259	186
6	125	Same	6.188					0.310				
7	103	Deficient + vitamin B_6 [2]	4.702	159	123	255	169	0.264	165	114	248	173
8	116	Same	5.774					0.295				
9	108	Deficient	4.991	93	70	153	98	0.273	72	51	126	87
10	98	Same	4.366					0.259				

[1] Bled and killed 4 hours after injection of L-valine-1-C^{14}. Organs of the 2 animals in each group receiving the same treatment were pooled for preparation of the subcellular fractions. N indicates nuclear fraction; Mito indicates mitochondrial fraction; Micro indicates microsomal fraction; S.F. indicates soluble fraction.

[2] Received 5 mg of pyridoxine 48 hours before injection of L-valine-1-C^{14}.

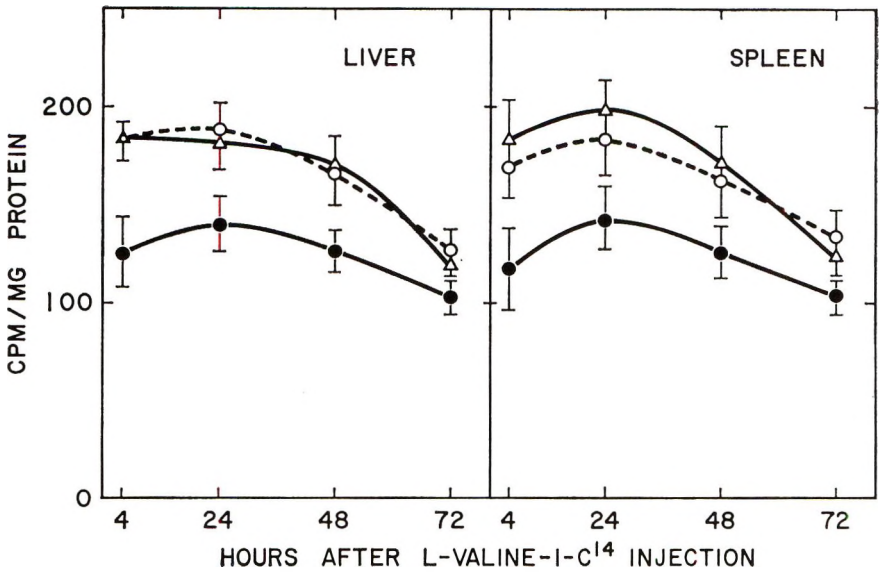

Fig. 1 Disappearance of L-valine-1-C^{14} from proteins of liver and spleen. Each point represents the average value of 2 to 4 animals. Inanition controls, △——△——△; pyridoxine-deficient rats that received 5 mg of pyridoxine 24 hours before valine injection, ○----○----○; pyridoxine-deficient rats, ●——●——●.

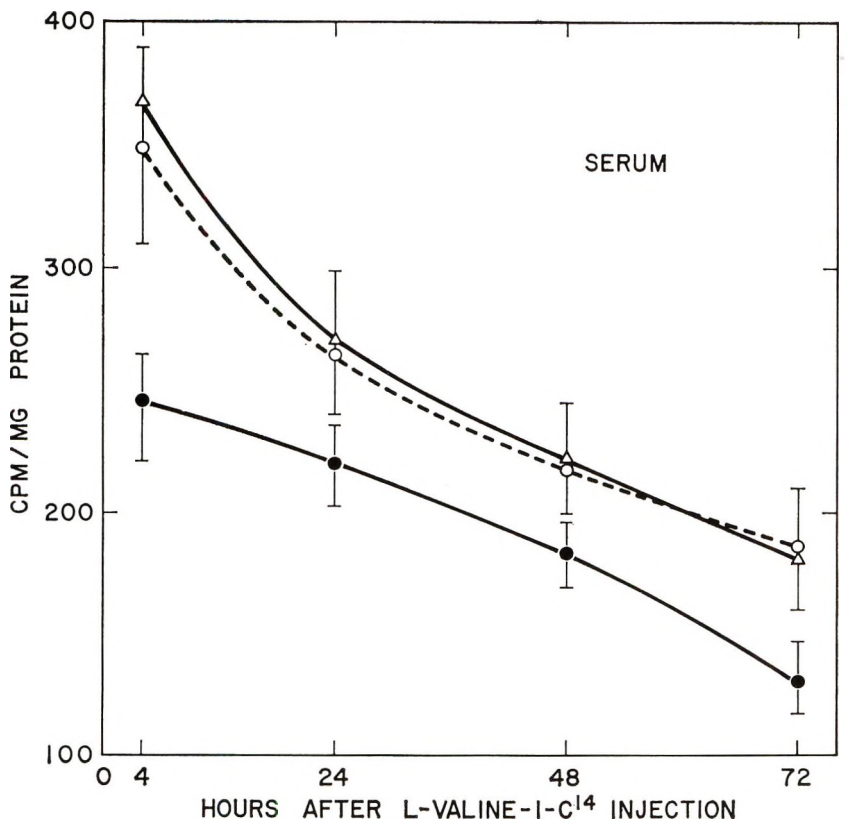

Fig. 2 Disappearance of L-valine-1-C^{14} from serum proteins. Each point represents the average value of 2 to 4 animals. Notations and treatment of animals same as in figure 1.

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Effect of Zinc Nutrition upon Uptake and Retention of Zinc-65 in the Chick[1]

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ABSTRACT The metabolism of zinc has been investigated using zinc-65 retention in the tissues of 4-week-old cockerels fed either zinc-deficient or zinc-sufficient diets. All tissues from the deficient chicks retained radiozinc to a much greater degree than controls, with the activity increasing greatly during a 32-hour study period. Correlation coefficients derived by computer analysis showed that zinc-65 retention in red blood cells had a high positive correlation with all tissues examined, especially for the sufficient group. Plasma retention exhibited a low negative correlation for both dietary supplement groups. Stable zinc analyses demonstrated that most organs remain constant in zinc content in deficiency states; only bone, liver and duodenum showed a lower stable zinc content in deficiency than in sufficiency. A zinc deficiency syndrome occurring elsewhere in human males, characterized by dwarfism, rough skin, delayed epiphyseal closure, hypogonadism, and failure in the development of secondary sex characteristics, was compared with the deficiency observed in these cockerels. The observations that uptake and retention of radioactive zinc is markedly greater in zinc deficient animals, as compared with that which occurs in those receiving adequate dietary zinc, have important implications, not only in normal nutrition, but also in terms of radioactive zinc-65 from nuclear fission.

Despite all of the research that has been carried out on zinc as a growth factor in swine (1,2), poultry (3–5), cattle (6) and man (7–9), there is little understanding of how this trace element functions (10). In the present investigation, studies were undertaken to determine the effects of the status of zinc nutrition upon stable zinc content and zinc-65 uptake and retention in most of the important tissues and organs of the chick. The studies have bearing not only on the metabolism of stable zinc but also on the movements of radiozinc pools.

MATERIALS AND METHODS

The basic diet described by Zeigler et al. (5) was supplemented with 5 or 60 ppm of zinc as the chloride. Each diet was offered to a group of chicks ad libitum in stainless steel containers. Distilled water was available from plastic and glass fountains. The chicks were housed in lacquered, electrically heated batteries.

Hatch-mate, one-day-old White Cornish × White Rock cross-bred cockerels, produced by hens receiving a commercial breeder ration, were fed the diets. At the end of 4 weeks, 16 cockerels were selected from each group so that the individual variation in weight from the treatment mean was minimal. The chicks grown with the 5 ppm zinc diet exhibited gross signs of zinc deficiency and averaged 240 g, with a range of 196 to 266 g. The cockerels fed the 60 ppm zinc diet appeared normal in all respects and averaged 580 g, with a range of 540 to 620 g. Feed and water were removed 8 hours prior to injection of the radioisotope.

The injection solution, containing 125 μc of zinc-65[5] and 1.0 mg of zinc/ml, both as chloride, was administered intramuscularly at the level of 10 μc/100 g of body weight. Two cockerels from each treat-

Received for publication November 18, 1963.

[1] Aided in part by grants CA 03952, National Institutes of Health, U.S. Public Health Service, and RH 00042, Division of Radiological Health, Bureau of State Services, U.S. Public Health Service.

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[5] The $Zn^{65}Cl_2$ was obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee.

ment group were killed by an intracardiac injection of sodium pentobarbital solution at 0.25, 0.5, 1, 2, 4, 8, 16 and 32 hours after administering the isotope. Tissue samples were then removed, washed in saline, identified, frozen and subsequently weighed and counted.

Prior to killing, 5 ml of blood were removed from the wing vein of each chick with a heparinized syringe. From each sample 1-ml aliquots were removed, centrifuged and the plasma decanted. The red blood cells were resuspended and centrifuged 3 times in 3 ml of saline, and the liquid portion decanted into the plasma. Additional washings had little effect on the radioactivity in the centrifuged red blood cell fraction. The plasma, together with the washings, was made up to 15 ml, and an aliquot counted.

The radioactivity of the respective aliquots or samples was determined in a deep-well scintillation counter filled with a 3.8-cm sodium iodide crystal, and the counts corrected for decay. Correlation coefficients relating retention values of blood to other tissues were determined by computer analysis.

Tissues for the stable zinc analyses were obtained from 5 cockerels of each treatment group. The analyses were conducted in duplicate on pooled dry-ashed tissue samples. The mineral fraction was then taken up in dilute hydrochloric acid and analyzed using the dithizone method of Vallee (11).

RESULTS

Data on zinc-65 retention, expressed as percentage of injected dose per gram tissue, are presented graphically in figure 1. The figure is divided into 3 sections according to high, moderate and low retention. Average deviations of all tissues from cockerels grown on 60 ppm zinc supplement were small. Approximately one-half of the tissues from cockerels receiving 5 ppm of zinc also showed small average deviations, but bone, duodenum, kidney, liver, muscle, pancreas and testis occasionally showed up to 50% variation. These results illustrate the changes in the radiozinc distribution with time. All tissues examined, except plasma, showed a gradual buildup in activity during the 32-hour study period. Zinc deficiency caused a markedly increased uptake of zinc-65 in all tissues as compared with that in the tissues of chicks receiving adequate dietary zinc.

The zinc-65 uptake of the red blood cells showed the same increase with time as that exhibited by all other tissues and organs. The plasma levels of radiozinc reached a plateau early and then showed a gradual decrease with time. This is demonstrated by the correlation coefficients shown in table 1 which were calculated by computer analysis from the data presented in figure 1. These coefficients show that the zinc-65 retention for all tissues was negatively correlated with plasma but was positively correlated with red blood cells. The negative coefficients for plasma show that radiozinc levels had a greater inverse relationship with the zinc-deficient than with the sufficient tissues.

The stable zinc content of wet tissues, the percentage retention of the dose of zinc-65 after 32 hours, and the specific activities calculated from these values for both groups of chicks are presented in table 2. These results show that the stable zinc content only of bone, liver and duodenum are markedly decreased by dietary zinc deficiency. The dietary zinc level had little influence on the stable zinc concentrations of the other tissues. The testis of zinc-deficient chicks actually contained a higher zinc concentration than the testis of chicks with adequate zinc. The greater concentration of stable zinc noted in the spleen of the deficient chicks may have been dependent upon the hemo concentration state within the spleen at the time the analyses were conducted since assays on 2 samples showed very high zinc content and 2 other samples showed a low zinc content. In spite of this, all tissues from the zinc-deficient chicks, including the testis, showed similar marked increases in zinc-65 retention and specific activities as compared with the tissues of chicks receiving adequate zinc.

DISCUSSION

The experimental and practical aspects of zinc deficiency have been extensively studied in both plants and domestic animals. In 1955, Tucker and Salmon (1) showed that zinc deficiency in swine

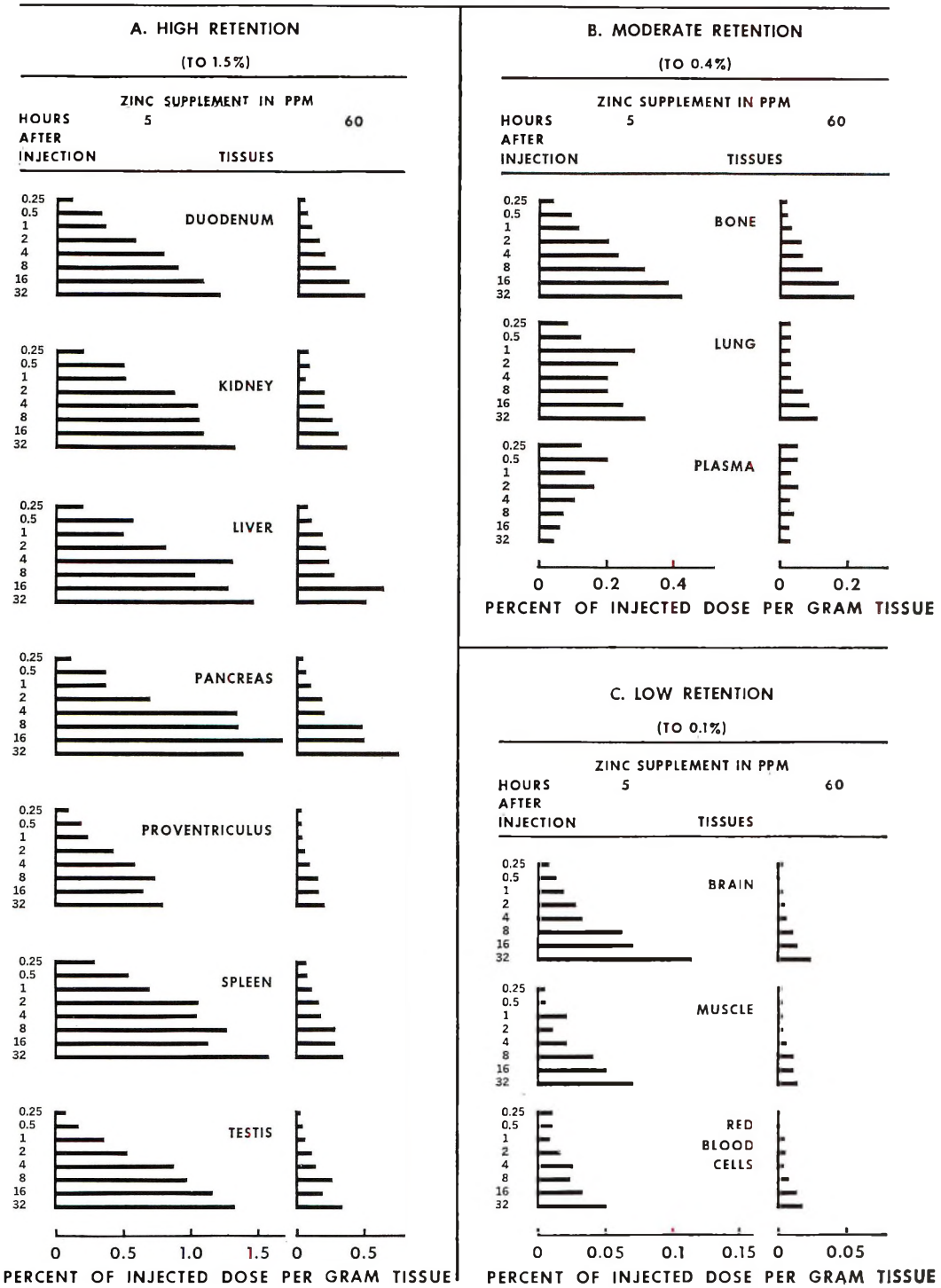

Fig. 1 Zinc-65 retention in four-week-old cockerels.

TABLE 1

Correlation coefficients of zinc-65 retention studies in cockerels

Tissue	Plasma		Red blood cells	
	5 ppm	60 ppm	5 ppm	60 ppm
Blood plasma	—	—	−0.745	−0.613
Red blood cells	−0.745	−0.613	—	—
Bone	−0.810	−0.574	0.895	0.979
Brain	−0.830	−0.525	0.956	0.939
Duodenum	−0.790	−0.620	0.876	0.970
Kidney	−0.689	−0.474	0.836	0.892
Liver	−0.691	−0.656	0.820	0.924
Lung	−0.532	−0.516	0.667	0.974
Muscle	−0.875	−0.573	0.903	0.916
Pancreas	−0.771	−0.549	0.725	0.953
Proventriculus	−0.793	−0.582	0.810	0.911
Spleen	−0.700	−0.597	0.854	0.906
Testis	−0.851	−0.525	0.871	0.869

TABLE 2

Effect of dietary zinc content on stable zinc content and zinc-65 retention of tissues 32 hours after zinc-65 injection

Tissue	60 ppm dietary zinc supplement			5 ppm dietary zinc supplement			Increase in specific activity in deficient chicks
	Zinc content	Injected Zn^{65} retention	Specific activity	Zinc content	Injected Zn^{65} retention	Specific activity	
	μg/g fresh tissue	*% of dose/g fresh tissue*	*% of dose/ μg Zn*	*μg/g fresh tissue*	*% of dose/g fresh tissue*	*% of dose/ μg Zn*	*%*
Bone	40.2	0.22	0.005	12.6	0.42	0.033	660
Kidney	35.8	0.35	0.0097	34.6	1.32	0.038	390
Proventriculus	29.7	0.19	0.006	31.9	0.79	0.025	420
Duodenum	27.6	0.48	0.017	19.6	1.20	0.061	368
Pancreas	27.2	0.75	0.027	22.0	1.39	0.063	230
Testis	26.6	0.33	0.012	35.2	1.33	0.038	320
Liver	26.0	0.50	0.019	16.8	1.46	0.087	460
Spleen	22.1	0.33	0.015	37.5	1.58	0.042	280
Lung	16.1	0.11	0.007	14.7	0.31	0.021	300
Brain	12.1	0.02	0.002	13.1	0.11	0.008	400
Muscle	7.6	0.01	0.001	10.8	0.07	0.006	600

resembles the spontaneous and endemic disease of this species, parakeratosis. Furthermore, the growth retardation and dermatitis observed in this disease were prevented by dietary zinc supplementation (12, 13). Similar studies in other animals also have associated zinc deficiency with poor feed efficiency; disorders of feathers, hair and coat; delayed sexual maturity, sterility and loss of fertility; poor bone development and hock enlargement; and embryonic malformations (10).

The form in which zinc is administered, and the presence of other substances in the diet, can aid or interfere with the availability of zinc. For example, the phytic acid in soybean meal apparently chelates some of the zinc (13, 14), whereas certain chelating agents such as EDTA and natural chelates aid the utilization of zinc (15, 16). The basal diet used in this experiment contained soybean protein, and even though analysis showed a stable zinc content of 23 ppm, much of this is not utilized (5). Studies on ionic and chelated forms of zinc administered intravenously to mice have shown that Zn^{65} EDTA and Zn^{65} DTPA may be rapidly excreted by the kidney, whereas $Zn^{65}Cl_2$ is slowly excreted by the intestine (17). Thus, the form of administered zinc influences both the absorption and excretion of this element and may regulate, in part, the body zinc content.

In the present study, the objective was to learn how zinc deficiency affects the stable zinc content and isotopic uptake of various tissues. The results show that zinc deficiency had the greatest effect upon the stable zinc content of bone, liver and duodenum. These tissues also showed the greatest changes in specific activity. The stable zinc content of the other tissues was not appreciably influenced by dietary zinc content. This is consistent with the severe reduction in growth rate which occurs with zinc deficiency and indicates that zinc is an integral part of most tissue growth. However, all the tissues from the zinc-deficient chicks showed marked increases in zinc-65 uptake as compared with the zinc-sufficient chicks. This observation is in agreement with the work of Furchner and Richmond (18) who reported that increasing the dietary uptake of stable zinc inhibits whole body retention of radiozinc.

Recently, Prasad et al. (7–9) described a deficiency syndrome in human males in Egypt characterized by dwarfism, delayed epiphyseal closure, rough, hyperpigmented skin, hypogonadism and lack of secondary sex characteristics. These symptoms are largely similar to those observed in zinc deficiency states in experimental animals and appear to respond to a marked degree to zinc therapy. Studies with zinc-65 have shown a more rapid plasma zinc disappearance and a greater retention of zinc in these subjects than in controls.

Prasad et al (8) reported that the hypogonadism in the Egyptian dwarfs was associated with a low urinary follicular stimulating hormone (FSH) level and suggested that perhaps this was related to anterior pituitary hypofunction conditioned by zinc deficiency. The observations in the present study that the testes of chicks have an especially high affinity for zinc may be indicative of a more direct function of zinc in the testis.

In the Egyptian dwarfs as in the zinc-deficient chicks of the present study, the zinc-deficient state is accompanied by a higher retention of radiozinc as compared with that which occurs in normal animals or humans consuming adequate amounts of stable zinc. This observation has important implications, not only in normal nutrition, but also in terms of radioactive zinc-65 from nuclear fission.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Mrs. Susan Black.

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Effect of Selenium, Sulfur and Sulfur Amino Acids on Nutritional Muscular Dystrophy in the Lamb[1]

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ABSTRACT Eighty-six ewes and 125 lambs were assigned at parturition to 6 dietary treatments to test the addition of combinations of Se as Na_2SeO_3 and S as Na_2SO_4 to the diet of the ewe and cystine and methionine when given to the lamb on the incidence of nutritional muscular dystrophy (NMD) in the lambs. Se given at the level of 0.17 ppm during lactation had no significant effect on clinical incidence of NMD but reduced the number of lambs with elevated SGOT values. Dietary sulfur (0.33%) increased the clinical incidence of NMD and when given in combination with Se prevented any beneficial effect of Se. No significant effects were observed due to either cystine or methionine administration at the level and frequency tested.

Nutritional muscular dystrophy (NMD, white muscle disease, stiff-lamb disease) in the lamb is not a simple dietary deficiency. Vitamin E (1), selenium (2, 3) and muscular activity (4) have been shown to be factors in the etiology of the disease. Dystrophogenic factors have been proposed in legume hay (5) and raw kidney beans (6). Sulfur may also be a factor, since sulfate decreases selenium uptake in certain plants (7) and alleviates selenium toxicity in microorganisms (8) and rats (9). Andersson (10) reported that muscular dystrophy in calves in West Finland is associated with soils rich in sulfides. Muth et al. (11) reported the addition of 0.053% sulfur as Na_2SO_4 to the ration of ewes decreased the effectiveness of 0.1 ppm added selenium in preventing NMD in lambs, although these results were not conclusive.

The effect of sulfur amino acids on NMD in the lamb has received little attention, even though certain relationships have been established in NMD in the chick (12) and turkey poult (13) and in liver necrosis in the rat (14). Kradel et al. (15) produced NMD in rabbits by feeding ethionine. Erwin et al. (16) reported that a hydroxy analogue of methionine did not prevent NMD in lambs fed purified diets (coconut oil-torula yeast) but did prevent NMD in chicks fed the same diets.

The study reported herein was designed to test the effect of sulfur and selenium combinations as well as the sulfur amino acids on NMD in the suckling lamb.

METHODS

Eighty-six western white-faced crossbred ewes were randomly assigned to 6 dietary treatments at lambing and continued on trial for 8 weeks. A total of 13 to 15 ewes was assigned to each treatment along with their 20 to 22 lambs. Each group contained essentially an equal number of single and twin lambs and Hampshire and Dorset sired lambs.

The 6 dietary treatments were as follows: 1) Basal ration of 1,370 g of trefoil grass mixed hay grown on the same field as hay used in previous studies (6) and 900 g of raw cull kidney beans (*Phaseolus vulgaris*) per ewe per day; 2) hay-bean basal plus 0.17 ppm selenium as Na_2SeO_3 to the ewes' diet; 3) hay-bean basal plus 0.33% sulfur as Na_2SO_4 to the ewes' diet; 4) hay-bean basal plus 0.17 ppm selenium as Na_2SeO_3 plus 0.33% sulfur as Na_2SO_4 to the ewes' diet; 5) basal plus L-cystine 3 times weekly to the lambs; 6) basal plus DL-methionine 3 times weekly to the lambs. The cystine and methionine were

Received for publication November 26, 1963.
[1] This investigation was supported in part by Public Health Service Research Grant NB 03517 02 from the National Institutes of Health.
[2] The authors are indebted to Dr. H. W. Allaway, Director, ARS, Plant, Soil and Nutrition Laboratory, Ithaca, New York, for the selenium and sulfur analyses and to the Department of Veterinary Pathology, New York State Veterinary College for autopsy analyses of several animals.

given orally by capsule at the rate of 1 g/lamb/dose for the first 2 weeks and 2 g thereafter. The selenium and sulfur were given to the ewes in 90 g of dextrose/ewe/day. Equal amounts of dextrose were given those ewes not receiving selenium or sulfur. All ewes were fed the basal ration for approximately the last 2 months of gestation. All animals were housed in groups for the duration of the experiment. Water and trace mineralized salt were available ad libitum.

Criteria included growth, death, incidence of clinically affected lambs and blood serum glutamic-oxalacetic transaminase (SGOT) levels. SGOT values above 200 Sigma-Frankel units were considered to be "elevated" and the associated lambs to have some degree of muscular dystrophy (18). The lambs were individually weighed bi-weekly and blood samples were taken by jugular puncture at the same interval. "Clinically affected" lambs were judged by visual appraisal for characteristic stiffness or weakness and the SGOT values determined by a colorimetric procedure.[3] All animals that died were autopsied and examined histologically for the characteristic "Zenkers" degeneration of striated muscle.

The selenium content of the feeds was determined by fluorimetric analysis. Sulfur determinations were made by X-ray fluorescence using a helium pathway and a quartz crystal.[4] Values were determined from a standard curve based on X-ray fluorescence measurements of samples of alfalfa, red clover and a sedge of known sulfur content.

RESULTS AND DISCUSSION

The results of the criteria measured on the lambs are summarized in table 1 and the selenium and sulfur content of the feeds are shown in table 2. The percentage of lambs with elevated SGOT levels at the bi-weekly intervals is depicted in figure 1.

No significant differences were observed in weight gains among any of the treatments.

Selenium, added at the level of 0.17 ppm to the ewes' diet was relatively ineffective in reducing the incidence of NMD. Other workers (3, 17) have reported the general effectiveness of 0.1 ppm when added to the ewes' diet, whereas research in this laboratory has indicated 1.0 ppm may not be completely effective, probably due to a proposed dystrophogenic factor present in kidney beans (6). In this trial, selenium was fed to the ewes only during lactation

[3] Sigma Technical Bulletin 505, Sigma Chemical Company, St. Louis.

[4] Lazar, V. A., unpublished method. U.S. Plant, Soil and Nutrition Laboratory, Ithaca, New York.

TABLE 1

Effect of selenium, sulfur and sulfur amino acids on weight gain, clinically affected lambs and SGOT levels

Treatment	No. of lambs	Avg wt gain (birth to 8 weeks)	Death loss		Clinically affected lambs		Lambs with elevated SGOT [1]	
		kg	*no.*	*%*	*no.*	*%*	*no.*	*%*
1. Basal ration (1370 g hay + 900 g kidney beans/ewe/day)	22	9.8	3	13.7	6	27.3	16	72.7
2. Basal ration + 0.17 ppm Se as Na_2SeO_3	21	9.3	0	0	5	23.8	10	47.6
3. Basal ration + 0.33% S as Na_2SO_4	21	10.6	3	14.2	10	46.6	17	81.0
4. Basal ration + 0.17 ppm Se and 0.33% S	20	10.5	3	15.0	4	20.0	15	75.0
5. Basal ration + cystine to the lamb	20	9.2	2	10.0	3	15.0	14	70.0
6. Basal ration + methionine to the lamb	21	10.1	1	4.8	6	28.5	18	90.0

[1] Values of over 200 Sigma-Frankel units.

TABLE 2

Selenium and sulfur content of diet ingredients

Ingredients	Se	S
	ppm	%
Mixed hay	0.03	0.17
Kidney beans	0.06	0.16
Cystine	0.65	—
Methionine	0.10	—

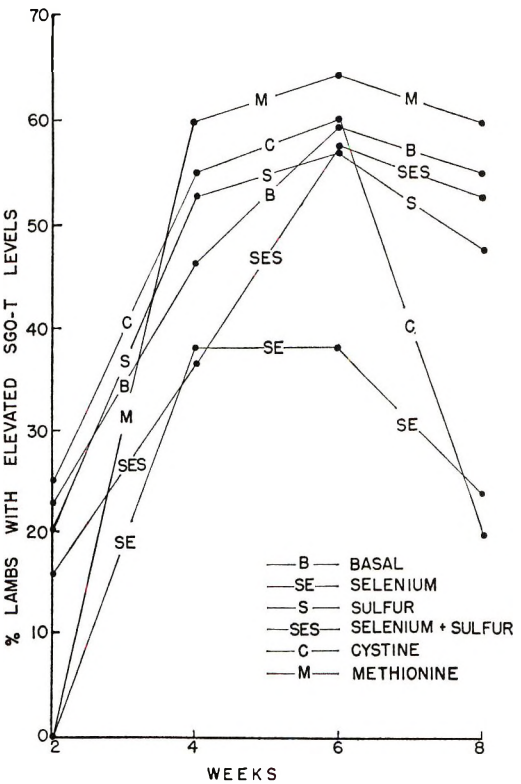

Fig. 1 The effect of dietary treatments on SGOT levels in lambs.

and not during gestation, whereas in past experiments in this laboratory animals have been on experimental treatment for approximately the last 30 days of gestation as well as lactation. The level of 0.17 ppm Se used in this experiment was, however, effective in reducing but not preventing the number of lambs with elevated SGOT values. The addition of 0.33% sulfur to the ewes' diet increased the number of clinically affected lambs over the basal group, but it did not increase the number of lambs with elevated SGOT values.

When both selenium and sulfur were added to the ewes' diet (treatment 4) the number of clinically affected lambs was lower than when only sulfur was added and the beneficial effect of selenium in maintaining normal SGOT values and preventing death was negated. These results indicate sulfur may be a further complicating factor in NMD in lambs by acting as an antagonist to selenium. However, the ration with the added sulfur contained 0.5% total sulfur which is much higher than the 0.2 to 0.3% which would be expected in most rations, although values up to 0.7% have been reported (18).

The results on treatments 5 and 6 in which cystine and methionine were given to the lambs indicate no statistically significant effects in any criteria measured. However, the incidence of clinically affected lambs was lower in the cystine group than any other group and the decreased number of lambs with elevated SGOT values at 8 weeks indicate cystine may have had some beneficial effect but was inconclusively tested in this experiment.

The breed of the sire (Hampshire or Dorset) and type of birth (single or twin) did not affect the incidence of clinically affected lambs or number of lambs with elevated SGOT levels. However, if one twin became clinically stiff or had an elevated SGOT level, the other twin had a greater chance ($P < 0.05$) of also being affected than that of an unrelated lamb within the same group.

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The Role of Dispensable Amino Acids in the Nutrition of the Rat

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ABSTRACT The effects of modifying the dispensable amino acid and sodium bicarbonate content of purified amino acid diets on the growth rate and feed consumption of male weanling rats were studied. Growth rate and feed consumption were significantly increased when an amino acid diet containing the hydrochloride forms of arginine, histidine and lysine and the salt mixture of Jones and Foster was supplemented with sodium bicarbonate. The omission of asparagine, all or a part of the glutamic acid or proline resulted in significant decreases in weight gain. Weight gains tended to be lowered, though not significantly, by the omission of serine and glycine. The effects on feed consumption were comparable to those on weight gains. The growth-promoting qualities of casein lost in acid hydrolysis were restored by the addition of asparagine and indispensable amino acids known to be affected by acid hydrolysis. The complete amino acid diets and the casein hydrolysate supplemented with asparagine appeared to be nutritionally equivalent to intact casein.

In earlier studies in this laboratory (1) purified diets, containing several mixtures of amino acids reported in recent literature, were found to be inferior to casein in supporting growth of weanling rats. The nature of these results suggested that factors other than the levels of indispensable amino acids might have been limiting in these diets. Two factors have been studied in experiments reported in this paper, namely, the effect of the addition of sodium bicarbonate to amino acid diets containing the hydrochlorides of certain amino acids and the qualitative requirement of certain dispensable amino acids for maximal growth of the rat.

Many workers have added sodium bicarbonate to amino acid diets to "buffer" the hydrochloride present. Studies by Zeigler[1] showed that the growth rate of chicks fed purified diets was favored by mineral mixtures having the lowest chloride level and the highest carbonate level. Additional studies indicated that the growth depression from high chloride levels could be altered by varying the level of sodium and potassium in the diet. Similar studies by Nesheim et al. (2) have confirmed these observations. It was noted in work at this laboratory (1) that slightly lower rates of gain resulted when a salt mixture formulated to meet NRC requirements for the rat (3) was substituted for the Jones and Foster mineral mixture (4) in an amino acid diet (experiment 2). The NRC mixture supplied a smaller amount of sodium and a lower sodium-to-chloride molar ratio. Also, lower growth rates coincided with the substitution of the hydrochlorides of arginine and histidine for the hydrochloride-free forms without increasing the level of sodium bicarbonate (diet E-1 in experiments 2 and 3 of preceding reference). The results from the addition of sodium bicarbonate to this diet are reported in the present paper.

Some qualitative aspects of the dispensable amino acid nutrition of the rat have been discussed in recent reviews (5–6). Rose et al. (7) reported that a mixture of dispensable amino acids was superior to a single source of "dispensable-N," but they were unable to show a significant effect on growth from the omission of individual dispensable amino acids from the mixture although glutamic acid appeared to have some effect. Others have noted that glutamic acid in the presence of other dispensable amino acids affects growth of rats (8, 9). Sauberlich (10) reported the formulation of an amino acid

Received for publication October 24, 1963.

[1] Zeigler, T. R. 1962 Investigations of the dietary requirements for maximum gain of chicks fed purified-type diets containing adequate quantities of all recognized essential nutrients. Ph.D. Thesis, Cornell University, Ithaca, New York.

diet which supported a high rate of growth equal to that obtained with a 20% casein diet. This amino acid mixture was chosen as the basal mixture for a series of studies on the qualitative requirements of rats for dispensable amino acids. This mixture appeared to offer the advantage of being capable of supporting a rather high growth rate, an obvious requirement of a basal mixture for the demonstration of dispensable amino acid requirements for maximal growth. Furthermore, this mixture supplied a high level of "dispensable-N" from excess L-isomers (11) and D-isomers of indispensable amino acids as well as dispensable-N from several dispensable amino acids which make qualitative changes possible resulting in only minor changes in the total dispensable-N supplied by the diet. Thus, studies are reported in this paper where effects on growth rate and feed consumption were noted in rats following the alteration of the dispensable amino acid content of this mixture.

EXPERIMENTAL

The following procedure was followed in each experiment. Male weanling Holtzman rats were placed in individual wire cages in a constant-temperature room (23 ± 1°) and fed a commercial diet[2] for 2 or 3 days after they were received. The rats were then divided into weight groups and randomly assigned to diets within each group. Diets were prepared prior to the experiments and stored in a refrigerator (4°) until used. The diets and tap water were supplied ad libitum. Fresh diet was added daily and feed consumption recorded. The rats were weighed every 1 or 2 days. Experiment 1 was of 21 days' duration, and experiments 2 and 3 were 16 days long.

The composition of the diet and the basal amino acid mixtures are shown in tables 1 and 2, respectively. The various modifications are indicated in the tables of results. Amino acid mixture A is similar to that of Rechcigl et al. (12) and mixture B to that of Sauberlich (10).

The data were treated statistically by the method of Duncan (13) and tested at the 0.05 level of significance.

TABLE 1

Composition of experimental diets

	% of diet
Casein, casein hydrolysate or amino acid mixture	( )[1]
Hydrogenated vegetable oil[2]	14.0
Mineral mixture[3]	4.0
Vitamin mixture[4]	2.2
Anti-oxidant[5]	0.0125
Antibiotic (chlortetracycline)	( )[6]
Sucrose	( )[7]

[1] Casein was "vitamin-free" casein and the hydrolysate was a salt-free acid hydrolysate of casein, both purchased from General Biochemicals Inc. Diet 7 contained 14% casein supplemented with 0.18% DL-methionine and diet 8 contained 20% casein supplemented with 0.26% DL-methionine. Diets 14 and 15 contained 21.6% casein hydrolysate (adjusted from 20% to allow for water of hydrolysis and was supplemented with 0.26% DL-methionine, 0.10% cystine, 0.50% DL-tryptophan, 1.25% DL-threonine and 1.60% $NaHCO_3$. Diet 15 was further supplemented with 0.60% L-asparagine. For composition of amino acid mixtures see table 3.

[2] Crisco, Procter and Gamble, Cincinnati.

[3] Jones and Foster (4). Purchased from General Biochemicals Inc., Chagrin Falls, Ohio.

[4] "Vitamin Fortification" Mixture, Nutritional Biochemicals Corporation, Cleveland.

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

[6] Added to diet 4 at a level of 0.0001%.

[7] Added as required to complete mixture to 100%.

RESULTS AND DISCUSSION

Experiment 1. The results of a study on the effects of adding sodium bicarbonate to an amino acid diet are summarized in table 3. When the diet containing amino acid mixture A with arginine, histidine and lysine in the hydrochloride form (diet 1) was supplemented with levels of 2 or 4% sodium bicarbonate, weight gains and feed consumption were significantly increased in all periods. The differences between the 2 and 4% levels were not significant in any period. Since the design of this experiment did not include a direct comparison of amino acid mixtures with and without amino acid hydrochlorides supplemented with sodium bicarbonate, a definite conclusion cannot be drawn concerning what part of the response was due to balancing the hydrochlorides with sodium bicarbonate. It appeared possible that part of the response could have been the result of buffering this rather acid diet containing 12.4% of the dicarboxylic acid, glutamic acid. It is apparent that certain factors related to the mineral content of this amino acid diet have affected its utilization for growth. Such factors should be

[2] Big Red Laboratory Animal Feed, G. L. F. Feed Store, Ithaca, New York.

TABLE 2

Composition of amino acid mixtures [1]

Amino acid	Mixture A [2]	Mixture B [3]
	% of diet	*% of diet*
Indispensable amino acids		
Arginine·HCl	0.93	0.80
Histidine·HCl·H_2O	0.38	1.00
Isoleucine	0.46	3.00 (50% L-, 50% D-allo-)
Leucine	0.85	2.00
Lysine·HCl	1.25	1.85
Methionine	0.22	0.80 (DL-)
Cystine	0.20	0.30
Phenylalanine	0.48	1.30 (DL-)
Tyrosine	0.38	0.80
Threonine	0.51	1.50 (DL-,allo-free)
Tryptophan	0.10	0.50 (DL-)
Valine	0.72	2.80 (DL-)
Dispensable amino acids		
Alanine	—	0.60 (DL-)
Aspartic acid	—	0.60
Asparagine	—	0.60
Glutamic acid	12.39	4.00
Glycine	—	0.40
Proline	—	0.50
Serine	—	0.50 (DL-)
Sodium bicarbonate	( ) [4]	1.60

[1] Amino acids purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. L-Isomers used except as indicated.
[2] Amino acid mixture of Rechcigl et al. (12). Used in diets 1, 2 and 3.
[3] Amino acid mixture of Sauberlich (10). Indispensable mixture used in all other amino acid diets. Variations in the basal dispensable amino acid mixture are indicated in the diet descriptions in the tables of results.
[4] 0.58% $NaHCO_3$ was added to this mixture in diet 1, 2.58% in diet 2 and 4.58% in diet 3.

considered when comparing amino acid diets with protein diets.

The other treatments of this experiment included a comparison of the basal amino acid mixture B (diet 4) with a casein diet and studies of the effect of altering the asparagine and aspartic acid content of this mixture. The original design included the basal amino acid mixture B with and without antibiotics since antibiotics had been included in the Sauberlich diet (10) but the basal diet without antibiotic treatment was lost because of a technical error. A comparison of the basal diet with antibiotic versus the modified basal without antibiotic (diet 4 vs. diets 5 and 6) appears to be valid in view of the results of experiment 2 (diet 4 vs. diet 9) in which no effect from antibiotic was observed. Weight gain and feed consumption of rats receiving amino acid mixture B tended to be lower than that of rats receiving 14% casein diets but the differences were not significant. Removing both aspartic acid and asparagine from the diet resulted in significantly lower weight gains in the first week and for the entire experiment. The differences in feed intake were not significant. Weight gains of rats receiving diets where asparagine was replaced with an additional 0.6% aspartic acid were significantly lower than those of rats fed the basal diet in all periods. Feed intake was significantly lower in the second and third week and during the entire experiment.

The observations on weight gains and feed consumption of rats receiving the higher level of aspartic acid tend to support the comment of Sauberlich (10) that aspartic acid appears to have a "toxic" effect at higher levels of supplementation. Maximal growth rates of rats receiving either the casein diet (7a and 7b) or diets containing amino acid mixture B were attained by the second week of the experiment. Therefore, further experiments reported here were reduced to a 16-day duration.

Experiment 2. The results of the previous experiment indicate that asparagine plays a significant role in the level of per-

TABLE 3

Effects of adding sodium bicarbonate to amino acid diets and the effects of altering the asparagine and aspartic acid content on rat growth and feed consumption (daily averages) [1] *(exp. 1)* [2]

Diet no.	Description	First week		Second week		Third week		Entire experiment	
		Wt gain	Feed intake	Wt gain	Feed intake	Wt gain	Feed intake	Wt gain	Feed intake
		g	*g*	*g*	*g*	*g*	*g*	*g*	*g*
1	Mixture A, 0.58%, $NaHCO_3$	0.96[d]	7.1[e]	1.89[e]	8.0[d]	2.7[e]	9.4[d]	1.84[e]	8.2[d]
2	Mixture A, 2.58%, $NaHCO_3$	1.93[e]	8.5[be]	3.53[d]	10.8[e]	4.6[b]	13.7[bc]	3.18[b]	10.7[c]
3	Mixture A, 4.58%, $NaHCO_3$	2.03[e]	8.4[be]	4.21[cd]	11.8[e]	4.6[b]	14.5[abc]	3.62[b]	11.6[c]
4	Mixture B, antibiotic added	4.69[a]	10.2[a]	6.10[ab]	13.7[ab]	6.0[a]	16.5[ab]	5.60[a]	13.5[ab]
5	Mixture B, aspartic acid and asparagine omitted	3.60[b]	9.5[ab]	5.24[bc]	12.2[bc]	5.0[ab]	14.7[abc]	4.61[b]	12.1[bc]
6	Mixture B, asparagine omitted and aspartic acid level increased to 1.2%	3.33[b]	8.9[ab]	5.07[bc]	11.5[c]	4.1[b]	12.5[c]	4.18[b]	10.9[c]
7a	14% Casein	5.92[a]	10.5[a]	6.33[a]	14.0[a]	6.4[a]	17.0[a]	6.01[a]	13.8[a]
7b	14% Casein	5.53[a]	10.6[a]	6.86[a]	14.7[a]	6.3[a]	17.3[a]	6.26[a]	14.2[a]

[1] Treatment means without a common letter in their superscript are significantly different ($P < 0.05$).
[2] Six rats/treatment, average initial weight of 55 g.

formance supported by the basal amino acid mixture B. This experiment was conducted to study further the role of asparagine and aspartic acid in this mixture with a more complete experimental design and larger numbers of rats. The diets were not kept isonitrogenous. The results are summarized in table 4. Weight gains of rats receiving all of the diets without asparagine were significantly lower in the first 8-day period and for the combination of the 2 periods than for any of those receiving the other diets used in this experiment. There were no other significant differences in weight gains; however, weight gains tended to be lower during the second period of the experiment in rats receiving diets without asparagine as well as in those receiving the diets containing twice the basal level of asparagine and twice the basal level of aspartic acid and asparagine.

The feed intake of rats receiving the diet without aspartic acid or asparagine was significantly lower than that of rats receiving the basal diet in the first period and for the combined periods. The feed intake of rats receiving the diet without asparagine in the presence of 1.2% aspartic acid was significantly lower than that of rats receiving the basal diet during the second period.

No differences in performance of rats receiving the casein diet, amino acid basal diet, or the basal diet with antibiotic were apparent in this experiment. The amino acid basal diet without aspartic acid (diet 11) supported better average performance than any of the other diets tested. The importance of asparagine in amino acid mixture B for optimal weight gains and feed intake of weanling rats is apparent from this experiment. The optimal level of asparagine appears to be less than 1.2% of the diet. There is no evidence that the aspartic acid in this mixture was of any benefit. Rather, the higher level of aspartic acid appeared to suppress weight gains and feed consumption and the best performance was obtained in the absence of a dietary supply of aspartic acid.

Experiment 3. The previous observations reported here prompted an investigation into whether the growth-promoting properties of casein lost during acid hy-

TABLE 4

Further studies on the effects of altering the asparagine and aspartic acid content of an amino acid diet on rat growth and feed consumption (daily averages) [1] *(exp. 2)* [2]

Diet no.	Description	First 8 days		Second 8 days		Entire experiment	
		Wt gain	Feed intake	Wt gain	Feed intake	Wt gain	Feed intake
		g	*g*	*g*	*g*	*g*	*g*
8	20% Casein	5.78[a]	11.2[ab]	6.64[a]	14.1[ab]	6.21[a]	12.7[abc]
4	Mixture B, antibiotic added	5.58[a]	11.2[ab]	6.74[a]	15.2[a]	6.16[a]	13.2[ab]
9	Mixture B	5.38[a]	11.3[ab]	6.48[a]	14.6[ab]	5.93[a]	13.0[ab]
10	Mixture B, aspartic acid and asparagine levels both increased to 1.2%	5.50[a]	11.5[a]	6.11[a]	14.6[ab]	5.81[ab]	13.0[ab]
11	Mixture B, aspartic acid omitted	5.91[a]	11.6[a]	6.80[a]	15.4[a]	6.36[a]	13.5[a]
12	Mixture B, aspartic acid omitted and asparagine level increased to 1.2%	5.76[a]	11.7[a]	6.23[a]	14.6[ab]	5.99[a]	13.1[ab]
13	Mixture B, asparagine omitted	4.24[b]	10.4[bc]	5.95[a]	13.6[bc]	5.09[bc]	12.0[bc]
6	Mixture B, asparagine omitted and aspartic acid level increased to 1.2%	4.28[b]	10.4[bc]	6.01[a]	12.5[c]	5.14[bc]	12.2[bc]
5	Mixture B, asparagine and aspartic acid omitted	4.06[b]	10.0[c]	5.90[a]	13.4[bc]	4.98[c]	11.7[c]

[1] See footnote 1 of table 3.
[2] Ten rats/treatment, average initial weight of 66 g.

drolysis could be restored by the addition of asparagine to the hydrolysate. The results of a study of the effect of the addition of asparagine to a casein hydrolysate diet are summarized in table 5.

The hydrolysate was supplemented with tryptophan, threonine and cystine to account for possible losses during hydrolysis (14) and with methionine. Supplementation of the hydrolysate diet with asparagine resulted in significantly higher weight gains and feed consumption in both experimental periods. The asparagine-supplemented hydrolysate diet supported performance equal to that obtained with the 20% casein diet.

Table 5 shows a summary of the results of altering the glutamic acid, proline, glycine, serine and alanine content of amino acid mixture B. The diets were not kept isonitrogenous and aspartic acid was omitted from the basal diet on the basis of results in the previous experiment.

Modifications of the basal diet which resulted in a significant lowering of weight gains included omitting proline in the first period and combined periods and all or 2 percentage units of glutamic acid in the first period. Lower weight gains approaching significance ($P = 0.06$ to 0.07) were observed for the combined periods in rats receiving the glutamic acid-free, 2% glutamic acid, serine-free and glycine-free diets. Feed intake was significantly lower than that of the basal diet in rats receiving the glutamic acid-free diet in the first period and combined periods and the 2% glutamic acid, proline-free and serine-free diets for the combined periods.

In table 6, a summary is presented of the effects on growth rate and feed consumption of removing individual dispensable amino acids from the aspartic acid-free amino acid diet and comparisons with the 20% casein diet. Average weight gains in the first half of the experiment were appreciably affected by the omission of asparagine, glutamic acid, proline, one-half of the glutamic acid and glycine, in this order, from the basal diet. The effects of omitting serine and adding 0.6% aspartic acid were relatively small and further studies are required before the significance of these differences may be evaluated. The high rate of formation of

TABLE 5

Effects of the addition of asparagine to an acid hydrolysate of casein and the effects of altering the glutamic acid, alanine, proline, serine and glycine content of an amino acid diet on rat growth and feed consumption (daily averages) [1] *(exp. 3)* [2]

Diet no.	Description	First 8 days		Second 8 days		Entire experiment	
		Wt gain	Feed intake	Wt gain	Feed intake	Wt gain	Feed intake
		g	*g*	*g*	*g*	*g*	*g*
8	20% Casein	5.99^{ab}	10.0^{ab}	7.99^{a}	15.4^{a}	6.99^{a}	12.7^{ab}
14	Casein acid-hydrolysate, indispensable amino acids added	4.04^{f}	8.8^{c}	6.44^{b}	13.2^{b}	5.24^{d}	11.0^{c}
15	Casein acid-hydrolysate, indispensable amino acids and 0.6% asparagine added	6.29^{a}	10.3^{a}	7.46^{ab}	15.0^{a}	6.75^{ab}	12.7^{ab}
11	Mixture B, aspartic acid omitted	5.61^{abc}	10.3^{a}	7.05^{ab}	15.4^{a}	6.36^{abc}	12.8^{a}
16	Mixture B, aspartic acid and glutamic acid omitted	4.34^{def}	8.8^{c}	7.16^{ab}	14.1^{ab}	5.76^{cd}	11.4^{c}
17	Mixture B, aspartic acid and 2.0% glutamic acid omitted	4.79^{de}	9.5^{abc}	6.71^{b}	13.9^{ab}	5.75^{cd}	11.7^{bc}
18	Mixture B, aspartic acid and alanine omitted	5.33^{bcd}	9.5^{abc}	6.93^{ab}	14.2^{ab}	6.06^{bcd}	11.9^{abc}
19	Mixture B, aspartic acid and proline omitted	4.66^{def}	9.5^{abc}	6.33^{b}	13.8^{ab}	5.49^{d}	11.6^{bc}
20	Mixture B, aspartic acid and serine omitted	5.16^{cd}	9.5^{abc}	6.44^{b}	14.0^{ab}	5.79^{cd}	11.7^{bc}
21	Mixture B, aspartic acid and glycine omitted	4.95^{cde}	9.6^{abc}	6.73^{b}	14.4^{ab}	5.84^{cd}	12.0^{abc}

[1] See footnote 1 of table 3.
[2] Ten rats/treatment, average initial weight of 60 g.

TABLE 6

Summary of the effects of modifying the dispensable amino acid content of an amino acid diet on the growth rate and feed consumption by weanling rats and comparisons with casein diets (values expressed as % difference from amino acid mixture B with aspartic acid omitted, diet 11) [1]

Modification	Experiment	Diet no.	First 8 days		Second 8 days	
			Wt gain	Feed intake	Wt gain	Feed intake
Asparagine omitted	2	5	−28.3	−10.3	−12.5	−11.3
Glutamic acid omitted	3	16	−22.6	−14.8	+ 1.6	− 8.5
2% Glutamic acid omitted	3	17	−14.6	− 7.4	− 4.8	− 9.6
Proline omitted	3	19	−16.9	− 8.2	−10.2	−10.3
Glycine omitted	3	21	−11.8	− 7.3	− 4.5	− 6.4
Serine omitted	3	20	− 8.0	− 7.7	− 8.7	− 9.3
Alanine omitted	3	18	− 5.0	− 7.5	− 1.7	− 7.7
0.6% Aspartic acid added	2	9	− 9.0	− 3.0	− 5.1	− 4.6
20% Casein	2	8	− 2.2	− 3.9	− 2.4	− 8.1
20% Casein	3	8	+ 6.8	− 2.5	+13.3	0

[1] The coefficients of variation in these experiments for weight gains and feed consumption were approximately 0.15 and 0.10, respectively.

alanine through transamination (15, 16) may account for the lack of response to omitting this amino acid from the basal diet. Likewise, the extensive metabolism of glutamic acid during the absorption process when present in the intestine at lower concentrations (15) may explain the relatively high glutamic acid requirement for maximal early growth. Based on the criterion of growth rate during the second 8-day period the rat appears to be able to adapt within a short period of time to the lack of glutamic acid and glycine in the diet and to a lesser extent to the lack of asparagine and proline when receiving a diet containing a relatively high level of "dispensable-N" (an estimated 2% of the diet). The adaptation which is often observed when rats are fed an amino acid diet may thus be due in part to inadequate levels of certain dispensable amino acids.

In experiments 2 and 3 the consumption of the basal amino acid diet equaled or exceeded that of the 20% casein diet, whereas the consumption of diets devoid of certain of the dispensable amino acids was lower. These results indicate that the primary factor influencing the consumption of amino acid diets is their nutritional adequacy. It appears that the amino acid diet used here with adequate levels of dispensable amino acids is nutritionally equivalent to an intact casein diet containing a similar level of nitrogen and that the nutritional properties of casein lost in acid hydrolysis may be restored by the addition of indispensable amino acids and asparagine which are altered in the process of acid hydrolysis. The extension and application of these observations should make possible more definitive studies on the optimal protein and nitrogen nutrition of the mammalian organism.

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Rachitogenic Activity of Soybean Fractions[1]

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ABSTRACT Isolated soybean protein and raw soybean meal were shown to possess growth-depressing and rachitogenic properties when used in a glucose-soybean protein-type diet for turkey poults to 4 weeks of age. Vitamin D_3 was supplied at the level of 880 ICU/kg. The rachitogenic effects of isolated soybean protein could be largely overcome by an eightfold increase in vitamin D_3 supplementation or by autoclaving the isolated soybean protein. However, maximal growth and bone ash were obtained only when heated soybean meal was included in the diet. The growth-depressing and rachitogenic effects of raw soybean meal were only partially overcome with a tenfold increase in vitamin D_3 supplementation, whereas autoclaving the meal overcame both effects.

Several workers have reported rachitogenic effects of natural products. Grant and O'Hara (1) observed extracts of green oats and permanent pasture grasses to be rachitogenic, whereas dried grasses were antirachitic. The rachitogenic substance was equally distributed in alcohol soluble and insoluble phases and moved with carotene in a chromatogram. Pure β-carotene or synthetic vitamin A at many times the requirement levels for rats inhibited calcification in the presence of vitamin D, and it was assumed that β-carotene was the rachitogenic factor. Raoul et al. (2) also demonstrated the presence of a rachitogenic substance in green oats, and in grasses and cabbage. This material was soluble in benzene and was purified by distillation and chromatographic procedures.

Coates et al. (3) showed that a fraction of fresh liver which was soluble in an ether-petroleum-alcohol mixture (2:2:1) was rachitogenic for rats largely because of its interference with calcium absorption. Wasserman et al. (4) demonstrated that for chicks, free dietary L-lysine reduced absorption of calcium from the gastrointestinal tract. For rats, L-lysine improved absorption.

A previous report from this laboratory (5) showed soybean meal and water extracts of soybean meal and raw soybean flakes to possess antirachitic and growth-promoting activity for turkey poults fed a purified diet of the glucose-isolated soybean protein type. The diet contained all known nutrients in quantities assumed to be adequate, that is, all vitamins were supplied at 2 to 5 times the NRC (6) suggested requirements. A high level of isolated soybean protein was shown to be rachitogenic, suggesting that raw soybeans might also contain this factor. Subsequent studies have demonstrated this to be the case and their results will be described.

EXPERIMENTAL

The batteries used for brooding were in a temperature-controlled basement room with incandescent lighting. Mixed sex turkey poults of the BBB variety were fed to one week of age the basal diet previously described (5), but with vitamin D added at a level of 880 ICU/kg (5). Three groups of 8 to 10 poults each, equalized as to weight, were then randomly selected and fed each of the experimental diets as described in the tables shown. Feed and water were supplied ad libitum, and feed consumption and mortality data were obtained. At 4 weeks of age the poults were weighed as groups and 5 poults from each group, selected randomly, were killed for tibia ash determinations by the AOAC (7) method. Statistical analyses were conducted according to Duncan's multiple range test (8).

Received for publication October 21, 1963.

[1] Scientific Paper no. 2381, Washington Agricultural Experiment Stations, Pullman, project no. 1578. This investigation was supported by funds provided for biological and medical research by the State of Washington Initiative no. 171.

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RESULTS

Data from the first experiment (table 1) confirmed the previous work (5) and showed that an increase in protein level from 30 to 39% had little effect upon growth but significantly reduced bone ash and increased the visible signs of rickets with the low level of vitamin D_3. Leg weakness and a condition resembling perosis were observed as early as 2 weeks of age with this group (no. 3). However, with the higher level of vitamin D_3 there was no difference in bone ash between the 2 levels of protein.

Autoclaving the soybean protein allowed for greatly increased growth and bone ash values. A reduction in the growth response from vitamin D_3 was obtained even with the heated protein. Use of the autoclaved protein, together with the addition of soybean meal, increased bone ash so that the higher level of vitamin D_3 gave no significant effect in this respect over the lower level. The data obtained with the commercial soybean meal supplement also confirm the previous work (5) showing antirachitic properties in soybean fractions. Normal bone ash is about 46% for poults under these conditions in this laboratory.

In the second and third experiments, diets containing 30 and 40% protein, respectively, were used. The results (table 2) show that raw soybean meal depressed growth and reduced bone ash. Only where two-thirds or more of the protein was supplied by the raw soybean meal was the rachitogenic effect evident, however. Bone ash data, unfortunately, are not available for the groups containing intermediate levels of raw soybean meal. Although there was a significant growth response to vitamin D_3 with the 20:40 heated-to-raw groups, vitamin D_3 counteracted only a small portion of the growth-depressing effect of raw soybean meal. With 80% raw soybean meal in experiment 3, there was also a trend toward improved growth and bone ash with increased levels of vitamin D_3. Bone ash values of above 50% for poults were considerably above normal, although 80% levels of soybean meal are not commonly used.

Further evidence showing destruction of the rachitogenic property of soybean protein by heating was obtained in experiment 4 (table 3). Although the commercial soybean meal and raw soybean meal did not improve growth significantly with the low level of vitamin D_3, there was a significant improvement in bone ash with these supplements. Also, with the high level of vitamin D_3, raw soybean meal gave a significant increase in bone ash. Autoclaving the raw soybean meal gave significant improvements in both criteria at the low level of vitamin D_3, and significantly improved bone ash with the high level of the vitamin.

Experiments 5, 6 and 7 were conducted to determine whether there were any differ-

TABLE 1

Effect of protein level, autoclaved protein and heated soybean meal on the response of poults to vitamin D_3 (exp. 1)

	Treatment	Level of vitamin D_3	4-Week avg wt	Bone ash [1]
		ICU/kg	*g*	%
1	30% Protein, raw [2]	880	341 [ab 3]	34.0 [bc 3]
2	30% Protein, raw	7040	427 [bc]	40.6 [d]
3	39% Protein, raw [2]	880	329 [a]	30.2 [a]
4	39% Protein, raw	7040	466 [c]	40.9 [de]
5	As 3, autoclaved [4]	880	436 [c]	37.1 [c]
6	As 3, autoclaved	7040	508 [c]	44.7 [f]
7	As 3 with HSBM,[5] 10%	880	443 [c]	34.9 [bc]
8	As 3 with HSBM, 10%	7040	498 [c]	44.3 [ef]
9	As 5 with HSBM, 10%	880	469 [c]	41.0 [def]
10	As 5 with HSBM, 10%	7040	480 [c]	44.7 [f]

[1] Percentage of dry, defatted bone.
[2] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis. Diet as described previously (5) adjusted to 100% with glucose.
[3] The same small letters indicate no significant difference at the 5% level of probability.
[4] Soy protein autoclaved at 120° for 30 minutes.
[5] Commercial heated soybean meal, 50% protein, General Mills, Inc., Minneapolis.

TABLE 2

Effect of graded levels of heated soybean meal vs. raw soybean flakes on the response of poults to vitamin D

Treatments [1]		Level of vitamin D_3	4-Week avg wt	Bone ash [2]
HSBM	RSBF			
%	%	*ICU/kg*	*g*	%
		Experiment 2		
60 [3]	0 [4]	880	452 [e 5]	47.4 [c 5]
60	0	4400	444 [e]	46.9 [bc]
60	0	8800	428 [e]	47.2 [c]
50	10	880	429 [e]	—
50	10	4400	445 [e]	—
50	10	8800	414 [de]	—
40	20	880	379 [cd]	—
40	20	4400	374 [c]	—
40	20	8800	363 [c]	—
20	40	880	308 [a]	—
20	40	4400	359 [bc]	—
20	40	8800	360 [bc]	—
—	60	880	290 [a]	43.6 [a]
—	60	4400	302 [a]	42.5 [a]
—	60	8800	321 [ab]	44.6 [ab]
		Experiment 3		
80 [6]	—	880	407 [b]	51.9 [b]
80	—	1760	424 [b]	50.6 [b]
80	—	3520	417 [b]	52.2 [b]
—	80 [3]	880	229 [a]	43.4 [a]
—	80	1760	245 [a]	44.7 [a]
—	80	3520	277 [a]	46.2 [a]

[1] HSBM indicates heated soybean meal, and RSBF, raw soybean flakes; diets similar to those described previously (5) as to mineral and vitamin content, otherwise 60 or 80% raw or heated soybean meal, 28 or 8% glucose and 5% corn oil.
[2] Percentage of dry, defatted bone.
[3] Commercial heated soybean meal, 50% protein, General Mills, Inc., Minneapolis.
[4] Raw soybean brew flakes (meal), Central Soya Company, Inc., Fort Wayne, Indiana.
[5] The same small letters indicate no significant difference at the 5% level of probability.
[6] Soybean brew flakes autoclaved at 120°C for 30 minutes.

TABLE 3

Effect of heated, raw and autoclaved soybean meal on the response of poults to vitamin D (exp. 4)

Treatment	Level of vitamin D_3	4-Week avg wt	Bone ash [1]
	ICU/kg	*g*	%
Basal	880	375 [a 2]	32.0 [a 2]
Basal	3520	427 [abc]	41.2 [bc]
HSBM,[3] 10%	880	437 [abc]	41.7 [bcd]
HSBM, 10%	3520	518 [d]	44.2 [cde]
RSBF,[4] 5%	880	410 [ab]	42.9 [bcde]
RSBF, 5%	3520	479 [bcd]	45.8 [e]
ASBF,[5] 5%	880	470 [bcd]	39.7 [b]
ASBF, 5%	3520	501 [cd]	45.2 [de]

[1] Percentage of dry, defatted bone.
[2] The same small letters indicate no significant difference at the 5% level of probability.
[3] Commercial heated soybean meal, 50% protein, General Mills, Inc., Minneapolis.
[4] Raw soybean brew flakes (meal), Central Soya Company, Inc., Fort Wayne, Indiana.
[5] As 3, autoclaved at 120°C for 30 minutes.

ences in potency of different sources of vitamin D_3 and to study the effects on rickets when supplementing the basal diet with materials such as ethylenediaminetetraacetic acid (EDTA), inositol, various types of protein, phytic acid and phosphorus. The summarized data for these studies are shown in table 4. The poults in experiment 5 apparently were not as deficient in vitamin D_3 as in previous work

TABLE 4

Effects of source of vitamin D, EDTA, inositol, source of protein, sodium phytate or phosphorus upon poult growth and calcification

	Treatment	Source of protein	4-Week avg wt	Bone ash [1]
			g	%
		Experiment 5		
1	Source A [2]	C-1 [3]	460	40.3 [c 4]
2	Source B [5]	C-1	463	40.5 [c]
3	As 1 + EDTA,[6] 500 ppm	C-1	453	37.4 [a]
4	As 2 + EDTA, 500 ppm	C-1	446	39.3 [abc]
5	As 1, without inositol [7]	C-1	489	40.0 [bc]
6	As 1	casein-gel [8]	494	46.6 [d]
7	As 6 + sodium phytate, 1%	casein-gel [8]	481	45.6 [d]
		Experiment 6		
8	As 1	C-1	329	30.2
9	As 1 + 0.2% phosphorus [9]	C-1	339	33.0
		Experiment 7		
10	As 1	C-1	333 [A]	29.6 [A]
11	As 6	casein-gel	484 [B]	43.4 [B]

[1] Percentage of dry, defatted bone.
[2] Fixtdee, vitamin D_3 supplement to supply 880 ICU/kg, Dawes Laboratories, Inc., Chicago.
[3] ADM C-1 Assay Protein, 44% + 0.5% glycine and 0.7% hydroxy methionine analogue.
[4] The same small letters indicate no significant difference at the 5% level of probability; the same large letters indicate no significance at the 1% level.
[5] Nu-Rovamix-AD_3 325/325, to supply 880 ICU/kg, Hoffmann-LaRoche, Inc., Nutley, New Jersey.
[6] Ethylenediaminetetraacetic acid.
[7] Otherwise supplied to all other diets at 1.1 g/kg diet.
[8] 35% Casein + 7.5% gelatin.
[9] As 85% H_3PO_4.

or in experiments 6 and 7, as evidenced by the superior growth and bone ash with the basal diets (treatments 1 vs. 8 and 10). Although the poults were in a similar physical arrangement for all studies, experiment 5 was conducted in a different battery room from all of the other studies. It is possible that more ultra-violet rays from the sun could have leaked into this room.

The vitamin D_3 from source A was equal in potency to that of source B for growth and bone ash. Leaving inositol out of the diet had no significant effect on either criteria. In contrast, adding EDTA depressed bone ash with source A but had no significant effect with source B of vitamin D_3. In experiment 5, replacing the isolated soybean protein with casein and gelatin greatly improved bone ash and appeared to increase growth. Highly significant improvements in growth and bone ash were obtained in experiment 7 with this substitution. Although in experiment 5 there was a slight depression in bone ash with sodium phytate, the difference was not significant. Adding 0.2% phosphorus had no effect on growth and improved bone ash only slightly in experiment 6.

DISCUSSION

These results demonstrated that the basal diet was rachitogenic, largely due to the isolated soybean protein. It was also shown that raw soybean meal possesses rachitogenic properties, but isolated soybean protein appeared to be a much more potent source of the activity. This could be explained on the basis that the raw soybean meal contained both antirachitic and rachitogenic factors that to some extent masked each other, whereas the isolated protein contained only the rachitogenic factor. On the other hand, the heated or autoclaved soybean meals possessed little of the rachitogenic factor and retained the antirachitic factor. The rachitogenic factor appears to be labile to heat whereas the antirachitic factor is heat stable.

Coming from soybean meal, the rachitogenic factor could not be β-carotene as demonstrated by Grant and O'Hara (1) although it might be the same as that present in green oat leaves shown by

Raoul et al. (2). Body stores of vitamin D_3 or other accessory factors (as perhaps the antirachitic factor) or a combination of these, present at the time dietary treatments are initiated, undoubtedly influence the magnitude of responses to both rachitogenic and antirachitic factors.

The mechanism for interference of normal calcification by raw isolated soybean protein is not known. Tying up phosphorus by phytic acid appears to be ruled out as an explanation of the results, since it should not be destroyed by heating at 120°C for 30 minutes, and since sodium phytate addition was not detrimental when added to the casein-gelatin diet. Even though dietary inositol might be converted to phytic acid, omitting it from the diet had no effect on growth or bone ash. Davis et al. (9) have shown that isolated soybean protein contains a component which combines with zinc, manganese and copper so that the dietary requirement for these minerals is increased. Adding EDTA reduced the requirement. In the present study, EDTA, if anything, aggravated the condition. In the previous work (5), extra manganese or dicalcium phosphate did not prevent the rachitic effects, and in the present study, phosphorus was completely ineffective. Biological and chemical assays (5) had also demonstrated that the vitamin D_3 supplements used contained within 15% of the their stated potency of vitamin D_3.

Possibly soybean meal, inadequately heated, is one of the contributing factors in the increased reports of field rickets in turkey poults. As shown here in experiments 2 and 3, vitamin D did not completely overcome the rachitogenic effect of raw soybean meal, even when fed at 10 times the suggested requirement level. However, with properly heated soybean meal, the level of vitamin D_3 suggested by the NRC (6), 880 ICU/kg, was adequate for maximal growth and bone development.

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