Influence of Dietary Vitamin A on Carotenoid Utilization, Nitrogen Retention and Energy Utilization by the Chick [1,2]

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ABSTRACT The effect of feeding high dietary levels of vitamin A palmitate on the utilization of carotenoids, nitrogen retention and energy utilization by the chick was studied. Five-week-old chicks were fed diets supplemented with 4,400, 26,400, 158,400, and 475,200 IU of vitamin A/kg of diet for a 3-week experimental period. Yellow corn was the dietary source of carotenoids. A consistent decrease in the carotenoid content of serum, liver and skin was obtained with the increase in vitamin A level of the diet. Significant ($P < 0.01$) negative correlation coefficients were calculated when values of serum, liver, and skin carotenoids were plotted against logarithm of dietary vitamin A level. A consistent decrease in the absorption of carotenoids was found when the output of carotenoids in the excreta was measured. Absorption of carotenoids was determined by the conventional total collection method and by the chromic oxide indicator technique. The results obtained by the 2 methods were essentially similar. Both N retention and metabolizable energy of the diets were depressed with excess vitamin A supplementation. An adverse effect of excess body storage of vitamin A on the utilization of carotenoids was also noted.

High dietary levels of vitamin A have been reported to depress the utilization of carotene and other carotenoid pigments. Deuel et al. (1, 2) noted a marked decrease in the carotene content of milk following the administration of large doses of vitamin A to cows. In a later study, Mattson and Deuel (3) observed a similar decrease in blood and liver carotenoids of chicks in the presence of an excessive vitamin A intake. More recently, Dua and Day (4) demonstrated the adverse effect of high levels of vitamin A on skin and serum carotenoids of chicks. The mechanism of the manner in which vitamin A depresses the utilization of these pigments has not been explained. Deuel et al. (2) postulated that vitamin A increases the destruction of carotene in the tissues through the development of a new enzyme system.

An excessive intake of vitamin A has also been shown to affect the utilization of other nutrients. The administration of 10,000 IU of vitamin A/g of body weight to rachitic rats resulted in a negative nitrogen balance.[4] Similarly, Nerurkar and Sahasrabudhe (5) reported a decrease in N retention in young rats fed 40,000 IU of vitamin A/day.

The investigations reported herein were undertaken to study further the effect of high dietary levels of vitamin A on the utilization of carotenoids together with its effect on N retention and energy utilization by the chick.

EXPERIMENTAL

Two experiments were conducted using 5-week-old male Vantress × Arbor Acre chicks. The chicks had previously been fed a carotenoid-free diet (a white corn-soybean meal starting diet). The chicks were housed as groups in rearing batteries with raised wire-screen floors, with the room temperature held at approximately 25°. Feed and water were supplied ad libitum. Individual body weights were obtained at the beginning and end of the test periods. Feed consumption records were obtained by groups.

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[2] Presented in part before the meeting of the Poultry Science Association, August 1965 (Poultry Sci., 44: 1365, 1965, abstract).

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[4] Maddock, C. L., and S. B. Wolbach 1950 Nitrogen, phosphorus and calcium metabolism in the rachitic rat given excessive doses of vitamin A. Federation Proc., 9: 337 (abstract).

In experiment 1, the basal diet consisted of the following: (in per cent) yellow corn, 61.16; soybean meal (50% protein), 27.80; poultry by-products meal (55% protein), 2.50; dried whey (18% protein), 2.50; vegetable oil,[5] 3.00; defluorinated rock phosphate (14% P, 34% Ca), 1.50; limestone, 0.50; sodium chloride, 0.35; chromic oxide, 0.35; and the following supplements/kg of diet: vitamin D_3, 1200 ICU; α-tocopheryl acetate, 13.2 IU; vitamin B_{12}, 9.9 μg; and (in mg/kg) riboflavin, 4.4; Ca pantothenate, 8.8; niacin, 35.2; manganese sulfate, 141; and choline chloride, 325. This diet was supplemented with 4 levels of stabilized vitamin A palmitate:[6] (IU/kg) 4,400, 26,400, 158,400, and 475,200. Each dietary treatment was fed to duplicate groups of birds (10/group) for a 3-week experimental period. Yellow corn was used as the dietary source of carotenoids. All diets, by analysis, contained approximately 21 mg of total carotenoids/kg of diet.

During the last week of the experiment the feces which fell onto pans covered with waxed paper were collected for 3 consecutive days. A record of feed intake during the collection period was also maintained. Each day's fecal collection was stored in a refrigerator. Fecal collections for 3 days were composited, sampled for moisture immediately and portions were frozen for future analysis.

At the termination of the test period the birds were bled by cardiac puncture, killed by disjunction of the neck and the livers and feet were removed. Within each dietary group, serum, liver and toe-web skin samples from each of 5 birds were pooled for determination of carotenoids.

A second experiment was conducted to determine the effect of previous storage of vitamin A in the body on subsequent utilization of carotenoids. Duplicate groups of chicks (12/group) were fed a carotenoid-free basal diet from 5 to 8 weeks of age, supplemented with the same dietary levels of vitamin A as used in the first experiment. The basal diet was similar to the one used in the first experiment except that white corn replaced yellow corn and chromic oxide was omitted. At 8 weeks of age the chicks were weighed, 2 chicks/lot were killed for estimating liver storage of vitamin A and the remaining chicks were fed the basal diet containing yellow corn with a normal level of vitamin A (2,640 IU/kg). At 10 weeks of age carotenoids in serum, liver and skin were determined.

The analytical procedure of determining carotenoids of feed samples was essentially that of Blessin (6) with the exception that a Goldfish fat extraction apparatus rather than Butt extraction tubes was used to extract the pigments. Feces and liver samples were saponified with alcoholic KOH and carotenoids were then extracted with hexane. The optical density of the extract was measured at 445 mμ against a hexane blank in a Beckman DU spectrophotometer. For the determination of skin carotenoids, samples were obtained by removing discs of skin from the toe-web area by means of a sharp cork borer (7-mm diameter). The skin discs from 5 birds were pooled in Erlenmeyer flasks (50-ml glass stoppered) containing 10 ml of acetone. The discs were kept in the acetone solution for 48 hours in the dark, with frequent shaking. The solutions were filtered and the volume was adjusted to 10 ml prior to measuring the optical density of each solution at 445 mμ. Serum carotenoids were determined by the method of Grau and Klein (7). In each case, the concentrations of carotenoids were determined by reference to a standard calibration curve established for pure β-carotene[7] at 445 mμ. The carotenoid content of the skin was expressed as micrograms/100 cm^2 of toe-web area.

Liver vitamin A was estimated by the method of Ames et al. (8). Energy determinations were made on feed and feces samples using a Parr oxygen bomb calorimeter. Dietary and fecal N were determined by the macro-Kjeldahl method, and chromic oxide was determined essentially according to the method of Czarnocki et al. (9). Metabolizable energy values were corrected for N retention (10).

Statistical examinations of the data were made using analysis of variance, and treatment differences were separated using

[5] Wesson Oil, distributed by Wesson Oil Sales Company, Fullerton, California.

[6] Vitamin A palmitate in gelatin, 30,000 IU/g. Additions to feed based on declared potency. From Chas. Pfizer & Co., Inc., New York 10017.

[7] Eastman Organic Chemicals no. 3702.

TABLE 1

Effect of vitamin A levels on the performance and utilization of carotenoids (exp. 1)

Vitamin A level	Avg wt		Feed [1] gain	Carotenoids [2]		
	Initial (5-week)	Final (8-week)		Serum	Liver	Toe-web skin
IU/kg	*g*	*g*		*μg/ml*	*μg/g*	*μg/100 cm²*
4,400	711	1449	2.36	6.1 [a 3]	3.8 [a 3]	118 [a 3]
26,400	718	1424	2.41	5.5 [a]	2.8 [ab]	108 [a]
158,400	721	1463	2.39	2.1 [b]	1.2 [b]	48 [b]
475,200	730	1434	2.42	1.3 [b]	1.0 [b]	41 [b]

[1] Feed-to-gain ratio during the experimental period.
[2] Each value represents the average of 4 separate determinations (samples from 5 birds were pooled for each determination).
[3] Means within each column having different superscripts are significantly different ($P < 0.01$) by Duncan's multiple range test (11).

Duncan's multiple range test (11). In experiment 1 the data were also evaluated by means of regression analysis.

RESULTS

Supplemental vitamin A (4,400, 26,400, 158,400, and 475,200 IU/kg of diet) did not significantly affect body weight gain or feed utilization in experiment 1 (table 1). Similarly, no significant effect on body weight or feed gain was obtained among dietary treatments in the second experiment at any of the feeding periods; consequently, the performance data from the second experiment are not presented.

Increasing the vitamin A level of the diet resulted in a consistent decrease in the carotenoid content of serum, liver and skin (table 1). At the two highest levels of vitamin A supplementation a significant decrease ($P < 0.01$) was obtained in the serum and skin carotenoids as compared with the other 2 groups. Liver carotenoids in the groups fed 158,400 and 475,200 IU of vitamin A/kg of diet were significantly lower ($P < 0.01$) than those in the group supplemented with 4,400 IU of vitamin A.

Table 2 shows the effect of dietary levels of vitamin A on the absorption of carotenoids as measured during the last week of the experiment; the absorption represents the difference between intake and output of carotenoids in the excreta and is expressed as percentage of intake. The absorption values were calculated using both the conventional total collection and the chromic oxide indicator ratio technique. The results indicate a consistent decrease in the absorption of carotenoids as the dietary vitamin A level was increased. With the total collection method, the absorption of carotenoids in the group fed the highest level of vitamin A was significantly depressed ($P < 0.05$) as compared with the groups supplemented with 4,400 and 26,400 IU of vitamin A. With the chromic oxide ratio method, the ab-

TABLE 2

Effect of vitamin A levels on the absorption of carotenoids (exp. 1)

Vitamin A level	Total intake of carotenoids/ group [1]	Absorption of carotenoids	
		Total collection method	Chromic oxide ratio method
IU/kg	*mg*	%	%
4,400	370	61.9 [a 2]	66.5 [a 2]
26,400	360	51.2 [ab]	50.3 [b]
158,400	373	44.4 [bc]	49.3 [b]
475,200	360	36.7 [c]	39.0 [c]
SE [3]		±2.6	±2.1

[1] Intake during the experimental period, calculated from the total feed consumed; values are averages of duplicate groups.
[2] Means within each column having different superscripts are significantly different ($P < 0.05$) by Duncan's multiple range test (11).
[3] SE of the general mean.

TABLE 3

Effect of vitamin A levels on nitrogen retention and metabolizable energy of the diets (exp. 1)

Vitamin A level	N retention	Metabolizable energy
IU/kg	%	*kcal/g*
4,400	64.3 [a 1]	3.365 [A 1]
26,400	57.4 [ab]	3.314 [A]
158,400	61.1 [ab]	3.368 [A]
475,200	50.2 [b]	3.138 [B]
SE [2]	±2.7	±0.015

[1] Duncan's multiple range test (11): means within each column having different small and large superscripts are significantly different at $P < 0.05$ and $P < 0.01$, respectively.
[2] SE of the general mean.

sorption of carotenoids in the group fed the highest level of vitamin A was significantly lower ($P < 0.05$) as compared with all other groups. There was no significant difference in the absorption values obtained with the 2 methods.

The intake of carotenoids, calculated from the total feed consumed, was essentially similar in all groups (table 2).

The effect of vitamin A on N retention and metabolizable energy of the diets is shown in table 3. These values were calculated using only the chromic oxide ratio technique. N retention and metabolizable energy tended to be depressed with the increase in dietary vitamin A level. At the highest level of vitamin A supplementation, N retention was significantly lower

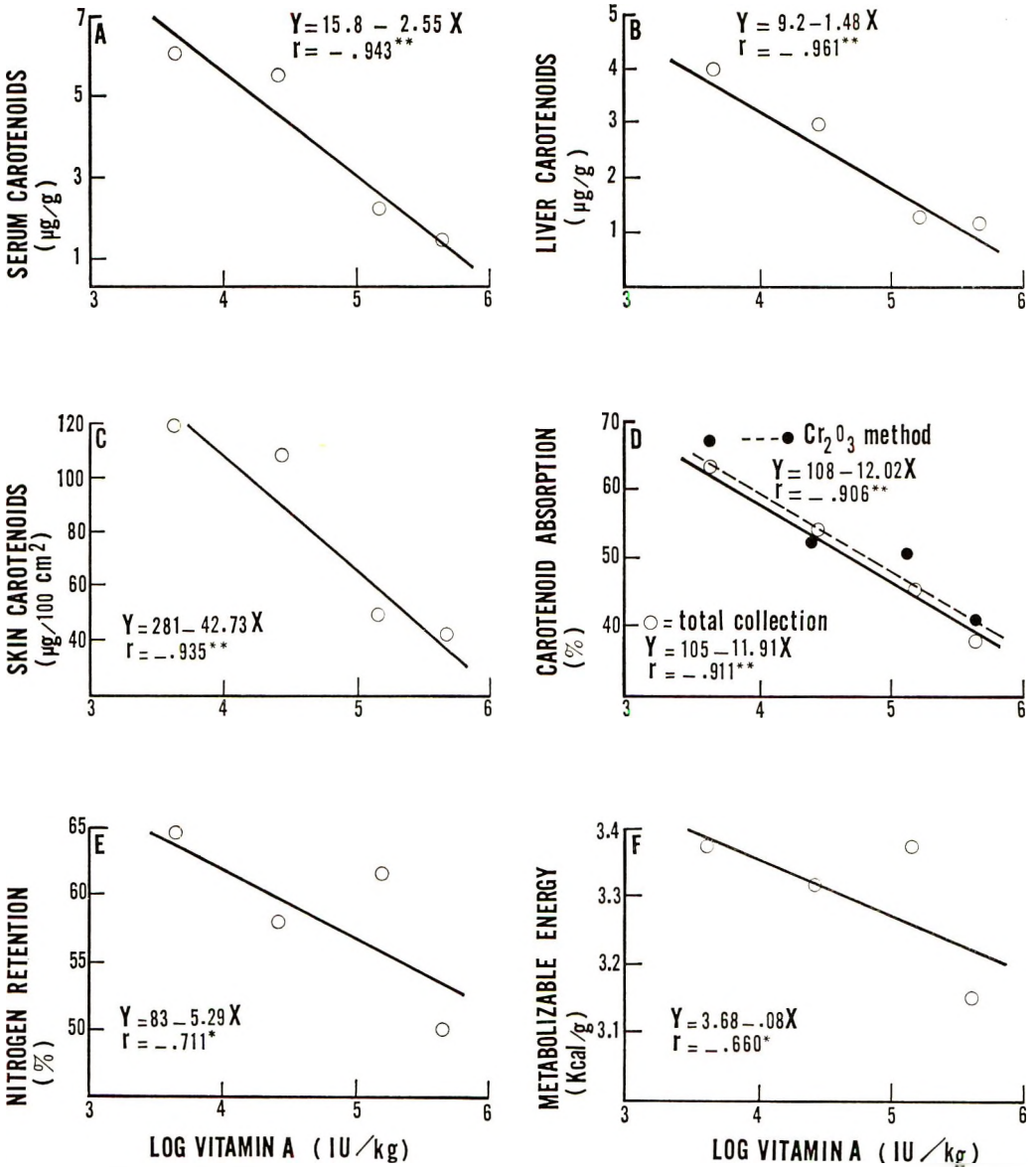

Fig. 1 Relationship between logarithm of vitamin A level and (*A*) serum carotenoids, (*B*) liver carotenoids, (*C*) skin carotenoids, (*D*) absorption of carotenoids, (*E*) N retention, and (*F*) metabolizable energy (exp. 1). Asterisks indicate level of significance: * = $P < 0.05$, and ** = $P < 0.01$.

TABLE 4

Effect of previous feeding of vitamin A on subsequent utilization of carotenoids (exp. 2)

Dietary [1] vitamin A level	Liver vitamin A [2]	Results, 10 weeks		
		Carotenoids [3]		
		Serum	Liver	Skin
IU/kg	*μg/g*	*μg/ml*	*μg/g*	*μg/100 cm²*
4,400	54	4.8 [a 4]	3.3 [a 4]	63 [a 4]
26,400	212	4.8 [a]	3.6 [a]	64 [a]
158,400	1,323	4.4 [ab]	3.1 [ab]	55 [b]
475,200	4,234	3.3 [b]	2.4 [b]	41 [c]

[1] These levels were fed in a carotenoid-free diet to duplicate groups (12 chicks/group) from 5 to 8 weeks of age.

[2] Average of 4 birds/treatment at 8 weeks of age.

[3] From 8 to 10 weeks all chicks were fed a yellow corn basal containing 2,640 IU of vitamin A/kg. Each value represents the average of 4 separate determinations (samples from 5 birds were pooled for each determination).

[4] Means within each column having different superscripts are significantly different ($P < 0.05$) by Duncan's multiple range test (11).

($P < 0.05$) as compared with the group supplemented with 4,400 IU of vitamin A. Metabolizable energy of the diet was significantly depressed ($P < 0.01$) in the group fed the highest level of vitamin A as compared with all the other groups.

The data from experiment 1 were also evaluated by regression analysis techniques and are shown in figure 1. The results were plotted against the logarithm of vitamin A level and their respective prediction equations and correlation coefficients were calculated. These data demonstrate negative correlations between dietary vitamin A level and the different criteria studied.

The results from the second experiment are shown in table 4. Liver vitamin A of the representative birds removed from each treatment at 8 weeks, as expected, increased with each increase in the dietary vitamin A and was proportional to the vitamin A content of the diet. The results from carotenoid analyses at 10 weeks show that body storage of vitamin A also exerted an adverse effect on the utilization of these pigments. Carotenoids of serum, liver and skin were significantly lower ($P < 0.05$) in the group which had previously been fed the diet supplemented with highest vitamin A level and had the highest liver storage of vitamin A as compared with the groups which were fed 4,400 and 26,400 IU of vitamin A/kg of diet.

DISCUSSION

The adverse effect of high dietary levels of vitamin A on the utilization of carotenoids is in agreement with previous reports (1–4). Our results indicate that vitamin A interferes with the absorption of carotenoids from the intestinal tract. These results are in contrast with those of Mattson and Deuel (3) who failed to find a difference in the absorption of carotene and xanthophyll in chicks fed 9,000 IU of vitamin A/day from shark-liver oil. In the present study, total carotenoids in feed and feces were also separated into main fractions, carotene and xanthophyll. Xanthophyll constitutes the main fraction of carotenoids in yellow corn which was the only source of dietary carotenoids used in this study. With the quantity of samples taken for fecal analysis of carotenoids, the amount of carotene separated was very small and could not be estimated accurately; xanthophyll was the major fraction separated and when the values were calculated separately for the absorption of xanthophyll, essentially the same relative values as those for the absorption of total carotenoids were obtained.

Since carotenoids are unstable during long storage periods, feed samples were analyzed for carotenoids at the start and at the end of the experimental period to check for any possible destruction of carotenoids. No appreciable loss of carotenoids was observed in the feed during the 3-week experimental period.

Chromic oxide as an index material has been used successfully in carotene absorption studies (12). In the present study, good agreement was obtained between the conventional total collection and the chromic oxide ratio technique when applied to the absorption of carotenoids. The

values determined by the chromic oxide ratio technique were, in general, higher than those determined by the total collection method.

Decreased N retention due to high vitamin A supplementation is in agreement with the results of Nerurkar and Sahasrabudhe (5) with young rats. It appears that the high dietary levels of vitamin A may produce a generalized phenomenon that affects the overall metabolism of the body.

The adverse effect of vitamin A was greater on carotenoids than on N retention and metabolizable energy (fig. 1). Since vitamin A and carotenoids are structurally related, this would indicate a competition between the two for absorption from the intestinal tract. Such a competitive relationship may exist even after absorption, according to data obtained in the second experiment. Rubin and Bird (13) also noted an inhibitory effect of previous vitamin A feeding on subsequent utilization of carotenoids.

Whether high levels of vitamin A exert a direct or indirect effect, involving some physiological or biochemical mechanism(s), on the utilization of carotenoids and other nutrients cannot be explained from the present data. Chanda et al. (14) reported that thyroxine reduced and thiouracil increased the fecal excretion of carotene in cows. A decreased metabolism rate and thyroid size has been reported as a result of high vitamin A administration in rats (15). It is possible that the effect of excess vitamin A is mediated through the thyroid gland. Several suggestions have been made of a possible role of this vitamin on membrane permeability. In a recent review article, Dingle and Lucy (16) concluded that many of the widespread effects of excess vitamin A appear to result indirectly from its actions on the lipoprotein membranes of the cell.

LITERATURE CITED

1. Deuel, H. J., Jr., N. Halliday, L. F. Hallman, C. Johnston and A. J. Miller 1941 The production of high vitamin A milk by diet. J. Nutr., 22: 303.
2. Deuel, H. J., Jr., L. F. Hallman, C. Johnston and F. Mattson 1942 The effect of a high vitamin A intake on the blood and milk carotene of Holstein and Guernsey cows. J. Nutr., 23: 567.
3. Mattson, F. H., and H. J. Deuel, Jr. 1943 Studies on carotenoid metabolism. III. The effect of a high vitamin A diet on the carotenoid metabolism of chickens. J. Nutr., 25: 103.
4. Dua, P. N., and E. J. Day 1964 Effect of high dietary vitamin A levels on the utilization of carotenoids by broilers. Poultry Sci., 43: 1511.
5. Nerurkar, M. K., and M. B. Sahasrabudhe 1956 Metabolism of calcium, phosphorus and nitrogen in hypervitaminosis A in young rats. Biochem. J., 63: 344.
6. Blessin, C. W. 1962 Carotenoids of corn and sorghum. 1. Analytical procedure. Cereal Chem., 39: 236.
7. Grau, C. R., and N. W. Klein 1957 Sewage-grown algae as a feedstuff for chicks. Poultry Sci., 36: 1046.
8. Ames, S. R., H. A. Risley and P. L. Harris 1954 Simplified procedure for extraction and determination of vitamin A in liver. Anal. Chem., 26: 1378.
9. Czarnocki, J., I. R. Sibbald and E. V. Evans 1961 The determination of chromic oxide in samples of feed and excreta by acid digestion and spectrophotometry. Can. J. Animal Sci., *41:* 167.
10. Sibbald, I. R., J. D. Summers and S. J. Slinger 1960 Factors affecting the metabolizable energy content of poultry feeds. Poultry Sci., 39: 544.
11. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.
12. Kreula, M. S. 1946 Absorption of carotene from carrots in man and the use of the quantitative chromic oxide indicator method in the absorption experiments. Biochem. J., *41:* 269.
13. Rubin, M., and H. R. Bird 1946 The apparent antagonism between vitamin A and carotenoids in the fowl. Science, *103:* 584.
14. Chanda, R., H. M. Clapman, M. L. NcNaught and E. C. Owen 1952 The digestibility of carotene by the cow and the goat as affected by thyroxine and thiouracil. Biochem. J., 50: 95.
15. Sadhu, D. P., and S. Brody 1947 Excess vitamin A ingestion, thyroid size and energy metabolism. Amer. J. Physiol., *149:* 400.
16. Dingle, J. T., and J. A. Lucy 1965 Vitamin A, carotenoids and cell function. Biol. Rev., *40:* 422.

Effects of Dietary Modifications on Response of the Duckling to Aflatoxin [1,2]

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ABSTRACT The purpose of this investigation was to determine the short-term effects of dietary modifications on the response of ducklings to aflatoxin, a mold contaminant found in certain natural food products. In the presence of aflatoxin, dietary supplements of 4.0% of methionine, 1.0% of arginine or 0.8% of lysine, as individual additions, depressed weight gain but decreased mortality. The addition to the diet of 1.0% arginine and 0.8% lysine with, but not without, aflatoxin sharply decreased weight gain and increased mortality. The addition of glutathione or cysteine to the diet as sources of sulfhydryl groups had no effect on toxicity. Autoclaving aflatoxin-contaminated peanut meal decreased toxicity and markedly increased weight gains of ducklings over a 9-day period.

During an investigation of field outbreaks of a toxic disease (1) which resulted in the loss of over 100,000 turkey poults in England in 1960 and 1961, it was observed that ducklings were very sensitive to the toxic factor responsible for the condition later designated as turkey "X" disease. Subsequent studies revealed that toxicity was associated with the peanut meal included in the diet of the animals. The peanut meal used in the animal feeds associated with the original outbreak in England was shipped to England from Brazil. During the same period peanut meal grown in Africa and in India was found to contain a toxin which resulted in the same clinical syndrome and histopathologic lesions in animals as had been observed in England. Shortly thereafter investigations revealed that peanut meal produced in the United States also contained a toxin (2) that induced a toxicosis identical to that observed in England, Africa and India. It is now known that the toxic factor involved in all of the aforementioned outbreaks is a complex of metabolites, referred to collectively as aflatoxin and elaborated by certain strains of the mold *Aspergillus flavus* (3). When these strains of mold grow on natural food products such as peanuts, cottonseed meal, soybean meal and cereal grains, their metabolites (aflatoxin) contaminate the product. There are 4 major fractions in the complex, designated B_1, B_2, G_1 and G_2 according to their characteristic fluorescence and R_F values on a thin-layer chromatogram. The chemical structures of the various fractions have been identified (4) and the crude complex as well as the primary fraction (B_1) are now known to be carcinogenic to several species of animals (2, 5, 6).

The day-old duckling played an important role in the isolation and chemical characterization of the toxin complex because of its sensitivity and rapid response, as manifested by liver parenchymal cell damage and bile duct proliferation (6).

Richardson et al. (7) observed that dietary supplements of arginine and lysine decreased the toxicity of moldy feed to poults. Renewed interest in the toxicity of moldy feeds and the widespread use of vegetable proteins for both human and animal consumption prompted a study of the effects of dietary supplements on the response of the duckling to aflatoxin-contaminated feeds and to purified aflatoxin. Observations of the effects of dietary additions of amino acids or sources of sulfhydryl groups and of autoclaved peanut meal were included in our studies. Results

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[2] This is contribution no. 887 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts.

of these investigations form the basis for this report.

MATERIALS AND METHODS

One-day-old white male Pekin ducklings were used in all experiments. They were placed in a battery brooder 24 to 30 hours after hatching, with feed and water supplied ad libitum. Purified aflatoxin, isolated by methods described previously (6), was intubated daily in 0.1 ml volume of propylene glycol; control ducklings were given only a 0.1 ml daily ration of propylene glycol. Feed-grade peanut meal was purchased on the open market and its aflatoxin content was determined by chemical fluorescence and bioassay techniques (6). Some samples of the peanut meal were extracted with hot methanol for 3 periods of 4 hours each with the solvent exchanged after each period of extraction, a procedure known to remove most of the contaminating aflatoxin. Other samples of the raw peanut meal were autoclaved under steam pressure at 121° for 30 minutes in stainless steel pans without additional water. Additional samples of peanut meal were washed 3 times with tap water, dried and ground, then mixed into the diet. The wash water was evaporated and the dried residue was added as 10% of the diet. The composition of the diets is shown in table 1.

Necropsy was performed on all ducklings. The tissues were routinely processed for microscopic study.

RESULTS

Experiment 1 was designed to study the effects of adding methionine, arginine and lysine to diets containing toxic peanut meal. A diet with nontoxic peanut meal (basal diet D) was fed to control groups. Data shown in table 2 represent 2 or 3 trials for each dietary treatment with results combined. The addition of methionine, arginine or lysine individually to the diet depressed weight gain slightly but decreased mortality However, when lysine and arginine were added together, three-fourths of the ducklings succumbed before the end of 1 week and all were dead before the end of the 2-week period. The apparent reduction in bile duct cell proliferation in groups fed supplemental arginine plus lysine is misleading because the unusual type of morphologic alteration observed under these conditions was difficult to evaluate.

Experiment 2 tested the effects of autoclaved peanut meal on duckling growth; in addition, an attempt was made to deter-

TABLE 1

Dietary composition

	Diets				
	A	B	C	D	E
			% of diet		
Casein	20.0	10.0	10.0	10.0	10.0
Peanut meal no. 4 [1]	—	50.0	35.0	—	—
Peanut meal no. 10 [2]	—	—	—	50.0	—
Methanol-extracted peanut meal [3]	—	—	—	—	35.0
Gelatin	8.0	—	—	—	—
Sucrose	49.7	22.0	37.0	49.7	37.0
Cellulose	4.0	—	—	—	—
Salts [4]	5.0	5.0	5.0	5.0	5.0
Vitamin mix [5]	3.0	3.0	3.0	3.0	3.0
DL-Methionine	0.3	0.0	0.0	0.0	0.0
Cottonseed oil [6]	10.0	10.0	10.0	10.0	10.0

[1] Contained 3.0 ppm of aflatoxin when purchased.
[2] Contained no detectable aflatoxin.
[3] Continuous extraction with hot methanol, 3 periods of 4 hours each with solvent changed between periods of extraction; aflatoxin was not detected by usual assay.
[4] Wesson salts. Wesson, L. G., Science, 75: 339, 1932.
[5] The vitamin mix supplied the following in milligrams/kg of diet: thiamine·HCl, 8.0; riboflavin, 4.0; pyridoxine·HCl, 4.0; Ca pantothenate, 20.0; folic acid, 10.0; nicotinic acid, 50.0; inositol, 250.0; biotin, 0.2; *dl-α*-tocopheryl acetate, 110; menadione, 10.0; vitamin A acetate, 10,000 IU; vitamin D_3, 2500 IU; vitamin B_{12} and choline chloride added in aqueous solution, 50 μg and 0.2%, respectively, when diet was mixed.
[6] Wesson Oil, Wesson Oil Sales Company, Fullerton, California.

mine whether aflatoxin could be destroyed or removed by autoclaving or by water extraction. Results are shown in table 3. When the diet contained 1.5 ppm aflatoxin (diet B) weight gain was only half that of the controls (diet D); autoclaving the toxic meal reversed this effect. The toxic effect was not entirely eliminated because bile duct proliferation was observed in all ducklings fed the autoclaved peanut meal and chemical assay confirmed the presence of residual aflatoxin in the diet. Compared with those fed nonautoclaved toxic meal, average body weight after 9 days was higher in all 3 of the groups fed the autoclaved contaminated peanut meal. Highest body weights were achieved by ducklings fed basal diet D, which contained nontoxic, autoclaved peanut meal.

Experiment 3 was designed to test the effects of the addition of single and combined amino acids and aflatoxin to semipurified (casein) diets. Results are shown in table 4. The addition of arginine or of lysine to the diet without aflatoxin depressed body weight but mortality occurred only in those groups given aflatoxin; the addition of 2% instead of 1% of arginine to the semipurified diet appeared to be toxic. As observed with the

TABLE 2

Effects of supplemental amino acids on ducklings fed aflatoxin-contaminated peanut meal (exp. 1)

Dietary treatment	No. of ducklings [1]	Dietary aflatoxin	Avg 2-week wt	2-Week mortality	Bile duct proliferation [2]
		ppm	*g*		
Basal diet D	15	0.0	263	0/15	0.0
Basal diet B	10	1.5	143	6/10	34.0
Basal diet B + 4% methionine	15	1.5	112	3/15	24.0
Basal diet B + 1.0% arginine	15	1.5	118	1/15	32.0
Basal diet B + 0.8% lysine	15	1.5	121	3/15	34.0
Basal diet B + 1.0% arginine + 0.8% lysine	20	1.5	— [3]	20/20	20.0

[1] Numbers represent 2 or 3 trials in each dietary treatment, results of which are combined; the total is presented in the table.
[2] Graded 0 to 4 according to severity ranging from none (0) to severe (4), multiplied by 10 and averaged.
[3] Losses began at 5 days with 15/20 dead by day 7and 20/20 dead by day 12.

TABLE 3

Effects of autoclaving and water-washing peanut meal on toxicity of ducklings to aflatoxin (exp. 2) [1]

Dietary treatment	No. of ducklings	Dietary aflatoxin	Avg 9-day wt	9-Day mortality	Bile duct proliferation
		ppm	*g*		
Basal diet D	10	0.0	242	0/10	0.0
Basal diet B	14	1.5	119	10/14	24.0
Basal diet B (autoclaved peanut meal)	10	0.5	289	0/10	20.0
Basal diet B (autoclaved peanut meal)	15	—	320	0/15	15.0
Basal diet B (autoclaved peanut meal)	10	—	328	0/10	18.0
Basal diet B + water-extracted peanut meal	10	1.0	135	0/10	20.0
Basal diet D + dried, water extract of peanut meal no. 4	12	0.3	338	0/12	12.0
Basal diet D (autoclaved peanut meal)	10	0.0	410	0/10	0.0

[1] Data represent combined results of 2 trials.

casein-peanut meal diets, the addition of both arginine and lysine to the diet had adverse effects only in the presence of aflatoxin.

Experiment 4 examined the effects of sources of sulfhydryl groups and of amino acids on bile duct hyperplasia and mortality in ducklings with and without aflatoxin. Results are shown in table 5. The addition of cysteine or glutathione to the toxic diet had no appreciable effect on the response of the ducklings. When the toxin-containing diet was supplemented with both arginine and lysine, toxicity was increased in a manner similar to that observed previously. The addition of arginine and lysine, as single or combined supplements, to the nontoxic casein-peanut meal diet had no effect on growth or mortality. This is in agreement with the results obtained when semipurified casein diets were supplemented with arginine and lysine.

Histopathology. The response of ducklings to aflatoxin is degeneration of parenchymal cells and proliferation of bile duct cells (figs. 1–4). In paraffin preparations hepatic cells are severely vacuolated, a result of the accumulation of large quantities of lipid as shown by staining frozen sections with Oil Red O. The young duckling normally has a significant amount of lipid in its liver, but this is increased when the animal is exposed to aflatoxin. Proliferation of bile duct cells under the influence of aflatoxin originates in the peri-

TABLE 4

Effect of amino acids and aflatoxin with purified diets on response to aflatoxin by ducklings (exp. 3) [1]

Dietary treatment	No. of ducklings	10-Day body wt	10-Day mortality	Bile duct hyperplasia
		g		
Basal diet A	20	200	0/20	0.0
Basal diet A + 1% arginine	10	192	0/10	0.0
Basal diet A + aflatoxin [2]	10	188	0/10	24.0
Basal diet A + 1% arginine + aflatoxin [2]	10	180	1/10	30.0
Basal diet A + 2% arginine	10	159	0/10	0.0
Basal diet A + 2% arginine + aflatoxin [2]	10	145	2/10	26.0
Basal diet A + 0.8% lysine	10	185	0/10	0.0
Basal diet A + 0.8% lysine + aflatoxin [2]	10	165	1/10	24.0
Basal diet A + 0.8% lysine + 1.0% arginine	15	182	0/15	0.0
Basal diet A + 0.8% lysine + 1.0% arginine + aflatoxin [2]	20	136	8/20	20.0

[1] Data represent 2 or more trials, results of which are combined; the total is presented in the table.
[2] Two micrograms of aflatoxin intubated daily for 5 days.

TABLE 5

Effect of amino acids, sulfhydryl compounds and aflatoxin in ducklings (exp. 4)

Dietary treatment	No. of ducklings	Aflatoxin	12-Day wt	12-Day mortality	Bile duct hyperplasia
		ppm	*g*		
Basal diet C	10	1.0	143	0/10	22.0
Basal diet C + 1% cysteine	10	1.0	121	0/10	26.0
Basal diet C + 1.75% glutathione	10	1.0	135	2/10	30.0
Basal diet C + 1.0% arginine + 0.8% lysine	15	1.0	142	3/15	30.0
Basal diet E	5 [1]	0.0	281	0/5	0.0
Basal diet E + 1% arginine	10	0.0	284	0/10	0.0
Basal diet E + 0.8% lysine	10	0.0	285	0/10	0.0
Basal diet E + aflatoxin	5 [1]	— [1]	225	1/5	18.0
Basal diet E + 1% arginine + 0.8% lysine	10	0.0	296	0/10	0.0
Basal diet E + 1% arginine + 0.8% lysine + aflatoxin	10	— [2]	180	6/10	20.0

[1] One trial only; all others represent 2 trials with results combined.
[2] Two micrograms of aflatoxin B_1 intubated daily for 5 days.

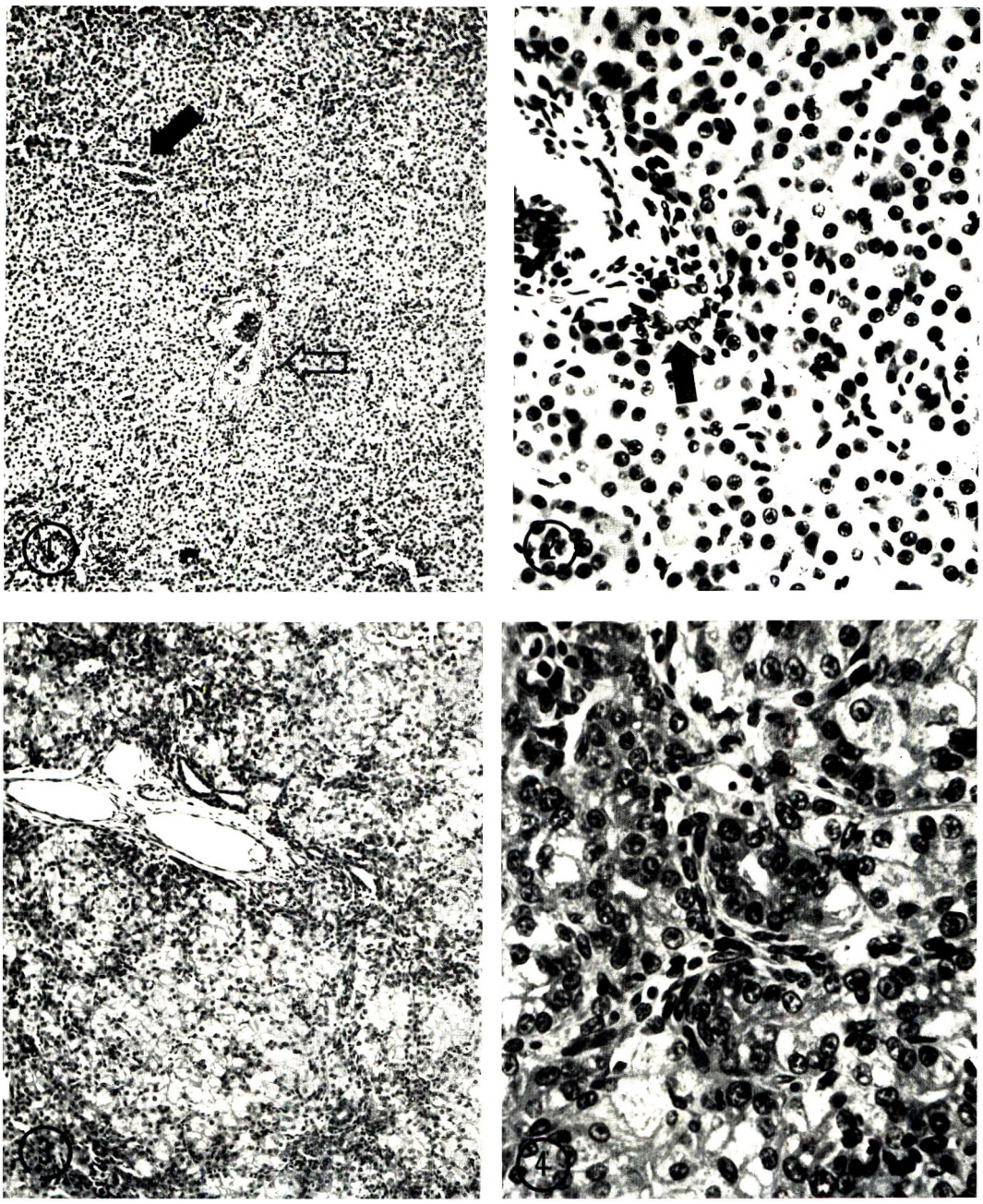


Fig. 1 Normal duckling liver illustrating portal (open arrow) and central (black arrow) areas of lobules. H & E. × 75.

Fig. 2 Higher magnification of figure 1 showing appearance and arrangement of parenchymal cells and 1 bile duct (arrow). H & E. × 376.

Fig. 3 Section of liver from duckling exposed to aflatoxin. Note vacuolation of parenchymal cells and proliferating bile duct cells radiating out from portal zone. H & E. × 188.

Fig. 4 High magnification of figure 3. Enlarged, vacuolated parenchymal cells forming cyclindrical cords can be seen scattered among proliferating bile duct cells. H & E. × 639.

portal zone and radiates toward the centrolobular zone (fig. 3). The lobule is involved to a variable degree, depending on the concentration of aflatoxin and the length of exposure. After a single dose of aflatoxin, diffuse bile duct cell proliferation begins within 48 hours and increases progressively to 4 days; it then reaches a plateau and begins to regress after 5 to 7 days. When exposure is continuous, as it is with contaminated feed, bile duct cell hyperplasia proceeds in a more orderly fashion and attempts to form duct-like structures are often seen in sections (fig. 4).

In contrast with the response of ducklings to aflatoxin under normal dietary conditions, the addition of arginine and lysine to diets concomitant with aflatoxin exposure resulted in a different morphologic alteration. Instead of the typical periportal radiation of bile duct cells the entire lobule was severely affected and liver cells assumed a duct-like arrangement (figs. 5 and 6), some of which resembled bile ducts. The morphologic appearance strongly suggested a metaplastic change from parenchymal to bile duct cells.

DISCUSSION

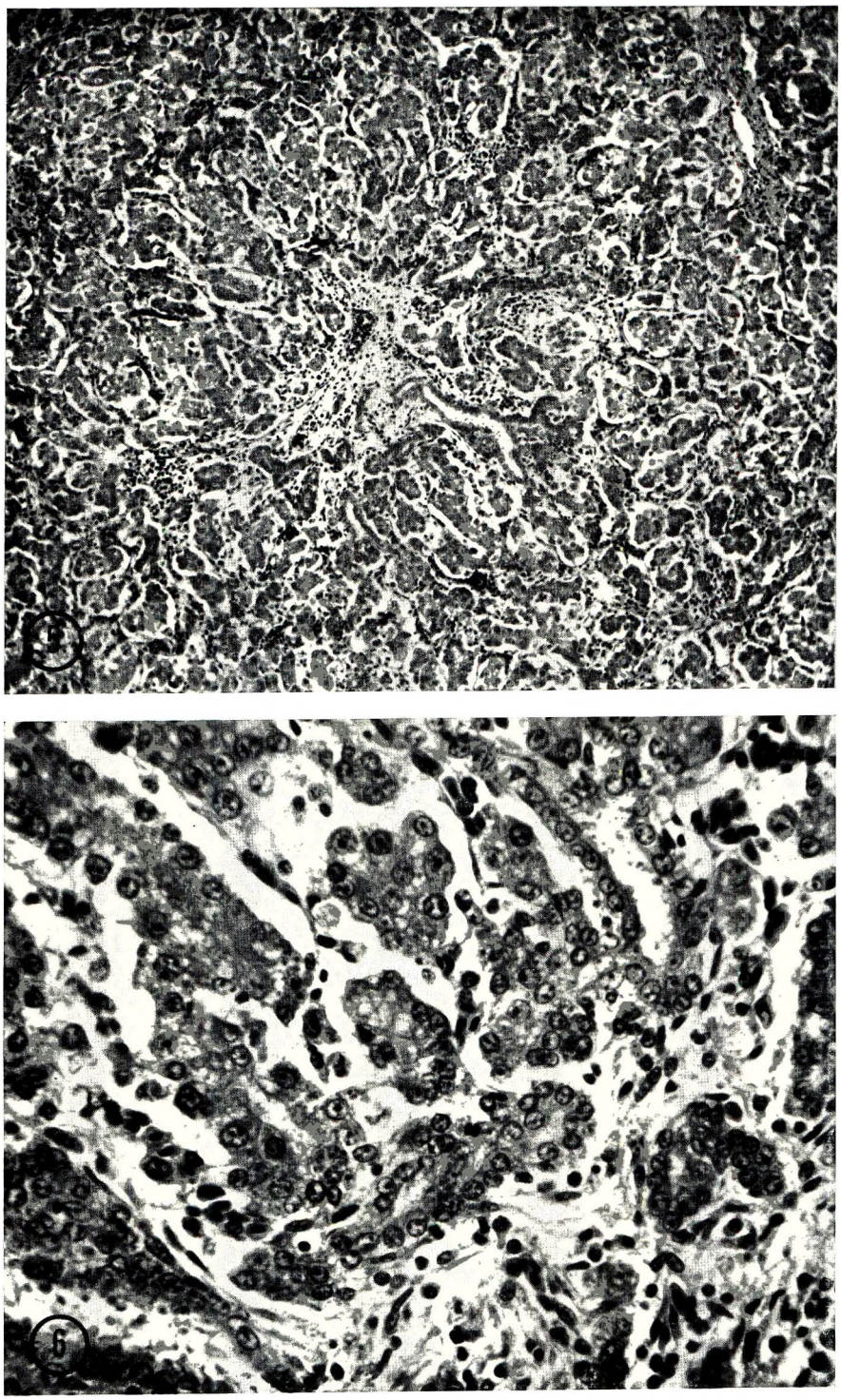
It has been established by a number of laboratories that the duckling is highly sensitive to the acute effects of aflatoxin. Results of these experiments demonstrate conclusively that feeding supplemental arginine and lysine increased the sensitivity of the duckling to aflatoxin. This was also true when these 2 amino acids were added to casein diets and the duckling was intubated with aflatoxin. Addition of the amino acids to the same diets without superimposing aflatoxin had no effect on growth and did not affect liver morphology. The reason for this unusual response is not clear; it may have been an imbalance or a toxicity or it may have been related in some way to the increased work load presented to the liver by the addition of these dibasic amino acids to the diet. Increased deamination and transamination as a result of amino acid additions to the diet exposed the already damaged liver to a higher level of nitrogenous products and imposed a demand for detoxication greater than that associated with aflatoxin alone. However, this does not offer a complete explanation since the addition of 4% methionine, roughly equivalent to arginine-lysine additions in amino nitrogen, resulted in a much less severe response. The experiments of Squibb et al. (8) are of interest in this respect; these investigators fed 4 times the normal dietary requirement of lysine to chicks and reported that this level not only failed to increase cellular levels of this amino acid but tended to depress levels of other amino acids. Liver weight, as a percentage of body weight, was unaffected in the normal chick by increased dietary lysine, but liver size in chicks infected with tuberculosis increased linearly with increasing levels of dietary lysine. Moreover, when tuberculosis was concomitant with increased dietary lysine, the only significant changes in free amino acids of the liver were in total lysine and arginine, both of which were decreased. These conditions also permitted a more severe infection with tuberculosis.

Recently, Jones (9) has reported that excessive dietary lysine may induce an arginine deficiency in chick plasma and muscle. There appeared to be an antagonism between these 2 dibasic amino acids under his experimental conditions.

It seems clear from results shown in table 3 that autoclaving the contaminated peanut meal destroyed or eliminated a part of the aflatoxin activity. It must also be concluded, based on weight gains, that autoclaving the peanut meal increased its efficiency as a food factor. Washing the contaminated peanut meal with water removed a part of the aflatoxin, some of which was observed in the dried, particulate residue after the water was evaporated. It is not likely that any of the toxin was actually in aqueous solution; instead

Fig. 5 Section of liver from duckling fed diet containing supplements of 1.0% of arginine and 0.8% of lysine. Note arrangements of parenchymal cells into cords and duct-like structures and lack of characteristic bile duct cell hyperplasia as shown in figure 4. H & E. × 140.

Fig. 6 High magnification of figure 5 illustrating unusual architectural arrangement of liver cell cords of duckling exposed to aflatoxin concomitant with dietary supplements of arginine and lysine. Cells form duct-like structures in some areas with what appears to be continuity between parenchymal cells and bile duct cells. H & E. × 680.



Figures 5–6

it was probably attached to the particulate fraction as a suspension in the water.

The addition of sources of sulfhydryl groups failed to decrease the toxicity to the duckling, indicating that the toxin has little effect on active sulfhydryl groups and that it must act through some mechanism other than the inactivation of sulfhydryl-containing enzymes.

Although results of our work do not parallel those of Richardson, we were working with ducklings exposed to known amounts of a well-defined toxin, whereas Richardson fed poults diets which were contaminated by unidentified molds. The exact nature of the contaminants and toxins or of either alone was unknown. Therefore, it is impossibe to compare results because of these factors and because of a difference in species.

The significant observation in this investigation was the profound increase in sensitivity of ducklings to aflatoxin when they were fed concomitant dietary supplements of arginine and lysine. This unusual pathologic change deserves further study.

LITERATURE CITED

1. Allcroft, R., and R. B. A. Carnaghan 1963 Toxic products in groundnuts. Biological effects. Chem. Ind., *2*: 50.
2. Newberne, P. M., W. W. Carlton and G. N. Wogan 1964 Hepatomas in rats and hepatorenal injury in ducklings fed peanut meal or *Aspergillus flavus* extract. Pathol. Veterinaria, *1*: 105.
3. DeIongh, H. R. K., R. O. Vees, C. B. Barrett and W. O. Ord 1962 Investigations of the factor in groundnut meal responsible for "turkey X" disease. Biochim. Biophys. Acta, *65*: 548.
4. Asao, T., M. M. Buchi, M. M. Abdel-Kader, S. B. Chang, E. L. Wick and G. N. Wogan 1965 The structures of aflatoxins B_1 and G_1. J. Amer. Chem. Soc., *87*: 882.
5. Newberne, P. M. 1965 Carcinogenicity of aflatoxin-contaminated peanut meal. In: Mycotoxins in Foodstuffs, ed., G. N. Wogan. Massachusetts Institute of Technology Press, Cambridge, Massachusetts, p. 187.
6. Newberne, P. M., G. N. Wogan, W. W. Carlton and M. M. Abdel-Kader 1964 Histopathologic lesions in ducklings caused by *Aspergillus* cultures, culture extracts and crystalline aflatoxin. Toxicol. Appl. Pharmacol., *6*: 542.
7. Richardson, L. R., S. Wilkes, J. Godwin and K. R. Pierce 1962 Effect of moldy diet and moldy soybean meal on the growth of chicks and poults. J. Nutr., *78*: 301.
8. Squibb, R. L., H. Seigel and M. Solotorovsky 1965 Protein metabolism in livers of chicks fed deficient to excess quantities of protein and lysine and infected with tuberculosis. J. Nutr., *86*: 133.
9. Jones, J. D. 1964 Lysine-arginine antagonism in the chick. J. Nutr., *84*: 313.

Alterations in Liver Enzyme Activities and Blood and Urine Metabolite Levels during the Onset of Thiamine Deficiency in the Dairy Calf [1,2]

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ABSTRACT A combination of physiological, clinical and enzymatic criteria was used to characterize the onset of thiamine deficiency in male Holstein calves fed a purified diet lacking thiamine. Deficiency symptoms appeared between 27 and 48 days after eliminating thiamine from the diet and 24 to 45 days after thiamine excretion had decreased to below 0.05 mg/day. Concomitantly with the appearance of overt deficiency symptoms, blood pyruvate and lactate levels increased from 1 and 15 mg/100 ml to 5 and 100 mg/100 ml, respectively; urinary pyruvate excretion increased from 15 mg/day to over 50 mg/day. Average blood hemoglobin and hematocrit levels were significantly depressed, 16 and 22%, respectively. The activities of transketolase and anaerobic pyruvate dehydrogenase decreased to 50 and 30% of control values. Aldolase activity increased at the time of the clinical symptoms relative to that of controls, whereas malic enzyme, isocitrate dehydrogenase, and glucose-6-phosphate dehydrogenase activities apparently were not altered. Refeeding thiamine corrected all the deficiency symptoms.

One of the major problems when attempting to study metabolic alterations accompanying thiamine deficiency is that of establishing when the deficiency has a direct, specific effect upon metabolism. This is important because if the deficiency develops to the point at which acute, gross symptoms become apparent, metabolism may have become altered by nonspecific factors such as reduced food intake.

Many clinical tests with varying degrees of reliability have been devised as means for assaying adequacy of thiamine intake. Individual variation has limited the usefulness of some of these methods, especially those involving relationships between thiamine intake and excretion levels (1–3) or blood levels (4–6). Others based on metabolic indexes such as levels of blood pyruvate and lactate (7), the "carbohydrate index" (8) or activity of erythrocyte transketolase (9) may involve uncertainties under particular circumstances (10).

Results are presented in this paper from a study of thiamine deficiency in calves when normal dietary loads were maintained. Several criteria, including urinary and blood metabolite levels and activities of several key enzymes, were used to characterize the sequence of events associated with the development and onset of a metabolic thiamine deficiency.

MATERIALS AND METHODS

Animals and diet. Male Holstein calves one week of age and weighing between 36 and 45 kg were obtained from either the University herd, the stockyards, or local dairymen. They were kept in individual circular wire pens, fitted with urine collection pans, and housed in an air conditioned room maintained at 20 to 22°.

Ten calves were used in the study. Two were used in a preliminary trial to evaluate the diet and the clinical and enzymatic techniques to be used, and 4 pairs of animals were used in the main part of the study.

A purified diet (table 1) similar to that used by Johnson et al. (11) was fed at 12-hour intervals. The lard was emulsified in water, stabilized with soya lecithin and

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[2] Data from a thesis submitted by the senior author in partial fulfillment of requirements for the Doctor of Philosophy degree.
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TABLE 1

Composition of the diet [1]

	Weight %
Vitamin-free casein, micro-pulverized	22.6
Lard [2]	9.9
Dextrose [3]	30.9
Lactose [4]	30.9
Salt mix IV + cobalt [5]	4.8
Supplementary salt mix [6]	0.3
Fat-soluble vitamins [7]	—

[1] Blended with water to 11.7% solids and fed at 12 kg/100 kg live weight/day.
[2] Silver Leaf, Swift and Company, Chicago, added as an emulsion (25%) in water and stabilized with 1% lecithin. The mixture was heated to 90° and homogenized at 125 kg/cm².
[3] Cerelose 2001, Corn Products Company, New York.
[4] Edible lactose grade B, Foremost Dairies, Inc., San Francisco.
[5] Phillips, P. H., and E. B. Hart 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109*: 657. The salt mixture contained: (in per cent) dipotassium phosphate, 32.2; calcium carbonate, 30.0; sodium chloride, 16.7; magnesium sulfate (hydrate), 10.2; calcium phosphate ($CaHPO_4 \cdot 2H_2O$), 7.5; ferric citrate, 2.75; manganese sulfate, 0.51; potassium iodide, 0.08; copper sulfate, 0.03; zinc chloride, 0.025; and cobalt chloride, 0.005. Obtained in premixed form from Nutritional Biochemicals Corporation, Cleveland.
[6] MgO, 9.70%; $CuSO_4 \cdot 5H_2O$, 0.53%; $MnSO_4 \cdot 5H_2O$, 1.54%; $ZnSO_4 \cdot 7H_2O$, 0.60%; and K_2HPO_4, 87.61%.
[7] 3,600,000 IU vitamin A and 450,000 IU vitamin D Quadrex 20, Napco Chemical Company, Harrison, New Jersey); 100 IU vitamin E acetate (α-tocopherol powder, 250 IU vitamin E/g); and 2.5 mg menadione/kg diet.

TABLE 2

B-vitamin mixture [1]

	mg/100 ml
Thiamine·HCl	122.9
Riboflavin	245.8
Pyridoxine·HCl	122.9
Ca pantothenate	491.6
Choline·Cl	49,166.0
Folic acid	83.3
Biotin [2]	1.9

[1] Fed at a rate of 1.0 ml/kg liquid diet.
[2] Taken up in minimum 95% ethanol before added to the mixture.

stored separately from the mixed, dry non-fat ingredients until feeding. The liquid diet was prepared by mixing the powder and fat emulsion in water (38°) to 11.75% solids and fed at levels of 12 kg/100 kg of live weight. A vitamin B mixture (table 2) was added to the diet just before it was offered in nipple pails.

The level of vitamin intake shown in table 2 was selected after results of the preliminary study indicated that the level of vitamin B supplementation used previously (11) was inadequate. At this intake urinary thiamine excretion was very low and blood metabolite levels became elevated after 2 weeks (figs. 1 and 2). Therefore, the vitamin intake was adjusted to that shown in table 2.

During the course of the preliminary trial it was noted that the activities of several enzymes changed with age. Thereafter, control and deficient animals were paired by age and weight. Both animals were fed the complete diet and after they

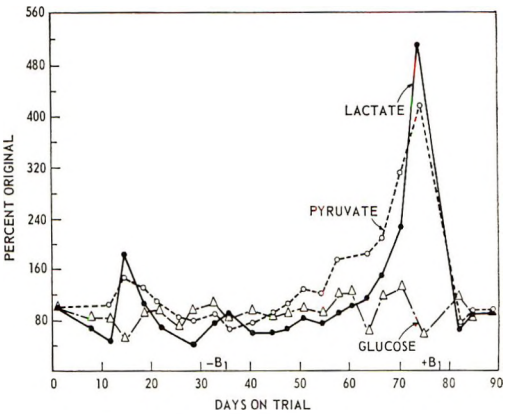

Fig. 1 Relative blood metabolite levels. The vitamin B supplement doubled on day 15. Thiamine removed from diet on day 35 ($-B_1$) and added on day 75 ($+B_1$). The data plotted are the averages of 2 animals, presented as percentage of the values on day 1. The average values on day 1 were 160, 15, and 1 mg/100 ml for glucose, lactate, and pyruvate, respectively.

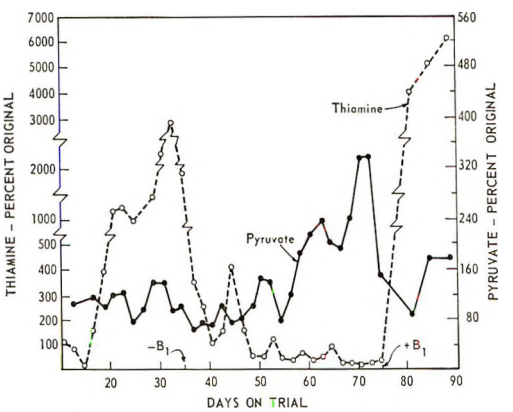

Fig. 2 Relative urinary excretion levels of thiamine and pyruvate. Vitamin B supplement doubled on day 15. Thiamine removed from diet on day 35 ($-B_1$) and added on day 75 ($+B_1$). Data plotted are the averages of 2 animals, presented as a percentage of the values on days 10 and 11. The average daily excretion levels at that time were 42 μg and 14 mg, respectively, for thiamine and pyruvate.

had adjusted to it, thiamine was removed from the vitamin B supplement of the deficient member of the pair. When anorexia developed as a result of the deficiency the animals were force-fed in order to maintain the same relative dietary loads in the deficient and control animals.

Clinical methods. Twice weekly, 2 hours after the morning feeding, jugular blood was collected. Microhematocrit values were calculated after blood was centrifuged at 5,000 × *g* for 10 minutes. Hemoglobin was determined by 2 methods: the one described by Evelyn (12) and the cyanmethemoglobin method as outlined by Benjamin (13). Crystalline bovine hemoglobin was used as a reference standard in the latter method. Glucose was determined by the method of Klein and Weissman (14) and lactate by the method outlined by Barker and Summerson (15). Pyruvic acid was determined by a modification of the method described by Bonting (16). By these methods the recovery of glucose, lactate, and pyruvate standards added to whole blood was 95 to 100%.

On alternate days total urine was collected and analyzed for pyruvic acid by the same procedure as that for blood. Urinary thiamine was determined by the method of Mickelsen et al. (17). Recovery of added thiamine was 95 to 99%.

Enzymatic methods. The enzymes listed in table 3 were selected as indicators of thiamine-dependent metabolism and pentose cycle, Embden-Meyerhof, and related metabolic activities. Liver samples for the enzymatic assays were obtained weekly by biopsy 2 hours after the morning feeding. A biopsy tool of 5-mm inside diameter × 34-cm long (18) was inserted through a small incision made either in the eleventh or twelfth intercostal space, and a liver sample weighing 0.5 to 1.0 g was removed. The liver sample was homogenized in 9 volumes of ice cold 0.14 N KCl in a Potter-Elvehjem-type homogenizer. A portion of the homogenate was removed for the pyruvate dehydrogenase assay and the remainder was centrifuged at zero to 4° for 30 minutes at 27,000 × *g*. The supernatant was removed and used as the enzyme source for all assays except pyruvate dehydrogenase.

All assays except pyruvate dehydrogenase were made in a Beckman model DU monochromator fitted with a Gilford model 2000 multiple sample absorbance recording system. The cuvette chamber temperature was maintained at 30 ± 1°. All the direct spectrophotometric assays were shown to be linear with respect to enzyme concentration over the range of rates reported. The details of the individual assay systems are shown in table 3. Some modifications of the original methods were made to obtain maximal enzyme activities. The pH optimum for the assay of calf transketolase activity was found to be 7.8 rather than 9.0 as reported for the rat enzyme (19). The transketolase and pyruvate dehydrogenase assays were made with and without preincubation of the enzyme with thiamine pyrophosphate (10 to 20 μg/ml) to determine whether decreases in enzyme activities were due to decreases in active enzyme or to incomplete cofactor saturation.

The pyruvate dehydrogenase assay system used was a modification of that developed by Monfoort (20) but included the use of acetaldehyde as suggested by Juni and Heym (21). Using the levels of ingredients listed in table 3 the reaction was carried out as described below. All components were pipetted into a 50-ml Erlenmeyer flask, which was then capped with a rubber serum stopper. The flask was evacuated and flushed with nitrogen 4 or 5 times and incubated for 3 hours at 37°. The reaction was stopped with 1 ml of 5% trichloroacetic acid, heated for 3 minutes on a steam bath, and chilled. One milliliter of water was added to the contents and the mixture was centrifuged at 27,000 × *g* for 20 minutes. A 4-ml aliquot of supernatant was removed and aerated for 12 to 15 hours to eliminate the adverse effect of acetaldehyde on the color reaction (22). One-fourth milliliter of 1 N NaOH was added to each of the test tubes and the volume was adjusted to 6 ml with distilled water. Acetoin was measured by the method devised by Westerfeld (23). Enzyme activity was estimated from a standard curve because early experiments with this system indicated that acetoin production was not linearly related to enzyme concentration. The pH optimum of

TABLE 3

Enzyme methods

Enzyme	Assay mixture	Volume	Wave length	Reference
		ml	*mμ*	
Glucose-6-phosphate dehydrogenase; 0.01 ml of 10% homogenate	Glycylglycine, 5.0 μmoles, pH 7.5; TPN, 0.1 μmole; magnesium chloride, 1.0 μmole; glucose-6-phosphate, 0.6 μmole	0.3	340	(35)
Isocitric dehydrogenase; 0.01 ml of 2% homogenate	Glycylglycine, 4.6 μmole, pH 7.5; TPN, 0.093 μmole; magnesium chloride, 0.93 μmole; manganese chloride, 1.0 μmole; isocitrate, 2.0 μmole	0.3	340	(41)
Malic enzyme; 0.03 ml of 10% homogenate	Tris-HCl, 44.0 μmole; pH 7.5; TPN, 0.3 μmole; manganese chloride, 0.5 μmole; L(+)-malic acid, 1.5 μmole, pH 7.0	0.3	340	(41)
Transketolase [1]; 0.03 ml of either 5 or 10% homogenate	Potassium arsenate, 5.1 μmole, pH 7.8; glycine, 8.1 μmole, pH 7.8; cysteine, 6.0 μmole, pH 7.8; DPN 1.5 μmole; magnesium chloride, 1.0 μmole; glyceraldehyde-3-phosphate dehydrogenase, 1.5 units; ribose-5-phosphate, 1.0 μmole	0.3	340	(19)
Aldolase; 0.01 ml of 2% homogenate	Potassium arsenate, 5.1 μmole, pH 7.8; glycine, 8.1 μmole, pH 7.8; cysteine, 6.0 μmole, pH 7.8; DPN, 1.5 μmole; magnesium chloride, 1.0 μmole; glyceraldehyde-3-phosphate dehydrogenase, 15 units; fructose 1,6 diphosphate, 2.0 μmole	0.3	340	(36)
Anaerobic pyruvic dehydrogenase; 0.5 ml of 10% whole homogenate	Sodium phosphate, 180 μmole, pH 7.5; sodium pyruvate, 40 μmole; acetaldehyde, 20 μmole	3.0	525	(text)

[1] Xylulose-5-phosphate was not available. This assay system may be designated better as "pentose phosphate metabolizing enzymes" (16).

TABLE 4
Requirements of the anaerobic pyruvic dehydrogenase system

Assay mixture	Relative rate
Complete system	100
Complete system minus TPP [1]	59
Complete system minus homogenate	14
Complete system minus TPP and homogenate	12
Complete system minus TPP and acetaldeyhde	13
Complete system minus TPP and pyruvate	20
Complete system minus TPP, pyruvate, and acetaldehyde	12

[1] Thiamine pyrophosphate; 10 μg/flask when added.

the assay was broad; therefore, a pH of 7.5 was used. The requirements of the pyruvate dehydrogenase assay are presented in table 4. Enzyme, pyruvate, and acetaldehyde were required and thiamine pyrophosphate did not catalyze the reaction in the absence of enzyme.

RESULTS

After the adjustment in vitamin intake the diet appeared to be satisfactory, because the 2 calves used in the preliminary experiment gained an average of 0.36 kg/day over the 90 days of the experiment. The control calves used in the later phase of the experiment gained 0.27 kg/day. The levels of blood pyruvate and lactate in control calves were normal throughout the trial.

Results from the preliminary trial are shown in figures 1 and 2. The pattern of thiamine excretion after the adjustment in vitamin intake (fig. 2) indicated that the tissues were becoming saturated with thiamine, since the urinary levels increased steadily even while the intake remained reasonably constant. When thiamine was removed from the diet on day 35 its excretion decreased markedly and remained less than 10% of normal for 20 to 25 days before deficiency symptoms became apparent. Urinary pyruvate excretion had increased threefold by day 36 after removal of thiamine from the diet. Blood pyruvate and lactate levels remained normal for a considerable period of time and then increased markedly to about 400 and 500% of initial values after the deficient diet had been fed for 40 days. Anorexia developed in both animals by day 36 and 2 to 3 days later the heart developed a distinct arrhythmia. Breathing was affected so that the animals respired rapidly 4 to 5 times and then rested for a short period. Each expiration appeared to require considerable effort and was accompanied by a groan. During this period profuse lacrimation and a continual grinding action of the jaws were observed in both animals.

In the preliminary trial, liver "transketolase" activity (see note table 3) had decreased to approximately 70% of original by day 40 after thiamine had been removed from the diet. The deficiency did not appear to affect the activity of glucose-6-phosphate dehydrogenase or isocitrate dehydrogenase. However, malic enzyme activity was markedly reduced by the time that the animals exhibited the high blood metabolite levels. On the basis of substantial increases in levels of blood lactate and blood and urinary pyruvate, and reductions in liver "transketolase" activity, the animals were diagnosed as being deficient on day 40 after thiamine was removed from the diet (day 75 of the trial). All deficiency symptoms disappeared completely after the animals were supplemented with thiamine.

The clinical and enzymatic results from studies with 4 pairs of animals are presented in figures 3, 4, 5, and 6. The average values for the 4 pairs of animals were expressed as percentages of control values and were plotted from a common origin arbitrarily called day zero, which was the day that animals were diagnosed as thiamine deficient. The relative levels of blood pyruvate and lactate (fig. 3) increased markedly at onset on the deficiency, whereas the levels of glucose appeared to be unaffected. At the time of the deficiency, the excretion of thiamine was below 1% of control values, whereas that of pyruvate was 6 times that in control calves (fig. 4). The data shown for the period following the addition of thiamine (day zero) represent only one pair of animals since 2 pairs were killed to complete an isotope experiment [4] and one

[4] Benevenga, N. J., R. L. Baldwin, M. Ronning and A. L. Black. Pyruvate metabolism in thiamine-deficient calves (to be published).

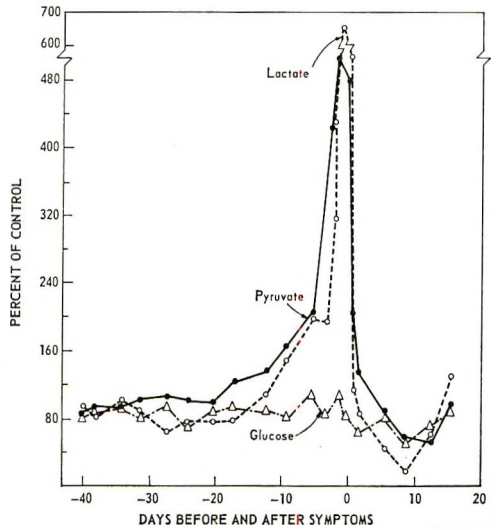

Fig. 3 Relative blood metabolite levels as a percentage of each animal's respective control. Averages of 4 deficient-control pairs. Day 0 is the day of diagnosed deficiency. Average control values on day — 40 were 160, 13, and 1 mg/100 ml, respectively, for glucose, lactate, and pyruvate.

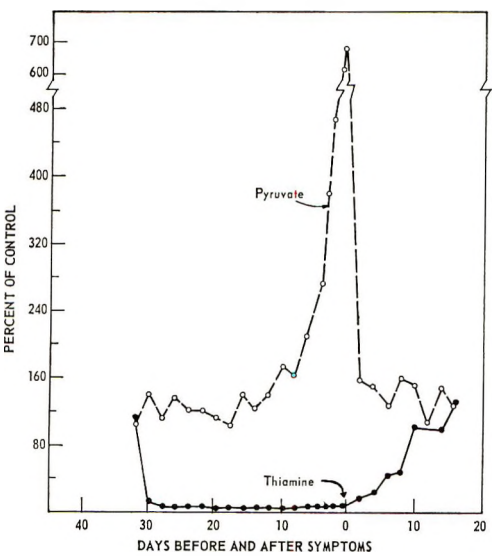

Fig. 4 Relative levels of urinary metabolites as a percentage of each animal's respective control. Averages of 4 deficient-control pairs. Day 0 is the day of diagnosed deficiency. Average control values on day 32 were 15 mg/day and 900 (300–2000) μg/day for pyruvate and thiamine, respectively.

animal died during biopsy. The data presented in figures 1, 3, and 4 suggested that the metabolic breakdown, once initiated, became critical within 3 to 5 days.

Average hemoglobin and hematocrit levels decreased 16 and 22%, respectively, by the time calves became deficient. These values were significantly different from the initial values at the 2 and 5% levels of probability, respectively, when analyzed as paired data. Differential cell counts were not changed from those of normal animals.

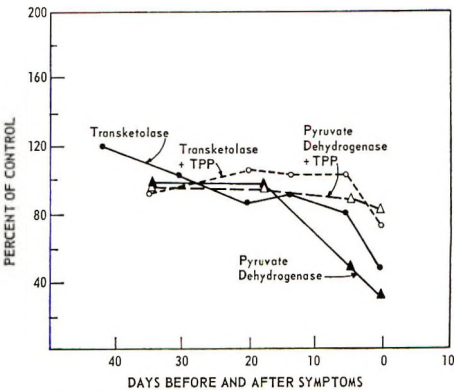

Fig. 5 Relative liver enzyme activities as a percentage of control. Transketolase (TK) and pyruvate dehydrogenase (PVAD) and transketolase (TK + TPP) and pyruvate dehydrogenase (PVAD + TPP) after incubation with thiamine pyrophosphate. Day 0 is the day of diagnosed deficiency.

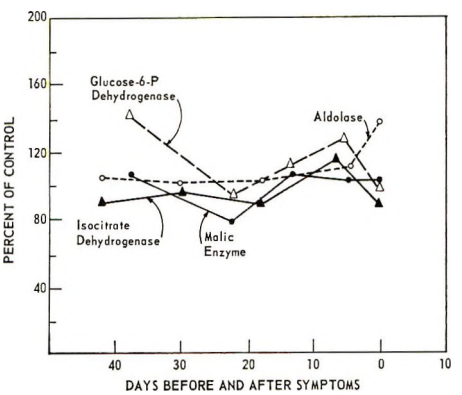

Fig. 6 Relative activities of liver aldolase (ALD), glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME), and isocitrate dehydrogenase (ICD) as a percentage of control. Day 0 is the day of diagnosed deficiency.

The activities of the thiamine-requiring enzymes were substantially reduced at the time of the deficiency. "Transketolase" and pyruvate dehydrogenase activities were 49 and 32%, respectively, of control values (fig. 5). The major factor causing this reduction in activity appeared to be the limitation of cofactor since these values were 74 and 83% of the control values when the homogenates were preincubated with thiamine pyrophosphate. Of the non-thiamine-requiring enzymes only the activity of aldolase appeared to be affected, since its activity was 140% of control values (fig. 6).

On the basis of all criteria, based on increased blood pyruvate and lactate levels it appeared that the calves became metabolically deficient in thiamine between 27 and 48 days after thiamine was removed from their diets. This was substantiated by other clinical criteria and by decreased activities of transketolase and pyruvate dehydrogenase.

DISCUSSION

Thiamine deficiency symptoms observed in this study were similar to those reported for a number of other species. Anorexia has been reported in the deficient calf (11, 24), lamb (25), horse (26), chick (27), pig (28), and rat (29). Bradycardia and heart abnormalities have been associated with thiamine deficiency (23, 25–28); however, there is a question whether bradycardia is a specific symptom resulting primarily to a thiamine deficiency (27, 28), or whether it is caused by lowered feed intake since chicks force-fed thiamine-deficient diets rarely have exhibited this symptom (27). Heart arrhythmia was observed in all deficient calves even though the effects of food intake were prevented by force-feeding. A respiratory abnormality developed in four of the deficient calves and appeared to be similar to that reported in thiamine-deficient horses (26). The respiratory problem observed may have been due to acidosis caused by high lactate and pyruvate levels in the blood.

The most marked changes that occurred in the deficient calves were the increases in blood lactate and blood and urinary pyruvate. Blood pyruvate increased from a normal of 1 mg/100 ml to an average close to 5 mg/100 ml. Similar high blood pyruvate concentrations have been reported for a large number of species (11, 24, 26, 28, 30–33). In this study blood lactate levels of deficient calves increased from a normal of 15 mg/100 ml to an average of 100 (85–200) mg/100 ml. These levels were considerably higher than those previously reported for the calf, 40 mg/100 ml (24) and dog, 60 mg/100 ml (32). Urinary pyruvate excretion increased from a normal of about 15 to an average of 80 (50–140) mg/day, elevations similar to those reported previously for deficient calves (11, 24) and lambs (25).

The length of time required for the animals to become deficient was about the same as that reported previously for calves (11, 24) and lambs (25) but slightly longer than that required for young pigs (30). There appeared to be no relationship between the level of urinary thiamine at the time of removal of thiamine from the diet and the length of time before the appearance of clinical symptoms. However, there was a close and positive correlation between the length of time that calves were fed the control diet and the time required to deplete the animals of their thiamine stores to the point at which deficiency symptoms became manifest. Furthermore, there was a positive correlation between the length of time that the control diet had been fed and the period of time elasped between the time when daily urinary thiamine excretion had decreased below 50 μg/day and the day when the deficiency was definitely diagnosed. These observations suggested that the amount of thiamine stored depended on the lentgh of time that the control diet had been fed.

The deficiency affected the activity of liver pyruvate dehydrogenase to a greater extent than the activity of "transketolase" (fig. 5). The depression in liver "transketolase" activity was similar to that observed in rat liver (19, 34, 35). However, the actual depression of "transketolase" activity may have been greater than that observed due to the nature of the assay system employed (19).

Decreases in liver pyruvate dehydrogenase activity similar to those shown in fig-

ure 5 have been observed in the livers of thiamine-deficient rats (31, 36, 38) and in breast and heart muscles of pigeons fed thiamine-free, high carbohydrate diets (20). A major portion of the activity was restored when thiamine pyrophosphate was added to the medium. The percentage stimulation of pyruvate dehydrogenase (pyruvate dehydrogenase + thiamine pyrophosphate)/pyruvate dehydrogenase × 100 was not positively correlated significantly with time the diet was fed to control animals but was ($P < 0.01$) in the deficient ones, indicating that the stimulation did not change in the control, whereas it increased with time in the deficient calves. The average percentage stimulation of pyruvate dehydrogenase by thiamine pyrophosphate was significantly greater ($P < 0.02$) for the deficient calves (460%) relative to that of their controls (180%) at the time of diagnosed deficiency. The nearly twofold stimulation of pyruvate dehydrogenase in control calves by thiamine pyrophosphate indicated that the apoenzyme was only about 50% saturated with cofactor within the cell. Similar stimulations have been observed in heart and breast muscle of pigeons deficient in thiamine (20, 38).

In contrast with pyruvate dehydrogenase, tranketolase activity of liver extracts from normal calves was not uniformly affected by preincubation with thiamine pyrophosphate. However, since the observed depression of liver transketolase activity in the deficient calves was reversed by preincubation with thiamine pyrophosphate it appeared that cofactor saturation of the transketolase apoenzyme was also lower in deficient than control calves.

The activity of glucose-6-phosphate dehydrogenase was expected to decrease along with that of "transketolase" because "transketolase" and glucose-6-phosphate dehydrogenase activities have been reported to increase and decrease together in rats in various physiological conditions (19) including thiamine deficiency.[5] The absence of a decrease in glucose-6-phosphate dehydrogenase in the calves may have been related to the length of time that the animals were metabolically deficient in thiamine. The calves had just entered the period of deficiency when sampled, whereas the rats had been allowed to lose weight for 3 to 4 days before the activities of the enzymes were evaluated. What appeared to be an increase in aldolase activity (fig. 6) may reflect an increased ability to metabolize glucose via the Embden-Myerhof pathway, and may indicate an adaptive response to a decrease of pentose cycle activity as a result of the depression in "transketolase" activity. Alternatively, increased activity of the Cori cycle may be indicated.

The data presented in figures 3, 4 and 5 indicate that the activity of pyruvate dehydrogenase decreased to about 50% of normal before elevated pyruvate levels were observed; however, this does not establish a direct cause-and-effect relationship between liver pyruvate dehydrogenase activity and blood lactate and pyruvate levels. Alterations in muscle metabolism could be the cause of elevated metabolite levels. The rate of loss of pyruvate dehydrogenase activities from liver and muscle may not be similar since it has been shown that heart loses pyruvate dehydrogenase activity faster than breast muscle in pigeons fed a thiamine-free diet (20).

The results of this study indicate that comprehensive studies of thiamine-deficient animals require the use of many techniques. Many of the indexes of thiamine deficiency are not effective when considered alone. For example, thiamine excretion reached a minimum a long time before the animals exhibited clinical symptoms of thiamine deficiency, and cellular thiamine pyrophosphate levels can decrease markedly before an effect on enzyme activity is observed (34–41). Also, enzyme activities decreased significantly before elevations of lactate and pyruvate in body fluids were observed.

LITERATURE CITED

1. Jolliffe, N., R. Goodhart, J. Ginnis and J. K. Cline 1939 The experimental production of vitamin B_1 deficiency in normal subjects. The dependence of the urinary excretion of thiamine on the dietary intake of vitamin B_1. Amer. J. Med. Sci., *198:* 198.
2. Mason, H. L., and R. D. Williams 1942 The urinary excretion of thiamine as an index of the nutritional level. Assessment of the

[5] Benevenga, N. J., and R. A. Freedland 1965 Effect of a thiamine deficient diet on the activity of non-thiamine requiring enzymes in rat liver. Federation Proc., 24: 690 (abstract).

value of a test dose. J. Clin. Invest., *21:* 247.

3. Mickelsen, O., W. D. Caster and A. Keys 1947 A statistical evaluation of the thiamine and pyramin excretions of normal young men on controlled intakes of thiamine. J. Biol. Chem., *168:* 415.
4. Burch, H. B., O. A. Bessey, R. H. Love and O. H. Lowry 1952 The determination of thiamine and thiamine phosphates in small quantities of blood and blood cells. J. Biol. Chem., *198:* 477.
5. Baker, H., O. Frank, J. J. Fennelly and C. M. Leevy 1964 A method for assaying thiamine status in man and animals. Amer. J. Clin. Nutr., *14:* 197.
6. Bessey, O. A. 1954 Evaluation of vitamin adequacy: Blood levels. Methods of evaluation of nutritional adequacy and status, Quartermaster Food and Container Institute, Chicago, p. 59.
7. Stotz, E., and O. A. Bessey 1942 The blood lactate-pyruvate relation and its use in experimental thiamine deficiency in pigeons. J. Biol. Chem., *143:* 625.
8. Horwitt, M. K., and O. Kreisler 1949 The determination of early thiamine-deficiency status by estimation of blood lactic and pyruvic acids after glucose administration and exercise. J. Nutr., *37:* 411.
9. Brin, M. 1962 Erythrocyte transketolase in early thiamine deficiency. Ann. N. Y. Acad. Sci., *98:* 528.
10. Pearson, W. N. 1962 Biochemical appraisal of nutritional status in man. Amer. J. Clin. Nutr., *11:* 462.
11. Johnson, B. C., T. S. Hamilton, W. B. Nevens and L. E. Boley 1948 Thiamine deficiency in the calf. J. Nutr., 35: 137.
12. Evelyn, K. A. 1936 A stabilized photoelectric colorimeter with light filters. J. Biol. Chem., *115:* 63.
13. Benjamin, M. M. 1961 Outline of Veterinary Clinical Pathology. Iowa State University Press, Ames, pp 56–57.
14. Klein, B., and M. Weissman 1953 New color reagent for determination of hexoses. Determination of glucose in blood serum. Anal. Chem., 25: 771.
15. Barker, S. B., and W. H. Summerson 1941 The colorimetric determination of lactic acid in biological material. J. Biol. Chem., *138:* 535.
16. Bonting, S. L. 1955 Colorimetric determination of pyruvic acid and other α-keto acids in submicrogram quantities. Arch. Biochem. Biophys., *58:* 100.
17. Mickelsen, O., H. Condiff and A. Keys 1945 The determination of thiamine in urine by means of the thiochrome technique. J. Biol. Chem., *160:* 361.
18. Hughes, J. P. 1962 A simplified instrument for obtaining liver biopsies in cattle. Amer. J. Vet. Res., 23: 1111.
19. Benevenga, N. J., W. J. Stielau and R. A. Freedland 1964 Factors affecting the activity of pentose phosphate metabolizing enzymes in rat liver. J. Nutr., 84: 345.
20. Monfoort, C. H. 1955 The disappearance of pyruvic decarboxylase and α-ketoglutaric decarboxylase from pigeon muscles on thiamine-deficient diets. Biochim. Biophys. Acta, *16:* 219.
21. Juni, E., and G. A. Heym 1956 Acyloin condensation reactions of pyruvic oxidase. J. Biol. Chem., *218:* 365.
22. Yatco-Manzo, E., F. Roddy, R. G. Yount and D. E. Metzler 1959 The catalytic decarboxylation of pyruvate by thiamine. J. Biol. Chem., *234:* 733.
23. Westerfeld, W. W. 1945 A colorimetric determination of blood acetoin. J. Biol. Chem., *161:* 495.
24. Blaxter, K. L., and J. A. F. Rook 1955 Energy and carbohydrate metabolism in magnesium-deficient calves. Brit. J. Nutr., *9:* 121.
25. Draper, H. H., and B. C. Johnson 1951 Thiamine deficiency in the lamb. J. Nutr., *43:* 413.
26. Carroll, F. D., H. Goss and C. E. Howell 1949 The synthesis of B vitamins in the horse. J. Animal. Sci., 8: 290.
27. Sturkie, P. D. 1954 Effects of acute thiamine deficiency on the electrocardiogram of the chick. Poultry Sci., 33: 508.
28. Wintrobe, M. M., R. Alcayaga, S. Humphreys and R. H. Follis, Jr. 1943 Electrocardiographic changes associated with thiamine deficiency in pigs. Johns Hopkins Hosp. Bull., 73: 169.
29. Veen, M. J. 1963 Anorexia and gastric emptying in thiamine-deficient rats. Can. J. Biochem. Physiol., *41:* 826.
30. Forbes, R. M. 1952 Blood pyruvate as a criterion of thiamine deficiency of growing swine. J. Animal Sci., *11:* 761.
31. Gubler, C. J. 1961 Studies on the physiological functions of thiamine. I. The effects of thiamine deficiency and thiamine antagonists on the oxidation of α-keto acids by rat tissues. J. Biol. Chem., 236: 3112.
32. Chesler, A., E. Homburger and H. E. Himwich 1944 Carbohydrate metabolism in vitamin B_1 deficiency. J. Biol. Chem., *153:* 219.
33 Lofland, H. B., Jr., H. O. Goodman, T. B. Clarkson and R. W. Prichard 1963 Enzyme studies in thiamine-deficient pigeons. J. Nutr., 79: 188.
34. Fennelly, J., O. Frank, H. Baker and C. M. Leevy 1964 Transketolase activity in experimental thiamine deficiency and hepatic necrosis. Proc. Soc. Exp. Biol. Med., *116:* 875.
35. Brin, M. 1962 Effects of thiamine deficiency and of oxythiamine on rat tissue transketolase. J. Nutr., *78:* 179.
36. Oshima, T., J. Hayashi, A. Maekawa, K. Ito, T. Suzuki and Y. Sahashi 1960 Decrease of apoenzyme activities in vitamin-deficient animals. I. Decrease of pyruvate-apooxidase

activity in vitamin B_1-deficient rats. J. Vitaminol., 6: 117.
37. Wright, R. C., and E. M. Scott 1954 Pyruvate and α-ketoglutarate metabolism in thiamine deficiency. J. Biol. Chem., 206: 725.
38. Franken, J. F., and F. P. Stapert 1954 Restoration of pyruvate breakdown in pigeon muscle homogenates impaired by thiamine deficiency. Biochim. Biophys. Acta, *14*: 293.
39. Glock, G. E., and P. McLean 1953 Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J., 55: 400.
40. Colowick, S. P., and N. Kaplan, eds. 1955 Methods in Enzymology, vol. 1. Academic Press, New York, pp. 310, 699, 739.
41. Olson, R. E., O. H. Pearson, O. N. Miller and F. J. Stare 1948 The effect of vitamin deficiencies upon the metabolism of cardiac muscle *in vitro*. I. The effect of thiamine deficiency in rats and ducks. J. Biol. Chem., *175*: 489.

Comparative Effects of Dihydrotachysterol$_2$ and Dihydrotachysterol$_3$ in Chicks [1]

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ABSTRACT Crystalline dihydrotachysterol$_2$ (DHT_2) was compared with vitamins D_2 and D_3 and with dihydrotachysterol$_3$ (DHT_3) over a wide dose range in chicks fed a rachitogenic diet. Effects on mineral metabolism were evaluated by following changes in serum calcium and phosphorus levels and in percentage bone ash. Both DHT_2 and DHT_3 possessed potent antirachitic and toxic actions, with a very narrow therapeutic range. DHT_3 was only very slightly more potent than DHT_2 in chicks in marked contrast with the tenfold potency difference of the parent molecules, vitamins D_3 and D_2, in this species.

The potency difference in chicks between both antirachitic (1) and hypercalcemic (2) doses of vitamins D_2 and D_3 has been systematically re-investigated using crystalline sterols given daily in an oral dosing regimen. Although minor qualitative differences were noted, the overall conclusion of these studies was that vitamin D_3 was more potent than vitamin D_2 in the chick by a factor of about 10:1 at both low and high dose levels. This potency ratio is lower than the ratios given in any of the earlier studies referred to previously (1), in which the values for the ratio range from 15:1 to 400:1. The use of crystalline vitamins, however, was not specified except in one instance.

In the present study, these experiments have been extended to include crystalline dihydrotachysterol$_2$ (DHT_2) and dihydrotachysterol$_3$ (DHT_3) which are reduction products, chemically related to vitamins D_2 and D_3, respectively. As evident from the structural formulas shown in figure 1, the reduction of a vitamin D to a dihydrotachysterol is characterized by conversion of the C-10 methylene group to a methyl group with an accompanying stereochemical rotation of the ring (A) on which it resides. No change occurs in the side chain, which is the only feature that distinguishes vitamin D_2 and DHT_2 from vitamin D_3 and DHT_3, respectively. The vitamin D_2 side chain is that of ergosterol, whereas vitamin D_3 has the same side chain as cholesterol, with no double bond between C_{22} and C_{23} and no methyl group on C_{24}.

While DHT_2 and DHT_3 have not yet been chemically identified in natural sources, owing to the extremely small concentrations of any of the calcemic sterols in most biological materials, practically all of the available evidence concerning the distribution of these sterols in nature has been obtained by biological assay methods. However, the high concentration in certain fish liver oils has permitted chemical identification of vitamin D in such sources. Although the DHT's are probably not present in food nor formed in vivo, their investigative importance resides in their structural similarity to vitamin D and their extremely potent hypercalcemic activity.

Dihydrotachysterol$_2$ was originally introduced clinically as a substitute for parathyroid hormone owing to its hypercalcemic action (3). Preparations available for clinical use in this country [2] were formerly prepared by reduction of vitamin D_2 irradiation mixtures, required standardization by bioassay, and were found upon physicochemical analysis (4) to consist mainly of dihydrovitamin D_2-II a sterol

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[2] Hytakerol, marketed by Winthrop Laboratories, New York (see references 4 and 5).

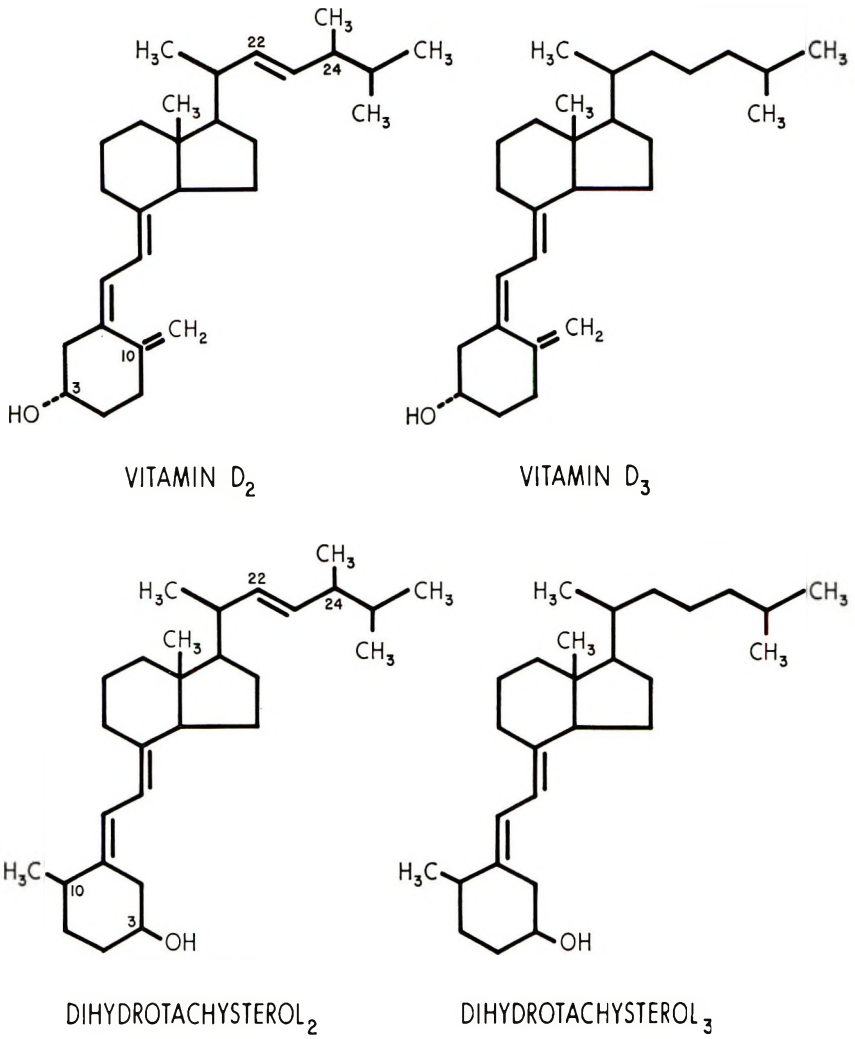

Fig. 1 Structural formulas of vitamin D_2, vitamin D_3 dihydrotachysterol$_2$ and dihydrotachysterol$_3$.

possessing only one-fifth to one-sixth the hypercalcemic potency of DHT_2 in rats and dogs (5). Therefore it is extremely difficult to properly evaluate the earlier studies of "dihydrotachysterol" action in view of the fact that the particular preparation used (Hytakerol,[3] AT-10) may have been variable in potency (4) and perhaps consisted, in fact, of something other than crystalline DHT_2.

Nevertheless, an interesting aspect of "DHT" action reported for chicks (6) deserves confirmation and extension. Despite the significant potency difference in the chick between vitamins D_2 and D_3 (which differ chemically only in their side chains), it was observed (6–8) that the potency of DHT_2 more closely resembled that of vitamin D_3 than of vitamin D_2, as if the reduction of ring B in the sterol nucleus counteracted, to a large degree, the reduction in potency caused by the side chain. The present availability of both crystalline DHT_2 and DHT_3 allows

[3] See footnote 2.

definite resolution of this question. Experiments conducted for this purpose are described in the present report.

METHODS

The general experimental approach is similar to that used previously in studies with crystalline vitamins D (2). Male, White Leghorn chicks (Mt. Hope Queens) were received at one day of age and separated into groups of 8 to 14 animals. All chicks were kept in brooders in an air-conditioned room from which all ultraviolet irradiation was excluded by covering windows and overhead lighting with red cellophane and were fed a commercial "rachitogenic chick test diet" (1.43% calcium, 1.10% phosphorus)[4] and distilled water ad libitum.

After being fed this diet for 21 days, each chick was intubated daily with 0.1-ml doses of sesame oil with either nothing added (rachitic control) or containing graded doses of crystalline sterols.[5] Treatment with the sterols was continued for either 14 or 21 days. Periodically the body weights of each chick were recorded.

Food, but not water, was withdrawn 24 hours prior to killing. The chicks were decapitated and blood was collected from the severed neck. Serum calcium and serum phosphorus levels were analyzed by methods described previously (1). The 2 tibia from each chick were dissected out; one was analyzed for percentage of bone ash, the other was fixed in absolute ethanol for histological examination or microradiography, although results of the latter study are not given here. For bone ash analysis each tibia was broken in half and the bones from each group were placed together in a Soxhlet thimble, extracted for 48 hours with absolute ethanol and for an equal period of time with diethyl ether. The bones were then placed in a dessicator for 24 hours and carefully weighed. From this weighing, an average extracted bone weight was calculated. The bones were then dried in platinum dishes at 100°, flamed over a Meker burner to burn off the major fraction of volatile material, and ashed in a muffle furnace at 750° for 16 hours. The percentage of tibial ash weight was determined for each group using the formula: % bone ash = $\left(\frac{\text{ash weight}}{\text{extracted bone weight}} \times 100\right)$.

Two comparative studies were conducted. In the first study, DHT$_2$ was compared with vitamins D$_2$ and D$_3$ at daily dose levels ranging from 0.25 μg to 2.5 mg/day in logarithmic increments using a 14-day treatment period. In the second study, DHT$_2$ and DHT$_3$ were compared with each other at daily doses of 0.1 μg to 100 μg over a 21-day treatment period.

RESULTS

Comparative lethal effects of DHT$_2$, DHT$_3$ and vitamins D$_2$ and D$_3$. Survival curves are shown in figure 2 based on results from both series of comparative studies. Data are shown for groups of 8 chicks which, after being fed the rachitogenic diet for 3 weeks, were intubated daily with either 0.1 mg of DHT$_2$ or DHT$_3$ for a maximal period of 21 days, or given a higher dose of 2.5 mg DHT$_2$ or vitamins D$_2$ or D$_3$, with killing of survivors on the fourteenth day.

The results indicated that high doses of DHT$_2$ were considerably more toxic than either vitamins D$_2$ or D$_3$ and that DHT$_3$ was comparable to or slightly greater than DHT$_2$ in toxicity.

Comparison of DHT$_2$ with vitamins D$_2$ and D$_3$. Data are plotted in figure 3 for the various quantitative parameters measured or calculated for the 3 sterols in the experiment in which chicks were treated for 14 days.

Referring to the data on percentage bone ash, 10 IU (0.25 μg) of vitamin D$_2$ were not curative of rickets, whereas 0.25 μg DHT$_2$ gave even better percentage of bone ash values than vitamin D$_3$. Consideration of the data on extracted tibia weights, serum calcium levels and final body weights also suggests that DHT$_2$ was as antirachitic as vitamin D$_3$. Serum phosphorus levels with DHT$_2$ treatment, how-

[4] General Biochemicals, Inc., Chagrin Falls, Ohio. This diet is composed of: (in per cent) alfalfa meal, 5.5; soybean meal, 15.4; bone meal, 4.4; yellow corn, 34.4; wheat flour middlings, 28.4; dried skim milk, 9.4; cottonseed oil, 1.0; iodized salt, 1.0; and bone charcoal, 0.5. Our lot of diet contained 1.43% calcium and 1.10% phosphorus by analysis (namely, a calcium-to-phosphorus ratio of 1.3).

[5] Crystalline Vitamins D$_2$ and D$_3$, DHT$_2$ and DHT$_3$ were manufactured by Philips-Duphar, Weesp, Netherlands. DHT$_3$ was supplied through the courtesy of the manufacturer.

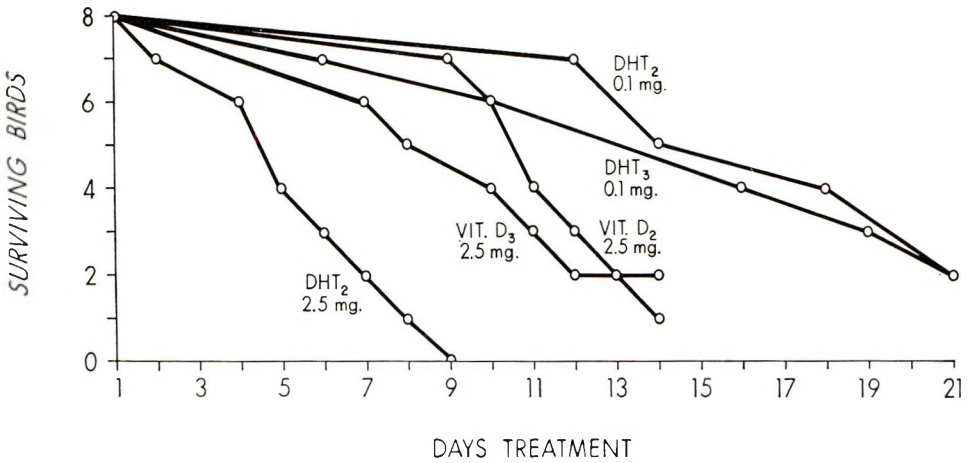

Fig. 2 Survival curves. Treatment begun after chicks had received rachitogenic diet for 21 days. Dose indicated is the daily dose.

ever, were not similar to those exhibited by vitamin D_3-treated birds and, at the 0.25-μg dose, the serum phosphorus level for the DHT_2-treated chicks was closer to that for the same dose of vitamin D_2. This observation is reflected by the serum Ca × P products. As the dose of sterols was increased, the anticipated fall in serum phosphorus level occurred earlier with DHT_2 and vitamin D_3 than with vitamin D_2, again indicating the resemblance between those 2 sterols.

Comparison of DHT_2 with DHT_3. Little quantitative difference was observed between crystalline DHT_2 and DHT_3 in their effects on the various quantitative parameters, as illustrated in figure 4 for the doses ranging from 0.1 to 100 μg administered daily for 21 days.

On the basis of growth and bone weights, the peak response was obtained with the daily dose of 10 μg. Doses below this level did not produce complete healing of the rachitic state as demonstrated by the lower percentage bone ash values and lower Ca × P products.

That the dihydrotachysterols have an extremely narrow therapeutic range is obvious because the toxic manifestations became evident at doses immediately higher than the 10-μg level. At 25 μg, growth and bone weights started decreasing, denoting toxicity, and serum phosphorus levels fell sharply at the 100-μg dose.

As pointed out previously for vitamins D_2 and D_3 (2) the serum calcium levels and percentage bone ash values were poor indicators of toxicity, showing relatively little response with toxic doses of the sterol. An important observation, however, was that with DHT treatment, the serum calcium levels remained relatively normal at a dose giving obvious toxicity (e.g., 25 μg) and then suddenly increased to over 16 mg/100 ml at the higher dose of 100 μg.

DISCUSSION

Several important points regarding the physiologic and pharmacologic actions of DHT_2 and DHT_3 in chicks are presented in this study. A major species difference between the chick and the rat in their response to the dihydrotachysterols is the powerful antirachitic activity shown by DHT_2 and DHT_3 in chicks. In rats, crystalline DHT_2 and DHT_3 have been reported (9) to be only 0.5% and 0.9% as active as vitamins D_2 or D_3 (both equipotent in rats). However, the dihydrotachysterols are more toxic and hypercalcemic than equivalent weights of the vitamins D in both rats (5, 9, 10) and in chicks as shown in this study.

The serum calcium level was a relatively poor indicator of the toxicity of DHT. It has been pointed out previously that this was true for rats (5) owing presumably to renal damage, which caused

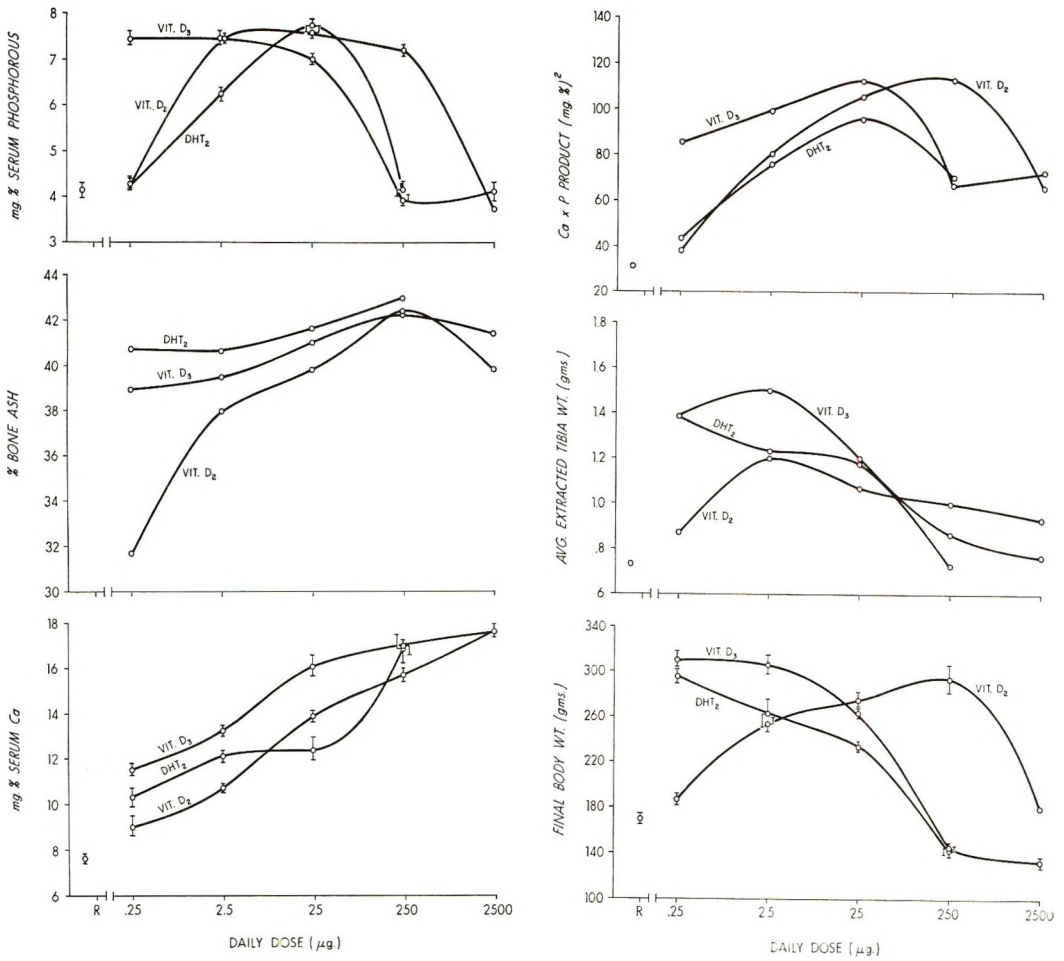

Fig. 3 Dose (log scale) of vitamins D_2 and D_3 and DHT_2 vs. (*top left*) serum phosphorus level; (*middle left*) percentage bone ash; (*bottom left*) serum calcium level; (*top right*) Ca × P product; (*middle right*) tibia weight; and (*bottom right*) final body weight. *R* refers to rachitic controls (means ± SE).

elevated serum phosphate and urea nitrogen levels. In chicks, however, hypophosphatemia rather than hyperphosphatemia was observed at high dosages of DHT; thus the serum phosphorus response to toxic doses of these sterols was different in the 2 species.

The serum calcium response to the dihydrotachysterols followed a step-by-step pattern as shown in figures 3 and 4. At DHT doses between 1 and 25 μg/day, the serum calcium remained at a plateau level of about 12 mg/100 ml and then increased rapidly to 16 to 17 mg/100 ml at doses of 100 to 250 μg/day. This is in distinct contrast with the serum calcium response to vitamins D_2 or D_3 which followed a rather smooth curve increasing gradually with dosage (2).

The explanation for the sudden rise in serum calcium level in response to the higher DHT doses could be that the mechanism by which these sterols evoke hypercalcemia is critically dose-dependent, and quite different from the mechanism by which low doses stimulate increased intestinal calcium absorption. In large measure the antirachitic action of the lower doses of DHT in chicks must be the result of a stimulation of intestinal calcium

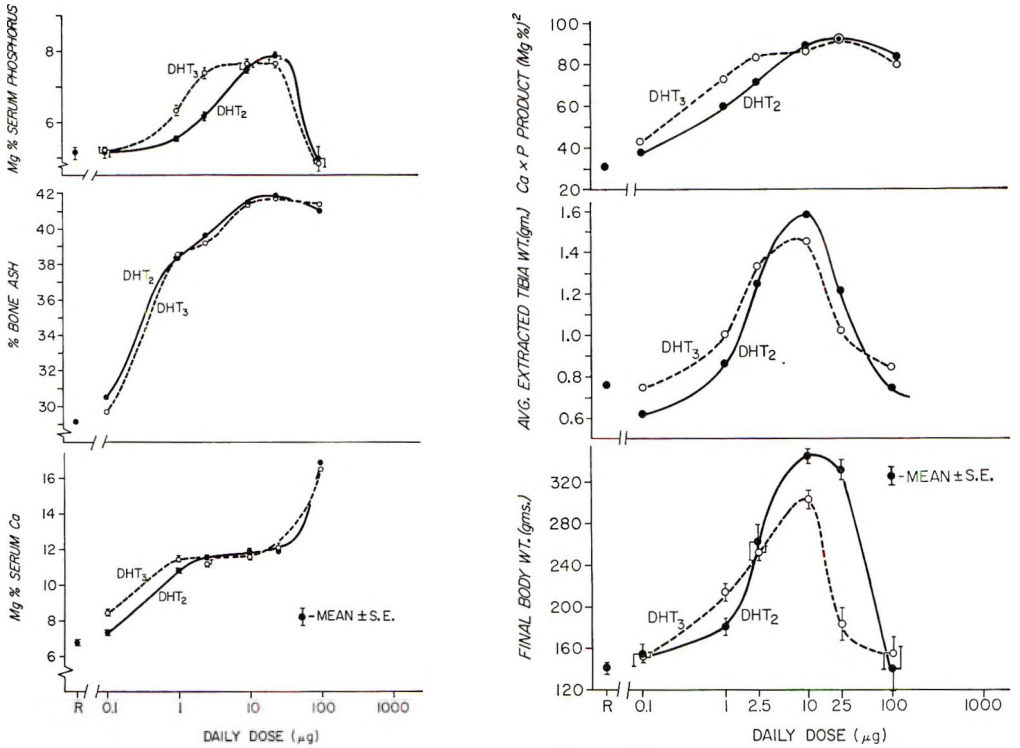

Fig. 4 Dose (log scale) of DHT_2 and DHT_3 vs. (*top left*) serum phosphorus level; (*middle left*) percentage bone ash; (*bottom left*) serum calcium level; (*top right*) Ca × P product; (*middle right*) tibia weight; and (*bottom right*) final body weight. *R* refers to rachitic controls.

transport; increased rates of appearance in serum of orally administered ^{45}Ca have been noted in DHT as well as vitamin D_3-treated chicks compared with rachitic controls (11). However, a direct bone action of DHT at low doses should also be considered (12).

Higher doses of the sterols produce toxic effects by hyperstimulation of intestinal calcium absorption and excessive bone resorption. The resultant tendency towards hypercalcemia may produce adverse effects either directly or indirectly (e.g., renal damage). These calcium movements result in decreased growth and smaller bones, despite high serum Ca × P products and normal percentage bone ash values.

It is at the higher dose levels that the response of the chick to DHT differs so much from its response to vitamin D. Previous studies in dogs (5) indicate a much more rapid induction and cessation of the hypercalcemic response to DHT_2 as compared with vitamin D_2. While the present data do not allow conclusions to be drawn about rates of action in chicks, the fact that DHT toxicity occurred at doses immediately above those which were beneficial is consistent with such a rapid action. Conceivably vitamin D toxicity is more the consequence of a cumulative effect and therefore its therapeutic range is greater. Nevertheless, it is equally probable that the mechanism for DHT toxicity differs in some important qualitative manner from that for vitamin D toxicity.

One possibility is that vitamin D might undergo a reduction in vivo which converts it into a hypercalcemic agent structurally related to, or identical with, DHT, thus accounting for the slower onset of vitamin D action. Although this possibility is difficult to explore directly, the indirect evidence obtained to date has not supported this hypothesis. For example,

no metabolic inhibitors have been found which would inhibit vitamin D action without having a similar inhibitory action against DHT (e.g., actinomycin D and *o,p'*-DDD). A converse result would have suggested that such a possibility might exist.

In conclusion, it has been shown that DHT$_2$ and DHT$_3$ are antirachitic in the chick, possessing nearly equal potencies both at this level and at higher toxic doses. Thus, the effect of alterations in the side chain which give rise to such dramatic potency differences (vitamin D_3 > vitamin D_2) in the chick are abolished when the sterol nucleus is chemically reduced to give the dihydrotachysterols.

LITERATURE CITED

1. Chen, P. S., Jr., and H. B. Bosmann 1964 Effect of vitamins D_2 and D_3 on serum calcium and phosphorus in rachitic chicks. J. Nutr., 83: 133.
2. Chen, P. S., Jr., and H. B. Bosmann 1965 Comparison of the hypercalcemic action of vitamins D_2 and D_3 in chicks and the effect on tetracycline fixation by bone. J. Nutr., 87: 148.
3. McLean, F. C. 1941 Activated sterols in the treatment of parathyroid insufficiency. J. Amer. Med. Ass., *117*: 609.
4. Terepka, A. R., P. S. Chen, Jr. and B. Jorgensen 1961 The nature of Hytakerol^R (AT-10) and its comparison with crystalline dihydrotachysterol. Endocrinology, 68: 996.
5. Chen, P. S., Jr., A. R. Terepka and C. Overslaugh 1962 Hypercalcemic and hyperphosphatemic actions of dihydrotachysterol, vitamin D_2 and Hytakerol^R (AT-10) in rats and in dogs. Endocrinology, 70: 815.
6. McChesney, E. W. 1943 Studies of calcium and phosphorus metabolism in the chick. I. The comparative effect of vitamins D_2 and D_3 and dihydrotachysterol given orally and intramuscularly. J. Nutr., 26: 81.
7. McChesney, E. W. 1943 Studies of calcium and phosphorus metabolism in the chick. II. Relative antirachitic effectiveness of vitamins D_2 and D_3 and dihydrotachysterol administered parenterally. J. Nutr., 26: 487.
8. Correll, J. T., and E. C. Wise 1943 The comparative toxicity of calciferol, AT-10, and cod liver oil concentrate for chicks. J. Nutr., 26: 641.
9. DeMan, T. J., and J. R. Roborgh 1959 The hypercalcemic activity of dihydrotachysterol$_2$ and dihydrotachysterol$_3$ and of the vitamins D_2 and D_3. I. Comparative experiments on rats. Biochem. Pharmacol., 2: 1.
10. Roborgh, J. R., and T. J. DeMan 1960 The hypercalcemic activity of dihydrotachysterol$_2$ and dihydrotachysterol$_3$ and of the vitamins D_2 and D_3 after intravenous injection of the aqueous preparations. II. Comparative experiments on rats. Biochem. Pharmacol., 3: 277.
11. Sallis, J. D., and E. S. Holdsworth 1962 Influence of vitamin D on calcium absorption in the chick. Amer. J. Physiol., 203: 497.
12. Bauer, G. C. H., A. Carlsson and B. Lindquist 1955 Evaluation of accretion, resorption, and exchange reactions in the skeleton. Kungl. Fysiol. Sallskap. Lund Forhandl., 25: 1.

Effect of an Essential Fatty Acid Deficiency in Rats on the Incorporation in vitro of Palmitate-1-^{14}C and Linoleate-1-^{14}C into Liver Glycerolipids [1]

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ABSTRACT In an attempt to learn more about the nature of the biochemical alterations occurring in an essential fatty acid deficiency, the incorporation, in vitro, of palmitate-1-^{14}C and linoleate-1-^{14}C into several glycerolipids has been measured in whole homogenate, microsomes, and microsomes plus supernatant, of livers from normal and essential fatty acid deficient rats. When either the homogenate or the microsomes plus supernatant were used as enzyme, most of the incorporation of palmitate-1-^{14}C and linoleate-1-^{14}C occurred in the triglycerides. When the microsomal fraction was used, however, relatively little radioactivity was observed in the triglycerides, and most of the ^{14}C uptake from both fatty acids occurred in the phosphatidylethanolamines. The incorporation of the fatty acids into the several lipids studied clearly was altered by the imposition of an essential unsaturated fatty acid deficiency. These effects differed depending upon the fatty acid used as precursor, the specific type of glycerolipid considered, and the portion of the liver used as enzyme.

Studies reported earlier (1–3) led to the suggestion that liver mitochondria from rats maintained with a diet deficient in the essential fatty acids (EFA) were structurally less stable than those from normal rats, and that part, at least, of the structural instability relates to the lipid portion of the lipoprotein of the mitochondrion. Whether this presumed site of a defect is associated with altered respiratory-linked processes in EFA-deficient livers (4–8) by way of altered phospholipids is not known. Marked changes occur in the fatty acid composition of phospholipids in deficient mitochondria (Mt) (9), however, which makes them suspect, especially in view of phospholipid involvement in electron transfer mechanisms (10).

In an effort to gain a better understanding of the metabolism of individual glycerolipids, and particularly of the phospholipids, in livers of rats reared with a diet devoid of the essential fatty acids, a study was made of the incorporation, in vitro, of palmitate-1-^{14}C and linoleate-1-^{14}C into triglycerides, phosphatidylethanolamine phosphatidylserine, phosphatidylinositol, and phosphatidylcholine. Fed and fasted animals were used, and whole liver homogenate (Hom), liver microsomes (Mc) and microsomes plus cytoplasmic supernatant (McS) were used as enzymes.

EXPERIMENTAL METHOD

Male rats of the Holtzman strain, weaned at 18 days of age, were maintained with either a semipurified diet containing corn oil at a level of 5% (6), or with an EFA-deficient diet in which hydrogenated coconut oil was substituted for corn oil in equivalent amounts. After the animals had been fed these diets for 12 weeks (11) they were placed in one of four experimental groups and fed as follows: (a) the control corn-oil diet until killed, (b) the control diet and fasted for 24 hours before killing, (c) the EFA-deficient diet until killed, (d) the EFA-deficient diet and fasted 24 hours prior to killing. The fatty acid composition of the diets (table 1) was determined by gas-liquid chromatography of the methyl esters (9).

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TABLE 1
Composition of diets

	Normal diet	Hydrogenated coconut oil diet
	%	%
Vitamin-free casein [1]	21	21
Non-nutritive fiber [2]	16	16
Salt mix, USP XIV	4	4
Dextrose	53	53
Vitamin mix [3]	1	1
Corn oil	5	—
Hydrogenated coconut oil [4]	—	5
	g	*g*
Composition of fatty acids:		
Lauric	0	35.7
Myristic	0	27.8
Palmitic	17.5	16.3
Stearic	2.0	16.0
Oleic	26.6	4.3
Linoleic	51.6	0
Linolenic	2.4	0
Total	100.1	100.1

[1] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[3] Contained (in g/kg vitamin mix): vitamin A conc (200,000 USP units/g), 9.93; vitamin D conc (400,000 USP units/g), 0.55; α-tocopherol, 11.05; ascorbic acid, 99.3; *i*-inositol, 11.05; choline chloride, 165.6; menadione, 4.96; *p*-aminobenzoic acid, 11.05; niacin, 9.93; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.63; biotin, 0.044; folic acid, 0.198; vitamin B_{12}, with mannitol 0.1%, 2.98 (Vitamin Diet Fortification Mixture in Dextrose, Nutritional Biochemicals Corporation).
[4] Hydrol, Durkee's Famous Foods, Cleveland, Ohio.

The animals were decapitated with a guillotine and livers were removed rapidly and placed in a beaker immersed in ice. After weighing, the livers were forced through a perforated Lucite press to remove connective tissue. The livers were suspended in Tris-KCl buffer at a pH of 7.4 and homogenized in an all-glass Potter-Elvehjem homogenizer.

The Hom and Mc fractions were separated according to the method as described elsewhere (12). The supernatant, after removal of the Mt by centrifugation at 12,000 × *g* for 30 minutes, was designated as the McS.

The components of the incubation mixture (table 2) were added to 50-ml Erlenmeyer flasks in the order given. The incubations were carried out in air in a Dubnoff metabolic incubator, with mechanical agitation, for 20 minutes at 37° (13). The reactions were stopped by the addition of methanol to the flasks. Zero-time controls were used in which methanol was added immediately following the addition of enzyme preparation to the incubation mixtures.

The lipids were extracted by the method of Johnson and Kerur (12). The phospholipids were precipitated 3 times in the cold with acetone, and finally dissolved in petroleum ether. The triglycerides were separated by 2-stage thin-layer chromatography on aluminum oxide according to the method of Lederkremer and Johnson (13) and the phospholipids were separated by 2-dimensional thin-layer chromatography on Silica Gel H, as described by Johnson and Lederkremer (14). The plates were sprayed with 2′,7′-dichlorofluorescein and the spots containing the lipids were located under ultraviolet light, transferred to scintillation counting vials, and radioactivity estimated as described previously (13, 14). Protein determinations were made on all enzyme systems by a modified biuret method.[3]

RESULTS

When either the whole homogenate or the microscomes plus supernatant were used as enzyme, most of the incorporation of palmitate-1-^{14}C and linoleate-1-^{14}C occurred in the triglycerides. When the microsomal fraction was used, however, relatively little radioactivity was found in the triglycerides and most of the ^{14}C uptake from both fatty acids occurred in the phosphatidylethanolamines (tables 2 and 3).

Effect of an essential fatty acid deficiency (fed rats). The incorporation of the fatty acids into the several lipids studied, clearly was altered by the imposition of an essential fatty acid deficiency. Moreover, the effects differed depending on the fatty acid used as precursor, the specific type of glycerolipid considered, and the portion of the liver used as enzyme. For example, EFA-deficient Hom incorporated 82.8 mμmoles of palmitate-1-^{14}C into the phosphatidylcholines and 19.2 mμmoles into the phosphatidylserines, whereas control liver Hom incorporated 47.0 and 38.1 mμmoles, respectively, into these lipids (table 2).

When McS was used as enzyme, more palmitate-1-^{14}C (57.4 mμmoles) was in-

[3] Personal communication, Dr. Albert L. Lehninger.

TABLE 2

Incorporation of palmitate-1-^{14}C into glycerolipids of liver cell fractions of essential fatty acid-deficient rats [1]

	Fed		Fasted	
	Normal	EFA-deficient	Normal	EFA-deficient
	mμmoles/100 mg protein		*mμmoles/100 mg protein*	
Homogenate				
Phosphatidylethanolamine	30.7 ± 7.3 [2]	45.2 ± 17.2	41.5 ± 13.7	47.9 ± 34.9
Phosphatidylserine	38.1 ± 18.9	19.2 ± 6.2	19.8 ± 13.7	12.2 ± 9.8
Phosphatidylinositol	27.9 ± 7.5	27.0 ± 6.3	24.6 ± 9.6	20.4 ± 13.7
Phosphatidylcholine	47.0 ± 4.4	82.8 ± 33.7	44.8 ± 15.5	45.8 ± 32.1
Triglycerides	383.4 ± 128.2	457.8 ± 156.1	337.4 ± 107.1	298.0 ± 119.4
Microsomes plus supernatant				
Phosphatidylethanolamine	29.4 ± 7.8	23.6 ± 10.8	28.0 ± 9.3	24.5 ± 18.8
Phosphatidylserine	57.4 ± 34.4	15.0 ± 13.4	17.9 ± 16.3	7.9 ± 6.0
Phosphatidylinositol	19.7 ± 13.8	11.3 ± 9.5	9.6 ± 6.2	8.3 ± 6.2
Phosphatidylcholine	20.4 ± 7.7	22.0 ± 15.7	19.9 ± 12.2	26.9 ± 22.2
Triglycerides	310.4 ± 131.4	312.1 ± 261.1	327.7 ± 110.4	286.8 ± 199.2
Microsomes				
Phosphatidylethanolamine	145.0 ± 39.1	189.7 ± 93.6	199.4 ± 95.0	219.0 ± 140.8
Phosphatidylserine	25.5 ± 31.7	27.8 ± 24.2	19.3 ± 12.3	20.4 ± 15.9
Phosphatidylinositol	17.0 ± 21.2	28.8 ± 25.0	15.3 ± 14.9	22.0 ± 20.0
Phosphatidylcholine	37.3 ± 13.3	134.2 ± 95.9	47.5 ± 35.7	83.9 ± 68.9
Triglycerides	32.2 ± 7.5	57.5 ± 21.2	21.8 ± 9.8	45.3 ± 17.8

[1] Incubated for 20 minutes at 37° in medium which contained 0.33 g liver, 20 μmole $MgCl_2$, Tris-KCl buffer of pH 7.4 with 25 μmole Tris and 69.3 μmole KCl, 20 μmole KH_2PO_4 with pH of 7.4, 50 μmole glucose, 10 μmole Na-α-ATP, 1.5 μmole Na-α-GP, 5 μmole cozymase, 1 μmole CoA, 2 μmole nonradioactive linoleic or palmitic acid, 0.1 ml palmitate-1-^{14}C or linoleate-1-^{14}C, and 1 ml rat liver homogenate, microsomal fraction plus supernatants or microsomal fraction. Specific activity of 0.1 ml palmitate-1-^{14}C and linoleate-1-^{14}C ranged from 353,000 to 675,000 cpm. Figures are based on 6 experiments for each of the 4 dietary conditions.
[2] Mean ± SD.

TABLE 3

Incorporation of linoleate-1-^{14}C into glycerolipids of liver cell fractions of essential fatty acid-deficient rats [1]

	Fed		Fasted	
	Normal	EFA-deficient	Normal	EFA-deficient
	mμmoles/100 mg protein		*mμmoles/100 mg protein*	
Homogenate				
Phosphatidylethanolamine	33.3 ± 8.4 [2]	40.4 ± 17.1	26.7 ± 11.7	40.6 ± 18.0
Phosphatidylserine	16.4 ± 6.8	9.5 ± 5.2	7.3 ± 4.1	6.6 ± 4.3
Phosphatidylinositol	46.4 ± 18.4	26.5 ± 6.1	26.9 ± 8.2	24.2 ± 6.1
Phosphatidylcholine	42.9 ± 23.3	80.6 ± 48.8	40.8 ± 32.8	46.6 ± 15.3
Triglycerides	286.9 ± 170.2	224.1 ± 109.1	320.2 ± 196.1	276.6 ± 145.9
Microsomes plus supernatant				
Phosphatidylethanolamine	32.1 ± 18.8	26.6 ± 16.9	21.0 ± 17.1	21.1 ± 9.4
Phosphatidylserine	24.5 ± 22.3	8.7 ± 7.2	9.6 ± 5.7	6.0 ± 4.5
Phosphatidylinositol	24.6 ± 19.8	12.2 ± 5.0	13.4 ± 7.8	7.8 ± 4.6
Phosphatidylcholine	15.7 ± 10.8	18.2 ± 12.5	6.4 ± 5.9	9.9 ± 5.1
Triglycerides	214.0 ± 147.4	155.4 ± 137.6	226.0 ± 127.4	146.7 ± 106.8
Microsomes				
Phosphatidylethanolamine	185.7 ± 114.4	123.5 ± 56.5	103.2 ± 66.4	131.4 ± 67.2
Phosphatidylserine	31.5 ± 15.1	21.7 ± 12.2	23.3 ± 20.5	11.7 ± 4.5
Phosphatidylinositol	30.8 ± 13.8	24.2 ± 11.3	13.8 ± 8.4	13.0 ± 7.8
Phosphatidylcholine	57.4 ± 48.5	68.3 ± 33.9	38.2 ± 53.5	35.0 ± 18.4
Triglycerides	33.0 ± 18.6	43.6 ± 22.3	26.0 ± 15.1	30.8 ± 10.7

[1] See table 2 for experimental conditions.
[2] Mean ± SD.

corporated into the phosphatidylserine of normal rats than in that of deficient animals (15.0 mμmoles), and no differences were observed in the other 3 phospholipids. Employing Mc as enzyme, the only difference observed between the normal and deficient tissues in palmitate incorporation into the phospholipids was an increased uptake of the fatty acid into the phosphatidylcholines of the latter (37.4 as compared with 134 mμmoles). The only significant differences noted in the incorporation of either of the fatty acids into triglycerides of normal and EFA-deficient enzyme systems occurred in the Mc, when palmitate-1-^{14}C incorporation was higher in enzymes from EFA-deficient rats (57.5 mμmoles) than from normal rats (32.2 mμmoles) (table 2).

Less linoleate-1^{14}C was incorporated into the phosphatidylinositol of deficient liver homogenates than was found in the normal (table 3). When either McS or Mc were used as enzyme, however, no differences were observed between normal and essential fatty acid-deficient tissues in the incorporation of linoleate-1-^{14}C into any of the lipids studied.

Effect of fasting. The effect of a 24-hour fast was, in general, to reduce the amount of radioactivity incorporated into the glycerolipids, regardless of the enzyme system used. In a few instances, however, fasting affected radioactive uptake into the lipids of EFA-deficient tissues differently from that observed in normal tissues (tables 2 and 3).

Using McS from control animals, we observed a depressed incorporation of palmitate-1-^{14}C into phosphatidylserine with fasting (57.4 vs. 17.9 mμmoles) and no change in linoleate-1-^{14}C incorporation. No differences were observed with Mc as enzyme, and no differences were observed between fed and fasted enzyme systems in the incorporation of either palmitate-1-^{14}C or linoleate-1-^{14}C into triglycerides.

Effect of the kind of fatty acid. In experiments using normal homogenate from fed rats as enzyme, the incorporation of palmitate-1-^{14}C was higher than that of linoleate-1-^{14}C into phosphatidylserine of both normal tissue (38.1 vs. 16.4 mμmoles) and deficient tissue (19.2 vs. 9.5 mμmoles), and into triglycerides of deficient homogenates (457.8 vs. 224.1 mμmoles). Linoleate, however, was incorporated faster than palmitate (46.4 vs. 27.9 mμmoles) into phosphatidylinositol of normal homogenate. When McS from fasted normal rats were used, more palmitate-1-^{14}C than linoleate-1-^{14}C was incorporated into lecithins (19.9 mμmoles and 6.4 mμmoles, respectively).

DISCUSSION

The results presented appear to be in conflict with the observations of Stein and Stein (15), who reported that an essential fatty acid deficiency in rats was accompanied by an increased incorporation of both linoleate and palmitate into the total phospholipids of either heart perfused in vivo or diaphragm used in vitro, and the 2 fatty acids were incorporated to approximately equal extents. In the present experiments, on the contrary, differences were observed between linoleate and palmitate incorporation into several lipids, and the incorporation of fatty acids was both decreased and increased in deficient tissues, depending upon the lipid considered and the enzyme preparation used. The differences in results may be related to either (or both) the fact that different tissues were used, or that specific phospholipids were examined in the present study, whereas total phospholipids were examined in the experiments of Stein and Stein. Thus, in the latters' experiments, differences existing in the metabolism of individual phospholipids may have been obscured.

One of the characteristics of an essential fatty acid deficiency in rats is a decreased amount of linoleic acid in total liver lipids, which is observed in microsomes as well (16). When deficient animals are refed a diet containing linoleate, this acid rapidly appears in the liver lipids, and for this reason as well as a consideration of the observations of Stein and Stein (15) we expected linoleate to be incorporated into the several lipids faster than was palmitate. With the exception of fatty acid incorporation into the inositols of homogenates, wherever differences were noted in the incorporation of the 2 acids used, more radioactivity was incorporated when palmitate was used than when lino-

leate was used. Whether this reflects an attempt to regain a condition of normalcy is not known. However, in an earlier study, most of the liver mitochondrial phospholipid species prepared from deficient rats contained less palmitate then was noted in corresponding lipids from normal rats (9).

Collins (17, 18) postulated that lecithins containing less arachidonic acid incorporated ^{32}P faster than other lecithins, and suggested that phospholipids containing this acid lend stability to membranes. Presumably, the increased ^{32}P specific activity of lecithins and ethanolamines of deficient rats was associated in their experiments with less stable membranes.

The increased incorporation of palmitate-1-^{14}C into lecithins of homogenates and microsomes in EFA-deficient animals observed in the present experiments possibly might be accepted as evidence favoring the Collins' hypothesis that the deficiency leads to an increased turnover of phospholipids in membranes. This connection between the present experiments and his may be in question, as he observed an increased ^{32}P incorporation into phosphatidylethanolamines as well as into lecithins in deficient animals (17), and in the present experiments we failed to observe any differences in the incorporation of either palmitate or linoleate into phosphatidylethanolamines in EFA deficiency. It may be of significance in this respect, however, that whereas the lecithins of deficient liver mitochondria contain only a trace of arachidonic acid, the phosphatidylethanolamines still contain relatively large amounts (9). It appears that more information is needed concerning the role of specific phospholipid classes in membrane functions before the significance of these observations can be assessed.

LITERATURE CITED

1. Johnson, R. M. 1963 Swelling studies on liver mitochondria from essential fatty acid deficient rats. Exp. Cell Res., *32:* 118.
2. Ito, T., and R. M. Johnson 1964 Effects of a nutritional deficiency of unsaturated fats on rat liver mitochondria. J. Biol. Chem., *239:* 3901.
3. Johnson, R. M. 1963 Adenosine triphosphatase and ATP-Pi exchange in mitochondria of essential fatty acid deficient rats. J. Nutr., *81:* 411.
4. Klein, P. D., and R. M. Johnson 1954 Phosphorus metabolism in unsaturated fatty acid-deficient rats. J. Biol. Chem., *221:* 103.
5. Tulpule, P. G., and J. N. Williams, Jr. 1955 Study of the role of essential fatty acids in liver metabolism. J. Biol. Chem., *217:* 229.
6. Levin, E., R. M. Johnson and S. Albert 1957 Mitochondrial changes associated with essential fatty acid deficiency in rats. J. Biol. Chem., *228:* 15.
7. Smith, J. A., and H. F. DeLuca 1964 Structural changes in isolated liver mitochondria of rats during essential fatty acid deficiency. Cell Biol., *21:* 15.
8. Hayashida, T., and O. W. Portman 1960 Mitochondria dehydrogenase activity of rat liver deficient in essential fatty acids. Arch. Biochem. Biophys., *91:* 206.
9. Johnson, R. M., and T. Ito 1965 Effects of a nutritional deficiency of unsaturated fats on the distribution of fatty acids in rat liver mitochondrial phospholipids. J. Lipid Res., *6:* 75.
10. Fleischer, S., G. Brierley, H. Klouwen and D. B. Slautterback 1962 Studies of the electron transfer system. XLVII. The role of phospholipids in electron transfer, J. Biol. Chem., *237:* 3264.
11. Aaes-Jørgensen, E. 1961 Essential fatty acids. Physiol. Rev., *41:* 1.
12. Johnson, R. M., and L. Kerur 1963 The incorporation in vitro of palmitate-^{14}C into glycerolipids by various cell components. Biochim. Biophys. Acta, *70:* 152.
13. Johnson, R. M., and J. M. Lederkremer. The incorporation of palmitate-1-^{14}C in vitro into several glycerolipids by normal and regenerating rat liver fractions. Arch. Biochem. Biophys., in press.
14. Lederkremer, J. M., and R. M. Johnson 1965 Purification of neutral lipid fractions by thin-layer chromatography on aluminum oxide. J. Lipid Res., *6:* 572.
15. Stein, O., and Y. Stein 1964 Metabolism in vitro of palmitic and linoleic acid in the heart and diaphragm of essential fatty acid-deficient rats. Biochim. Biophys. Acta, *84:* 621.
16. Klein, P. D., and R. M. Johnson 1954 A study of the onset of unsaturated fatty acid deficiency in subcellular particles of rat livers. Arch. Biochem. Biophys., *48:* 380.
17. Collins, F. D. 1962 Phospholipid metabolism in essential fatty acid deficient rats. Biochem. Biophys. Res. Commun., *9:* 289.
18. Collins, F. D. 1963 Studies on phospholipids. 9. The composition of rat-liver lecithins. Biochem. J., *88:* 319.

Cycad Flour Used by Guamanians: Effects Produced in Rats by Long-term Feeding [1]

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ABSTRACT Male and female rats were fed diets containing home-made cycad flour for about 23 months to determine whether the flour was carcinogenic. This cycad flour was processed by Guamanians for human consumption. Continuous ingestion of nutritionally adequate diets containing 1.5, 5.0 or 10.0% home-made cycad flour did not affect growth rates of rats significantly. However, a sudden drop of environmental temperatures caused a greater loss of body weights of transient nature in the treated rats than in the controls. Activity of the male rats measured by revolving cages was not significantly different between the control and treated rats. At the termination of the experiment, 11 of 45 experimental rats had died or were moribund, whereas all of the 15 control rats appeared to be in good health. Tumors and hyperplastic processes in organs of the endocrine system as well as reticulum cell sarcomas found at autopsy at the termination of investigation were thought to be related to the advanced age of the animals rather than to the ingestion of processed cycad flour. No neoplastic lesions were observed such as those noted previously following long-term ingestion of unprocessed cycad flour. These observations suggest that the local customs of preparing cycad flour by soaking, if properly executed, removes the carcinogenic substance effectively.

Several reports have appeared since 1963 describing acute toxic and late carcinogenic effects of unprocessed, dried and ground seeds of *Cycas circinalis* when fed to rats and guinea pigs as part of the diet (1, 2). The agent in the seed which caused tumor development in feeding experiments was the glucoside, cycasin (β-D-glucosyloxyazoxymethane), its aglycone (methylazoxymethanol) being also active after intraperitoneal injection and in germfree rats (3, 4).

The native population in Guam uses flour prepared from the seeds (kernels) of *Cycas circinalis* for food. Guamanians routinely try to rid the cycad kernel of its toxic component by prolonged soaking in water and subsequent drying in the sun. Observations by Whiting (5) indicate that considerable fermentation occurs during the soaking process. Since the washed and dried pieces of cycad seeds are usually ground and stored for later use in homes, examining the processed flour for possible harmful effects in feeding experiments in animals seemed important.

EXPERIMENTAL PROCEDURES

Fourteen shipments of processed flour prepared by the natives in Guam were obtained from local markets representing widely different areas on the island.[2] The samples were combined, added to the basal diet in concentrations of 1.5, 5.0, 10.0% and fed to groups of 15 rats consisting of 7 or 8 males and 8 or 7 females of the Osborne-Mendel strain. Another group of 15 rats served as controls and was fed only the basal diet. The basal diet routinely used in our laboratory has been described (6). The rats were started on the experiment when they were 4 weeks old. They were housed in individual suspended wire-bottom cages and were fed their respective diets and water on an ad libitum basis.

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[2] We thank Drs. L. T. Kurland and M. G. Whiting of the Institute of Neurological Diseases and Blindness, Bethesda, Maryland, for the generous supply of processed cycad flour, which was collected through the kindness of P. A. Hansen, Animal Industries, Department of Agriculture, Agana, Guam.

Body weights were recorded at weekly intervals throughout the 23 months of experiment.

Since these studies were initiated (6) to determine whether there was any relation between the ingestion of cycad by the natives in Guam and their high incidence of amyotrophic lateral sclerosis, an attempt was made to evaluate the neuromuscular condition of the animals after they had been fed the cycad for about 6 weeks. One indicator of motor function was postulated to be spontaneous activity and it was hypothesized that animals with any disturbances interfering with locomotion would show a reduction in spontaneous activity.

To evaluate spontaneous activity, the males in all groups were placed in activity cages [3] and left for one week to become familiar with their new environment. Spontaneous activity was then recorded daily for all male rats during the seventh and eighth week of the experiment.

Complete autopsies were performed on all rats including those that died or were moribund during the experiment and those that survived the duration of the investigation. Sections of tissues of all internal organs and all endocrine glands were prepared for microscopic study.

Body weight and spontaneous activity data were examined by analysis of variance (7).

RESULTS AND DISCUSSION

No significant difference in body weight gain was noted between the experimental and control groups although a greater depression in body weight was observed in all experimental groups than in the control group during a sudden drop of the temperature in the animal quarters. The effect was more severe on all female experimental animals than the control females ($P < 0.01$). The difference in the male animals was not significant ($P > 0.05$) (table 1). When the temperature in the laboratory was corrected, all animals regained their lost weight in about 2 months.

The average voluntary activity was not significantly different ($P > 0.05$) among the groups of male rats. The averages were 2457, 1773, 2526, and 1763 revolutions per rat per day for rats fed diets containing zero, 1.5, 5.0 and 10% processed cycad flour, respectively.

During the course of the experiment, 2 rats from each experimental group died and none in the control group. Three of them (1 female each from the 3 experimental groups) had extensive pulmonary infections, one male fed 1.5% cycad flour diet had a reticulum cell sarcoma probably arising from the submandibular region with widespread metastases; one other

[3] We are grateful to Dr. W. D. VanHuss of the Human Energy Laboratory, Michigan State University, East Lansing, for the use of the revolving drum cages.

TABLE 1

Body weights and mortality in controls and rats fed cycad flour prepared by the natives in Guam

Measurements	Weeks on experiment	Sex [1]	Processed kernels in basal diets, %			
			0	1.5	5.0	10.0
Body wt, g	0	M+F	77.4	76.1	77.7	77.2
	32	M	480	493	471	467
		F	292	295	307	308
	64	M	552	595	546	520
		F	330	322	344	343
	104	M	533	609	566	529
		F	371	310	375	404
Wt loss associated with low room temperature, g		M	28	44	41	57
		F	9	22	20	23
No. rats dead before end of experiment			0	2	2	2
No. rats killed before termination [2]			0	2	1	2

[1] M = male, F = female; 7 M and 8 F were fed 0 or 5.0% and 8 M and 7 F were fed 1.5 or 10.0% processed cycad flour diets.
[2] Killed because of severe weight loss and moribund condition.

TABLE 2

Incidence of hyperplastic lesions in endocrine glands [1]

Site	Controls		1.5% cycad flour		5% cycad flour		10% cycad flour	
	M [2]	F [3]	M	F	M	F	M	F
Anterior pituitary	3/7	6/8	3/8	3/7	3/7	2/8	1/8	3/7
Thyroid	2/7	1/8	2/8	1/7	0/7	0/8	1/8	1/7
Adrenal cortex	2/7	2/8	2/8	2/7	0/7	3/8	1/8	4/7
Adrenal medulla	1/7	1/8	0/8	0/7	3/7	0/8	0/8	0/7
Pancreas	0/7	0/8	0/8	0/7	0/7	0/8	1/8	0/7
Breast	1/7 [4]	1/8	0/8	1/7	0/7	0/8	0/8	2/7
Testis	0/7	0/0	0/8	0/0	0/7	0/0	0/8	0/0
No endocrine lesions	2/7	2/8	4/8	2/7	3/7	4/8	4/8	1/7

[1] The table includes pathologic data from all rats whether found dead or killed.
[2] M = male; values in table indicate number of rats affected over number of rats used.
[3] F = female; values in table indicate number of rats affected over number of rats used.
[4] Fibroma subcutaneous tissues of chest wall; no glandular tissue involved.

male fed 10% cycad flour diet had a large islet cell adenoma of the pancreas as the only important lesion and the sixth, a female (5% cycad flour diet) was autolyzed to such an extent that detailed study was impossible.

An additional 5 rats were killed because of their moribund condition associated with marked weight loss. One of these (male, 5% cycad diet) had severe pneumonia, three (male and female, 1.5% and female, 10% cycad diet) had large tumors of the pituitary gland and in one (male, 10% cycad diet), no pathologic lesion was found.

Thirty-four of the original 45 rats on the experimental diets and all 15 control rats were still living when the trial was terminated 713 to 717 days after its start. Many adenomas or foci of adenomatous hyperplasia or both in the organs of the endocrine system were found (table 2). However, these observations have been noted frequently in old rats of other experiments as well as in the present experiment and, therefore should not be attributed to the experimental treatments. Furthermore, primary tumors of liver, kidneys and intestinal tract, characteristic of cycad toxicity, were not found. These organs have been thus far the most frequent sites of tumor development in rats fed unprocessed cycad material and the glucoside (1, 8).

Other observations included 3 (male, 1.5%; male and female, 5% cycad diet) reticulum cell sarcomas which were limited to the lung and the one mentioned before, originating in the neck nodes with metastatic spread to lung, liver, lymph nodes, brain and bone marrow. A tumor in the anterior mediastinum, consistent with a histologic diagnosis of thymoma, was found at the termination of the experiment in a male fed 10% cycad flour diet. Lymphosarcomas have been observed in our laboratory in old rats of the same strain serving as controls in other experiments. They have also been reported in the literature as occurring spontaneously in old rats of the Osborne-Mendel strain (9–11).

LITERATURE CITED

1. Laqueur, G. L., O. Mickelsen, M. G. Whiting and L. T. Kurland 1963 Carcinogenic properties of nuts from *Cycas circinalis* L. indigenous to Guam. Nat. Cancer Inst., *31:* 919.
2. Spatz, M. 1964 Carcinogenic effect of cycad meal in guinea pigs. Federation Proc., *23:* 1384.
3. Laqueur, G. L. 1964 Carcinogenic effects of cycad meal and cycasin, methylazoxymethanol glycoside in rats and effects of cycasin in germfree rats. Federation Proc., *23:* 1386.
4. Matsumoto, H., and F. M. Strong 1963 The occurrence of methylazoxymethanol in *Cycas circinalis* L. Arch. Biochem., *101:* 299.
5. Whiting, M. G. 1963 Toxicity of Cycads. Econ. Bot., *17:* 270.
6. Campbell, M. E., O. Mickelsen, M. G. Yang, G. L. Laqueur and J. C. Keresztesy 1966 Effects of strain, age and diet on the response of rats to the ingestion of *Cycas circinalis*. J. Nutr., 88: 115.

7. Dixon, W. J., and F. J. Massey 1957 Introduction to Statistical Analysis. McGraw-Hill Book Company, New York.
8. Laqueur, G. L. 1965 The induction of intestinal neoplasm in rats with the glycoside cycasin and its aglycone. Virchows Arch. Pathol. Anat., *340:* 161.
9. Nelson, A. A., and H. J. Morris 1941 Reticulum cell lymphosarcoma in rats. Arch. Pathol., *31:* 578.
10. Fitzhugh, O. G., A. R. Bourke, A. A. Nelson and J. P. Frawley 1959 Chronic oral toxicity of four stearic acid emulsifiers. Toxicol., Appl. Pharmacol., *1:* 315.
11. Snell, K. 1964 Spontaneous lesions in the rat. In: The Pathology of Laboratory Animals, chapt. 10, compiled and edited by W. E. Ribelinand J. R. McCoy. Charles C Thomas, Springfield, Illinois.

Protein Requirements of Growing-Finishing Cattle and Lambs [1]

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ABSTRACT Data were summarized which indicate that the protein requirements of growing-finishing cattle and lambs are a function of body weight, body weight gain and digestibility of the protein in the ration. Daily digestible protein requirements can be expressed by the following equations:

$$\text{cattle, DP} = 2.79\ W_{kg}^{0.75}\ (1 + 1.905\ G)$$
$$\text{lambs, DP} = 2.79\ W_{kg}^{0.75}\ (1 + 6.02\ G)$$

where DP is the amount of digestible protein (g), $W_{kg}^{0.75}$ is the metabolic mass and G is the daily gain (kg). Ideally, the above equations need to be modified such that body weight and weight gain can be replaced with carcass weight and gain of a specified composition.

Protein requirements of livestock are thought to be a function of many variables and not a specific figure for all conditions. Yet, requirements as listed in the various NRC publications (8–10), are specific quantities, expressed either as a percentage of the ration or as an amount per animal per day. This may be a simple way of expressing protein requirements but is probably an over-simplification of the facts. A major need exists in nutrition research for further quantification of nutrient requirements in terms of all the variables which may cause these requirements to vary. Once the quantitative importance of each of these variables is determined, then the ability to formulate rations with predictable results will be enhanced.

Three variables known to affect the amount of protein required in a ration are body weight, rate of body gain and digestibility of the protein in the ration. It was the purpose of the work in this paper to attempt to integrate these 3 variables into a single protein requirement equation for growing-finishing cattle and lambs.

METHODS

Requirements listed in the NRC publications (8–10), were used as a source of data for the theoretical derivation of the relationship that was being sought. This was then applied to some experiments where protein intake was thought to be a limiting variable.

RESULTS

Total daily crude protein and digestible protein requirements listed for both growing-finishing beef cattle and growing dairy cattle weighing in excess of 100 kg were plotted as shown in figure 1. Both crude and digestible protein requirements, expressed per unit of metabolic mass ($W_{kg}^{0.75}$) per day, are plotted against the daily rate of gain shown for the respective protein intakes. Assuming that protein requirements are a function of body weight and rate of gain, the equations expressing these relationships are as follows:

(1) $CP = 5.86\ W_{kg}^{0.75}\ (1 + 0.924\ G)$ $\quad r = 0.88$

(2) $DP = 2.79\ W_{kg}^{0.75}\ (1 + 1.905\ G)$ $\quad r = 0.97$

where *CP* and *DP* are grams of crude and digestible protein per day, respectively, *W* is the body weight and *G* is the daily rate of gain, both in kilograms. As the correlation coefficients indicate, both relationships are quite good ($P < 0.01$, $n = 26$). Standard errors of estimate are 1.06 and 0.61 g per unit of metabolic mass, respectively, for equations 1 and 2.

Similar computations for growing-finishing lambs (data shown in figure 2) give the following relationship for daily crude and digestible protein requirements as a

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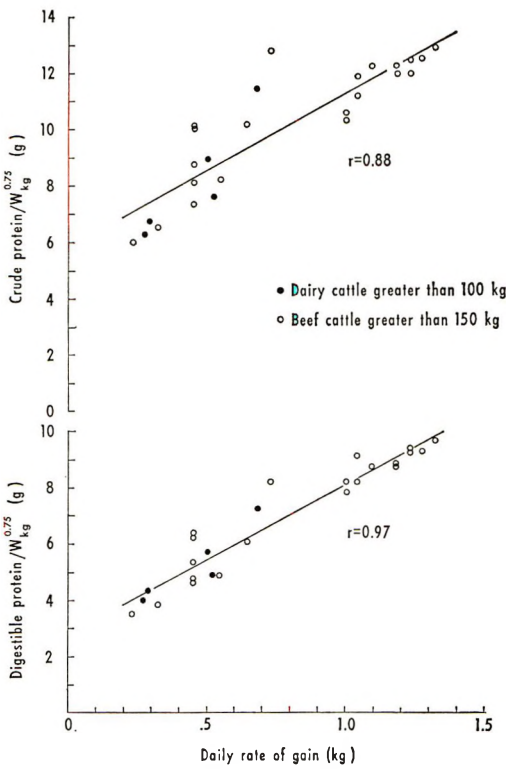

Fig. 1 Daily crude and digestible protein requirements per unit of metabolic mass ($W_{kg}^{0.75}$) for growing-finishing cattle.

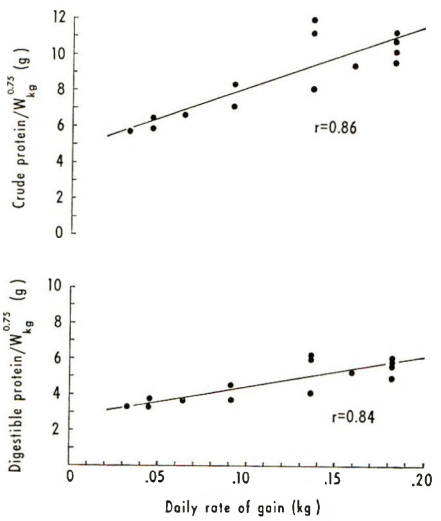

Fig. 2 Daily crude and digestible protein requirements per unit of metabolic mass ($W_{kg}^{0.75}$) for growing-finishing lambs.

function of metabolic mass and rate of gain:

(3) $CP = 4.88\ W_{kg}^{0.75}\ (1+6.64\ G)$ $r = 0.86$

(4) $DP = 2.79\ W_{kg}^{0.75}\ (1+6.02\ G)$ $r = 0.84$

using the same symbols as in the case of the cattle data. Both relationships are quite good ($P < 0.01$, $n = 14$). Standard errors of estimate are 1.15 and 0.63 g per unit metabolic mass, respectively, for equations 3 and 4.

Two cattle experiments by Thompson et al. (12, 13) were evaluated in terms of equation 1 (table 1). Here, in all cases where crude protein was thought to be limiting in relation to the energy which was fed, the calculated requirement was greater than the amount fed. It also appears that additional protein could have been effectively fed in treatment 2 in 1962. In a cattle experiment reported by Clanton et al. (2), the calculated digestible protein requirement (equation 2) is greater than the amount which was fed in the ration where digestible protein was thought to be limiting (table 2).

In both of these reports, when rations were fed which were deficient in protein, the cattle gained at a greater rate than expected from their protein intake. This may be the result of conservation of circulating urea (7), a lower maintenance requirement (5), or possibly to greater deposition of fat and less protein.

Digestible protein requirements of lambs (equation 4) can be compared with that found experimentally (11) as follows:

$$DP = 1.54\ W_{kg}^{0.75}\ (1+10\ G)$$

In this work, the maintenance requirement for digestible protein ($1.54\ W_{kg}^{0.75}$) was lower than Armsby's figure (1) of $2.78\ W_{kg}^{0.75}$. The coefficient for body weight gain, $1.54\ W_{kg}^{0.75}$ (10 G), is similar to that shown in this paper, $2.79\ W_{kg}^{0.75}$ (6.02 G), however. Both coefficients calculate close to $16\ GW_{kg}^{0.75}$.

DISCUSSION

In comparison with the conclusions of Armsby (1), the maintenance requirement for digestible protein of cattle and lambs (equations 2 and 4) is in good agreement (2.78 and 2.79 g/$W_{kg}^{0.75}$, respectively). With increasing rate of gain, digestible protein requirements increase for cattle

TABLE 1

Calculated crude protein required in comparison with the amount of crude protein fed [1]

Year	Treatment no.	Avg wt	Daily gain	TDN fed	Crude protein	
					Fed	Required
		kg	*g*	*kg*	*g*	*g*
1961	1 [2]	270	727	4.31	454	653
	2	280	868	4.31	727	720
	3	259	655	3.18	727	608
	4 [2]	260	573	3.18	454	580
1962	1 [2]	234	600	4.09	454	600
	2	312	1040	4.55–5.45	727	853
	3	254	877	4.09	727	675
	4	216	350	3.18	454	440

[1] Thompson et al. (12, 13).
[2] Indicates treatments where protein was thought to be limiting in the ration fed.

TABLE 2

Calculated digestible protein required in comparison with the amount fed [1]

Treatment no.	Avg wt	Daily gain	Digestible energy fed	Digestible protein	
				Fed	Required
	kg	*g*	*megcal*	*g*	*g*
1	173	109	9.08	168	159
2 [2]	181	200	9.22	136	191
3	179	200	9.32	295	186
4	196	368	10.77	295	250

[1] Clanton et al. (2).
[2] Indicates treatment where protein was thought to be limiting in the ration fed.

and lambs according to the relationship shown in equations 2 and 4, respectively.

Four NRC protein requirement values (9) were omitted in the relationships shown in this paper. They were those shown for 23-, 45-, 68- and 91-kg dairy calves. The listed crude and digestible protein requirements for these calves were very much higher than are indicated by equations 1 and 2 and probably reflect a high protein requirement for growth relative to their metabolic mass.

Protein requirements have little meaning unless energy requirements have been satisfied. Crampton (4) has discussed the relationship which exists between the protein and energy requirements of animals. Since the NRC requirement tables have expressed energy requirements of beef cattle and lambs in a form similar to that shown in equations 1 through 4 from the work of Garrett et al. (6), it is possible to calculate the protein-to-energy ratio required in the ration of cattle and lambs gaining a given amount of weight per day (table 3).

These ratios are well in line with those proposed by Crampton (4). Thus, rations which are formulated to provide energy for a specific rate of gain can also be formulated to supply sufficient digestible protein by providing the indicated protein-to-energy ratio required for that rate of gain. The data presented here indicate that this ratio is more a function of the rate of gain rather than the portion of mature weight which has been attained as presented by Crampton (4). At 100% of mature body weight, where the rate of body

TABLE 3

Protein-to-energy ratios required in the rations of growing-finishing cattle and lambs

	Daily gain	Digestible protein (g) / Digestible energy (megcal)
	kg	
Cattle	(maintenance)	20.8
	0.5	24.7
	1.0	26.4
	1.5	27.2
	2.0	27.8
Lambs	(maintenance)	20.4
	0.1	21.4
	0.2	21.9
	0.3	22.2
	0.4	22.4

weight gain would be zero, the indicated ratio would be 20.8 and 20.4, respectively, for cattle and lambs, which compares well with the inter-species maintenance value of 19 to 20 proposed by Crampton (4).

It is of interest to note the marked increase in apparent protein-to-energy ratio required by cattle with an increasing rate of gain in contrast with that required by lambs. A ratio of 22 has been found to be the optimal ratio for growing-finishing lambs (11).

The relationships presented above have two major criticisms. First, protein requirements shown in the NRC publications were used as the basis for derivation of the equations. Any consistent bias in these requirements would be reflected in the equations. This appears unlikely in the case of cattle since both the dairy and beef cattle requirement tables were used. A second criticism is the use of body weight and weight gain as the determining factors. Body weight gain, as measured in the live animal, is composed of gastrointestinal fill, water, protein, fat and bone. Thus, the ideal expression would relate protein requirements to specific increases in carcass mass of a certain composition. Such a method has been introduced by Combs (3). Development of this method of stating the protein requirements of cattle and lambs should yield very useful expressions.

LITERATURE CITED

1. Armsby, H. P. 1917 The Nutrition of Farm Animals. The Macmillan Company, New York.
2. Clanton, D. C., D. R. Zimmerman and R. C. Albin 1964 Protein, energy for wintering heifer calves. Nebraska Agr. Exp. Station Beef cattle Progr. Rep., Lincoln, Nebraska, p. 19.
3. Combs, G. F. 1964 Predicting amino acid requirements of chicks based on growth rate, body size and body composition. Federation Proc., *23:* 46.
4. Crampton, E. W. 1964 Nutrient-to-calorie ratios in applied nutrition. J. Nutr., *82:* 353.
5. Elliott, R. C. 1963 A preliminary study of protein requirements of African cattle. J. Agr. Sci., *61:* 417.
6. Garrett, W. N., J. H. Meyer and G. P. Lofgreen 1959 The comparative energy requirements of sheep and cattle for maintenance and gain. J. Animal Sci., *18:* 528.
7. Houpt, T. R. 1959 Utilization of blood urea in ruminants. Amer. J. Physiol., *197:* 115.
8. National Research Council, Committee on Animal Nutrition 1963 Nutrient requirements of beef cattle, pub. 1137. National Academy of Sciences — National Research Council, Washington, D. C.
9. National Research Council, Committee on Animal Nutrition 1956 Nutrient requirements of dairy cattle, pub. 464. National Academy of Sciences — National Research Council, Washington, D. C.
10. National Research Council, Committee on Animal Nutrition 1964 Nutrient requirements of sheep, pub. 1193. National Academy of Sciences — National Research Council, Washington, D. C.
11. Preston, R. L., D. D. Schnakenberg and W. H. Pfander 1965 Protein utilization in ruminants. I. Blood urea nitrogen as affected by protein intake. J. Nutr., *86:* 281.
12. Thompson, G. B., R. L. Preston and H. B. Hedrick 1961 Winter feeding for quality beef production. Missouri Agr. Exp. Station Spec. Rep. 5, Columbia, Missouri, p. 10.
13. Thompson, G. B., H. B. Hedrick, R. L. Preston and L. M. Schake 1962 Winter feeding for quality beef. Missouri Agr. Exp. Station Spec. Rep. 14, Columbia Missouri, p. 1.

Alterations in the Metabolism of 2-^{14}C-Thiazole-labeled Thiamine by the Rat Induced by a High Fat Diet or Thyroxine [1]

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ABSTRACT This study was designed to test the hypothesis that dietary fat "spares" thiamine by reducing its metabolic destruction. Fourteen adult rats of the Sprague-Dawley strain, of similar nutritional backgrounds, were fed a "low" fat (11% calories from fat) diet and injected daily with 50 μg 2-^{14}C-thiazole-labeled thiamine for 8 weeks. For 10 days, seven of the group were then fed a "high fat" diet (63% calories from fat) isocalorically with the remainder of the group that was continued on the "low-fat" regimen. Daily injections of radioactive thiamine were continued during this period. The rats fed the high fat diet excreted significantly more radioactive thiamine in the urine but significantly less $^{14}CO_2$ than did the controls. In a similar study, the administration of 20 μg of thyroxine daily to rats fed the "low fat" diet enhanced the excretion of $^{14}CO_2$ but did not alter the excretion of urinary radioactivity.

The thiamine-sparing effect of dietary fat has been the subject of many studies since its establishment in early feeding trials (1–3). Although comparable long-term feeding studies have not been made in the human, balance studies in infants (4) and studies of thiamine excretion by the uncontrolled diabetic (3) have supported this observation.

The general hypothesis that has emerged from these investigations is that an animal receiving a large portion of his calories from fat (or, in another way, a smaller portion of his calories from carbohydrate) has a lower thiamine requirement. The biochemical basis for this observation has been tacitly assumed to revolve around the fact that fatty acids can be converted to acetyl CoA without the participation of the pyruvic dehydrogenase system, a thiamine-requiring sequence that is obligatory for the entrance of the end-product of glycolysis (pyruvate) into the tricarboxylic acid cycle. For the concept to be correct, it must also be assumed that some thiamine is destroyed during its participation in the pyruvate dehydrogenase system.

Attempts to obtain experimental evidence in support of this hypothesis have met with failure. Studies of the rate of thiamine loss in animals fed thiamine-free diets of different fat content have yielded conflicting results. In 2 studies (6, 7) no differences were observed in the thiamine content of the tissues in rats fed high or low fat rations, whereas a higher tissue thiamine content in animals fed high fat rations was reported in two other studies (8, 9). All of these studies are subject to the general criticism that the precision of the thiamine methodology used may not have been sufficient to detect the small differences in thiamine content to be expected.

The presumed association of thiamine need with the basal metabolic rate has been discussed by Kleiber (10). The evidence for this association has been based largely on studies of the effect of thyroxine on thiamine requirements (11, 12) and on the rate of tissue depletion (7).[3] No studies of the effect of thyroxine on thiamine catabolism are recorded in the literature.

Long-term studies in this laboratory of the metabolism of ^{14}C-labeled thiamine in the rat afforded a unique opportunity to

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[3] Appledorf, H., and S. R. Tannenbaum 1965 Thyroxine-induced modifications in the thiamine deficient rat. Federation Proc., 24: 690 (abstract).

study the effects of high fat diets and thyroxine upon thiamine catabolism. The results of these studies are presented in this report.

MATERIALS AND METHODS

Fourteen adult rats of the Sprague-Dawley strain were fed a low fat thiamine-deficient diet and were injected daily with 50 μg of ^{14}C-thiazole-labeled thiamine [4] for 8 weeks. At this point they were divided into 2 groups of 7 rats by weight-pairing. One group received the regular thiamine-deficient diet containing 5% of fat and the other group received an isocaloric, isonitrogenous diet with an equal content of vitamins and minerals, but containing 40% of fat.

The regular "low fat" thiamine-deficient diet consisted of: (in per cent) vitamin-free casein, 18; hydrogenated vegetable oil,[5] 5; sucrose, 69; succinylsulfathiazole,[6] 1; HMW [7] salt mixture, 4; and nonnutritive fiber,[8] 3. The following amounts of vitamins were added per 100 g of the diet: vitamin A, 400 IU; vitamin D, 40 IU; and (in milligrams) α-tocopheryl acetate, 3; riboflavin, 0.8; niacin, 4.0; Ca pantothenate, 4.0; *p*-aminobenzoic acid 10.0; choline chloride, 100.0; menadione, 0.5; and vitamin B_{12}, 0.003→

The "high-fat" thiamine-deficient diet contained: (in per cent) vitamin-free casein, 26.0; hydrogenated vegetable oil,[9] 40%; sucrose, 25.6; succinylsulfathiazole, 1; HMW salt mixture, 5.4; and nonnutritive fiber, 3.0. The amount of vitamins added to this diet was 1.4 times that in the low fat diet described above. The control rats fed the low fat diet ad libitum consumed an average of 10 g of diet daily. The rats fed the high fat diet were pair-fed isocalorically with the control group and consequently received an average of 7.0 g of diet daily. The absolute daily intakes of nitrogen, calories, vitamins and minerals by the 2 groups were, within experimental error, almost identical but the high fat group received 63% of its calories from fat and the low fat group only 11%. Both groups received daily intraperitoneal injections of 50 μg of 2-^{14}C-thiazole-labeled thiamine.

The effect of thyroxine on thiamine metabolism was studied in 2 groups of 7 rats having similar nutritional backgrounds and similar weights. These animals received the low-fat thiamine-deficient diet and daily intraperitoneal injections of 50 μg of 2^{14}-C-thiazole-labeled thiamine for 8 weeks before the experiment. During the thyroxine studies, each rat in the experimental group received a daily subcutaneous injection of L-thyroxine sodium pentahydrate (20 μg of thyroxine) and the control rats were injected daily with saline solution. Both groups were fed the low fat thiamine-deficient diet (ad libitum) and were injected daily with 50 μg of ^{14}C-thiazole-labeled thiamine.

Details of the housing of the animals, collection of the excreta, and respiratory studies, desalting and chromatography of urine on Amberlite CG-50 columns and measurement of the radioactivity have been described previously (13).

RESULTS

Table 1 shows the urinary excretion of radioactive metabolites by rats fed the low and high fat diets during the 10-day experimental period. The rats fed the high fat diet excreted 50.8 ± 5.58% of their thiamine intake as metabolites and the rats fed the low fat diet excreted 43.7 ± 4.37% of their intake. This difference is significant ($P < 0.02$ by Student's t test).

The effect of feeding the high fat diet on the excretion of $^{14}CO_2$ is presented in table 2. The rats fed the high fat diet excreted about 15% less $^{14}CO_2$ than the control group. This difference is significant ($P < 0.01$).

To determine whether the high fat diet altered the excretion pattern of thiamine metabolites, the 10-day urine samples collected from each group were pooled and the metabolites were isolated by charcoal adsorption, elution and separation by column chromatography on Amberlite CG-50. The results are shown in figure 1. In both groups the radiactive metabolites were resolved into 5 peaks. Because the principal quantitative difference observed was in the

[4] Obtained from Nuclear-Chicago, Inc., Chicago. Specific activity = 5600 cpm/μg.
[5] Crisco, Procter and Gamble Company, Cincinnati.
[6] Nutritional Biochemicals Corporation, Cleveland.
[7] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutr., 14: 273.
[8] Alphacel, Nutritional Biochemicals Corporation.
[9] See footnote 5.

→ Column 1, line 30 should continue to read: biotin, 0.04; folic acid, 0.20 and pyridoxine, 0.50.

TABLE 1

Urinary excretion of ^{14}C-labeled thiamine metabolites by rats fed diets high or low in fat content [1]

Exp. day no.	Low fat diet		High fat diet	
	As vitamin B_1		As vitamin B_1	
	μg	*% of dose*	*μg*	*% of dose*
1	124.9	35.6	163.3	46.6
2	162.4	46.4	175.1	50.0
3	146.5	41.8	157.2	44.9
4	137.7	39.3	194.5	55.5
5	171.7	49.0	169.7	48.5
6	167.3	47.8	166.7	47.6
7	167.9	47.9	216.1	61.7
8	162.0	46.2	172.2 *	57.4
9	145.6	41.6	150.9 *	50.3
10	147.3	42.0	136.8 *	45.6
Mean ± SD:		43.7 ± 4.37 [2]		50.8 ± 5.58 [2]

[1] Each value is the total ^{14}C metabolite excretion expressed as thiamine of 24-hour pooled urine sample from 7 rats. Values with asterisks represent the excretions of 6 rats.
[2] Significantly different ($P < 0.01$).

TABLE 2

Effect of a high fat diet or the administration of thyroxine on the excretion of $^{14}CO_2$ derived from ^{14}C-thiazole-labeled thiamine [1]

Low fat diet		High fat diet		Thyroxine (low fat diet)	
As vitamin B_1		As vitamin B_1		As vitamin B_1	
μg	*% of dose*	*μg*	*% of dose*	*μg*	*% of dose*
7.3	14.6	5.9	11.8	7.9	15.8
6.8	13.6	6.2	12.4	7.9	15.8
6.6	13.2	5.7	11.4	7.6	15.2
6.5	13.0	6.1	12.2	7.5	15.0
		5.3	10.6		
Mean ± SD:					
6.8 ± 0.35	13.6 ± 1.24	5.8 ± 0.72	11.6 ± 1.43	7.7 ± 0.11	15.4 ± 0.72

[1] Each value represents the total radioactivity excreted by a single rat during one 24-hour period of the 10-day experiment. Rats fed the low fat and high fat diets were placed into the metabolism chamber on alternate days beginning on day 2 of the experimental period. The thyroxine-treated rats were placed into the metabolism cage on consecutive days following termination of the fat-feeding experiment. The values are expressed as micrograms of thiamine and as percentage of the daily dose.

size of peak 4 (free thiamine) (14), the tubes containing peak 4 (tubes 65–73) from each chromatographic separation were pooled and the solvent removed under vacuum at 40°. The residue was dissolved in 20 ml of water and analyzed for thiamine, using microbiological assay with *Lactobacillus viridescens* (15). The results revealed 273.9 μg of thiamine activity in peak 4 from the animals fed the high fat diet as compared with 171.6 μg in that of the low fat group.

Daily analysis of urine for radioactivity from thyroxine-treated animals and their controls during the 10-day experimental period showed no remarkable difference between the 2 groups. Conversely, measurement of the $^{14}CO_2$ excretion during the last 4 days of the experiment showed that the thyroxine-treated group excreted about 13% more $^{14}CO_2$ than did the controls ($P < 0.01$). These data are presented in table 2.

DISCUSSION

In this study, decreased production of $^{14}CO_2$ from radioactive thiamine by rats fed a high fat diet was associated with an increased urinary excretion of free thiamine. The excretion of other radioactive meta-

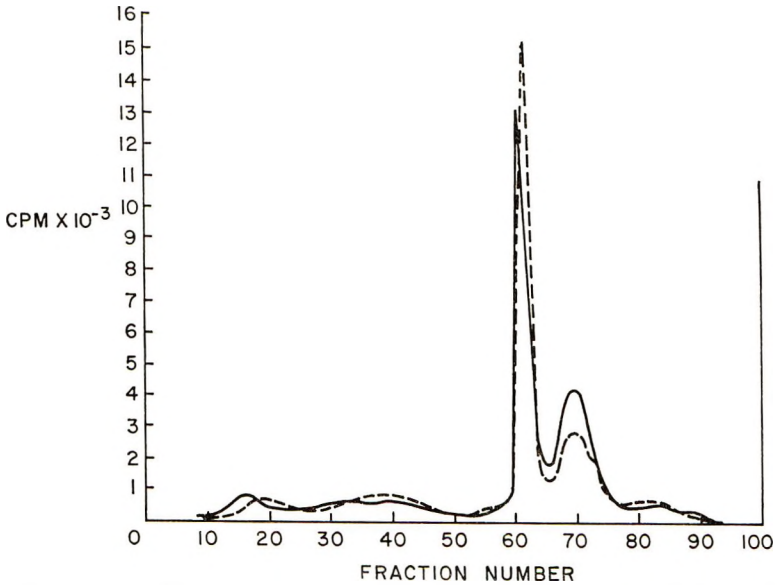

Fig. 1 Comparison of the chromatographic pattern of radioactive metabolities of a pooled 10-day urine collection of 7 rats fed the high fat and low fat diets, respectively. Exchanger, Amberlite CG-50 H^+ form (200–400 mesh), column size, 1 × 40 cm. The column was first eluted with 200 ml of water and then with 300 ml of a mixture of pyridine/acetic acid/water (75/10/915 v/v). Fraction size 5 ml, flow rate 60 ml /hour. *Continuous line* = rats fed high fat diet; *broken line* = rats fed low fat diet.

bolites of thiamine was not enhanced. These observations support the thesis that an alteration in the fat-to-carbohydrate ratio of the diet has a direct effect upon the metabolic need for thiamine. The data suggest that this effect is specifically concerned with the conservation of thiamine itself because the conversion of the 2-^{14}C-thiazole moiety of CO_2 may represent a "wear-and-tear" loss of thiamine as a direct consequence of its participation in metabolic reactions in the body (pyruvate dehydrogenase step?) or may be the result of unknown extra-metabolic reaction(s). The former hypothesis appears to be more reasonable because it is well-known that acetyl CoA can be generated from fat without the participation of the thiamine pyrophosphate, whereas this cofactor is required when acetyl CoA is derived from glycolysis. In any event, the substitution of fat for carbohydrate prevents the catabolic destruction of some thiamine and the term "sparing effect" appears to be an appropriate one.

Since the thiamine "spared" from the catabolic destruction by the high fat intake was excreted in the urine no actual nutritional advantage accrued to the rats in these studies. This observation does not detract from the biochemical interpretation, however, when it is considered that the experimental animals were in excellent thiamine nutriture. The fact that they were excreting significant quantities of radioactive thiamine in their urine (fig. 1) testifies to the adequacy of their tissue stores because assayable thiamine does not appear in rat urine until the tissue stores are 75 to 80% saturated (16). Under these circumstances it appears reasonable to assume that any "spared" thiamine would be excreted in the urine along with the excess already appearing there. Under more severe dietary conditions (i.e., thiamine deficiency) the "spared" thiamine would probably not be excreted but would be retained for metabolic purposes. Thus, a delay in onset of thiamine deficiency in the rat would be observed.

An alternate hypothesis for the thiamine-sparing effect of dietary fat deals with its possible effects upon the intestinal flora. This concept, first considered by Evans and Lepkovsky (1) has been recently re-evaluated by Meghal and Nath (17).

These workers concluded that dietary fat enhances the microbial synthesis of thiamine or its availability, or both. We consider intestinal microfloral effects to be an unlikely explanation for our observations because 1) the presence of succinylsulfathiazole in our diets depressed the intestinal microflora, 2) the screen-floor cages used permitted only limited access to the feces, and 3) the difference in thiamine excretion was evident within 24 hours of feeding the animals their respective diets (table 1). That bacterially synthesized ^{35}S-labeled thiamine does not appear in the heart and liver of rats during the 48-hour period following its synthesis has been well-demonstrated by Wostmann and Knight (18).

Of the other hypotheses proposed to explain the thiamine-sparing effect of fat, two (production of toxic material from carbohydrate (19); anti-convulsive effects of ketone bodies (6)) are without tangible experimental support, and a third (an effect of glycerol produced from the fat) has been tested and discarded by Jones (20).

Since it is known that thyroxine administration increases the thiamine requirement (11, 12, 21), it is to be expected that its effect on thiamine metabolism would be diametrically opposed to that induced by feeding a high fat diet. The CO_2 production data presented in table 1 are consistent with this anticipated effect. Since the major metabolic effect of thyroxine is to increase the basal metabolic rate and accelerate the reactions involved in energy production, it may be assumed that the increased breakdown of thiamine in thyroxine treated rats is the consequence of its participation in the normal metabolic reactions of the animal body rather than the result of a specific effect of thyroxine on thiamine catabolism. The fact that the requirements for several other vitamins are increased by thyroxine suggests further that this is probably a general effect of the hormone secondary to its effect on the metabolic rate rather than a specific effect on thiamine catabolism. Further evidence in favor of this idea is that other factors, such as physical activity which raise the basal metabolic rate, may also increase the thiamine requirement (3).

The percentage of radioactivity excreted in the urine by thyroxine-treated rats did not differ from that of the control animals. Since the former group showed a higher excretion of radioactive CO_2, it might be anticipated that there would be a concomitant decrease in the urinary excretion of radioactivity. It is possible that larger doses of thyroxine are necessary to produce any appreciable effect on the urinary excretion of thiamine especially when the tissue thiamine stores are adequate.

LITERATURE CITED

1. Evans, H. M., and S. Lepkovsky 1929 Sparing action of fat on the antineuritic vitamin B. J. Biol. Chem., *83:* 269.
2. Gregory, E., and J. C. Drummond 1932 A study of fat metabolism with special reference to nutrition on diets devoid of fat. Z. Vitaminforsch., *1:* 257.
3. Cowgill, G. R. 1934 The vitamin B requirement of man. Yale University Press, New Haven, Connecticut, p. 97.
4. Holt, L. E., Jr., and S. E. Snyderman 1955 The influence of dietary fat on thiamine loss from the body. J. Nutr., *56:* 495.
5. Lossy, F. T., G. A. Goldsmith and H. P. Sarett 1951 A study of test dose excretion of five B complex vitamins in man. J. Nutr., *45:* 213.
6. Kemmerer, A. R., and H. Steenbock 1933 A study of the sparing of fats on the vitamin B content of animal tissues. J. Biol. Chem., *103:* 353.
7. Gershoff, S. N., and D. M. Hegsted 1954 The failure of thyroxine and high fat diets to modify the rate of thiamine loss from the body. J. Nutr., *54:* 609.
8. Evans, H. M., and S. Lepkovsky 1935 The sparing action of fat on vitamin B. VIII. On the loss of vitamin B from the rat's tissues. J. Biol. Chem., *108:* 439.
9. Gruber, M. 1950 Nature of the vitamin B_1-sparing action of fat. Nature, *166:* 78.
10. Kleiber, M. 1945 Dietary deficiencies and energy metabolism. Nutr. Abstr. Rev., *15:* 207.
11. Himwich, H. E., W. Goldfarb and G. R. Cowgill 1932 Studies in the physiology of vitamins. XVII. The effect of thyroid administration upon the anorexia characteristic of lack of undifferentiated vitamin B. Amer. J. Physiol., *99:* 689.
12. Peters, R. A., and R. J. Rossiter 1939 Thyroid and vitamin B_1. Biochem. J., *33:* 1140.
13. Balaghi, M., and W. N. Pearson 1966 Metabolism of 2-^{14}C-thiazole-labeled thiamine by the rat. J. Nutr., *89:* 265.
14. Neal, R. A., and W. N. Pearson 1964 Studies of thiamine metabolism in the rat. I. Metabolic products found in urine. J. Nutr., *83:* 343.

15. Deibel, R. H., J. B. Evans and C. F. Niven, Jr. 1937 Microbiological assay for thiamine using *Lactobacillus viridescens*. J. Bacteriol., *74:* 818.
16. Pearson, W. N., W. J. Darby, Jr., E. Hung, M. Balaghi and R. A. Neal 1966 Excretion of metabolites of ^{14}C-pyrimidine-labeled thiamine by the rat at different levels of thiamine intake. J. Nutr., *89:* 133.
17. Meghal, S. K., and M. C. Nath 1965 Action of fats and linoleic acid on intestinal synthesis of thiamine in thiamine-deficient rats. Brit. J. Nutr., *19:* 189.
18. Wostmann, B. S., and P. L. Knight 1961 Synthesis of thiamine in the digestive tract of the rat. J. Nutr., *74:* 103.
19. Westerbrink, H. G. K. 1934 Uber das Verschwinden das vitamin B_1 sus einigen organen von Tauben bei vitamin-B_1-Freier, Kohlehydrat Freier, Fettreicher Nahrung, Nebst einer vergleichung des vitamin-B_1-Gehaltes von organen von ratten und Tauben. Arch. Neerl. Physiol., *19:* 116.
20. Jones, J. H. 1962 A striking difference in the thiamine-sparing action of fat on deficiencies produced by oxythiamine and pyrithiamine. J. Nutr., *78:* 353.
21. Cowgill, G. R., and M. L. Palmieri 1933 Studies in the physiology of vitamins. XXII. The effect of experimentally induced hyperthyroidism on the vitamin B requirement of pigeons. Amer. J. Physiol., *105:* 146.

Metabolism of a Plant Wax Paraffin (*n*-Nonacosane) in the Rat

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ABSTRACT Utilization of orally administered long-chain solid paraffin hydrocarbons by the rat was studied with ^{14}C-*n*-nonacosane (C_{29} H_{60}) as a tracer and evidence was presented which shows that these wax hydrocarbons from food are absorbed and metabolized by the rat. Nonacosane, the major paraffin of most plant waxes, is partly absorbed from the digestive tract, the major portion (75%) being excreted. Of the administered radioactivity, 4% appeared in respiratory CO_2 during the 5-day experimental period. Radioactivity was widely distributed in the many tissues and organs examined, the liver having the greatest amount (about 2% of the administered ^{14}C). Fractionation of total lipids from organs such as lung, heart, kidney and liver showed that paraffins accounted for less than 10% of the radioactivity. Gas-liquid chromatography of liver paraffins showed the presence of ^{14}C-nonacosane. About 20% of the radioactivity in the blood was found in the paraffins. Although ^{14}C was found in all classes of lipids in the liver, the major part of the radioactivity was in the phospholipids, almost all being in the fatty acid portion. Gas-liquid chromatography of the fatty acids derived from both the glycerides and phospholipids showed that most of the radioactivity present was present as C_{15}, C_{16}, C_{17}, C_{18} and C_{19} saturated fatty acids, the C_{17} being the most radioactive.

Recent reports have been noted which show saturated hydrocarbons to be present in human arterial tissues and plaques (1) and in lymph nodes, spleen and portal triads of liver in human autopsy and biopsy material.[1] Although no evidence was presented about the origin of these hydrocarbons, it was suggested that food products are a likely source. Long-chain hydrocarbons are widely distributed in animals (2) and plants (3). Crude and refined edible seed oils also contain a mixture of hydrocarbons (4). Although very complex mixtures of paraffins are often found in nature, normal saturated paraffins of the chain length C_{29} and C_{31} predominate in plants (3). For example, apple peel and common vegetables such as broccoli, cauliflower and cabbage contain normal paraffins of which about 90% is *n*-nonacosane (3, 5).

Extensive work has been carried out on the metabolism of various paraffins by microorganisms (6). In most cases hydrocarbons of medium-chain length (up to C_{18}) were studied, but there is ample evidence to show that microorganisms do utilize solid long-chain hydrocarbons (7). To the best of our knowledge nothing is known about the metabolic fate of the long-chain solid hydrocarbons that are generally present in the diet, although some work has been carried out with short-chain paraffins (8).

Results reported in this paper demonstrate that in the rat a solid paraffin normally found in food is absorbed, transported to various organs and metabolized, thus providing experimental evidence that the paraffins in animal organs may be at least in part derived from the diet.

EXPERIMENTAL

Radioactive *n*-nonacosane was isolated by the method recently reported (5) from brocolli leaves which had metabolized 1 mCi of 1-^{14}C-acetate for 4 days under light. Presumably the ^{14}C is present throughout the length of the molecule probably in alternate positions. About 2.4% of the ^{14}C administered to the leaf was recovered in the paraffin fraction, and this fraction was used as the substrate without further purification because about 90% was shown to be nonacosane (5).

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[1] Boitnott, J. K., and S. Margolis 1966 The increasing incidence of mineral oil in human tissues. Federation Proc., 25: 200 (abstract).

A 274-day-old male rat (600 g) of the Osborne-Mendel strain was fed for 5 days a diet consisting of ground dog meal [2] containing 1% of sulfaguanidine and 0.1 of neomycin sulphate (504 μg/g) to suppress the bacterial flora of the intestine as much as possible. Preliminary experiments had shown that when a few milligrams of ^{14}C-nonacosane were administered by stomach tube in 0.5 ml corn oil to a rat, most of the ^{14}C-nonacosane could be recovered from the feces, but measurable amounts of ^{14}C were observed in tissue lipids. To increase the absorption of the paraffin the following procedure was used in feeding the experimental animal. The radioactive paraffin (about 30 mg, 20.5 μCi) was dissolved in a small amount of pentane, and this solution was allowed to soak into about 10 g of warm (55°) dry dog food meal. After the pentane had evaporated completely, the radioactive food was mixed with warm (55°) 4% agar solution. The food was immediately divided into 5 approximately equal parts and allowed to harden. Each morning for 5 days, the rat was given one portion of the radioactive food after overnight starvation (16 hours). This was eaten completely with minimal spillage within 10 minutes. Subsequently throughout the day the rat received the ordinary dog food meal containing the antibacterial agents. The rat was kept in a large desiccator fitted with air inlet and outlet tubes throughout the experimental period except for the 10 minutes each day required for feeding the radioactive material. The respiratory CO_2 was collected in an alcoholic solution of ethylenediamine (10:1, v/v), the CO_2 trap being changed twice daily. Feces were collected on a wire screen placed in the desiccator and urine was collected at the bottom. The experiment was terminated 6 hours after feeding the last portion of the radioactive food. The animal was anesthetized with ether and the blood removed directly from the heart with a hypodermic syringe. Total lipids were extracted from blood, feces, urine, gut contents, various internal organs, abdominal fat pads, and portions of the subcutaneous fat and muscle with a 2:1 chloroform-methanol mixture (9).

Total lipid samples were placed on silica gel (10) columns (up to 2 mg lipid/g silica gel) and eluted successively with hexane, benzene, ether and methanol. Eluents (2 void volumes of each solvent) were collected in 10- or 20-ml portions, and the lipids located by assaying for ^{14}C. Lipid classes were identified by thin-layer chromatography (11), infrared spectroscopy (12), and phosphorus assays (13). Hexane eluted paraffins, benzene eluted cholesterol esters together with triglycerides, ether eluted mono- and diglycerides with free fatty acids, and finally, methanol eluted phospholipids.

Glyceride and phospholipid fractions of the liver lipids were saponified by refluxing under nitrogen with 20% alcoholic KOH for 5 hours. The fatty acids were then isolated, and methyl esters prepared with BF_3 as a catalyst. The methyl esters were purified by column chromatography on silica gel with 2% ethyl ether in ligroin (60–70°) as eluent.

A sample of oven-dried dog food was extracted with hexane in a soxhlet extractor for 4 hours; the lipid solution was evaporated to dryness, and paraffins were isolated by silica gel column chromatography as described.

Gas chromatographic analyses were made on a Perkin Elmer 810 gas chromatograph with a flame ionization detector. Fatty acids were analyzed on a 15% stabilized DEGS column and an Apiezon L column [3]; paraffins were analyzed on a 5% silicone rubber gum (SE-30) column.[4] Authentic samples of paraffins [5] and other lipid standards [6] were used for thin-layer and gas-liquid chromatography.

Radioactivity in the respiratory CO_2 was determined in aliquots of the ethanolic

[2] The manufacturer's analysis of this material is as follows: (in per cent) protein (min.), 25.80; fat (min.), 10.20; fiber (max.), 3.31; ash (max.), 9.90; N.F.E., 42.7; histidine, 0.57; methionine, 0.43; lysine, 1.34; tryptophan, 0.40; phenylalanine, 1.07; leucine, 2.04; threonine, 0.96; valine, 1.26; isoleucine, 1.14; calcium, 2.1; phosphorus, 1.4; sodium, 1.4; potassium, 0.8; magnesium, 0.5; and (in mg/kg) copper, 9.5; manganese, 5.3; cobalt, 2.2; iron, 167.4; zinc, 6.6; iodine, 1.5; vitamin B_1, 7.9; vitamin B_2, 6.8; vitamin B_6, 1.5; niacin, 30.4; pantothenic acid, 7.5; choline, 1270.9; and (in μg/kg) vitamin B_{12}, 29.1; (in USP units/kg) vitamin A, 11,674; vitamin D, 5,507; and (in IU/kg) vitamin E, 46.9 (Big Red Dog Food Meal, Agway, Inc., Syracuse, New York).

[3] From the Analytical Engineering Laboratory, Hamden, Connecticut.

[4] See footnote 3.

[5] Gift from Dr. B. J. Humphrey, Humphrey Chemical Co., North Haven, Connecticut.

[6] From The Hormel Institute, Austin, Minnesota.

solution of ethylenediamine carbonate in a liquid scintillation counter. Aliquots of lipid solutions, generally in ether, were similarly assayed for ^{14}C. In general, counting was done with 62% efficiency and a standard deviation not greater than 3%. The liquid scintillation mixture and the determination of ^{14}C in the thin-layer and gas-liquid chromatographic fractions have been described previously (5).

RESULTS AND DISCUSSION

About 75% of the administered radioactivity was recovered from the excretion products. Almost all of the excreted radioactivity was noted in unchanged hydrocarbons. When the paraffins recovered from the excretion products were subjected to gas-liquid chromatography, a pattern identical to the substrate hydrocarbon (5) was obtained together with a series of additional peaks corresponding to paraffins mostly smaller than C_{29} (see fig. 1). In the paraffin fraction isolated from the dog food diet, a series of hydrocarbons including C_{29} was detected by gas-liquid chromatography. The aditional hydrocarbons observed in the excretion products were presumably derived from the diet. Cattle manure for example has been reported to contain paraffins identical to those in pasture plants (14). It is clear that the major portion of the administered paraffin is excreted unchanged, unlike such substrates as 2:2 dimethyl stearic acid which is mostly absorbed (15). However such paraffins as hexadecane have been reported to be absorbed well (50 to 100 mg /day) (16). Most microorganisms utilize long-chain solid paraffins much less efficiently than shorter liquid paraffins (17).

A considerable amount of the paraffin eaten by the rat is metabolized as indicated by the distribution of ^{14}C in the various organs (table 1). The appearance of 4% of the administered ^{14}C in the respiratory CO_2 shows that a significant amount of the paraffin had undergone extensive metabolic breakdown by the end of the experimental period. Although the water-soluble metabolites were not investigated in detail, measurable amounts of ^{14}C could be detected in these fractions. Data in table 1 indicate that the ^{14}C from orally administered vegetable hydrocarbons is incorporated into the lipids of every organ examined. Liver had the greatest amount of ^{14}C, almost 2% of the ^{14}C eaten by the rat.

An increase in the unsaponifiable fraction of tissue lipids after the administration of hexadecane to animals has been reported (16). Hexadecane has been isolated from tissue lipids after prolonged feeding of this material (18). Accordingly the tissue lipids were fractionated by column chromatography and the results are shown in table 1. Small but significant amounts of ^{14}C were present in the paraffin fractions isolated from organs such as liver. This observation indicates that paraffins were taken up from the food and translocated through blood into various organs and probably metabolized there. The presence of large proportions of ^{14}C in the hydrocarbon fraction of the intestinal wall lip-

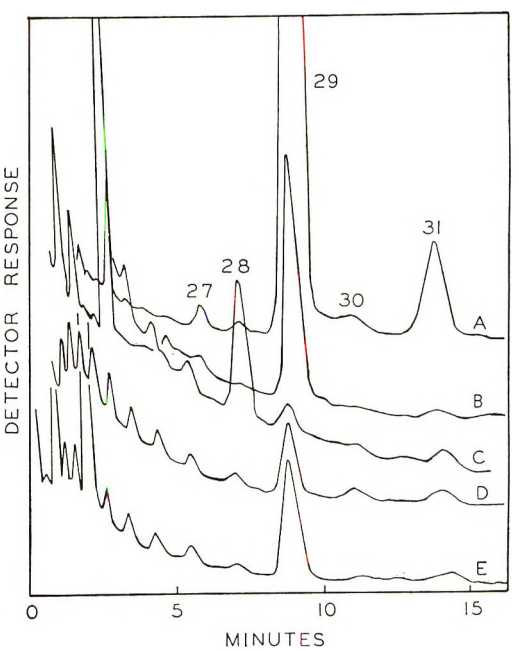

Fig. 1 Gas-liquid chromatography of the substrate paraffins (A) and the hydrocarbons isolated from liver (B) food (C), feces (D), and urine (E). Number on each peak represents the chain length. Paraffins shorter than C_{25} were not identified. Radioactivity distribution was identical in substrate, feces, gut contents, urine and liver. Experimental conditions: 122-cm (0.6-cm o.d.) (4-foot (0.25 in. o.d.)) coiled copper column, 5% silicone rubber gum (SE-30) on 80-90 mesh Anakrome ABS; column temperature 290°, carrier gas He at 45 ml/minute.

TABLE 1

Distribution of radioactivity in lipids of organs and excreta obtained from a rat metabolizing ^{14}C-nonacosane [1]

Fraction	Radioactivity in total lipids	Radioactivity in paraffins
	cpm	*% of total lipid/fraction*
Kidney	10,300	1.4
Blood [2]	28,000	18.0
Abdominal fat pads [3]	190,000	—
Liver	450,000	10.0
Muscle [4]	10,000	—
Heart	14,000	2.1
Lung	41,000	1.5
Brain	6,300	4.0
Testes	4,000	—
Gut contents	4,780,000	99.1
Urine [5]	850,000	98.0
Subcutaneous fat [6]	50,000	—
Feces	13,300,000	98.9
Intestinal wall [7]	116,000	62.0

[1] Of the 26.5×10^6 cpm fed to the rat only 0.6×10^6 cpm could be recovered from the spillage and food cups, 0.96×10^6 cpm appeared in the respiratory CO_2.
[2] All the blood that could be removed from the heart is included.
[3] All of the fat pads that could be dissected.
[4] A piece of the abdominal muscle was extracted to determine whether radioactive lipids were present in muscle and probably only less than 5% of the total muscle of the rat is represented in this fraction.
[5] Urine had some contact with the feces and hence some of the radioactivity could have been derived from the feces.
[6] Only a small portion of the subcutaneous fat was used to determine whether radioactive lipids were incorporated into this fraction. No accurate estimate can be made of total radioactivity in this fraction.
[7] Intestinal walls were washed with water before lipids were extracted. However, the possibility that traces of adsorbed paraffin were present cannot be ruled out with certainty.

ids, and the distribution of radioactivity in blood lipids (table 1) support this contention. Moreover, when the liver hydrocarbon fraction was further analyzed by gas-liquid chromatography, *n*-nonacosane could be detected (see fig. 1), and most of the ^{14}C was located in this paraffin just as it was in the original substrate. This demonstrates that food paraffins are translocated intact to the liver. The liver hydrocarbon fraction also contained some additional paraffins, similar in retention time to those present in the diet, feces and gut contents (fig. 1). It is likely, therefore, that other paraffins present in the food are absorbed from the gut just as is nonacosane. Thus it may be concluded that the paraffins from the diet are absorbed in the intestines and translocated via blood to various organs; a similar conclusion has been drawn in the case of short-chain paraffins also (8). In the light of recent observations that human organs and tissues such as arterial walls and plaques contain hydrocarbons of unknown origin (1),[7] the present results are highly significant, since a possible source of such material is now indicated. To ascertain to what extent the diet contributes to the hydrocarbon deposits in animal tissues, further studies on the composition of tissue hydrocarbons and the availability of these in the diet are needed.

Organs such as heart, kidney and lung, in contrast with the liver, contain much less radioactive lipid and in particular, extremely small amounts of radioactive paraffins. Probably the liver is the most active site of paraffin metabolism. The presence of radioactive lipids in other organs may be at least in part due to translocation of metabolites of hydrocarbon from the liver. Stetten (8) also concluded that hexadecane is metabolized largely in the liver.

As liver appears to be the organ in which ^{14}C from nonacosane is concentrated, the total lipids from this organ were fractionated on a silica gel column. From the distribution of activity shown in table 2, it is clear that nonacosane contributed its carbon atoms to every class of lipid examined. The phospholipid fraction had more radioactivity than all other fractions.

It has been reported that animals convert paraffins such as hexadecane into fatty acids of the same chain length (8), an observation similar to that made with microorganisms (6). To determine whether this is true for long-chain paraf-

TABLE 2

Radioactivity in rat liver lipids [1]

Lipid fraction	Radioactivity	
	cpm	*% of total*
Hydrocarbons	45,900	10.2
Cholesterol esters	3,600	0.8
Triglycerides	91,800	20.4
Mono- and diglycerides and free fatty acids	58,500	13.0
Phospholipids	250,200	55.6

[1] Total lipid was fractionated on a silica gel column; elution of lipids was followed by assays for ^{14}C and the eluted lipids were then identified by thin-layer chromatography on silica gel, phosphorus assay and infrared spectroscopy.

[7] See footnote 1.

fins such as nonacosane, the liver lipids were saponified, and the fatty acids were examined by thin-layer chromatography. Almost all of the radioactivity of the glyceride and phospholipid fractions was found to be in the fatty acids. When the methyl esters of fatty acids were analyzed on a diethylene glycol succinate column, the major part of the radioactivity could be located in the fatty acids that are present only in trace quantities. Comparison of the retention times of the radioactive fatty acids with those of authentic samples showed that these highly radioactive minor components are C_{17}, C_{19} and C_{15} (figs. 2 and 3). To avoid the possibility that unsaturated fatty acids are not well separated from the next higher homologue of the saturated series on the polyester column, the methyl esters were analyzed on an Apiezon L column. On this column unsaturated fatty acids precede the saturated ones. The results confirmed those obtained with the polyester column (see figs. 4 and 5). To make sure that the peaks of radioactivity and mass coincided, nonradioactive C_{15}, C_{17} and C_{19} were added to the radioactive samples and the mixture was chromatographed, care being taken to collect several fractions of the effluent during the elution of each single added fatty acid. The radioactivity collected was proportional to the mass of the component collected. The distribution of radioactivity in various fatty acids isolated from the phospholipids and glycerides is shown in figures 2, 3, 4, and 5. In both phospholipids and glycerides, the fatty acids which contain significant radioactivity are the C_{15}, C_{16}, C_{17}, C_{18}, and C_{19} acids. Heptadecanoic acid, which is present in the rat only in trace quantities was the most radioactive. This observation suggests that the C_{29} molecule, presumably after being oxidized to the corresponding acid, undergoes successive β-oxidation until the chain length becomes similar to those fatty acids (C_{16} and C_{18}) that are generally present in the organism. The presence of radioactivity in C_{15} and C_{19}

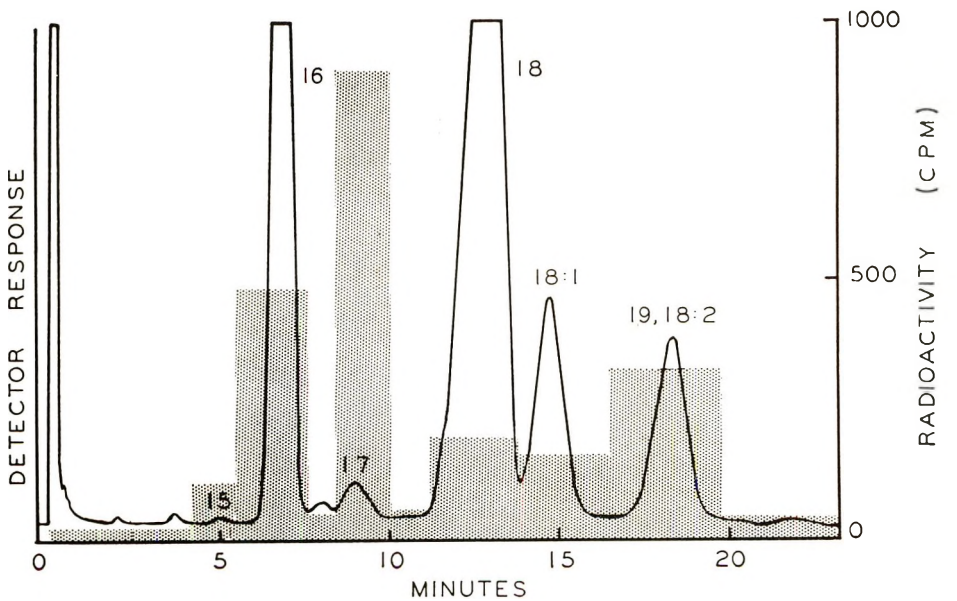

Fig. 2 Gas-liquid chromatography of the methyl esters of fatty acids isolated from the phospholipids of rat liver. Number on each peak represents chain length, the number that follows the colon is the number of double bonds. Nonradioactive methyl heptadecanoate added. Radioactivity represented by the bar graph; height of each bar represents the total radioactivity collected during the time represented by the width of the bar. Experimental conditions: 183-cm (0.6-cm o.d.) coiled copper column, 15% stabilized diethylene glycol succinate on 60–70 mesh Anakrome AB, column temperature 200°, carrier gas He at 67 ml/minute.

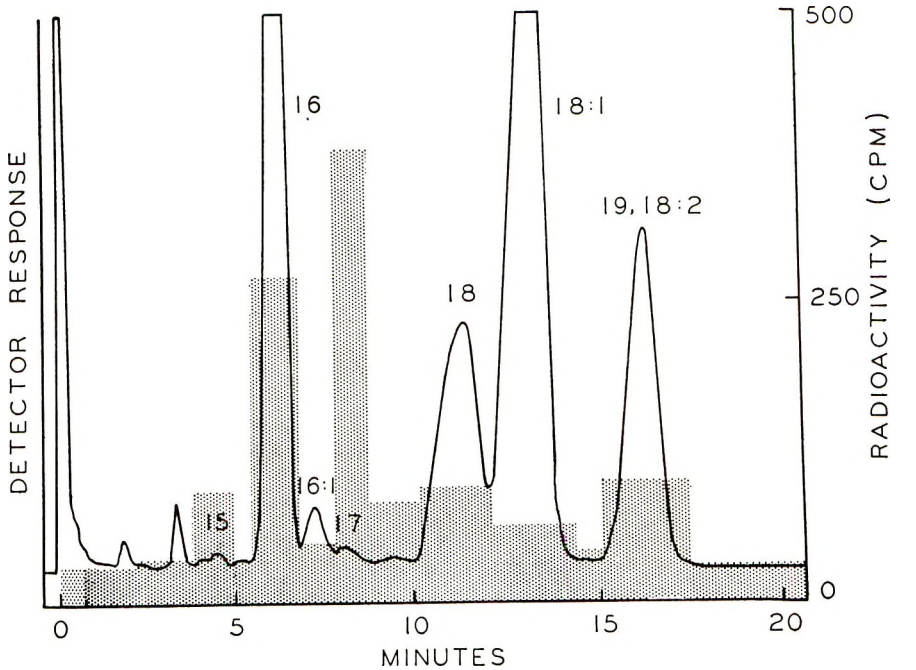

Fig. 3 Gas-liquid chromatography of the methyl esters of fatty acids isolated from the glycerides of rat liver. Experimental conditions and presentation of radioactivity identical to those in figure 2.

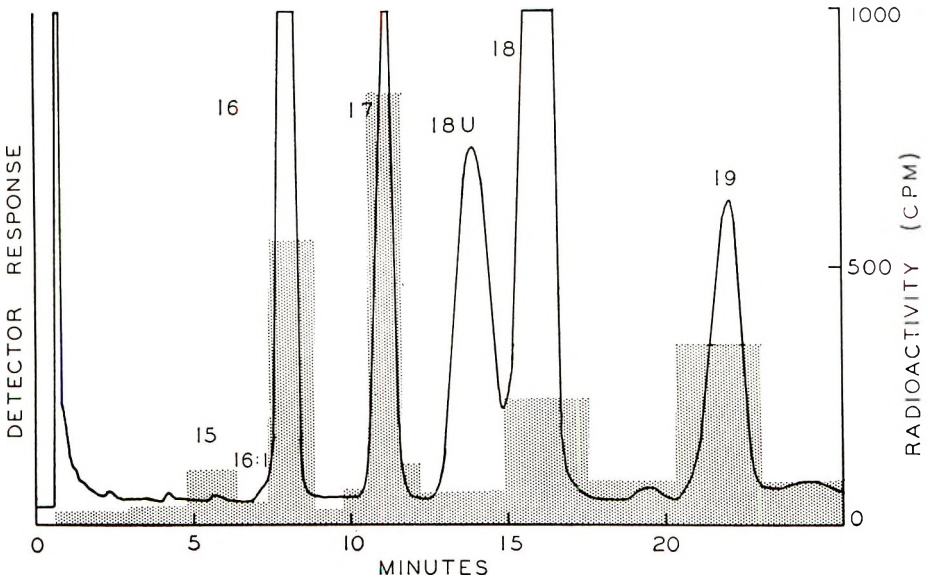

Fig. 4 Gas-liquid chromatography of the methyl esters of fatty acids isolated from the phospholipids of rat liver. Non-radioactive methyl esters of heptadecanoic acid and nonadecanoic acid added. Presentation of radioactivity in the same manner as in figure 2. Experimental conditions: 183-cm (0.6-cm o.d.) coiled copper column, 12% Apiezon L on 50–60 mesh Anakrome A, column temperature 250°, carrier gas He at 60 ml/minute. U = unsaturated.

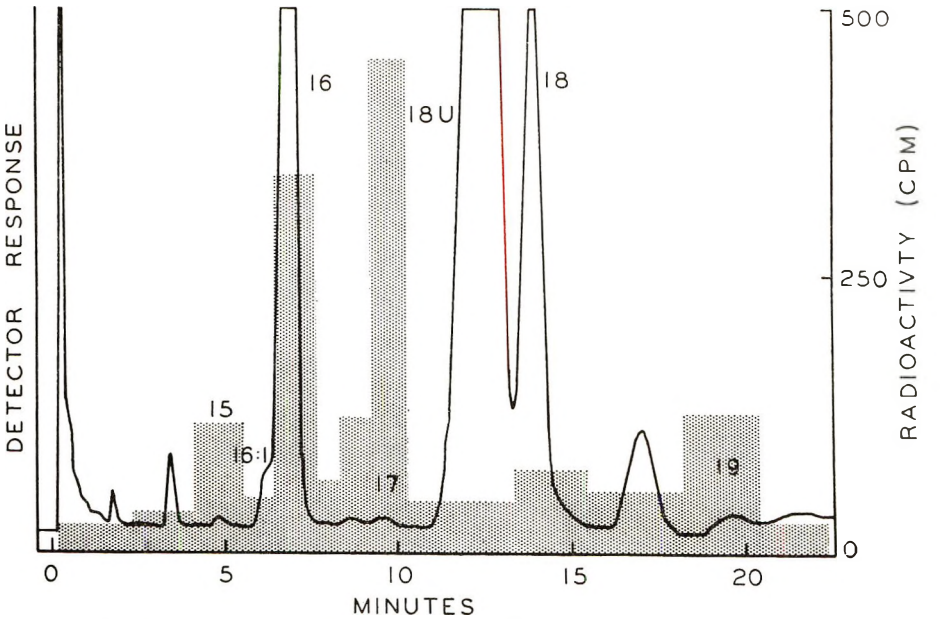

Fig. 5 Gas-liquid chromatography of the methyl esters of fatty acids isolated from the glycerides of rat liver. Experimental conditions same as in figure 3, presentation of radioactivity in the same manner as in figure 2.

acids support such a contention. In microorganisms the carbon chain of paraffins shorter than C_{16} are elongated to C_{16} or C_{18} during their incorporation into other lipids (6). Presumably carbon chains of paraffins much longer than C_{18} are shortened by β-oxidation to the vicinity of C_{16} or C_{18} before being incorporated into tissue lipids. Such a trend is indicated in the fatty acids produced by *Nocardia* grown on *n*-paraffins (19). The presence of radioactivity in palmitic acid may be explained by either α-oxidation of the C_{17} acid or by the synthesis of palmitic acid from acetate or other short-chain acids produced from the radioactive paraffin. Small amounts of radioactivity found in other fatty acids with even numbers of carbon atoms might have been from similar sources.

That hydrocarbons are utilized by animals, at least in small quantities, raises the question of their possible nutritive value. In a diet rich in vegetables, long-chain hydrocarbons (C_{27} to C_{31}) are generally present in small quantities. Our study with nonacosane, one of the hydrocarbons most commonly found in vegetables, indicates that the major part of the paraffins consumed by a rat is excreted and thus lack of absorption appears to limit their utilization. If the normal population of intestinal bacteria were present, the utilization of the paraffin might have been higher, since many species of bacteria can produce metabolites (6) that might be more easily absorbed than paraffins. However, when milligram quantities of radioactive cabbage leaf paraffin were administered in 0.5 ml of corn oil by stomach tube to a mature rat (no antibiotics used) most of the ^{14}C could be recovered from the feces as unchanged hydrocarbon. Under the same conditions, lipids such as 2,2-dimethyl stearic acid and shorter paraffins such as hexadecane were well taken up (15, 16, 18). The fact that paraffins of the pasture plants tend to accumulate in the manure (14) also indicates poor absorption in the digestive tract of cattle. Thus it appears that a major portion of the paraffin present in the food is excreted unabsorbed even when only a few milligrams of hydrocarbons are present in the food. A small increase in chain length of a paraffin often results in a dramatic de-

crease in the rate of utilization of that paraffin by organisms. For example, *Aspergillus versicolor* showed good-to-no growth as the chain length increased from C_{23} to C_{35} (17). Hence the results obtained with relatively small paraffins such as hexadecane cannot be extended to the long solid paraffins that are found in the diet as far as uptake and catabolism are concerned. From our results it may be concluded that the solid paraffins that are generally present in the diet cannot supply significant caloric value. But there can be little doubt as to the ability of animals to utilize, at least to a limited extent, the *n*-paraffins such as nonacosane usually present in the diet.

The observation of omnipresence of paraffins in nature raises the question whether these compounds in small quantities can play a functional role in nutrition in a manner yet to be elucidated.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Gazzarrini, F., and B. Nagy 1966 Saturated hydrocarbons in human femoral arterial tissues and plaques. Arch. Biochem. Biophys., *113:* 245.
2. Nicolaides, N. 1965 Skin lipids. II. Lipid class composition of samples from various species and anatomical sites. J. Amer. Oil Chem. Soc., *42:* 691.
3. Silva Fernandes, A. M., E. A. Baker and J. T. Martin 1964 Studies on plant cuticle. VI. The isolation and fractionation of cuticular waxes. Ann. Appl. Biol., *53:* 43.
4. Kuksis, A. 1964 Hydrocarbon composition of some crude and refined edible seed oils. Biochemistry, *3:* 1086.
5. Kolattukudy, P. E. 1965 Biosynthesis of wax in *Brassica oleracea*. Biochemistry, *4:* 1844.
6. McKenna, J. E., and R. E. Kallio 1965 The biology of hydrocarbons. Ann. Rev. Microbiol., *19:* 183.
7. Zobell, C. E. 1950 Assimilation of hydrocarbons by microorganisms. Adv. Enzymol., *10:* 443.
8. Stetten, D., Jr. 1943 Metabolism of a paraffin. J. Biol. Chem., *147:* 327.
9. Folch, J., M. Lees and G. H. Sloane-Stanley 1957 A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., *227:* 449.
10. Bulen, W. A., J. E. Varner and R. C. Burrell 1952 Separation of organic acids from plant tissues. Anal. Chem., *24:* 187.
11. Mangold, H. K. 1965 II. Thin layer chromatography of lipids. In: Thin Layer Chromatography, a Laboratory Handbook, ed., E. Stahl, Academic Press, New York, p. 147.
12. Freeman, N. K., F. T. Lindgren, Y. C. Ng and A. V. Nicholas 1957 Serum lipid analysis by chromatography and infrared spectrophotometry. J. Biol. Chem., *227:* 449.
13. Stewart, C. P., and E. B. Hendry 1935 The phospholipins of blood. Biochem. J., *29:* 1683.
14. Oro, J., D. W. Nooner and S. A. Wikström 1965 Paraffinic hydrocarbons in pasture plants. Science, *147:* 870.
15. Bergström, S. B., N. Bergström, N. Tryding and G. Westöö 1954 Intestinal absorption and metabolism of 2:2-dimethylstearic acid in the rat. Biochem. J., *58:* 604.
16. El Mahdi, M. A. H., and J. J. Channon 1933 The absorption of *n*-hexadecane from the alimentary tract of the rat. Biochem. J., *27:* 1487.
17. Hopkins, S. J., and A. C. Chibnall 1932 Growth of *Aspergillus versicolor* on higher paraffins. Biochem. J., *26:* 133.
18. Channon, H. J., and J. Devine 1934 The absorption of *n*-hexadecane from the alimentary tract of the cat. Biochem. J., *28:* 467.
19. Davis, J. B. 1964 Microbial incorporation of fatty acids derived from *n*-alkanes into glycerides and waxes. Appl. Microbiol., *12:* 210.

Requirements for Glucose and Amino Acids in Defined Media for Chick Embryos [1]

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ABSTRACT Experiments were conducted to determine the response of young chick embryos to amino acids and glucose in a defined medium for yolk-sac perfusion. As the concentration of glucose in the medium was increased from zero to 5.0 g/liter, the median survival of the embryos increased from 24 to 80 hours of perfusion. A similar increase in survival was obtained when amino acids in a medium containing 1.5 g of glucose/liter were increased from 0 to 4.8 g/liter. It is proposed that these effects may be attributable, in part, to changes in the total solute concentration of the medium. It has been shown, however, that glucose also exerts an effect on survival which is independent of solute concentration. When lysine or valine was omitted from the defined medium, there was a marked reduction in survival: the omission of lysine produced a smaller effect than the omission of valine. When these amino acids were omitted from the medium supplemented with 2.5 ml of yolk and 5 ml of egg white/liter, survival was not decreased, thus indicating the relative effectiveness of small amounts of yolk and egg white as sources of amino acids for the embryo.

The role of nutrition in embryonic development can be studied directly by replacing the natural food supply of the embryo with a defined medium. Such a replacement was attempted by Spratt (1), who observed that explanted one-day chick embryos quickly disintegrated when glucose was omitted from the agar-containing nutrient medium. Refinements in explantation techniques eventually permitted Klein et al. (2) to show for the first time that leucine and lysine are essential for development of the embryo.

Previous attempts to use defined media during yolk-sac perfusion have been unsuccessful (3), but yolk-sac perfusion of 4-day embryos with a defined medium containing small amounts of egg white or yolk permitted survival to 8 days of incubation (4). Best survival was obtained with media in which potassium and glucose concentrations were higher, and sodium and calcium concentrations lower, than in media used for tissue culture. Death followed after a few hours of perfusion with media lacking glucose, thus confirming the observations of Spratt (1).

In some experiments in which the effects of varying the glucose concentrations were studied,[2] survival was better with 5.0 g/liter than with 2.5 g or 1.0 g/liter. This most effective concentration is much higher than that of glucose normally present in the subgerminal fluid (approximately 1.0 g/liter) with which the embryo is in direct contact. The apparent high requirements for glucose plus the observation that egg white enters the yolk-sac during perfusion (3) suggested to us that the glucose concentration in the yolk-sac medium bore some relationship to the movement of egg white into the yolk-sac, or to the enhanced availability of egg white to the embryo. It appeared possible that this may have been a factor in our inability to demonstrate a requirement for amino acids in defined media in earlier studies (unpublished).

In the studies we shall discuss in the present paper, the requirements of chick embryos for glucose and amino acids were reinvestigated in an endeavor to construct a medium with which the requirements of embryos for specific amino acids could be studied. With the observation that amino acids could substitute effectively for a large portion of the glucose normally included in the media, it became possible, through the use of a low glucose-high

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[2] Peters, S. M., and C. R. Grau, unpublished data.

amino acid medium, to minimize the relative contribution of egg white and residual yolk to the protein nutrition of the embryos, and to study for the first time the effect of the lack of specific amino acids on the development of 4-day-old chick embryos.

MATERIALS AND METHODS [3,4]

White-shelled eggs from hybrid, egg-production stocks were obtained from a commercial hatchery, incubated for 3 days at 38°, and prepared with an opening from the yolk-sac to the exterior of the egg using an electrosurgical technique (3, 5). Yolk was flushed out at 3 days by passing a salts-glucose solution through a 20-gauge needle which was inserted into the egg through the opening. Flushing was repeated again at 4 days to remove residual yolk.

Equipment was sterilized by autoclaving and the media were sterilized by passage through membrane filters (Millipore, 0.22 mμ). In experiments in which yolk or egg white was included in the media, fresh yolk or egg white was collected under

[3] Dihydrostreptomycin sulfate was kindly furnished by Merck and Company, Rahway, New Jersey.

[4] Erythromycin glucoheptonate was kindly furnished by Eli Lilly and Company, Indianapolis, Indiana.

TABLE 1

Composition of the defined media; the flushing solutions and media were prepared with demineralized water

Component	(1) Salts-glucose flushing solution	(2) Defined medium	(3) Defined medium
		Glucose experiment	Amino acid experiments
	mg/liter	*mg/liter*	*mg/liter*
NaCl	4460	4460	4460
KCl	1700	1700	1700
$CaCl_2$	20	20	20
$MgCl_2 \cdot 4H_2O$	340	340	340
$MgH_2PO_4 \cdot H_2O$	200	200	200
$NaH_2PO_4 \cdot H_2O$	280	280	280
$NaHCO_3$	1100	1100	1100
D-Glucose			1500
Dihydrostreptomycin sulfate	851	851	851
Erythromycin glucoheptonate	13.4	13.4	13.4
Pyridoxine·HCl		1.0	1.0
Biotin		0.1	0.1
Folic acid		0.1	0.1
Niacin		1.0	1.0
Thiamine·HCl		1.0	1.0
Calcium pantothenate		2.0	2.0
Riboflavin		2.0	2.0
Cyanocobalamin		0.002	0.002
Inositol		10.0	10.0
Choline chloride		300	300
L-Alanine		60	120
L-Arginine·HCl		140	280
L-Aspartic acid		90	180
Glycine		60	120
L-Glutamic acid		190	380
L-Histidine		40	80
L-Isoleucine		100	200
L-Leucine		150	300
L-Lysine·HCl		140	280
L-Methionine		40	80
L-Phenylalanine		80	160
L-Proline		60	120
L-Serine		110	220
L-Threonine		90	180
L-Tryptophan		30	60
L-Valine		110	220
L-Cystine		30	60
L-Tyrosine		80	160

sterile conditions from unincubated eggs and was added directly to defined media which had been sterilized previously by filtration.

The perfusion of the medium into the yolk-sac, begun on the fourth day, was continued at 8-hour intervals at a rate of 30 ml/feeding. Perfused eggs were maintained in an atmosphere of 50% oxygen and 50% nitrogen (7). A diagram of the perfusion apparatus is given in figure 1.

The eggs were candled at each feeding period. Embryos that had died, as indicated by the appearance of the yolk-sac circulatory system, were removed and fixed in 10% formalin for observations and wet-weight determinations. Sterility

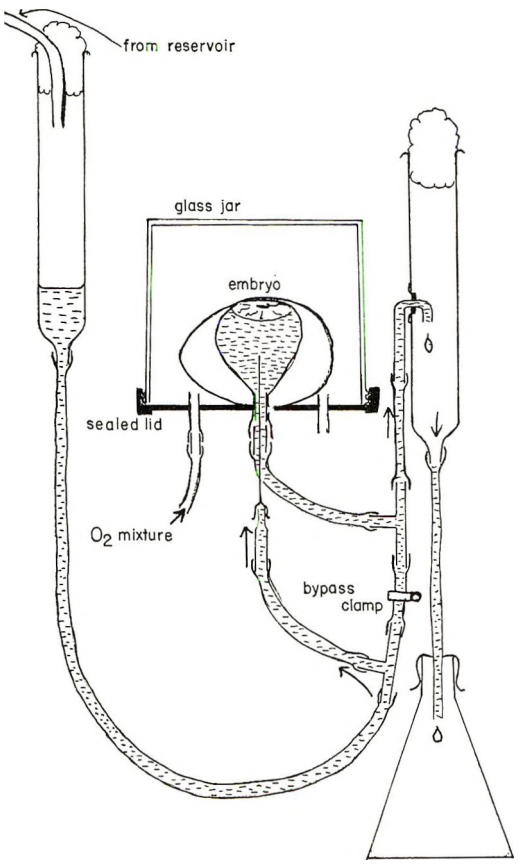

Fig. 1 Perfusion apparatus. The perfusion medium flows from a reservoir through the calibrated tube on the left into the egg. The fluid which is displaced from the yolk-sac passes through the observation tube on the right and is collected in the flask. The arrow in the tubing indicates the direction of fluid flow.

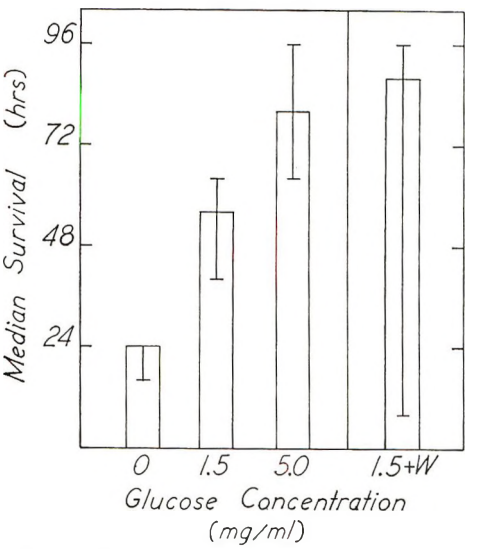

Fig. 2 Effect of glucose concentration on survival. Each bar represents the median survival in hours of perfusion for 6 embryos except the bar for the zero glucose group which represents 5 embryos. The vertical lines represent the range; W = egg white, 10 ml/liter.

was checked by plating a sample of the fluid from the yolk-sac on blood agar.

In the experiment concerned with glucose concentrations (fig. 2), the yolk was displaced at three and four days with the salts-glucose solution (table 1, column 1) containing 5.0 g of glucose/liter. The perfusion medium contained the concentrations of nutrients listed in table 1, column 2. Glucose was omitted, or included at 1.5 g or 5.0 g/liter. One group of embryos received 1.5 of glucose plus 10 ml of fresh egg white/liter.

To maintain a constant concentration of glucose, yolk was displaced with the salts-glucose solution (table 1, column 1) containing only 1.5 g of glucose/liter in the experiment concerned with amino acid concentration (fig. 3). In this experiment 4 groups of embryos were perfused with the defined medium (table 1, column 2) containing 0, 1.6, 3.2, or 4.8 g of amino acids/liter, corresponding to zero, one, two and three times, respectively, the concentrations of amino acids listed in table 1, column 2.

Earlier studies (3) have indicated that the osmolarity of the perfusion medium may be an important factor determining

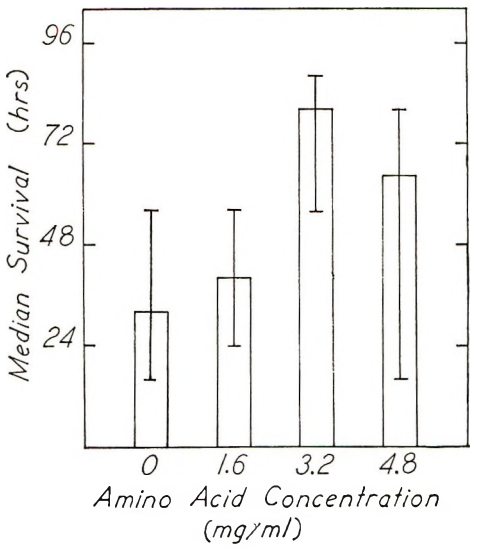

Fig. 3 Effect of amino acid concentration on survival. Each bar represents the median survival in hours of perfusion for 6 embryos. The vertical lines represent the range.

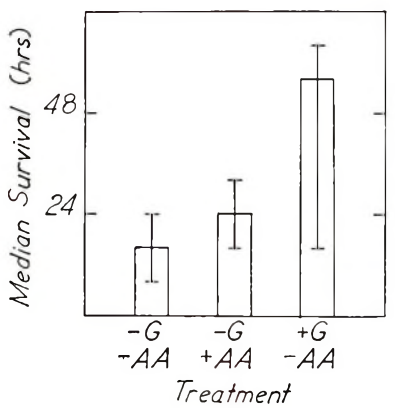

Fig. 4 Effects of isosmotic substitution of glucose and amino acids for salts in a defined medium lacking glucose and amino acids. Each bar represents the median survival, in hours of perfusion, for 6 embryos. The verticle lines represent the range; AA = amino acids; G = glucose.

embryo survival. Since the osmolarity of the media was not held constant in the 2 previous experiments as glucose and amino acid concentrations varied, the following experiment (fig. 4) was designed to determine the response of embryos to the addition of glucose and amino acids to a medium in which osmotic pressure was not a variable.

The basal medium contained no glucose or amino acids. The concentrations of salts and antibiotics were 1.15 times those listed in table 1, column 2; the vitamin concentrations, except for choline, were one-tenth of those listed. To maintain the osmolarity of the medium constant, the concentrations of salts and antibotics were 1.05 times those listed in table 1, column 2 when glucose or amino acids were added to the medium. One group of embryos received the basal medium; one group each received the medium supplemented with 4.2 g of glucose/liter or 3.2 g of amino acids/liter.

In the experiments in which lysine and valine were studied (figs. 5 and 6) in defined media, the yolk was displaced with the salts-glucose solution containing 1.5 g of glucose/liter (table 1, column 1). Lysine and valine were omitted from the medium containing 3.2 g of amino acids/liter (table 1, column 3), or were included in concentrations listed in table 1, column 3.

Lysine and valine were also studied in media supplemented with 2.5 ml of yolk plus 5.0 ml of egg white/liter (fig. 7). Yolk was displaced with the salts-glucose solution containing 5.0 g of glucose/liter (table 1, column 1). The perfusion me-

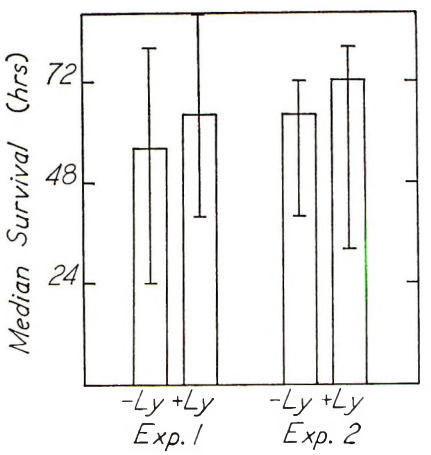

Fig. 5 Effect of lysine deficiency on survival. Each bar represents the median survival in hours of perfusion. The vertical lines represent the range. There were 7 embryos in the +Ly group of experiment 1 and the —Ly group of experiment 2, and 8 embryos in each of the other 2 groups. The +Ly medium contained 3.2 g of amino acids/liter.

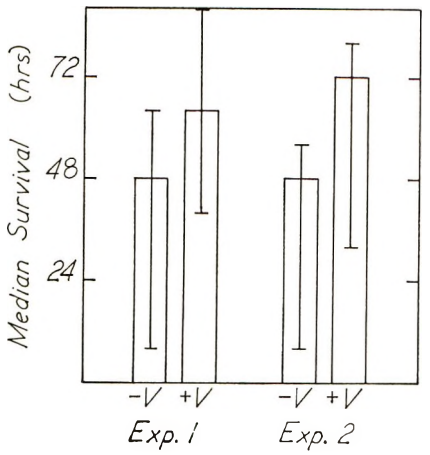

Fig. 6 Effect of valine deficiency on survival. Each bar represents the median survival in hours of perfusion. The vertical lines represent the range. Each bar represents 8 embryos except the bar for the +V group in experiment 1 which represents 7 embryos. The +V medium contained 3.2 g of amino acids/liter. V = valine.

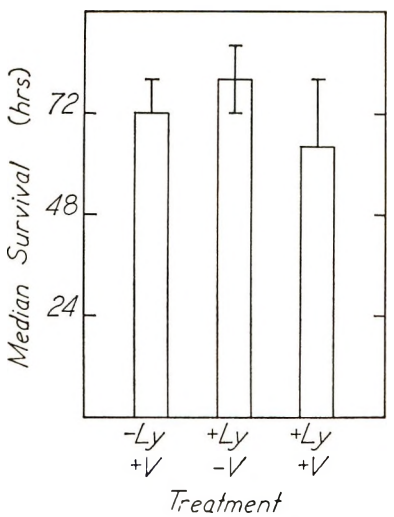

Fig. 7 Effect on survival of lysine and valine omission from the defined medium supplemented with 2.5 ml of yolk and 5.0 ml of egg white/liter. The first and third bars represent the median survival, in hours of perfusion, for 3 embryos; the second bar represents that for 4 embryos. Vertical lines represent the range. The +Ly +V medium contained 4.8 g of purified amino acids/liter. Ly = lysine; V = valine.

dium contained the concentrations of salts, glucose and antibiotics listed in table 1, column 3. In addition to the presence of yolk and egg white, this medium differed from the defined medium used in the previous lysine and valine experiments in amino acid and B-vitamin concentrations: The amino acid concentration was one and one-half times, and the B-vitamins, except choline, one-tenth, the levels used in those experiments.

In the glucose experiment, two of the embryos in each of the groups receiving 1.5 and 5.0 g of glucose/liter became contaminated. Although they are included in the data in figure 2, their omission would not have affected the median survival time of the 1.5 g/liter group, but would have increased the median survival time of the 5.0 g/liter group by 8 hours. Only four of the total of 95 embryos in all of the amino acid experiments were contaminated. Their omission would have increased the medium survival time by 8 hours for the groups which received the complete medium in experiment 1 (figs. 5 and 6).

RESULTS

When glucose was omitted from the medium containing 1.6 g of amino acids/liter (table 1, column 2), survival time was greatly reduced (fig. 2), in confirmation of previous reports (3). At a concentration of 1.5 g of glucose/liter, the median survival was increased to 56 hours, and at 5.0 g/liter, it was extended to 80 hours. In the group that received 10 ml of egg white/liter in the presence of only 1.5 g of glucose/liter, the median survival was extended to 88 hours, in contrast with 56 hours without the egg white.

There were no obvious abnormalities among embryos that were given any of the glucose levels. Weight comparisons are not meaningful because of the great differences in survival times between groups. There was considerable variation in survival times within treatments. The single embryo in the egg white-supplemented group that died during the first 8 hours clearly fell outside the range of others in the group, and its early death is probably attributable to injury during preparation for the experiment.

The striking effect of supplemental egg white on the defined medium containing only 1.5 g of glucose/liter may be explained on the basis of some special attributes of egg white (vitamins, minerals, or specific

proteins) or it may be the result of the contribution of egg white to the total amino acid and energy content of the medium. The defined medium contained 1.6 g of amino acids/liter; 10 ml of egg white contains about 1.1 g of protein, contributing significantly to the total of 2.7 g of amino acids. Thus it appeared possible that the medium containing 1.5 g of glucose/liter was deficient in amino acids, and that higher amino acid concentrations might prove beneficial. For this reason, the first amino acid experiments were conducted.

Omission of amino acids from the medium (fig. 3) did not reduce survival below that obtained with 1.6 g of amino acids/liter, which was the level used in the glucose experiment (fig. 2). The only differences between the treatment given the second groups of figures 2 and 3 was that in the glucose experiment, the yolk was displaced with the salts-glucose solution containing 5.0 g of glucose/liter. As the amino acid concentration was increased to 3.2 g/liter, the median survival of the embryos increased from 32 hours to 80 hours. The decrease to 64 hours when the amino acid concentration was increased to 4.8 g/liter probably was not significant. No gross differences in appearance were observed among the embryos perfused with the various concentrations of amino acids. In experiments prior to those reported in this paper the inclusion of varying levels of amino acids in a medium containing 5.0 g of glucose/liter had little effect on survival and had no effect on growth.

In the experiment concerned with the isosmotic substitution of glucose or amino acids into the defined medium (fig. 4), the median survival was 16 hours for embryos receiving the medium containing no glucose or amino acids. The addition of 3.2 g of amino acids/liter did not increase survival, but the addition of 4.2 g of glucose/liter increased the median survival to 56 hours.

The effect on survival which was obtained by varying the concentrations of amino acids (fig. 3) led to more critical experiments in which single amino acids were omitted from the defined medium. Two of these experiments are summarized in figures 5 and 6. In a preliminary experiment which is not presented in detail because one group of embryos became contaminated, the results were nearly identical to those obtained in these 2 experiments. In all 3 experiments the complete medium permitted the longest survival. Lysine omission produced less effect on survival than the omission of valine. There were no differences in weights between any of the groups of embryos in these experiments; all were markedly smaller than the controls.

When the medium contained 2.5 ml of yolk plus 5 ml of egg white/liter there was no depression in survival nor in embryo weights when either lysine or valine was omitted from the amino acid mixture (fig. 7).

In all of the studies reported, the growth of embryos perfused with defined media was much less than that of control embryos. The specific reasons for this are not known, but it is recognized that the medium did not supply all of the nutrients present in yolk, particularly lipids, fat-soluble vitamins and some minerals.

DISCUSSION

It is not possible from the experiments reported here to determine the nature of the need for glucose or amino acids in the yolk-sac perfusion medium. There is abundant evidence from tracer studies that embryos utilize free glucose,[5] and that explanted embryos disintegrate in the absence of glucose or some related compound (1). Neither this evidence nor that reviewed by Boell (8) precludes the possibility that energy sources other than glucose may be more important than glucose itself for the early development of the embryo.

In the present studies it appears possible that part of the effect on survival which was obtained by varying glucose and amino acid concentrations may be attributable to changes in the osmolarity of the media. When the median survival times of embryos which received the various treatments in the glucose and amino acid experiments (figs. 2 and 3) were plotted

[5] Klein, N. W. 1959 The metabolism of carbon-14-labeled nutrients by the chick embryo. Ph.D. Thesis, University of California, Davis.

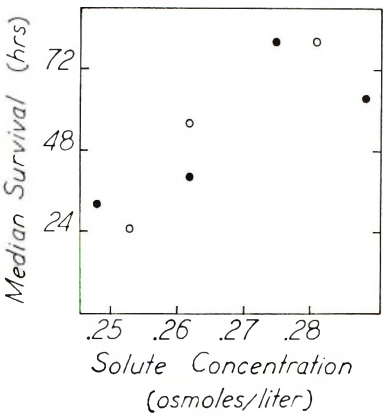

Fig. 8 Relationship between median survival, in hours of perfusion, and the calculated osmolarity of the medium. ○ = data from glucose experiment (fig. 2); ● = data from amino acid experiment (fig. 3).

against the osmolarity of the media (fig. 8), it became apparent that an increase in survival was associated with an increase in osmolarity. As the osmolarity increased from 248 to 288 milliosmols/liter, the median survival increased from a low of 24 hours to a high of 80 hours. Grau et al. (3), utilizing a more complex medium, reported that when the osmolarity of the medium was increased from approximately 158 to 317 milliosmols/liter,[6] the median survival of 4-day-old embryos increased from 5 hours to a peak of 95 hours.

The manner in which the osmolarity of the medium influences survival is uncertain, but it is possible that a large difference in osmotic pressure between the medium in the yolk-sac and egg white may favor the movement of egg white components across the vitelline membrane where they may be utilized by the embryo. The osmolarity of egg white is approximately 224 milliosmols/liter[7] which is much less than that of the defined media promoting maximal survival.

It was shown in one experiment, however, that glucose exerted an effect on survival which was independent of changes in the osmolarity of the medium (fig. 4). Further evidence for this is shown in the first groups of the glucose and amino acid experiments (figs. 2 and 3). Poor survival was obtained in the glucose experiment when glucose was omitted from the perfusion medium containing 1.6 g of amino acids/liter. Survival was considerably better when amino acids were omitted from the medium containing 1.5 g of glucose/liter in the amino acid experiments. The osmolarities of these 2 media were similar; 253 and 248 milliosmols/liter[8] for the glucose and amino acid experiments, respectively.

Thus, there is a special requirement for glucose in the defined medium which cannot be satisfied by substituting similar amounts of amino acids for glucose. It is not possible from the data presented here to determine the magnitude of this requirement, but it is proposed that it may be relatively low and that once the requirement has been met, the osmolarity of the medium may be the most important factor determining survival. Hence, amino acids and glucose are similarly effective in supporting maximal survival when added to a low glucose medium.

The special function of glucose in the perfusion medium is not clear. Since glucose and amino acids are the only nutrients included in the medium from which the embryo can derive a significant amount of energy, glucose may be specifically required as a readily available source of energy. Another possibility is that glucose enhances the utilization of egg white by the embryo. Feeney et al. (9) have presented evidence for a reaction in ovo between glucose and egg white proteins.

With the observation that a relatively high concentration of amino acids was required in the media containing 1.5 g of glucose/liter, it became possible to study the requirements of the chick embryo for single amino acids. The lack of tyrosine has resulted in degeneration of the neural retina and cessation of retinal pigment formation (6); the lack of methionine has resulted in similar retinal degeneration and in reduced embryo growth and survival.[9]

[6] The expected osmolarities have been calculated from the designated components of the medium. These, and the subsequent values reported in this paper, have not been adjusted for activity.

[7] Average value of egg white from 7 eggs determined by vapor pressure osmometry. Standard deviation = 8.6.

[8] See footnote 6.

[9] Grau, C. R., G. C. Matteson, R. E. Austic and P. Nieberg. The need for methionine in defined media for chick embryos, in preparation.

In the present studies, marked effects on survival were obtained when the defined medium lacked lysine or valine (figs. 5 and 6) but the complete medium did not permit good growth, and no differences in embryo weights were observed. Survival was shorter when all amino acids were omitted (fig. 3) than when either lysine or valine was omitted. This may be attributed, however, to the great reduction in the solute concentration of the media when all amino acids were omitted.

When yolk and egg white were present in the medium in low concentrations the omission of lysine or valine had no effect on growth or survival (fig. 7). The embryos were not severely deficient in lysine or valine when these amino acids were omitted from the defined medium. This is evident because the weights of the embryos were not different from those of the embryos perfused with the medium containing all of the amino acids. Thus, even the survival effect resulting from lysine or valine omission could be eliminated by the availability of small amounts of lysine and valine from yolk and egg white added to the medium.

The egg white which is left intact in the perfused egg is another potential source of amino acids for the embryo. The extent to which it is utilized is not known, but it may be sufficient to prevent the expression of more severe deficiencies when single amino acids are omitted from the defined medium. The relative importance of this source of amino acids, and of yolk and egg white added to the perfusion medium, may be reduced in further experiments by increasing the concentration of purified amino acids in the medium above 4.8 g/liter.

These experiments have demonstrated for the first time with yolk-sac perfusion techniques that normal nutrients of chick embryos can be replaced in part by defined media. The use of a low glucose medium containing relatively high concentrations of amino acids has made possible the study of specific amino acid deficiencies. Further improvements in this defined medium and in the perfusion techniques may permit even more meaningful studies of amino acids and other nutrients in relation to the development of chick embryos.

LITERATURE CITED

1. Spratt, N. T., Jr. 1948 Development of the early chick blastoderm on synthetic media. J. Exp. Zool., *107:* 39.
2. Klein, N. W., E. McConnell and B. J. Buckingham 1962 Growth of explanted chick embryos on a chemically defined medium and effects of specific amino acid deficiencies. Develop. Biol., *5:* 296.
3. Grau, C. R., H. I. Fritz, N. E. Walker and N. W. Klein 1962 Nutrition studies with chick embryos deprived of yolk. J. Exp. Zool., *150:* 185.
4. Grau, C. R., N. E. Walker, H. I. Fritz and S. M. Peters 1963 Successful development of chick embryos nourished by yolk-sac perfusion with calcium-low media. Nature, *197:* 257.
5. Grau, C. R., N. W. Klein and T. L. Lau 1957 Total replacement of the yolk of chick embryos. J. Embryol. Exp. Morphol., *5:* 210.
6. Grau, C. R., R. E. Austic and G. C. Matteson 1965 Degeneration of the eyes of tyrosine-deficient chick embryos. Science, *148:* 1743.
7. Grau, C. R., B. W. Wilson and G. C. Matteson 1965 Relation of oxygen and yolk to retinal pigmentation in chick embryos subjected to yolk-sac perfusion. Proc. Soc. Exp. Biol. Med., *119:* 544.
8. Boell, E. J. 1955 Energy exchange and enzyme development during embryogenesis. In: Analysis of Development, eds., B. H. Willier, P. A. Weiss and V. Hamburger. W. B. Saunders Company, Philadelphia, p. 520.
9. Feeney, R. E., J. J. Clary and J. R. Clark 1964 A reaction between glucose and egg white proteins in incubated eggs. Nature, *201:* 192.

Effect of Dietary Fat, Protein and Cholesterol on Atherosclerosis in Swine [1]

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ABSTRACT Three trials involving 48 pigs each were conducted to study the effects of high and low levels of energy intake from 2 fat sources (soybean oil versus tallow), protein level (18 versus 12%) and the addition of 1% cholesterol to the above fat sources, on serum cholesterol levels and the incidence of atherosclerotic lesions in the thoracic and abdominal aortas and coronary arteries. The feeding of diets containing tallow generally resulted in a trend toward higher serum cholesterol levels and increased incidence of lesions except in trial 1, where the coronary arteries of the pigs receiving the diets containing soybean oil had the greater incidence. Serum cholesterol levels were unaffected by the levels of energy intake. The effect of caloric intake on the incidence of lesions was inconsistent. The pigs fed the diets containing 12% protein had slightly elevated serum cholesterol levels as opposed to those fed the diets containing 18% protein. The effect of protein level on the incidence of lesions was inconsistent. Cholesterol addition to the diets significantly increased serum cholesterol levels and incidence of lesions as compared with that observed in the pigs on similar diets without cholesterol addition.

There is little doubt that lesions occuring in the aorta and coronary arteries of swine bear a close resemblance to atherosclerotic lesions in man (1, 2)[3] and that long-term application of dietary variables influences the prevalence and severity of these lesions (3, 4). The work reported herein was initiated to study the effect of fat source (soybean oil versus tallow), level of food intake, protein level and the effect of cholesterol addition to the diet on serum cholesterol levels and lesion development in the coronary arteries and in the thoracic and abdominal aortas of swine.

EXPERIMENTAL PROCEDURE

The pigs used were crossbreds consisting of Poland China, Landrace and Yorkshire breeding. Pigs were allotted at random from outcome groups, based on initial weight and sex, to a randomized complete block experimental design. The initial starting weights of the pigs in these trials ranged from 23.6 to 38.2 kg and averaged 32.7, 34.7 and 35.0 kg for trials 1, 2 and 3, respectively. The age of the pigs averaged 85, 101 and 98 days initially and the animals were on test for 140, 257 and 96 days for trials 1, 2 and 3, respectively. In trials 1 and 2 the pigs were fed a common corn-soybean diet in individual feeding pens for one week before the start of the trials. The composition of the experimental diets is shown in table 1. Stabilized [4] edible tallow and degummed [5] soybean oil were used as the fat sources. In trial 2 dehydrated, finely ground corn cobs replaced a part of the carbohydrate-containing ingredients in the diets to increase the proportion of metabolizable calories coming from the fat source. The corn cob material is highly indigestible, thus contributing little energy to the diet and in addition is highly absorptive which facilitates the inclusion of higher levels of fat in the diet mixture.

In trials 1 and 2, the pigs were fed individually twice daily, and in trial 3 the pigs were allowed feed ad libitum. Trials 1 and 2 included 8 and 6 individually fed pigs per treatment and trial 3 included 4 groups of 3 pigs per treatment. In trial 1, the pigs of one group were allowed to consume all the control (low fat) diet

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[1] Journal Paper no. J-5264 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project no. 1513.

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[3] Skold, B. H. 1962 Spontaneous atherosclerosis in the arterial system of aging swine. Ph.D. Thesis. Library, Iowa State University, Ames, Iowa.

[4] Tallow was stabilized with 0.01% citric acid, 0.01% butylated hydroxytoluene and 0.01% butylated hydroxyanisol.

[5] Phosphatides removed by steam washing and centrifugation.

TABLE 1
Composition of the experimental diets

	Trial 1		Trial 2		Trial 3	
	High fat	Control	18% protein	12% protein	Control	1% cholesterol
	%	%	%	%	%	%
Ground yellow corn	63.95	82.20	25.50	39.70	75.85	74.60
Fat source [1]	15.00	—	15.00	15.00	5.00	5.00
Ground corn cobs, dehydrated	—	—	25.00	25.00	—	—
Cholesterol	—	—	—	—	—	1.00
Soybean meal, 50% protein	16.45	13.25	30.05	15.65	14.55	14.80
Vitamin-antibiotic premix [2]	2.00	2.00	2.00	2.00	2.00	2.00
Calcium carbonate, 30% Ca	0.70	0.85	0.60	0.45	0.80	0.80
Dicalcium phosphate, 26% Ca, 18% P	1.30	1.10	1.25	1.60	1.20	1.20
Iodized salt	0.50	0.50	0.50	0.50	0.50	0.50
Trace mineral premix [3]	0.10	0.10	0.10	0.10	0.10	0.10
Total	100.00	100.00	100.00	100.00	100.00	100.00

[1] Degummed soybean oil or stabilized edible tallow. Samples of the soybean oil analyzed 801 mg, sitosterol/100 g and samples of the tallow analyzed 97 mg cholesterol/100 g.
[2] Contributed the following/kg of diet: 1760 IU vitamin A; 662.3 IU vitamin D_3; 4.4 mg riboflavin; 8.8 mg calcium pantothenate; 19.8 mg niacin; 22 mg choline chloride; 22 µg vitamin B_{12}; 44 mg chlortetracycline and 124.3 mg ethoxyquin (74.8 mg ethoxyquin in trial 1).
[3] Contributed the following elements in parts per million: Fe, 70.4; Cu, 4.8; Co, 1.7; Mn, 56.8; Zn, 81; K, 7.5.

they wanted in two 30-minute feeding periods daily. This level was considered as the high level of food intake and served as the basis for determining the level of caloric intake for the other groups. A second group was fed 70% of the high level of intake. The diets containing soybean oil or tallow were then fed in isocaloric amounts to the high or low intake levels of the control diet. In trial 2, one group of pigs was allowed to consume approximately full feed; this was increased step-by-step as the pigs matured, up to a maximal intake of 2.7 kg/day. The low intake group was fed 70% of the high level of intake.

Blood samples were taken at the start then bi-weekly throughout each trial. The samples were withdrawn, after a 16-hour fast period, from the anterior vena cava by the method described by Carle and Dewhirst (5). In trial 2, to investigate treatment effects in fasted and nonfasted animals, samples were collected after a 16-hour fast and at 2, 4 and 6 hours after feeding over a period of 4 bleeding times. These collections were made as follows: All pigs were bled after a 16-hour fast and then fed. After 2 hours, two of the replications were sampled; after 4 hours, two more were sampled; and after 6 hours, the remaining 2 replications were sampled. Total serum cholesterol determinations were carried out by using the method of an industrial laboratory.[6]

Upon termination of the trials, the pigs were slaughtered, and the heart with the aorta and the first few centimeters of the iliac arteries was carefully dissected from each carcass. The coronary arteries were dissected from the heart by the method described by Skold.[7] The aorta was divided into the thoracic and abdominal portions by transection at the hiatus aorticus of the diaphragm. In these studies, the abdominal aorta also included the first few centimeters of the external and internal iliac arteries. The aorta and coronary arteries were carefully stripped of any adventitial fat and tissue and were then opened longitudinally and pressed

[6] Technicon Instruments Corporation 1964 Total cholesterol. Technicon Auto Analyzer Methodology — Method File N-24. Technicon Instruments Corporation, Chauncey, New York.
[7] See footnote 3.

onto dry chipboard for fixing and gross staining by the method described by Holman et al. (6). Measurement of the total area involved with atherosclerotic lesions was determined by a method of tracing and planimetry similar to that described by Cranston et al. (7). No attempt was made to distinguish between plaques and fatty streaks; both were considered as lesions of atherosclerosis and included in the percentage of the total area involved.

The data collected from each experiment were statistically analyzed by variance methods as described by Snedecor (8).

RESULTS

Trial 1. This trial involved a 2×3 factorial arrangement of 2 levels of feeding (high caloric or low caloric intake) in combination with 3 types of diets (low fat control diet, diet containing 15% tallow or diet containing 15% soybean oil). A summary of the results is presented in table 2.

Feeding the control diet resulted in significantly ($P < 0.01$) lower serum cholesterol levels than feeding the diets with added fat. Pigs consuming the diets containing tallow had significantly higher ($P < 0.01$) serum cholesterol levels than those consuming the diets containing soybean oil. A significant ($P < 0.05$) fat source and level of feeding interaction was noted. The low intake with the tallow diet resulted in a higher serum cholesterol level than that with the high intake, whereas the serum cholesterol levels of the pigs fed the 2 levels of the diets containing soybean oil were similar. The differences in serum cholesterol levels among samples taken at different times during the experiment were statistically significant ($P < 0.01$). However, no consistent relationship of time on experiment and serum cholesterol levels was observed.

No statistically significant treatment effects were noted on the percentage of the total area of the right coronary artery affected with lesions. There was a trend, however, for the pigs on the lower level of intake to have a lesser area affected by lesions and for pigs fed the diets containing soybean oil to have a greater incidence of lesions than those fed the diets containing tallow or the control diet.

TABLE 2

Summary of the effects of level of feed intake and fat source on serum cholesterol levels and atherosclerotic lesions (trial 1)

Fat source	Control		Soybean oil		Tallow		$s_{\bar{x}}$ [1]
Level of intake	High	Low	High	Low	High	Low	
Avg feed intake, kg/day	2.44	1.62	1.92	1.37	1.91	1.34	
Energy intake, kcal/day [2]	7552	5014	7037	5021	7147	5014	
Avg gain, g/day	636	436	654	527	676	508	
Serum cholesterol, mg/100 ml	125	111	127	126	134	148	4.74
% of total area affected by atherosclerotic lesions:							
Right coronary	3.51	2.63	5.08	4.29	3.14	3.00	1.08
Left coronary	1.00	1.81	3.30	4.90	2.27	2.82	1.35
Thoracic aorta	0.63	0.63	0.42	1.02	0.46	1.38	0.39
Abdominal aorta	3.39	3.48	1.84	3.20	2.07	7.32	1.09

[1] SE of mean.
[2] Based on the metabolizable energy values of Diggs et al. (9).

The addition of fat to the diet resulted in a significantly higher ($P < 0.05$) incidence of lesions in the left coronary artery. Fat source or level of intake did not significantly affect the severity of lesions. The pigs had a greater incidence of lesions in the right coronary artery than in the left coronary artery.

No statistically significant effects of dietary variables on the production of atherosclerotic lesions in the thoracic aorta were noted. The animals on the low intake level tended to have a greater incidence of lesions than those on the high dietary intake level. In the abdominal aorta, pigs fed at the high energy intake level exhibited a significantly lower ($P < 0.05$) percentage of lesions than those in the low energy intake group. Fat source exhibited no significant effects on the severity of lesions. The pigs had a greater incidence of atherosclerotic lesions in the abdominal aorta than in the thoracic aorta.

Trial 2. This trial involved a $2 \times 2 \times 2$ factorial arrangement of 2 levels of feeding (high caloric or low caloric intake), 2 types of diet (diet containing 15% soybean oil or diet containing 15% tallow) and 2 levels of protein (12% or 18%). A summary of the results is presented in table 3.

Fat source had no statistically significant effect on serum cholesterol levels, although pigs fed the tallow diet tended to have higher serum cholesterol levels than those receiving the soybean oil treatment (120 vs. 110 mg/100 ml). Overall, the low intake groups had serum cholesterol levels similar to the high intake groups. The group receiving the diets containing 12% protein tended to have higher serum cholesterol levels than the group receiving diets containing 18% protein.

Time after feeding resulted in a significant increase ($P < 0.05$) in serum cholesterol levels, with the greatest increase generally coming between 4- and 6-hour bleeding periods. The average levels were 105 mg/100 ml following a 16-hour fast and 119 mg/100 ml at 6 hours after feeding. In the samples taken at 2, 4 or 6 hours after feeding, pigs receiving the diets containing soybean oil had signifi-

TABLE 3

Summary of the effects of level of protein, level of intake and fat source on serum cholesterol levels and atherosclerotic lesions (trial 2)

Protein level, %	18				12				$s_{\bar{x}}$[1]
Fat source	Soybean oil		Tallow		Soybean oil		Tallow		
Level of intake	High	Low	High	Low	High	Low	High	Low	
Avg intake, kg/day	2.29	1.63	2.28	1.62	2.28	1.63	2.28	1.61	
Energy intake, kcal/day[2]	6570	4676	6721	4776	6801	4862	6981	4930	
Avg gain, g/day	497	378	507	356	513	365	496	366	
Serum cholesterol, mg/100 ml	104	108	113	121	112	115	130	116	8.62
% of total area affected by atherosclerotic lesions:									
Right coronary	4.90	4.04	9.77	3.79	3.26	3.52	6.41	5.14	1.75
Left coronary	5.91	6.82	12.78	4.96	6.64	4.63	6.33	9.03	1.96
Thoracic aorta	1.52	0.61	5.07	1.69	0.58	3.22	3.89	9.14	3.62
Abdominal aorta	6.02	5.48	7.40	7.85	7.48	9.97	21.34	9.47	4.89

[1] SE of mean.
[2] See footnote 2, table 2.

cantly lower ($P < 0.05$) values than those pigs receiving the diets containing tallow.

The pigs fed the diets containing tallow showed a trend toward an increased incidence of lesions in the right and left coronary arteries, and pigs fed the diets containing 18% protein exhibited a trend toward an increased incidence of lesions over those fed the diets containing 12% protein. In the case of the left coronary artery, there was a significant interaction ($P < 0.05$) between protein level, fat source and level of feeding; pigs on the high intake level of the high protein diet, containing tallow, had the greater incidence of lesions. In the right and left coronary arteries, the pigs receiving the high level of intake had a greater incidence of lesions than those on the low intake level; however, the difference was not statistically significant. The pigs had a greater incidence of lesions in the left coronary artery than in the right coronary artery.

There were no statistically significant treatment effects on the incidence of lesions in the thoracic or abdominal aortas. The pigs had a greater incidence of lesions in the abdominal aorta than in the thoracic aorta. The feeding of low protein, low intake and tallow diets resulted in a greater incidence of lesions in the thoracic aortas than the feeding of high protein, high intake and soybean oil diets; however, the trend was not statistically significant. Similar differences were noted with the abdominal aorta, except that the pigs on the high intake level in this case had a greater incidence of lesions than those on the low intake level.

Trial 3. This trial involved a 2 × 2 factorial arrangement of 2 fat sources (5% tallow or 5% soybean oil) with and without the addition of 1% cholesterol to the diet. A summary of the results is presented in table 4.

Differences in serum cholesterol levels among samples taken at different times during the experiment were statistically significant ($P < 0.01$). The serum cholesterol levels increased linearly with time. The pigs fed the diets containing tallow had higher serum cholesterol levels than those fed the diets containing soybean oil; however, the difference was not statistically significant. The addition of cholesterol to the diet resulted in a significant increase ($P < 0.01$) in serum cholesterol levels over those observed in pigs fed the same diet treatment without the cholesterol inclusion. Although the source of the fat and cholesterol interaction was not significant, the addition of cholesterol to the diet containing tallow resulted in higher serum cholesterol levels than a similar cholesterol addition to the diet containing soybean oil.

The feeding of diets containing tallow resulted in a trend toward an increased incidence of lesions in the thoracic and abdominal aortas and in the right and left coronary arteries as compared with the incidence of lesions in the animals fed the diets containing soybean oil. The addi-

TABLE 4

Summary of the effect of dietary fat and cholesterol on serum cholesterol levels and atherosclerotic lesions (trial 3)

Fat source	Soybean oil		Tallow		$s_{\bar{x}}$ [1]
Cholesterol, 1%	Without cholesterol	With cholesterol	Without cholesterol	With cholesterol	
Avg feed intake, kg/day	2.87	2.82	2.66	2.78	
Energy intake, kcal/day [2]	9439	9176	8818	9118	
Avg gain, g/day	902	885	837	874	
Serum cholesterol, mg/100 ml	112	158	114	184	7.45
% of total area affected by atherosclerotic lesions					
Right coronary	1.30	9.34	2.76	12.49	2.67
Left coronary	1.10	8.12	2.14	14.47	2.34
Thoracic aorta	0.48	6.89	0.49	18.59	3.42
Abdominal aorta	1.34	6.24	1.32	8.47	1.19

[1] SE of mean.
[2] See footnote 2, table 2.

tion of 1% cholesterol to the diets resulted in a significantly greater ($P < 0.01$) incidence of lesions in the pigs fed these diets over the incidence of lesions in animals fed similar diets but without the inclusion of cholesterol. The fat source and cholesterol interaction was not statistically significant; however, the addition of 1% cholesterol to the diet containing tallow resulted in a greater incidence of lesions than a similar cholesterol addition to the diet containing soybean oil. The pigs had approximately the same incidence of lesions in the left coronary artery as in the right coronary artery. The pigs had a greater incidence of lesions in the thoracic aorta than in the abdominal aorta.

DISCUSSION

Lesion distribution. Lesions in the coronary arteries were generally confined to the first few centimeters of the right coronary artery and of the two main divisions of the left coronary artery. This result is in agreement with observations reported by Skold,[8] Downie et al. (2) and French et al. (10). In trial 1, the main branch of the right coronary arteries had a greater incidence of lesions than the two main branches of the left coronary arteries. In trial 2, however, the incidence was reversed. In trial 3, the percentage of total area involvement was similar for the right and left coronary arteries. The observation of a greater involvement of the two main branches of the left coronary than of the main branch of the right coronary is in accord with reports by Duff and McMillan (11) and Levene (12) who reported a greater incidence at this site in man. A greater involvement with plaque formation was noted in the abdominal aortas than in the thoracic aortas. This observation is in agreement with Schwartz and Mitchell (13), who noted similar results in man, and Skold [9] who noted similar observations in the pig. The plaques were most prevalent around the bifurcation of the aorta and the external and internal iliac arteries. In trials 1 and 2, the abdominal aorta had a greater percentage involvement with lesions than the thoracic; whereas, in trial 3, the thoracic aorta had the greater total area involvement of the two. Bragdon et al. (14) reported that lesions of experimentally produced atherosclerosis in swine were most prevalent in the thoracic aorta. Downie et al. (2) reported results similar to those of Bragdon et al. (14) but noted that, with age, the distribution of atherosclerotic lesions became more pronounced in the abdominal aorta. Trial 3 lasted 96 days, whereas in trials 1 and 2, the time was 140 and 257 days, respectively; hence, the lesser time on experiment could be responsible for the greater incidence of lesions in the thoracic than in the abdominal aorta, in trial 3. The observations in trials 1 and 2 agree with those reported by Stephenson et al. (15) who noted that, in man, the greatest concentrations of lesions were in the abdominal and common iliac arteries rather than in the thoracic aorta.

Fat source. In the comparison of soybean oil or tallow as the fat source in the diet, the pigs fed the diets containing tallow had slightly elevated serum cholesterol levels. In general, the pigs in trials 2 and 3 which received the diets containing tallow had a greater incidence of lesions than those fed the diets supplemented with soybean oil. In trial 1, however, lesions were more prevalent in the coronary arteries but not in the thoracic and abdominal aortas of the pigs receiving the diets containing soybean oil. Neither of these observations was statistically significant. Since the caloric intake per day from the fat sources was maintained fairly constant, it appears that the type and not the quantity of the fat source was important. The results obtained in trials 2 and 3 and trial 1 in part are consistent with work reported by Peifer and Lundberg [10] and Rowsell et al. (3) who reported that saturated fats give rise to a greater incidence of atherosclerosis than unsaturated fats and with Bragdon et al. (14) and Barnes et al. (16) who reported increased serum cholesterol levels resulting from feeding saturated fats. Barnes et al. (17) and Gresham et al. (18), however, noted no evidence that the type of fat in the diet influenced the level

[8] See footnote 3.

[9] See footnote 3.

[10] Peifer, J. J., and W. O. Lundberg 1957 Influence of specific fatty acids on the development of atherosclerosis in miniature pigs. Federation Proc., 16: 232 (abstract).

of serum cholesterol or the degree of atherosclerosis, respectively.

Energy intake. An increased incidence of lesions as the caloric intake was restricted was shown in trial 1. In trial 2, however, there was a trend for the animals on the high energy intake level to have the greater incidence of lesions. In trial 1, the energy effect was significant only in the case of the abdominal aorta, and in trial 2, the incidence of lesions in the thoracic aorta did not fit into the general trend. Serum cholesterol levels in each trial for the overall high and low levels of intake were similar. Peifer and Lundberg [11,12] reported no significant effects attributable to the total calories per se but that the effect of the caloric intake from the fat source was important; however, Hays et al.[13] observed that marked differences in caloric intake did significantly influence the serum cholesterol levels.

Protein level. No significant effects due to protein level were observed in trial 2, although there was a trend for the feeding of the low protein diet to elevate the serum cholesterol levels and result in a greater incidence of lesions in the thoracic and abdominal aortas. The feeding of the high protein diet resulted in a greater involvement in the case of the right and left coronary arteries. Barnes et al. (16) and Moreland et al. (19) reported that serum cholesterol levels were not influenced by protein level; however, Barnes et al. (20) did find that extremely low protein diets elevated the serum cholesterol levels. Hays et al.[14] reported that, in gestating pigs, a high protein intake elevated serum cholesterol levels and that the effect was greater with a higher level of energy intake.

Cholesterol addition. In trial 3, the addition of 1% cholesterol to diets supplemented with either soybean oil or tallow, resulted in significantly higher serum cholesterol levels and incidence of lesions than that noted in pigs fed similar diets without added cholesterol. These observations are in agreement with the reports of Downie et al. (2) and Moreland et al. (19). Reiser et al. (21) reported that unsaturated fat increased the absorption of cholesterol, whereas the saturated fat depressed the absorption. The results in this trial do not support the work of Reiser et al. (21) since cholesterol addition resulted in a greater incidence of lesions and higher serum cholesterol levels in animals fed the saturated fat source (tallow) as compared with the incidence of lesions and cholesterol levels in pigs fed the diet containing the unsaturated fat source (soybean oil).

Time of sampling after feeding. In trial 2, the serum cholesterol levels increased in a linear manner with time at 2, 4 and 6 hours after feeding. When the differences between treatments were compared at each sampling time with the corresponding serum cholesterol values obtained after a 16-hour fast and just prior to feeding, the differences were similar, regardless of sampling time. This suggests that treatment effects were no less evident in the fasted than in the nonfasted animal.

LITERATURE CITED

1. Jennings, M. A., H. W. Florey, W. E. Stehbens and J. E. French 1961 Intimal changes in the arteries of a pig. J. Pathol. Bacteriol., *81:* 49.
2. Downie, H. G., J. F. Mustard and H. C. Rowsell 1963 Swine atherosclerosis: the relationship of lipids and blood coagulation to its development. Ann. N. Y. Acad. Sci., *104:* 539.
3. Rowsell, H. C., H. G. Downie and J. F. Mustard 1958 The experimental production of atherosclerosis in swine following the feeding of butter and margarine. Can. Med. Ass. J., *79:* 647.
4. Rowsell, H. C., J. F. Mustard and H. G. Downie 1965 Experimental atherosclerosis in swine. N. Y. Acad. Sci., *127:* 743.
5. Carle, B. N., and W. H. Dewhirst 1942 A method for bleeding swine. J. Amer. Vet. Med. Ass., *101:* 495.
6. Holman, R. L., H. C. McGill, J. P. Strong and J. C. Geer 1958 Technics for studying atherosclerotic lesions. Lab. Invest., *16:* 42.
7. Cranston, W. I., J. R. A. Mitchell, R. W. R. Russell and C. J. Schwartz 1964 The assessment of aortic disease. J. Atheroscler. Res., *4:* 29.
8. Snedecor, G. W. 1956 Statistical Methods, ed. 5. The Iowa State College Press, Ames.

[11] Peifer, J. J., and W. O. Lundberg 1958 Influence of total calories, fat calories and fat unsaturation on blood lipids. Federation Proc., *17:* 288 (abstract).

[12] Peifer, J. J., and W. O. Lundberg 1962 Effects of long term feeding studies on the blood lipids of swine. Federation Proc., *21:* 281 (abstract).

[13] Hays, V. W., V. B. Mayrose, V. C. Speer and R. Getty 1964 Effect of dietary protein and fat on reproductive performance and cholesteremia in swine. Proc. of the 6th International Nutriiton Congress. E and S Livingstone Ltd., Edinburgh and London.

[14] See footnote 13.

9. Diggs, B. G., D. E. Becker, A. H. Jensen and H. W. Norton 1965 Energy value of various feeds for the young pig. J. Animal Sci., *24*: 555.
10. French, J. E., M. A. Jennings, J. C. F. Poole, D. S. Robinson and Sir H. Florey 1963 Intimal changes in the arteries of ageing swine. Proc. Roy. Soc. (London), *158*: 24.
11. Duff, G. L., and G. C. McMillan 1951 Pathology of atherosclerosis. Am. J. Med., *11*: 92.
12. Levene, C. I. 1956 The early lesions of atheroma in the coronary arteries. J. Pathol. Bacteriol., *72*: 79.
13. Schwartz, C. J., and J. R. A. Mitchell 1962 The morphology, terminology and pathogenesis of arterial plaques. Postgrad. Med. J., *38*: 25.
14. Bragdon, J. H., J. H. Zeller and J. W. Stevenson 1957 Swine and experimental atherosclerosis. Proc. Soc. Exp. Biol. Med., *95*: 282.
15. Stephenson, S. E., Jr., G. V. Mann, R. Younger and H. W. Scott 1962 Factors influencing the segmental deposition of atheromatous material. Arch. Surg., *84*: 49.
16. Barnes, R. H., E. Kwong, G. Fiala, M. Rechcigl, R. N. Lutz and J. K. Loosli 1959 Dietary fat and protein and serum cholesterol. I. Adult swine. J. Nutr., *69*: 261.
17. Barnes, R. H., E. Kwong, L. R. Mattick and J. K. Loosli 1961 Isomerized fat and serum cholesterol in swine. Proc. Soc. Exp. Biol. Med., *108*: 468.
18. Gresham, G. A., W. M. F. Leat, A. N. Howard and I. W. Jennings 1964 Pathological changes in pigs reared on semi-synthetic diets containing no fat, beef tallow and maize oil. Brit. J. Exp. Pathol., *45*(2): 128.
19. Moreland, A. F., T. B. Clarkson and H. B. Lofland 1963 Atherosclerosis in "minature" swine. Arch. Pathol., *76*: 203.
20. Barnes, R. H., E. Kwong, W. Pond, R. Lowry and J. K. Loosli 1959 Dietary fat and protein and serum cholesterol. II. Young swine. J. Nutr., *69*: 269.
21. Reiser, R., M. F. Sorrels and M. C. Williams 1959 Influence of high levels of dietary fats and cholesterol on atherosclerosis and lipid distribution in swine. Circulation, *7*: 833.

Growth Inhibition of Rats Fed Raw Navy Beans (*Phaseolus vulgaris*) [1]

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ABSTRACT Growth inhibition of rats fed raw navy beans and the action of antibiotics in overcoming it were investigated. The growth inhibitory action of a navy bean fraction isolated by sodium chloride extraction and precipitation by 0.75 ammonium sulfate saturation was also studied. More nitrogen and more of 5 amino acids studied were excreted in the feces of rats fed raw navy beans than in the feces of rats fed heated beans. Nitrogen and amino acid retention in the animals was increased by feeding antibiotics. The intestinal contents of rats fed raw navy beans contained more insoluble nitrogen, protein nitrogen and amino nitrogen and these were not changed by supplementation with antibiotics. Proteolytic activity in the intestine of animals receiving raw navy beans was similar to that of those receiving heated navy beans. Increased insoluble matter and proteolytic activity in the cecum and increased size of the cecum of rats receiving antibiotics suggest that the site of antibiotic action may be the cecum. No significant effect on nitrogen or amino acid absorption due to the inclusion of the navy bean fraction in the diet was observed. Growth inhibition of rats fed raw navy beans may, in part, be the result of endogenous loss of nitrogen and impaired absorption of amino acids.

Growth inhibition of experimental animals resulting from the ingestion of raw beans has been reported by various workers and is generally attributed to the toxic factor(s) present in beans (1). The well-known toxic factors of navy beans are the trypsin inhibitors and hemagglutinins; however, very little is known regarding their mechanism of action.

The present investigation was undertaken to examine the mechanism of growth inhibition of rats fed raw navy beans. Two experiments were included in an attempt to explain: i) the action of supplementary antibiotics (2); and ii) the growth inhibitory action of Fraction 4 isolated from navy beans as described previously (3). Various investigators have discussed the possible manner by which an antibiotic might exert its action on the nutritive value of beans (2, 4–8). Recently Goldberg and Guggenheim (5) suggested that the action of antibiotics is to reduce the decrease of intestinal "trypsin" in rats fed beans, and the studies of Barnes et al. (6–8) indicate that antibiotics protect the trypsin from bacterial degradation. The mechanism of action was investigated by studying nitrogen and amino acid absorption, nitrogen analyses of intestinal contents, and proteolytic activity of intestinal contents using young male albino rats.

EXPERIMENTAL

Preparation of diets. Navy beans of the Sanilac variety (protein content 24%) were used as a source of dietary protein at a level to supply 10% protein. The composition of the diet is given in table 1.

Supplements were added to the diets at the expense of starch at the following levels: antibiotics: 0.1% procaine penicillin + 0.1 streptomcyin sulfate; and Fraction 4 (F_4): 1% Fraction 4, a major growth inhibtory fraction, was isolated from navy beans by the procedure described previously (3).

Rat feeding test. Weanling male rats from the Michigan State University stock colony, 21 to 23 days old, were trained to eat the maximal amount of food at one time by a restricted feeding technique.

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TABLE 1

Composition of diet

	g/100 g diet
Navy beans, raw or heated [1] (24% protein; N × 6.25)	41.67
Hegsted salt mixture [2]	4.00
Vitamin fortification mix [3]	2.00
Corn oil	6.00
Cornstarch	46.33
Total	100.00

[1] Autoclaved at 121° for 5 minutes.

[2] Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138*: 459. Purchased from Nutritional Biochemicals Corporation, Cleveland.

[3] Purchased from Nutritional Biochemicals Corporation. Supplied the following in mg/100 g diet: vitamin A conc (200,000 units/g), 9.0; vitamin D conc (400,000 units/g), 0.5; α-tocopherol, 10.0; ascorbic acid, 90.0; inositol, 10.0; choline·Cl, 150.0; menadione, 4.5; *p*-aminobenzoic acid, 10.1; niacin, 9.0; riboflavin, 2.0; pyridoxine·HCl, 2.0; thiamine·HCl, 2.0; Ca pantothenate, 6.0; biotin, 0.04; folic acid, 0.19; and vitamin B_{12}, 0.0027.

The technique consisted of feeding the regular stock diet to the rats twice a day for one hour only. The hours of feeding were from 9:00 AM to 10:00 AM and 4:00 PM to 5:00 PM. Water was available to the rats all the time. After the initial period of training (usually 8 to 10 days), rats were divided into groups of 6 animals, and each group was equalized with respect to initial weight.

Nitrogen and amino acid absorption. The animals were fed the experimental diets for 7 days. Feces were collected daily on absorbant paper during the last 4 days of the feeding period and dried at 95° to 100°.

Food consumption was determined daily, and the animals were weighed at the end of the experiment. The nitrogen of feces and diets was determined by Kjeldahl procedure (9). Amino acids were assayed microbiologically (10) in the diets and feces after acid hydrolysis of the samples. Acid hydrolysis was carried out with 2.5 N HCl in an autoclave for 8 hours at 121°. *Leuconostoc mesenteroides* P-60 was used for assaying methionine, cystine and lysine, and *Lactobacillus arabinosus* 17/5 was used to assay leucine and valine.

Studies on intestinal contents of unadapted rats. The trained rats were given the experimental diets in the morning from 9:00 AM to 10:00 AM, and the food intake was recorded. Two hours after the feeding period ended, all the rats were killed by ether and dissected. The intestinal tract of the animal was excised from the adhering tissues and was exposed. Ligatures were made at the proper places to remove the small intestine, and cecum separately. The contents of these organs were washed out with distilled water and made up to 25 ml. The contents were then centrifuged in the cold, and the insoluble residue was separated from the soluble fraction of the contents.

Nitrogen was determined by the Kjeldahl procedure (9) on the insoluble and soluble fractions of the intestinal contents and were designated as insoluble nitrogen and soluble nitrogen, respectively. Protein nitrogen determinations were made on the TCA precipitate of the soluble fraction after addition of 5 ml of 10% TCA to 5 ml of the soluble fraction. Total nitrogen was calculated by adding insoluble nitrogen and soluble nitrogen together, and non-protein nitrogen was calculated by subtracting the protein nitrogen from the soluble nitrogen. Amino nitrogen (NH_2-N) was determined by the method of Northrop (11).

Proteolytic activity was determined on the intestinal and cecal contents (soluble fraction) by the casein digestion method of Kunitz (12), and the activity was expressed as an increase in optical density at 280 mμ against a blank prepared by the addition of 5% TCA to the substrate prior to the addition of enzyme (intestinal or cecal) solution.

Studies on the intestinal contents of adapted rats. The trained rats were given the experimental diets for a week. These rats were designated as adapted rats. At the end of the adaptation period, the rats were given the experimental diets for one hour in the morning and then killed 2 hours after the feeding time. Record of food consumption was noted during this feeding period. Collection of the intestinal contents, nitrogen analysis, and the proteolytic activity determinations were made as described above.

RESULTS

Nitrogen absorption. Table 2 shows the data regarding the growth rate, food intake, and nitrogen absorption by rats fed the experimental diets. The results obtained in this experiment were similar to those reported earlier (2, 3, 13), namely,

TABLE 2

Nitrogen absorption from navy beans as measured in rats

Group no.	Diet	Weight/day [1]	Food intake/day [1]	N absorbed [1,2]
		g	*g*	%
1	Raw beans	−1.11 [3] ± 0.15 [4]	4.75 [3] ± 0.21	43.5 [3] ± 4.0
2	Heated beans	1.80 ± 0.19	8.80 ± 0.39	80.9 ± 0.17
3	Raw beans + antibiotics	0.52 [3] ± 0.16	5.45 [3] ± 0.28	59.5 [3] ± 4.9
4	Heated beans + antibiotics	2.47 ± 0.22	8.38 ± 0.44	87.1 ± 1.0
5	Heated beans + fraction 4	1.19 [3] ± 0.21	7.46 [3] ± 0.29	75.5 [5] ± 2.1
6	Heated (raw beans + fraction 4)	2.75 ± 0.30	9.65 ± 0.51	83.5 ± 1.4

[1] Average of 6 rats.

[2] Calculated: $\frac{\text{Nitrogen intake} - \text{nitrogen excreted in feces}}{\text{Nitrogen intake}} \times 100$.

[3] Significant, $P < 0.01$, compared with appropriate treatment (comparisons between groups 1 and 2, 3 and 4, 5 and 6).

[4] SE of mean.

[5] Not significant, $P > 0.01$, compared wtih appropriate treatment.

that i) rats fed the raw navy bean diet lost weight and consumed less food when compared to those fed the heated navy bean diet; ii) the addition of antibiotics to the raw navy bean diet prevented weight losses in the rats; iii) Fraction 4, when included in the heated bean diet, was growth inhibitory; iv) nitrogen absorption from raw navy beans was poor;[3] and v) antibiotics improved nitrogen absorption.[4] Although the inclusion of Fraction 4 in the diet reduced the nitrogen absorption of heated navy beans when compared with the proper control diet, the difference was not statistically as great ($P > 0.01$) as that between raw and heated beans ($P < 0.01$) or between antibiotics supplemented raw and heated beans (table 2).

Amino acid absorption (table 3). The absorption of all amino acids studied here for rats fed the raw navy bean diet was much lower ($P < 0.01$) than that obtained with rats fed the heated navy bean diet. Antibiotic supplementation greatly improved the absorption of amino acids by rats fed raw navy beans. However, the values for the absorption of amino acids from the raw navy bean diet with added antibiotics were significantly lower than those for the heated navy bean diet with or without added antibiotics. No significant differences were observed in the absorption of amino acids when Fraction 4 either heated or unheated was added to the ration ($P > 0.01$).

Nitrogen analyses of the intestinal contents of unadapted and adapted rats. A separate experiment was designed as described in the Experimental section to study the nitrogen analyses of the intestinal contents of unadapted and adapted rats. The results are presented in table 4 and are expressed on the basis of percentage of total nitrogen intake in order to differentiate the dietary nitrogen from endogenous nitrogen. It is assumed that nitrogen present in the intestine in excess of actual intake represents the endogenous nitrogen. The data indicated that there was a higher percentage of ingested nitrogen in the intestinal contents of rats receiving the raw navy bean diet than in those of rats receiving the heated navy bean diet caused by more insoluble nitrogen and soluble nitrogen (more NH_2-N, non-protein nitrogen and protein nitrogen). The addition of antibiotics to the raw navy bean diet did not prevent the increased percentage of ingested nitrogen in the intestinal contents. Although the same picture regarding the nitrogen analysis was observed in adapted rats, a definite decrease in NH_2-N, non-protein nitrogen and protein nitrogen was observed. The values of nitrogen analyses were not significantly different for the diets con-

[3] Kakade, M. L. 1964 Growth inhibition of rats fed raw navy beans. Ph.D. Thesis, Michigan State University, East Lansing, Michigan.

[4] See footnote 3.

TABLE 3

Absorption of amino acids [1] from navy beans fed to rats

Group no.	Diet	Methionine	Cystine	Lysine	Leucine	Valine
1	Raw beans	21.8 [2] ± 4.9 [3]	36.6 [2] ± 5.3	58.8 ± 2.8	47.6 [2] ± 3.2	46.0 [2] ± 3.3
2	Heated beans	68.7 ± 2.3	80.6 ± 2.2	85.0 ± 1.6	85.7 ± 1.2	84.8 ± 2.1
3	Raw beans + antibiotics	66.5 [2] ± 2.8	55.5 [2] ± 7.3	70.7 [2] ± 2.5	62.9 [2] ± 2.0	61.5 [2] ± 3.0
4	Heated beans + antibiotics	93.2 ± 1.4	85.0 ± 1.6	93.0 ± 1.1	93.6 ± 1.1	91.6 ± 1.9
5	Heated beans + fraction 4	68.0 [4] ± 3.2	75.3 [4] ± 1.1	79.1 [4] ± 1.2	81.1 [4] ± 1.8	78.5 [4] ± 2.0
6	Heated (Raw beans + fraction 4)	74.8 ± 2.6	83.6 ± 2.3	84.5 ± 1.0	87.3 ± 1.2	83.0 ± 1.8

[1] Average of 6 rats expressed as per cent.
Calculated: $\frac{\text{Amino acid intake} - \text{amino acid excreted in feces}}{\text{Amino acid intake}} \times 100$.
[2] Significant, $P < 0.1$, compared with appropriate treatment.
[3] SE of mean.
[4] Not significant, $P > 0.01$, compared with appropriate treatment.

taining Fraction 4 (table 4, groups 5 and 6).

Proteolytic activity. The proteolytic activity of the intestine and cecal contents of unadapted and adapted rats is shown in figure 1. No significant difference in the activity of the intestinal contents of rats fed the experimental diets was observed. However, a higher proteolytic activity was observed in the cecal contents of the rats fed the diets containing raw navy beans or raw navy beans plus antibiotics or heated beans plus Fraction 4 when compared with that obtained with the proper control diets containing heated navy beans or heated navy beans plus antibiotics or heated (raw navy beans + Fraction 4). There was a marked increase in proteolytic activity of cecal contents of adapted rats when they received antibiotic supplementation. Also, the size of cecum of rats fed the diets with added antibiotics was four to five times greater than that of rats fed the diets without added antibiotics. As shown in figure 2, more insoluble matter, which probably represents the food particles, mucosal cells and microorganisms, was found in the cecum of rats receiving antibiotics supplementation than that of rats receiving no antibiotics.

DISCUSSION

The results presented in the present investigation indicate that the lower nitrogen and amino acid absorption from raw navy beans is the result of increased excretion of endogenous nitrogen and interference in amino acid absorption. The sources of endogenous nitrogen could be mucosal cells (14, 15), digestive secretions (16, 17), and plasma albumin passed into the lumen (18). The data presented in table 4 show more insoluble nitrogen in the intestines of rats fed raw navy beans than in those of rats fed heated beans indicating that there was more mucosal slough-off, more protein nitrogen suggesting that there was probably excessive enzyme secretions or that the fraction of navy bean protein was resistant to enzymatic attack, and more NH_2-N showing interference with amino acid absorption from the intestine. Similar observations were reported for soybeans by

TABLE 4

Nitrogen analysis of the intestinal contents of rats fed navy beans [1]

Group no.	Diet	Total N	Insoluble N	Soluble N	Protein N	Non-protein N	Amino N
		% of ingested nitrogen				*% of ingested nitrogen*	
		Unadapted rats					
1	Raw beans	146.1 [2] ± 16.3 [3]	43.3 [2] ± 4.5	102.7 [2] ± 11.8	18.6 [2] ± 2.2	84.0 [2] ± 9.8	50.2 [2] ± 7.7
2	Heated beans	39.9 ± 2.3	10.0 ± 1.0	29.8 ± 1.7	3.3 ± 0.4	26.5 ± 1.4	14.7 ± 1.0
3	Raw beans + antibiotics	140.0 [2] ± 18.3	40.0 [2] ± 6.9	100.0 [2] ± 12.6	17.0 [2] ± 2.4	83.1 [2] ± 10.0	45.7 [2] ± 4.9
4	Heated beans + antibiotics	39.4 ± 2.8	9.5 ± 0.9	29.9 ± 1.1	3.2 ± 0.3	26.6 ± 2.6	12.9 ± 1.3
5	Heated beans + fraction 4	42.4 [4] ± 3.6	9.1 ± 1.0	33.4 [4] ± 2.0	5.5 [4] ± 1.1	27.9 [4] ± 1.4	15.3 [4] ± 1.1
6	Heated (raw beans + fraction 4)	38.0 ± 2.0	10.4 ± 0.8	29.5 ± 2.1	4.8 ± 0.8	24.7 ± 2.1	13.9 ± 0.9
		Adapted rats					
1	Raw beans	113.3 [2] ± 11.0	41.4 [2] ± 4.9	72.2 [2] ± 7.3	14.8 [2] ± 1.5	57.4 [2] ± 6.5	35.8 [2] ± 4.7
2	Heated beans	44.7 ± 4.7	10.6 ± 1.8	34.1 ± 3.6	4.8 ± 0.6	28.3 ± 3.8	15.4 ± 0.9
3	Raw beans + antibiotics	115.0 [2] ± 12.2	38.9 [2] ± 3.3	76.4 [2] ± 6.1	15.6 [2] ± 1.5	60.8 [2] ± 7.3	35.0 [2] ± 3.6
4	Heated beans + antibiotics	54.3 ± 10.0	14.0 ± 3.2	40.3 ± 6.9	6.6 ± 1.3	33.6 ± 5.7	15.9 ± 4.0
5	Heated beans + fraction 4	64.1 [4] ± 5.3	19.5 [4] ± 2.2	44.6 [4] ± 6.1	7.6 [4] ± 0.4	37.0 [4] ± 2.1	21.8 [4] ± 0.9
6	Heated (raw beans + fraction 4)	53.3 ± 6.9	16.0 ± 1.1	36.8 ± 3.7	5.9 ± 0.2	30.9 ± 3.0	20.3 ± 1.2

[1] Values represent the mean of 6 rats.
[2] SE of mean.
[3] Significant, $P < 0.01$, compared with appropriate treatment.
[4] Not significant, $P > 0.01$, compared with appropriate treatment.

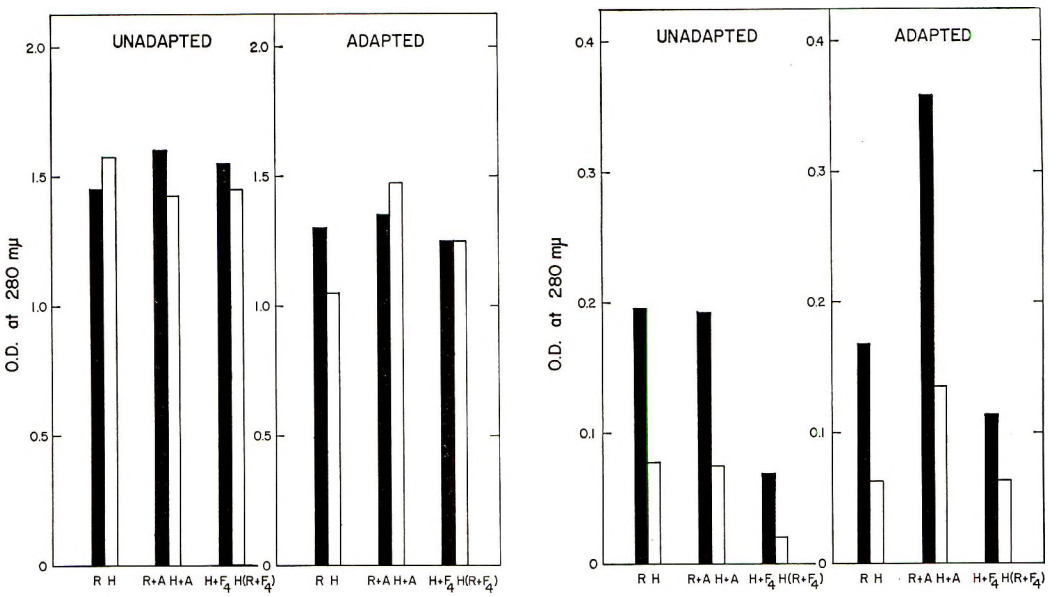

Fig. 1 Trypsin activity in the intestine (A) and cecum (B) of rats fed the experimental diets expressed as average of 6 rats. Key: R, raw navy bean diet; H, heated navy bean diet; R + A, raw navy bean diet + antibiotics; H + A, heated navy bean diet + antibiotics; H + F_4, heated navy bean diet + Fraction 4; H (R + F_4), heated (raw navy beans + Fraction 4) diet.

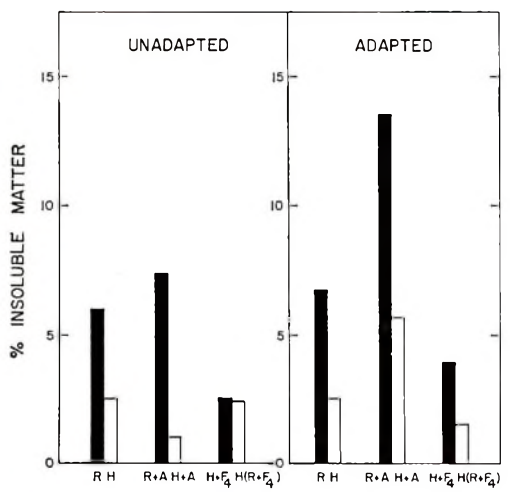

Fig. 2 Insoluble matter in the cecum of rats fed the experimental diets. Results are presented as a percentage of food intake. Key: R, raw navy bean diet; H, heated navy bean diet; R + A, raw navy bean diet + antibiotics; H + A, heated navy bean diet + antibiotics; H + F_4, heated navy bean diet + Fraction 4; H (R + F_4), heated (raw navy beans + Fraction 4) diet.

de Muelenaere (14) and Lepkovsky et al. (19).

According to the studies of de Muelenaere (14), it is the trypsin inhibitor of soybeans that brings about the increased mucosal slough-off, increased secretion of digestive enzymes, and the inhibition of amino acid absorption in rats. The presence of a trypsin inhibitor in navy beans would possibly explain the data in table 4.

The observation that raw navy beans interfere with the absorption of amino acids supports the hypothesis that the action of hemagglutinins is to combine with the intestinal cell linings, thereby interfering with the intestinal absorption of nutrients (1).

The results shown in figure 1A are in contrast with those of Goldberg and Guggenheim (5) who observed a decrease in the trypsin activity of intestine due to antibiotics. Our results (fig. 1B) tend to agree with those of Barnes et al. (8), who observed an increase in trypsin in the cecum and colon of rats fed raw soybeans. Barnes and co-workers (6, 7, 8) proposed that

the action of the antibiotic is to protect trypsin from bacterial degradation in the intestine and hence the high cystine content of trypsin is available to the animals through coprophagy. This is an attractive hypothesis supported by the fact that the beneficial effects of antibiotics supplementation is abolished when coprophagy is prevented (7). However, these authors state (8) that "the complete explanation of the beneficial effects of antibiotics added to the diets of rats receiving unheated soybeans has not as yet been obtained"; and since supplementation of methionine or trypsin completely failed to correct the growth depression in rats fed raw navy beans (13), the other possibilities still remain open to propose the mode of antibiotics action in improving the nutritive value of beans.

It appears that the beneficial effect of antibiotics added to the raw navy bean diet involves the participation of cecum, a conclusion which also has been reached by Nitsan and Bondi (20) in explaining the supplementary effect of antibiotics on soybeans. This would perhaps explain the present observations that antibiotics increased nitrogen and amino acid retention (tables 2 and 3) but had no effect on the nitrogen of small intestinal contents (table 4). Observations such as increased insoluble matter (fig. 2), increased tryptic activity, and increased size (observed in the present investigation) suggest that there was an increase of microflora in the cecum of rats fed raw or heated navy beans with added antibiotics. The stimulation of microbial growth in the intestinal tract of the animals fed diets supplemented with antibiotics has been reported by other workers, (20, 21). It is possible that the antibiotics promote the growth of certain microorganisms while suppressing the growth of others.

In the light of the above discussion, it can be suggested that increased microflora as a result of addition of antibiotics to the diets induced microbial activities such as detoxification, proteolysis, deamination, and protein synthesis which are beneficial to the animals. The question then remains why the addition of antibiotics to the raw navy bean diet did not promote growth of animals equal to that of those fed the heated navy bean diet. It may be that the site of antibiotic action, i.e., the cecum, is far away from the site (intestine) where efficient digestion and absorption of the nutrients take place.

Although trypsin inhibitors and hemagglutinins in navy beans can explain to some extent the results of the present study, the presence of other toxic factor(s) cannot be overruled. Kakade and Evans (3) isolated a major growth inhibitory factor from navy beans (Fraction 4) and observed no correlation between the growth inhibitor activity and trypsin inhibitor or of the inhibitor and the hemagglutin activity. The observations of the present investigation indicate that Fraction 4 inhibited the growth of rats by some unknown manner not revealed by the impaired absorption of amino acids and loss of endogenous nitrogen. The lower values reported for nitrogen and amino acid absorption caused by the addition of Fraction 4 to the diet than those of control diet containing heated Fraction 4, may in fact, be the result of the residual trypsin inhibitor or hemagglutinin activity present in Fraction 4 (3), or of both.

In conclusion, the growth inhibition of rats fed raw navy beans may in part be the result of low food intake, increased loss of endogenous nitrogen, and impaired absorption of amino acids. Toxic factors such as trypsin inhibitor and hemagglutinins present in navy beans might cause these effects. More work is needed to characterize the major growth-inhibiting factor contained in Fraction 4 and to examine the mechanism involved in the growth inhibition of rats resulting from Fraction 4.

LITERATURE CITED

1. Liener, I. E. 1962 Toxic factors in edible legumes and their elimination. Amer. J. Clin. Nutr., *11:* 281.
2. Kakade, M. L., and R. J. Evans 1964 Effect of methionine, vitamin B_{12} and antibiotics supplementation on protein nutritive value of navy beans. Proc. Soc. Exp. Biol. Med., *115:* 890.
3. Kakade, M. L., and R. J. Evans 1965 Growth inhibition of rats fed navy bean fractions. J. Agr. Food Chem., *13:* 450.
4. Borchers, R., D. Mohammad-Abadi and J. M. Weaver 1957 Antibiotic growth stimulation of rats fed raw soybean oil meal. J. Agr. Food Chem., 5: 371.

5. Goldberg, A., and K. Guggenheim 1964 Effect of antibiotics on pancreatic enzymes of rats fed soybean flour. Arch. Biochem. Biophys., *108:* 250.
6. Barnes, R. H., E. Kwong and G. Fiala 1965 Effect of penicillin added to an unheated soybean diet on cystine excretion in feces of the rats. J. Nutr., *85:* 123.
7. Barnes, R. H., G. Fiala and E. Kwong 1965 Prevention of coprophagy in the rat and the growth stimulating effects of methionine, cystine and penicillin when added to diets containing unheated soybeans. J. Nutr., *85:* 127.
8. Barnes, R. H., and E. Kwong 1965 Effect of soybean trypsin inhibitor and penicillin on cystine biosynthesis in the pancreas and its transport as exocrine protein secretion in the intestinal tract of the rat. J. Nutr., *86:* 245.
9. Association of Official Agricultural Chemists 1960 Official Methods of Analysis. Washington, D. C., p. 12.
10. Barton-Wright, E. D. 1953 The microbiology and assay of vitamin B complex and amino acids. Sir Isaac Pitman and Son, Ltd., London.
11. Northrop, J. H. 1926 A convenient method for the formol titration. J. Gen. Physiol., *9:* 767.
12. Kunitz, M. 1947 Crystalline soybean trypsin inhibitor. II. General properties. J. Gen. Physiol., *30:* 291.
13. Kakade, M. L., and R. J. Evans 1965 Nutritive value of navy beans (*Phaseolus vulgaris*). Brit. J. Nutr., *19:* 269.
14. de Muelenaere, M. J. H. 1964 Studies on the digestion of soybeans. J. Nutr., *82:* 197.
15. LeBlond, C. P., and B. E. Walker 1956 Renewal of cell populations. Physiol. Rev., *36:* 255.
16. Lyman, R. L., and S. Lepkovsky 1957 The effect of raw soybean meal and trypsin inhibitor diets on pancreatic enzyme secretion in the rat. J. Nutr., *62:* 269.
17. Haines, P. C., and R. L. Lyman 1961 Relationship of pancreatic enzyme secretion to growth inhibition in rats fed soybean trypsin inhibitor. J. Nutr., *74:* 445.
18. Campbell, R. M., D. P. Cuthbertson, W. Mackie, A. S. McFarlane, A. T. Phillipson and S. Sudsaneh 1961 Passage of plasma albumin into the intestine of the sheep. J. Physiol., *158:* 113.
19. Lepkovsky, S., F. Furuta, T. Koike, N. Hasegawa, M. K. Dimick, K. Krause and F. J. Barnes 1965 The effect of raw soybeans upon the digestion of proteins and upon the function of the pancreas of intact chickens and of chicken with ilostomies. Brit. J. Nutr., *19:* 41.
20. Nitsan, Z., and A. Bondi 1965 Comparison of nutritional effects induced in chicks, rats, and mice by raw soybean meal. Brit. J. Nutr., *19:* 177.
21. Sieburth, J. McN., J. J. Jezeski, E. G. Hill and L. E. Carpenter 1954 Some microbiological observations on the antibiotic-fed chick. Poultry Sci., *33:* 753.

Isolation of a Compound from Alfalfa Lipids that Inhibits Tocopherol Deposition in Chick Tissues [1,2,3]

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ABSTRACT A series of experiments was conducted to determine the chick tissue tocopherol-depressing activity of various fractions separated from alfalfa lipid extract. The methods of separation included precipitation from various solvents, column, and thin-layer chromatography. Liver and plasma tocopherol levels of chicks fed diets containing the various alfalfa fractions were used as the criteria of measurement. Two tocopherol-depressing fractions, an ethanol-soluble fraction, and an ethanol- and Skellysolve B-insoluble but acetone-soluble fraction, were isolated from the crude alfalfa lipid extract. The latter fraction was further subdivided by column chromatography and purified by thin-layer chromatography. Feeding the purified compound at levels of 0.02 and 0.00125% (200 and 12.5 ppm) of the diet resulted in a depression in liver storage of tocopherol of approximately 70 and 33%, respectively. This compound is dark green to black in color, and has a very firm but waxy consistency with a melting point of 114° and a molecular weight of 1060. The carbon, hydrogen, oxygen, and nitrogen content is 73.72, 8.36, 9.47, and 5.50%, respectively. The visible and infrared spectra indicate the possible presence of a prophyrin nucleus.

Alfalfa has been reported (1) to contain a relatively large amount of tocopherol that is primarily in the alpha- form. This tocopherol has been shown [5] to be less effective than an equivalent amount of pure *d*-α-tocopherol in preventing chick encephalomalacia. Later it was reported (2) that only approximately one-third of the tocopherol in alfalfa was utilized by the chick when liver storage of tocopherol was used as the measurement of response. This reduced utilization was subsequently found (3) to be caused by a material contained in the hot ethanol extract of alfalfa. The occurrence of this factor in the lipid extract was demonstrated by separating a distillate, which contained all of the tocopherol, from the remainder of the lipid material by molecular distillation and by feeding the partitioned materials separately and in combination with each other and pure *d*-α-tocopherol.

Studies involving the isolation of the components contained in the lipid extract of alfalfa have been carried out by precipitating fractions from a variety of solvents (4, 5), and more refined separations have been conducted by the use of column chromatography (6–8). Using a combination of column and thin-layer chromatography (TLC) as the separating medium, Thirkell and Tristam (9) were able to separate the lipid extract of lucerne into approximately 90 different components.

In preliminary work,[6] it was found that the unsaponifiable ethanol-soluble fraction of the crude alfalfa lipid extract contained a tocopherol-depressing factor for chicks. In the same study, 5 fractions of crude alfalfa lipid extract were obtained by precipitation

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[5] Singsen, E. P., L. M. Potter, R. H. Bunnell, L. D. Matterson, L. Stinson, S. V. Amato and E. L. Jungherr 1955 Studies on encephalomalacia in the chick. 6. The utilization of vitamin E from alfalfa meal and wheat middlings for the prevention of encephalomalacia. Poultry Sci., 34: 1234 (abstract).

[6] Pudelkiewicz, W. J., G. Olson and L. D. Matterson 1965 Nutritional studies on plant lipids. I. Use of a 24-hour chick bioassay to follow the isolation of a compound inhibitory to the deposition of α-tocopherol in tissue. Federation Proc., 24: 622 (abstract).

from a number of solvents, and three of these fractions contained some tocopherol-depressing activity.

With this preliminary information, a study was undertaken that had as its objectives: (a) to develop a reliable and short bioassay which would use minimal amounts of the separated fractions in order to follow the course of isolation of the active material; (b) to isolate and purify a tocopherol-depressing factor from the lipid extract of alfalfa [7]; (c) to determine the quantity of this factor required to give a significant depression of tocopherol levels in chick tissue; and (d) to determine some of the physical properties of this compound.

EXPERIMENTAL

Bioassay. Day-old White Plymouth Rock male chicks were placed in electrically heated wire batteries and fed a vitamin E-low basal diet (10) for from 15 to 18 days. The exact time period was adjusted to suit the needs of the experiment. The smallest and largest chicks, approximately 40% of the total, were then removed and the remaining chicks were distributed into experimental groups of 4 chicks each, of approximately equal weight. Four chicks provided sufficient pooled plasma and pooled liver for 2 or 3 tocopherol determinations. The isolated lipid fractions were then mixed into a reference tocopherol diet (40 or 80 mg of *d*-α-tocopherol [8]/kg) and fed for exactly 72 hours. A vitamin E-low basal diet and a reference tocopherol diet were included in each experiment. During preparation of the test diets, 2% of coconut oil [9] was added at the expense of the white corn meal. This oil reduced dustiness of the feed and acted as a carrier for the supplements. To equalize feed consumption during the 72-hour supplementation period, 50 g were fed each group of 4 chicks at zero, 12, 24, 36, and 48 hours and 100 g after 60 hours of supplementation for a total of 350 g. At the end of the test period, 4 ml of blood were obtained by heart puncture from each chick using heparin as the anticoagulant. The birds were then decapitated to exsanguinate the liver. Plasma tocopherol was determined by the method of Quaife and Biehler (11). Tocopherol determinations for excreta, liver, and the alfalfa extract were made as described previously for liver tocopherol (10). This method uses extraction, molecular distillation, and chromatography through Florex.[10] Total reducing material was determined by the ferric chloride-dipyridyl reaction. When the total reducing substances of the liver, plasma, or excreta of the chicks fed the basal diet were subtracted from the total reducing substances of the respective tissues of chicks fed the supplemented diets, essentially all of the residual reducing material was then assumed to be *d*-α-tocopherol, since this was the only form of tocopherol fed. Excreta collections were made as reported previously (3).

Fractional precipitation. The 2 primary objectives of the fractional precipitation were: (a) to separate the extract into a number of individual fractions, and (b) to concentrate as much of the tocopherol as possible into one fraction, thus leaving the other fractions essentially devoid of tocopherol. To the fractions devoid of tocopherol, a known amount of pure *d*-α-tocopherol was added when assaying for tocopherol-depressing activity. Five fractions of the alfalfa lipid extract were obtained by fractional precipitation using absolute ethanol, Skellysolve B, and acetone (fig. 1).

Fraction G_1 represents a fraction which is soluble in absolute ethanol at $-20°$ and contains approximately 50% of the dry matter of the original lipid extract. The tocopherol of alfalfa is present in this fraction, along with a major portion of the carotenoids.

Fractions G_2 and G_3 represent fractions which are soluble in hot ethanol but precipitate from ethanol when cooled to $-20°$. This portion of the extract repre-

[7] Obtained through the courtesy of Dr. K. W. Taylor, Agricultural Research Service, Western Utilization Research and Development Division, Albany, California. It is a 16-hour hexane extract (54°) of 1 ton of dehydrated alfalfa meal. The solvent was removed by vacuum distillation and contained approximately 90% dry matter when received. The tocopherol content was 3 mg/g of dry matter.

[8] Received through the courtesy of Dr. S. R. Ames, Distillation Products Industries, Rochester, New York.

[9] This is a refined, bleached and deodorized, tocopherol-low and sterol-free coconut oil, Product 1223, of the Drew Chemical Corporation, New York.

[10] Florex AA RVM 60/90 mesh, received through the courtesy of R. M. McCracken of the Floridin Company, New York.

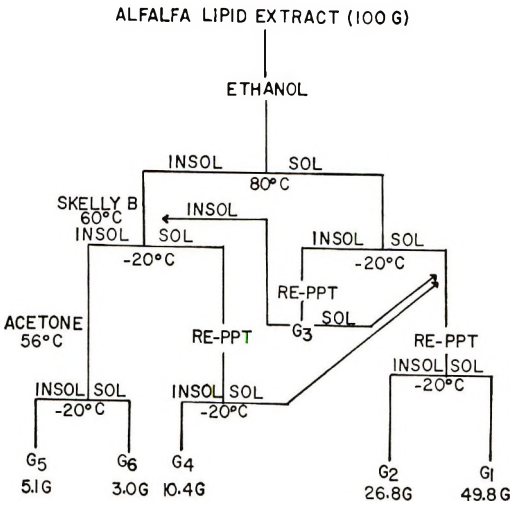

Fig. 1 Flow diagram for fractionation of alfalfa lipids.

sents about 27% of the total weight of the original extract. Fraction G_3, which was separated from G_2 by concentration and cooling to $-20°$, was previously observed [11] to have no activity and therefore was combined with fraction G_2 in this study.

Fraction G_1 is a fraction which is insoluble in ethanol. It is soluble in hot Skellysolve B, but precipitates relatively rapidly on cooling to $-20°$. This fraction represents slightly more than 10% of the original extract.

Fraction G_5 is a fraction which is insoluble in absolute ethanol, Skellysolve B, and acetone, but is soluble in chloroform. This fraction accounts for approximately 5% of the original lipid. After several precipitations from acetone, the material is nearly white and has a waxy consistency.

Fraction G_6 is a fraction that is insoluble in ethanol and insoluble in Skellysolve B between room temperature and $-20°$. However, it is soluble in acetone at this temperature and contains approximately 3% of the original lipid. This material, upon removal of solvent, is greenish-black in color and has a waxy appearance.

Each of the 5 fractions was analyzed for tocopherol. The G_1 fraction contained 93% of the tocopherol that was present in the lipid extract. The remaining 7% was in the G_2 fraction, whereas only a trace was found in fraction G_4. Fractions G_5 and G_6 were devoid of tocopherol.

Saponification procedure. The saponification procedure was a modification of the method described by Moore (12). Because of the low solubility of this material in ethanol, the quantity of solvent used was increased threefold.

Physical determinations. Melting points were determined using the Thiele melting point apparatus. Qualitative tests for nitrogen and sulfur were conducted by the sodium fusion method outlined by McElvain (13). Carbon, hydrogen, oxygen, and the molecular weight were determined commercially,[12] the latter by an ebullioscopic procedure using the Rieche's microapparatus, with butanone as the solvent. The ultraviolet (UV) and visible absorption spectra were made on a Cary Model 15 recording spectrophotometer. The infrared (IR) spectrum was made on a Perkin-Elmer Model 137 Infracord.

EXPERIMENTS AND RESULTS

The concentrations and standard deviations of the bioassay used gave blood plasma tocopherol levels of 1389 ± 80 (range 1276–1528) μg/100 ml and liver tocopherol levels of 18.2 ± 1.4 (range 15.8–20.1) μg/g when the diet contained 40 mg *d*-α-tocopherol/kg of diet. Ten replicates of the reference diet were used in the study. This bioassay was considered to be sufficiently reproducible to be used in these studies.

Experiment 1. We have recognized for some time that plant lipids can cause some difficulty in the analysis of tocopherol. The Emmerie-Engel colorimetric method for the determination of tocopherols is not specific, and numerous quinones present in plant tissue (14) would give a positive reaction when in the reduced state (15).[13] The reduced condition is brought about by chromatographing through a $SnCl_2$-washed adsorbent,[14] a purification procedure often used in the analysis of tocopherol. It was, therefore, felt desirable to obtain unequivocal proof that the decreased tocopherol content of tissues which occurred when

[11] See footnote 6.
[12] Conducted by the Clark Microanalytical Laboratory, Urbana, Illinois.
[13] Unpublished data about plastoquinone, W. J. Pudelkiewicz, 1964.
[14] See footnote 10.

alfalfa lipids were in the diet was caused by some component in the alfalfa lipid extract and not by erroneous tocopherol analysis of the extract.

In this experiment, zero, 1, 2, and 4% of alfalfa lipid extract, equivalent to zero, 37.5, 75, and 150 mg of tocopherol/kg of diet and pure *d*-α-tocopherol in equivalent amounts were added to the basal diet in a 4 × 4 factorial design. This design should reveal both quantitative and interaction effects resulting from the graded levels of the 2 variables. Coconut oil was not used in this experiment.

The data in table 1 show that at the zero level of alfalfa extract, the typical linear response to supplementation with pure *d*-α-tocopherol was obtained. As the amount of alfalfa extract was increased, a corresponding decrease in liver tocopherol occurred even though the alfalfa extract contributed 37.5, 75, or 150 mg of tocopherol/kg of diet. This observed decrease clearly indicated a detrimental effect of the alfalfa lipids on the deposition of tocopherol in the liver tissue. At the zero level of *d*-α-tocopherol, addition of the alfalfa extract resulted in only a very slight increase in liver tocopherol. Furthermore, when 150 mg of *d*-α-tocopherol/kg of diet were added in the presence of the alfalfa extract (last column), a significant decrease in liver tocopherol occurred. The contrast between 67.9 and 29.7 μg/g at the zero and 4% levels of alfalfa extract, respectively, is most striking.

Experiment 2. This experiment was designed to test the alfalfa lipid extract and 5 fractions separated by fractional precipitation for tissue tocopherol-depressing activity. In conjunction with this phase of the experiment, balance studies were conducted to determine whether the lower tocopherol content of the tissue, associated with certain fractions, could be related to a greater excretion of tocopherol.

The alfalfa lipid extract and fraction G_1 were analyzed for tocopherol and incorporated into the diet at levels that would give 40 and 80 mg of tocopherol/kg of diet, namely 1.3 and 2.6%, and 0.7 and 1.4% of the diet, respectively. The remaining 4 fractions were fed in amounts equivalent to the amount of each fraction contained in the alfalfa lipid extract fed.

The data presented in figure 2 indicate that 3 of the 7 treatments (alfalfa lipid extract, fractions G_1 and G_6) resulted in a sizable depression of liver tocopherol, whereas the remaining treatments corresponded favorably to the tissue tocopherol levels of the chicks receiving the standard tocopherol diet. The liver tocopherol levels obtained by feeding these 3 fractions at the 80 mg tocopherol level were 25.8, 39.2, and 46.3%, respectively, of the birds receiving the standard tocopherol reference diet. Plasma tocopherol plots (not shown) paralleled those of liver tocopherol.

TABLE 1

Effect of alfalfa lipids on the tocopherol content of liver tissue when an alfalfa lipid extract was fed with different combinations of d-α-tocopherol in a 72-hour bioassay.

Alfalfa extract [1]	*d*-α-Tocopherol, mg/kg of diet			
	0	37.5	75.0	150
%	μg/g liver		μg/g liver	
0	2.5	13.6	33.1	67.9
1	7.8	23.8	40.0	57.3
2	10.7	21.0	27.6	47.1
4	13.9	16.9	25.1	29.7

[1] Each 1% of alfalfa lipid extract is equivalent to 37.5 mg of apparent tocopherol/kg of diet.

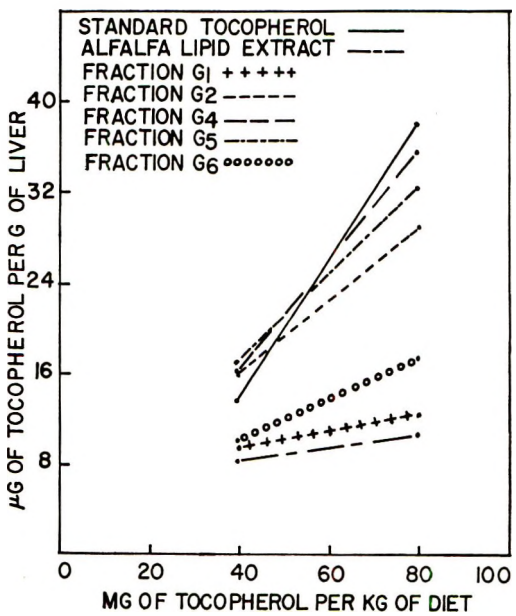

Fig. 2 Effect of feeding 2 levels of alfalfa lipid extract and its subfractions on chick liver tocopherol levels.

Analysis of the tocopherol content of the excreta (table 2) showed that the 2 fractions which caused the greatest depression in the tissue levels of tocopherol (G_1 and G_6) may have produced their depressing activity by different mechanisms. Incorporating the G_1 fraction into the diet caused a fourfold increase in the percentage of tocopherol intake excreted over that of the reference diet. Thus, it is possible that this fraction interfered with the intestinal absorption of tocopherol. However, supplementing the diet with the G_6 fraction did not increase the percentage of tocopherol intake excreted, suggesting that interference with absorption through the intestinal wall may not be the primary cause.

Based on the results of this experiment, it was assumed that at least 2 different compounds observed in the lipid extract of alfalfa were capable of producing a depression in chick tissue levels of tocopherol. Fraction G_6 was selected for further study primarily because it represented only 3% of the original extract, whereas fraction G_1 contained approximately 50% of the original material.

Experiment 3. This experiment consisted of a chromatographic separation of the G_6 fraction by the use of silicic acid (16) and Hyflo Supercel [15] in a 2:1 ratio. After drying at 110° for 24 hours, this mixture was further activated by the use of dehydrating washes as outlined by Barron and Hanahan (17). Seventy-five grams of the pretreated adsorbent mixture were added to a glass column 34 mm in diameter and 400 mm in length. The lipid mixture (236 mg of fraction G_6) was added to the column in 15 ml of benzene. A total of 40 cuts of approximately 25 ml each were collected from the column by the solvent sequence given below. Aliquots of each cut were spotted on plates coated with silica gel H. Then, on the basis of the similarity of major components that were observed either by the natural color of the components or made visible by UV light, the cuts were combined into 7 subfractions and are listed as G_6f_1 through G_6f_7. The order and quantity of eluting solvents was as follows: (a) 200 ml of petroleum ether (bp 65–110°), (b) 200 ml of 20% diethyl ether in petroleum ether, (c) 400 ml of 50% diethyl ether in petroleum ether, and (d) 200 ml of absolute methanol. Cuts 1–5 (G_6f_1) were clear at first and then yellow; 6–11 (G_6f_2, a gray band on the column) were dark green at first and then greenish-yellow; 12–15 (G_6f_3) contained a dark green band; 16–21 (G_6f_4) a reddish-brown band; 22–24 and 25–32 (G_6f_5 and G_6f_6, respectively) contained several light green and greenish-yellow bands; 33–40 (G_6f_7) dark green, and contained the strongly adsorbed material released by methanol. The room temperature was maintained below 20° during the chromatographic procedures to prevent the formation of air pockets. The 236 mg of the G_6 fraction applied to the column corresponded to the amount of it fed at the 80 mg tocopherol level in the preceding experiment. The 7 subfractions listed above were biologically assayed for tocopherol-depressing activity and the results are presented in table 3.

TABLE 2

Percentage of tocopherol intake excreted by chicks receiving alfalfa lipid extract and 5 major fractions

Treatment	Dietary tocopherol level	
	40 mg/kg	80 mg/kg
	%	%
Standard tocopherol [1]	10.6 [2]	7.0
Alfalfa lipid extract [3]	34.0	45.3
Fraction G_1	30.3	32.0
Fraction G_2 + tocopherol	10.7	20.2
Fraction G_4 + tocopherol	11.9	11.6
Fratcion G_5 + tocopherol	11.3	6.3
Fraction G_6 + tocopherol	9.4	5.8

[1] Where *d*-α-tocopherol was added to the diet it was incorporated at the rate of 40 mg and 80 mg/kg of diet.
[2] Each value represents an average of duplicate analyses of duplicate lots.
[3] Equivalent to 1.3 and 2.6% of the diet.

The plasma tocopherol levels of the birds receiving the reference diet were within the range expected at this level of dietary tocopherol intake. A large variation was observed in the plasma tocopherol levels of the birds receiving the various subfractions. Fraction G_6, and subfractions G_6f_3 and G_6f_4, resulted in a definite depression in the plasma tocopherol levels, whereas subfractions G_6f_2, G_6f_5, G_6f_6, and G_6f_7 caused no appreciable change when compared with the reference tocopherol diet. Frac-

[15] A diatomaceous earth product of Johns-Manville Company, New York.

TABLE 3

Tissue tocopherol-depressing activity of the subfractions of fraction G_6 separated by silicic acid column chromatography

Treatment	Plasma tocopherol	Liver tocopherol
	μg/100 ml	*μg/g*
Basal	143 [1]	2.0
Standard tocopherol [2]	2427	46.9
Fraction G_6 + tocopherol	1910	23.6
Subfraction G_6f_1 + tocopherol	3102	42.0
Subfraction G_6f_2 + tocopherol	2649	40.5
Subfraction G_6f_3 + tocopherol	1963	29.9
Subfraction G_6f_4 + tocopherol	1954	30.1
Subfraction G_6f_5 tocopherol	2324	35.4
Subfraction G_6f_6 + tocopherol	2515	39.1
Subfraction G_6f_7 + tocopherol	2778	40.2

[1] Each value represents an average of duplicate analyses.
[2] Pure *d*-α-tocopherol was incorporated at a level of 80 mg/kg of diet.

tion G_6f_1 resulted in an increased plasma tocopherol level; however, this increase was not reflected in the liver tocopherol level from those same birds. The liver storage of tocopherol in the birds that received subfractions G_6f_1, G_6f_2, G_6f_6, and G_6f_7 was within the expected range. This range, determined from 13 lots of chicks that consumed the standard tocopherol diet (80 mg/kg) in the entire series of experiments, was 40.1 μg/g of liver tissue with a confidence limit of ± 1.8 μg. The liver tocopherol level of the chicks fed the standard diet in the present experiment fell outside of the confidence interval; however, the plasma tocopherol level of the same birds was within the confidence interval. Incorporating fraction G_6 and subfractions G_6f_3 and G_6f_4 into the diet, resulted in a pronounced depression in liver storage of tocopherol, whereas subfraction G_6f_5 was only slightly below the normal range. From these results, it is evident that the tocopherol-depressing activity of the G_6 fraction is contained in its 2 subfractions, G_6f_3 and G_6f_4. Of the 2 depressing subfractions, G_6f_3 was found to be the least contaminated by other components on the basis of thin layer chromatographic analysis, and for this reason was selected for further purification.

Subfraction G_6f_3 contained a large amount of pigment that was dark green to black in color and appeared as a grey solution when diluted with chloroform. The consistency of this compound was tar-like but waxy. When this pigment was saponified, or removed by adsorption on activated charcoal,[16] the activity of these fractions was lost. Therefore, it was assumed that the depressing activity of the G_6f_3 subfraction was intimately associated with the pigment.

Experiment 4. This experiment consisted of separating the G_6f_3 subfraction into its components by TLC on silica gel H. The developing solvent for all assay and preparative work consisted of a 12:12:1 ratio of petroleum ether, diethyl ether, and ethanol. To prevent overloading of the plates, only approximately 3 mg of the parent lipid mixture could be applied to each plate. The developed chromatograms were observed to contain one major component, greenish-black in color, and several minor components. The major component was removed and designated as G_6f_3M. All minor components above the major one were collected as one (G_6f_3A), and all components below were collected as a separate one (G_6f_3L). The plasma and liver tocopherol content of the birds fed these components are presented in table 4.

The tissue tocopherol level of the birds fed the G_6f_3M component corresponds very

TABLE 4

Plasma and liver tocopherol levels of birds receiving components of subfraction G_6f_3 separated by thin-layer chromatography

Treatment	Plasma tocopherol	Liver tocopherol
	μg/100 ml	*μg/g*
Basal	222 [1]	1.7
Standard tocopherol [2]	2548	38.8
Subfraction G_6f_3 + tocopherol	1372	19.0
Component G_6f_3A + tocopherol	2516	42.1
Component G_6f_3M + tocopherol	1437	20.1
Component G_6f_3L + tocopherol	1959	27.6

[1] Each value represents an average of duplicate analyses.
[2] Pure *d*-α-tocopherol was incorporated at a level of 80 mg/kg of diet.

[16] Norite A, Pfanstiehl Chemical Company, Waukegan, Illinois.

closely with the value obtained from the original G_6f_3 subfraction. This relationship was expected since the G_6f_3M component theoretically contained all of the tocopherol-depressing substance contained in the parent G_6f_3 subfraction. It is apparent that the minor components making up fraction G_6f_3A contained no tocopherol-depressing activity. Because there was some streaking of the G_6f_3M component into the lower area of the TLC plate, a slight depression of both the plasma and liver tocopherol was expected from the G_6f_3L compounds; however, a sizable depression of tissue tocopherol resulted from this component. No explanation for this unexpected depression of tissue tocopherol is offered. It was concluded from this experiment that one of the factors contained in the alfalfa lipid extract that caused a depression in the tissue levels of tocopherol was the highly pigmented component designated as G_6f_3M. On the basis of one- and two-dimensional TLC, and by the use of 2 solvent systems, this component was assumed to be essentially pure; however, attempts to crystallize it from a variety of solvents failed.

Experiment 5. This experiment was designed to determine whether feeding graded levels of the G_6f_3M compound, which had been found to contain the tissue tocopherol-depressing activity, would result in a linear depression of liver and plasma tocopherol levels. Five levels of the G_6f_3M compound were fed, ranging from 0.2 to 200 mg/kg (ppm) of diet. Each level was supplemented with the standard amount (80 mg/kg) of pure *d*-α-tocopherol.

Incorporating the G_6f_3M component into the diet at the rate of 200 mg/kg of the diet resulted in an average liver tocopherol level of 13.0 μg/g of liver tissue, as compared with an average of 40.1 μg/g of liver tissue in the birds receiving the standard tocopherol diet. The incorporation of either 50 or 12.5 mg/kg of the G_6f_3M component resulted in a depression of tissue tocopherol levels of approximately 30%, and a slight reduction from the standard tocopherol level was noted when as little as 3.1 mg of the component were added.

To evaluate the quantitative relationship between the intake of the G_6f_3M component and the liver and plasma levels of tocopherol, the logs of the liver and plasma tocopherol levels were plotted against the log of the G_6f_3M intake/kg of diet (fig. 3). Increasing the level of the G_6f_3M component in the diet progressively depressed the tissue content of tocopherol. However, when the linearity of the treatment effects were tested with orthogonal comparisons, a significant quadratic effect ($P = 0.05$) was obtained. The tocopherol content of the excreta collected from the birds in this experiment showed no pronounced difference between treatments. These results confirmed those obtained for the parent compound, G_6, presented in table 3.

Results of chemical and physical determinations. On the basis of a stable melting point at 114° and a single spot on two-dimensional TLC with 2 solvent systems, it was assumed that the G_6f_3M compound was essentially pure. Figure 4 shows the visible spectrum of this compound. This spectrum contains 4 peaks between 500 and 700 mμ in addition to a major (Soret) band with a maximum at 415 mμ. This spectrum resembles those commonly associated with a porphyrin-type molecule (18). The UV spectrum of this compound had 2 minor peaks at 276 and 327 mμ. The infrared spectrum (fig. 5) contains some characteristics which resemble known porphyrin spectra (19).

Results of the elemental analyses for carbon, hydrogen, oxygen, and nitrogen are, respectively, 73.72, 8.36, 9.47, and 5.50% and total only 97.05%. Of the 2.95% unaccounted for, silicon and mag-

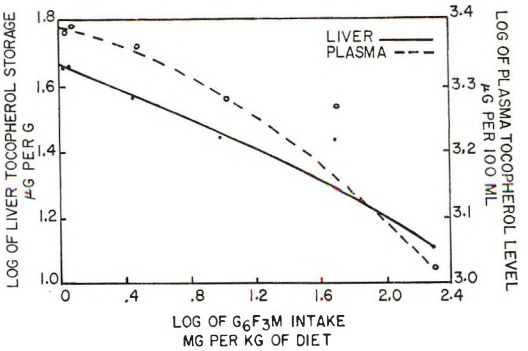

Fig. 3 Effect of feeding graded levels of alfalfa lipid fraction G_6f_3M on liver and plasma levels of tocopherol.

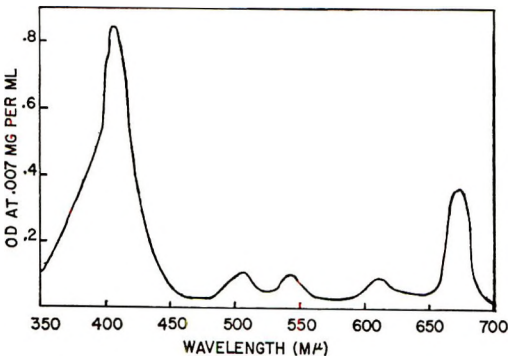

Fig. 4 Visible spectrum of alfalfa lipid fraction G_6f_3M in chloroform.

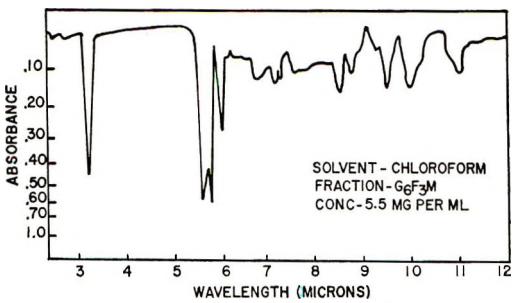

Fig. 5 Infrared spectrum of alfalfa lipid fraction G_6f_3M.

nesium were present in the ash in a ratio of 12:1 and are believed to be contaminants from column and TLC. Tests for sulfur and the halogens were negative. The molecular weight was 1060. It was estimated that there was approximately 4 mg of the G_6f_3M component/g of alfalfa lipid.

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LITERATURE CITED

1. Brown, F. 1953 The tocopherol content of farm feeding-stuffs. J. Sci. Food Agr., *4:* 161.
2. Bunnell, R. H. 1957 The vitamin E potency of alfalfa as measured by the tocopherol content of the liver of the chick. Poultry Sci., 36: 413.
3. Pudelkiewicz, W. J., and L. D. Matterson 1960 A fat-soluble material in alfalfa that reduces the biological availability of tocopherol. J. Nutr., *71:* 143.
4. Chibnall, A. E., E. F. Williams, A. L. Latner and S. H. Piper 1933 The isolation of *n*-triacontanol from lucerne wax. Biochem. J., 27: 1885.
5. Jackson, A. H., and F. A. Kummerow 1949 The lipids of dehydrated alfalfa leaf meal. J. Amer. Chem. Soc., *71:* 26.
6. Blair, E. H., H. L. Mitchell and R. E. Silker 1953 Isolation and characterization of constituents of alfalfa wax. Ind. Eng. Chem., *45:* 1104.
7. Cole, J. H. L., and J. B. Brown 1960 Chromatographic isolation of the original constituents of natural waxes. The composition of ouricuri wax. J. Amer. Oil Chem. Soc., *37:* 359.
8. Van Der Veen, J. W., and H. S. Olcott 1964 Nutrients in alfalfa. Lipids of dehydrated alfalfa (*Medicago sativa*). Agr. Food Chem., *12:* 287.
9. Thirkell, D., and G. R. Tristram 1963 The isolation of leaf components. II. J. Sci. Food Agr., *14:* 488.
10. Pudelkiewicz, W. J., L. D. Matterson, L. M. Potter, L. Webster and E. P. Singsen 1960 Chick tissue-storage bioassay of alpha-tocopherol: Chemical analytical techniques, and relative biopotencies of natural and synthetic alpha-tocopherol. J. Nutr., *71:* 115.
11. Quaife, M. L., and R. Biehler 1945 A simplified hydrogenation technique for the determination of blood plasma tocopherols. J. Biol. Chem., *159:* 663.
12. Moore, T. 1957 Vitamin A. Elsevier Publishing Company, New York, pp. 53–66.
13. McElvain, S. E. 1945 The Characterization of Organic Compounds. The Macmillan, Company, New York.
14. Thomson, R. H. 1957 Naturally Occurring Quinones. Academic Press, New York.
15. Pudelkiewicz, W. J., and L. D. Matterson 1960 Effect of coenzyme Q_{10} on the determination of tocopherol in animal tissue. J. Biol. Chem., *235:* 496.
16. Hirsch, J., and E. H. Ahrens, Jr. 1958 The separation of complex lipide mixtures by the use of silicic acid chromatography. J. Biol. Chem., *233:* 311.
17. Barron, E. J., and D. J. Hanahan 1958 Observation on silicic acid chromatography of the neutral lipides of rat liver, beef liver, and yeast. J. Biol. Chem., *231:* 493.
18. Falk, J. E., and J. B. Willis 1951 The infra-red spectra of porphyrins and their iron complexes. Australian J. Sci. Res., *4:* 579.
19. Aronoff, S. 1950 The absorption spectra of chlorophyll and related compounds. Chem. Rev., 47: 175.

Metabolizability and Nutritional Implications of L-Arabinose and D-Xylose for Chicks [1]

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ABSTRACT Inclusion of 40% L-arabinose and D-xylose in chicks' diet resulted in a severe depression of growth, feed efficiency, liver and gastrointestinal sizes and depletion in liver and muscle glycogen. Twenty per cent xylose also depressed growth significantly. From 3 experiments the average metabolizable energy values for arabinose and xylose at 20% dietary level were 3.06 and 2.54 kcal/g of pentose, respectively. The effects of graded dietary levels (10, 20 and 40%) of arabinose and xylose were compared. The data indicated that plasma uric acid was elevated at the two higher levels of each pentose. Plasma cholesterol was significantly higher among birds receiving a 40% xylose diet. Total reducing sugars in blood plasma were consistently higher for each level of arabinose, whereas xylose treatment at each level significantly increased the total reducing sugars in the plasma. Liver and muscle glycogen was proportionally depleted with increase in dietary pentose level. Glycogen in liver was more depleted among birds receiving 40% arabinose as compared with those fed the same level of xylose.

Bolton (1) has shown that the chick (2 to 5 weeks of age) digested pentosans (mainly of wheat by-products) to an extent of 5%, in contrast with sugar and starch which were fully digested. While it may be possible to improve the digestibility of pentosans through the use of enzymes or special processing procedures of feeding ingredients, or both, it seemed desirable first to examine the utilization of the constituent pentoses, namely, L-arabinose and D-xylose, by the chick.

It was the objective of this investigation to determine the influence of arabinose and xylose on the growth and certain metabolic characteristics of chicks and to determine the metabolizable energy of these carbohydrates.

EXPERIMENTAL

Male chicks (New Hampshire × Columbian) were reared in electrically heated battery brooders. Details of experimental periods and replication are shown in table footnotes. A randomized complete block design was used in each experiment. Statistical analysis of variance was made for each experiment and reported by means of Duncan's multiple range test (2). The composition of diets is shown in table 1. L-Arabinose, D-xylose and α-cellulose [3] were substituted at the expense of D-glucose (A.R.) in experiments 1 and 2, and D-glucose·H_2O in experiment 3; the stated percentage of each component was added on a dry-matter basis. Feed and water were supplied ad libitum.

Metabolizable energy values, including correction for nitrogen retention, for the test carbohydrates were determined using the equations of Potter et al. (3). In experiments 1 and 2, chromium sesquioxide and nitrogen were analyzed by the methods of Hill and Anderson (4) and AOAC (5), respectively, whereas in experiment 3 these were determined by a method developed in this laboratory,[4] using an automated technique employing a continuous digestor and autoanalyzer.[5] Samples of excreta were collected from each pen on the twelfth, thirteenth and fourteenth days of experiment 1, the seventh through twelfth days of experiment 2, and the fourth and fifth days of experiment 3. The excreta samples from each pen in each experiment were pooled, dried at 70°

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[3] Solka Floc (Brown Company, Berlin, N. H.), a purified wood cellulose containing at least 99.5% α-cellulose on a moisture-free basis.

[4] Unpublished data.

[5] Technicon Instruments Corporation, Ardsley, New York.

in a forced-draft oven, ground and together with samples of diets, assayed for gross energy using an adiabatic oxygen bomb calorimeter.[6]

TABLE 1

Composition of diets

	Exp. 1	Exp. 2	Exp. 3
D-Glucose·H_2O [1]	31.55	0	40
D-Glucose (A.R.)	20	20	0
Isolated soybean protein [2]	35	0	0
Corn, ground yellow	0	34.5	10.2
Soybean meal, dehulled	0	30.6	33.6
Constant ingredients	13.54 [3]	15.07 [4]	16.32 [5]

[1] Cerelose, Corn Products Company, New York.
[2] Promine, Central Soya, Inc., Chicago.
[3] Constant ingredients (%): cellulose (Solka Floc, Brown Company, Berlin, N. H.), 2; chromic oxide (30% bread), 1; DL-methionine, 0.75; glycine, 0.3; vitamin mix P-4 (per kg diet: thiamine·HCl, 20 mg; nicotinic acid, 50 mg; riboflavin, 12 mg; Ca D-pantothenate, 20 mg; vitamin B_{12}, (0.1% triturate), in mannitol, 20 mg; pyridoxine·HCl, 5 mg; biotin, 0.4 mg; folic acid, 4 mg; menadione, 2 mg; ascorbic acid, 50 mg; vitamin A palmitate (250,000 IU/g), 40 mg; vitamin D (30,000 ICU/g), 33.3 mg; *d*-α-tocopheryl acetate (250 mg/g), 80 mg; glucose·H_2O carrier), 0.20; mineral mixture P-6 (per kg diet: $CaHPO_4 \cdot 2H_2O$, 21.1 g; $CaCO_3$, 16.9 g; KH_2PO_4, 11.2 g; NaCl, 6.5 g; $MgSO_4$ (70–72%), 3.5 g; $FeSO_4 \cdot 7H_2O$, 333 mg; $MnSO_4 \cdot H_2O$, 333 mg; KI, 6.5 mg; $CuSO_4 \cdot 5H_2O$, 16.7 mg; $ZnCO_3$, 96 mg; $Na_2MoO_4 \cdot 2H_2O$, 5 mg; Na_2SeO_3, 0.22 mg), 6; corn oil (refined), 3; choline chloride (70%), 0.286.
[4] Constant ingredients: (%) fish solubles dried on soybean meal (100% equivalence), 3; alfalfa meal, dehydrated (17% protein), 2.5; dried whole whey, 3; corn oil, 2.0; dicalcium phosphate, 1.75; calcium carbonate, 1; methionine hydroxy analogue calcium salt (90%), 0.05; vitamin A (30,000 IU/g), 0.015; vitamin supplement (4.4 g riboflavin, 8.8 g calcium pantothenate, 19.8 g niacin and 22 g choline chloride/kg), 0.1; choline chloride (25%), 0.05; iodized salt, 0.5; vitamin D_3, (15,000 ICU/g), 0.005; vitamin E acetate (44 IU/g), 0.025; menadione sodium bisulfite — 63% USP (35.2 g/kg), 0.00625; vitamin B_{12} (44 mg/kg), 0.03; manganese sulfate (feed grade), 0.025; zinc sulfate (feed grade), 0.01; chromic oxide bread (30%), 1.
[5] Constant ingredients: (%) as in footnote 4, plus corn oil, 1; and dicalcium phosphate, 0.25.

At the end of the experimental period in experiment 3, plasma was prepared from heparinized blood samples from individual birds and pooled for each pen. Determinations of total plasma reducing sugars, cholesterol and uric acid in plasma were made by modified methods of Hoffman (6), Zlatkis et al. (7) and an industrial laboratory,[7] respectively, employing a continuous automated flow system. Liver and muscle glycogen analyses were made according to Carroll et al. (8).

RESULTS

Experiments 1 and 2. Both xylose and cellulose at 20% substitution for glucose depressed growth and efficiency of feed utilization in a highly significant manner (table 2). Arabinose depressed growth significantly in experiment 1. Feeding arabinose and xylose at the 20% dietary level resulted in higher gastrointestinal tract weights (including contents) as compared with either glucose or cellulose at the identical level. This was due mostly to increased water content.

Using the value for glucose as 3.64 kcal of metabolizable energy/g of dry glucose (9) for a reference, the determined values of metabolizable energy for arabinose, xylose and cellulose were 3.05, 2.52 and 0.05 kcal/g of respective carbohydrate, when the average results of both experiments were considered. Agreement between experiments was good, although diets were not similar.

[6] Parr Instruments Company, Moline, Illinois.
[7] Technicon Instruments Corporation, method N-13a.

TABLE 2

Performance of chicks as influenced by L-*arabinose and* D-*xylose and metabolizable energy of test carbohydrate* [1]

Diet variable (20% level) [2]	Wt gain		Gain/feed		ME of test carbohydrates [2]		GI tract wt/kg [3]
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 2
	g	*g*			*kcal/g*	*kcal/g*	*g*
D-Glucose	292 [a 4]	218 [a]	0.60 [a]	0.50 [a]	3.64 [a]	3.64 [a]	136 [a]
L-Arabinose	234 [b]	199 [ab]	0.58 [a]	0.47 [a]	3.13 [b]	2.97 [b]	192 [b]
D-Xylose	217 [bc]	141 [c]	0.47 [b]	0.41 [ab]	2.59 [c]	2.44 [c]	182 [c]
α-Cellulose	194 [c]	149 [bc]	0.45 [b]	0.35 [b]	0.06 [d]	0.03 [d]	159 [d]

[1] In experiment 1, treatment values are average of triplicate lots of one chick each; experimental period 7 to 21 days of age. In experiment 2, treatment values are average of duplicate lots of 3 chicks each; experimental period 14 to 27 days of age.
[2] ME indicates metabolizable energy; see experimental section for details.
[3] Values reported on wet basis per kilogram body weight and are adjusted means after removal of body weight variation by covariance analysis. Gastrointestinal tract weight with contents from anterior end of esophagus to posterior of rectum per kilogram of body weight.
[4] Treatment values followed by the same letter are not significantly different at $P < 0.05$.

TABLE 3

Effect of graded levels of dietary carbohydrates on performance of chicks including growth, chemical and physical changes, and metabolizable energy [1]

Diet variable [2]	Wt gain	Gain/feed	ME of test carbohydrate [2]	Liver wt/kg [3]	GI wt/kg [3]	Blood plasma/100 ml			Glycogen/100 g	
						Reducing sugars	Cholesterol	Uric acid	Liver	Muscle
	g		*kcal/g*	*g*	*g*	*mg*	*mg*	*mg*	*mg*	*mg*
D-Glucose	108.0 [a 4]	0.54 [a]	3.64 [a]	38.87 [a]	140.6 [ab]	256.9 [a]	140.2 [ab]	5.87 [a]	1583 [a]	783 [a]
10% L-Arabinose	107.3 [a]	0.51 [a]	3.25 [b]	40.37 [a]	171.6 [cd]	307.0 [ab]	127.6 [a]	7.77 [ab]	1514 [ab]	676 [ab]
20% L-Arabinose	101.5 [a]	0.48 [ab]	3.07 [b]	36.11 [a]	178.3 [d]	328.2 [ab]	155.9 [abc]	8.45 [bc]	1021 [bc]	581 [bcd]
40% L-Arabinose	32.8 [c]	0.24 [d]	0.06 [d]	18.41 [c]	81.5 [f]	288.1 [ab]	166.2 [bc]	8.11 [abc]	387 [d]	485 [cd]
10% D-Xylose	98.1 [a]	0.49 [a]	2.72 [c]	38.85 [a]	141.4 [ab]	378.0 [b]	161.7 [bc]	6.20 [ab]	1518 [ab]	705 [ab]
20% D-Xylose	81.3 [b]	0.42 [bc]	2.58 [c]	37.83 [a]	135.2 [a]	533.7 [c]	151.5 [abc]	8.57 [bc]	1152 [bc]	573 [bcd]
40% D-Xylose	26.1 [c]	0.21 [d]	—0.03 [d]	23.50 [b]	95.7 [f]	760.0 [d]	180.2 [c]	8.65 [bc]	797 [c]	422 [de]
20% α-Cellulose	100.4 [a]	0.41 [c]	0.16 [d]	37.92 [a]	158.7 [bc]	262.6 [a]	140.9 [ab]	9.49 [c]	935 [c]	645 [abc]
40% α-Cellulose	34.7 [c]	0.20 [d]	—0.09 [d]	23.19 [b]	108.6 [e]	271.9 [a]	138.8 [ab]	10.63 [c]	209 [d]	294 [e]

[1] Exp. 3: treatment values are average of triplicate lots of three chicks each. Experimental period was from 14 to 20 days of age.
[2] See Experimental section for details.
[3] See table 2, footnote 3.
[4] See table 2, footnote 4.

Experiment 3. Diets containing 20% arabinose and 10% xylose depressed growth and feed efficiency slightly but not significantly as did the next higher level (table 3). The results obtained due to the highest level of these pentoses were similar to those produced by the 40% cellulose control.

Liver and gastrointestinal tract weights were significantly lower due to the 40% dietary arabinose and xylose. However, the lower levels of arabinose resulted in significantly higher gastrointestinal tract weights when compared with the glucose control. The dietary level of cellulose at the 40% level also significantly lowered the liver and gastrointestinal tract weights.

Metabolizable energy values for arabinose and xylose at a 20% level of substitution were 3.07 and 2.58 kcal/g of test pentose, respectively, and were close to the values obtained earlier.

Inspection of the data pertaining to the amounts of total reducing sugars in blood plasma, and liver and muscle glycogen indicated that with reference to the 40% glucose control, each dietary level of the pentoses showed consistently an increase in the total reducing sugars in plasma and a decrease in the liver and muscle glycogen of chicks. Significant increases in the plasma reducing sugars were noted between each increasing level of xylose. For each 10% increase in the dietary xylose level, there was approximately an increase of 125 mg of total reducing sugars/100 ml of plasma. Arabinose levels did not reveal significant differences in total reducing sugars.

At the higher dietary levels of both pentoses, the liver glycogen was more depleted among the birds fed arabinose than those receiving xylose. There was less than one-half as much liver glycogen in livers of chicks receiving 40% arabinose as compared with those receiving the same level of xylose.

Plasma cholesterol values increased as a result of the higher levels of arabinose and all the levels of xylose in relation to glucose and cellulose controls. The only significant increase in plasma cholesterol resulted from the 40% xylose level.

Uric acid level in the plasma was elevated by the presence of pentoses and cel-

lulose in the diet. The higher levels of both pentoses resulted in a higher value for plasma uric acid as compared with their respective lower levels. The resultant increases in plasma uric acid due to the 20% and 40% cellulose diets were not significantly different from those resulting from the higher levels of dietary pentose.

DISCUSSION

Growth and efficiency of feed utilization owing to either of the pentoses at lower levels suggest some, although not statistically detectable, decrease in performance. Further studies, covering a longer experimental period, are required to determine whether these levels impair growth and (perhaps more importantly) physiological well-being.

There was little variation in the metabolizable energy (ME) values of carbohydrates among the 3 experiments. If it can be assumed that the effect of cellulose in reducing the rate of growth of chicks is a reflection only of energy deprivation, then the lesser available energies of arabinose and xylose may also be expected to limit growth. The expectation is partially substantiated by the observation that xylose yielded less ME and also showed greater growth depression effects at the 10% and 20% substitution levels than did arabinose. The observation that cellulose, xylose and arabinose all yielded zero ME at the 40% substitution level and at the same time resulted in more or less equivalent and severe growth depression also supports this energy limitation hypothesis.

Contrary to the above hypothesis, xylose depressed growth more than expected on the basis of apparent energy level as compared with cellulose. This suggests that xylose, despite having a higher ME value than cellulose, reduced the growth of chicks by yet another mechanism. Further investigations are needed to resolve this question.

That the apparent ME values for arabinose and xylose at the 40% level was zero kilocalories per gram, as compared with much higher values at lower pentose levels, requires consideration. Anderson (10) used 3 dietary levels of lactose, 10, 20 and 40%, in diets for growing chicks and reported a decrease in the ME value for lactose at the two higher dietary levels as compared with the lower level. The author attributed the decrease to the known laxative effect of lactose. Loss of xylose and arabinose at high levels may have resulted from decreased absorptive capability (including laxative effect) or increased urinary excretion. Loss in liver weight suggests the further possibility of alimentary excretion of energy-bearing components, namely, cholesterol and bile acids. It is also possible that precision of the ME determination was reduced because of abnormally low feed consumption and the longer time required to attain equilibrium.

At lower dietary levels, arabinose-fed birds had heavier cecums as compared with those receiving similar levels of xylose. This might explain the higher gastrointestinal weights of birds receiving lower arabinose levels. The heavier cecums contained mainly watery substance. A considerable reduction in the gastrointestinal weights of the birds fed the highest levels of both pentoses reflects the severe growth retardation and the low feed consumption.

The increase in plasma uric acid resulting from 20% and 40% cellulose and the higher levels of arabinose and xylose may be due to relative energy deficiency as related to the protein intake and consequently greater nitrogen metabolism. Increased uric acid in blood appears to be a good indicator of this occurrence.

Increase in the plasma cholesterol level as a result of the 40% xylose treatment may be the result of metabolic effects on cholesterol synthesis. As consistent increases in the cholesterol level were observed in relation to the higher dietary levels of arabinose and all the levels of xylose, pentoses may participate either in influencing cholesterol synthesis or inhibiting its excretion. Various workers have demonstrated that dietary carbohydrate may alter plasma cholesterol levels. Grant and Fahrenbach (11) reported that purified diets containing sucrose gave higher plasma cholesterol values than glucose.

Increases in the total reducing sugars in blood with increasing dietary xylose

suggest that the pentoses entering in the blood stream accumulate prior to excretion or utilization. Unpublished data indicate that the increased reducing activity in the plasma is caused by compounds other than glucose. Accordingly, it is unsafe to assume the total reducing activity as glucose.

The severe glycogen depletion in liver and muscle of chicks at 20% and 40% dietary levels of pentoses might be attributed to an interruption of glycogen synthesis. As a result, the endogenous glycogen could have been utilized by the chicks where the pentoses were not metabolized to glucose. Were the pentoses in question freely metabolized and converted to glucose by the chicks, glycogen should not have been depleted from the liver. This hypothesis can be further substantiated from the liver and muscle glycogen values among the birds fed cellulose. Since cellulose is not metabolized by chicks and the available energy to the bird, therefore, is lacking, the lower values of liver and muscle glycogen could be attributed to glycogenolysis. Also the depletion of liver glycogen could be related to the size of the liver as influenced by the pentose treatment. The ingestion of arabinose and xylose at 20% and 40% of the diet by chicks may in some way interfere with the enzyme systems participating in glycogenesis. Another possibility is that there is no effective route for the pentoses in the glycogen synthesis.

LITERATURE CITED

1. Bolton, W. 1955 The digestibility of the carbohydrate complex by birds of different ages. J. Agr. Sci., *46:* 420.
2. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw Hill Book Company, Inc., New York.
3. Potter, L. M., L. D. Matterson, M. W. Stutz and E. P. Singsen 1961 The metabolizable energy of feed ingredients for chickens (Progr. Rep. no. 10). University of Connecticut Experiment Station, Storrs, Connecticut.
4. Hill, F. W., and D. L. Anderson 1958 Comparison of metabolizable energy and productive energy determination with growing chicks. J. Nutr., *64:* 585.
5. Association of Official Agricultural Chemists 1955 Official Methods of Analysis, ed. 8. Washington, D. C.
6. Hoffman, W. S. 1937 A rapid photoelectric method for the determination of glucose in blood and urine. J. Biol. Chem., *120:* 51.
7. Zlatkis, A., B. Zak and A. J. Boyle 1953 A new method for direct determination of serum cholesterol. J. Lab. Clin. Med., *41:* 486.
8. Carroll, N. V., R. W. Longley and J. H. Roe 1956 The determination of glycogen in liver and muscle by use of anthrone reagent. J. Biol. Chem., *200:* 583.
9. Anderson, D. L., F. W. Hill and R. Renner 1958 Studies of the metabolizable and productive energy of glucose for the growing chick. J. Nutr., *65:* 561.
10. Anderson, D. L. 1955 The energy value of poultry feeds. Proc. Cornell Nutrition Conference for Feed Manufacturers, Ithaca, New York, p. 5.
11. Grant, W. C., and M. J. Fahrenbach 1959 Effect of dietary sucrose and glucose on plasma cholesterol in chicks and rabbits. Proc. Soc. Exp. Biol. Med., *100:* 250.