# Effects of the α-Hydroxy Analogues of Isoleucine, Lysine, Threonine and Tryptophan and the α-Keto Analogue of Tryptophan and the Level of the Corresponding Amino Acids on Growth of Rats [1]

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*ABSTRACT* The utilization of the α-hydroxy analogues of isoleucine, lysine, threonine and tryptophan and the α-keto analogue of tryptophan for growth was studied in male weanling rats fed purified amino acid diets. The amino acid mixtures used were those of Rechcigl et al. (10) and Sauberlich (11). The analogues were added to the diet at the expense of the corresponding amino acid. Rats fed the α-hydroxy analogue of isoleucine and the α-keto analogue of tryptophan gained less weight than those fed the corresponding amino acid at the same level. The use of tail cups to prevent coprophagy in one experiment had no apparent effect on utilization of the analogues. The α-hydroxy analogue of tryptophan was equal to tryptophan in promoting growth, whereas the α-hydroxy analogue of lysine and of threonine (prepared with either $KMnO_4$ or $H_2O_2$-$OsO_4$) resulted in weight loss. The levels of isoleucine, lysine, threonine and tryptophan in the complete amino acid mixture were shown to be greater than necessary for maximal body weight gain with the diet used. Rats fed the tryptophan-free diet were hyperexcitable and had convulsions, symptoms unlike those observed during deprivation of other amino acids. Casein was superior to either of the complete amino acid mixtures in promoting weight gain, feed consumption and gain per unit of feed.

Published work through 1939 concerning the utilization of amino acid analogues for the growth of the rat was reviewed by Jackson and Chandler (1). Work up to that time had demonstrated that the α-keto analogues of histidine and tryptophan and the α-hydroxy analogues of isoleucine, histidine and tryptophan would promote growth of rats fed diets low in or devoid of the corresponding amino acid. The α-hydroxy analogue of lysine failed to replace lysine for growth of rats fed a basal diet containing gliadin as the protein source (2).

Subsequent work has shown that successful replacement of amino acids by their corresponding α-keto acids in the diet of the rat is possible for methionine (3), valine (4), phenylalanine (5, 6), leucine (7), isoleucine (7) and arginine (8). Wood and Cooley (9) obtained essentially no differfence in growth when isoleucine, leucine, methionine, phenylalanine and valine were replaced, in a mixture of essential amino acids, by their corresponding α-keto acids.

The purposes of the work reported here were: 1) to test the utilization of the α-hydroxy analogues of isoleucine, lysine, threonine and tryptophan and the α-keto analogue of tryptophan for growth of rats fed the Rechcigl et al. (10) amino acid diet or the Sauberlich (11) amino acid diet (hereafter referred to as reference diets 1 and 2, respectively); 2) to more clearly refine the minimum level of the corresponding amino acids required to promote maximal weight gain in reference diet 2; and 3) to compare performance of rats fed reference diets 1 and 2 with that of rats fed a similar diet containing intact protein (casein plus methionine).

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## EXPERIMENTAL

Male weanling rats of the Holtzman (Sprague-Dawley) strain in experiments 1, 2, 3 and 5 and the Charles River strain in experiment 4 were assigned by weight outcome groups, after consuming a stock diet for 2 or 3 days following arrival, to the experimental diets and supplied feed and water ad libitum. They were housed individually in wire-bottom cages. Diets were kept refrigerated (+4°C) before use. Fresh feed was added to each feeder daily. Feed consumed was measured daily and body weights were recorded on alternate days throughout the 21-day experiments. The basal amino acid diet used in experiments 1 and 2 was that used by Rechcigl et al. (10) and that used in experiments 3 through 5 was essentially Sauberlich's (11). The diet composition is shown in table 1, along with that of the casein plus methionine diet. The composition of the basal amino acid mixtures is shown in table 2. The amino acid analogues were provided by and prepared in an industrial laboratory.[2] Each analogue tested replaced entirely the corresponding amino acid, and the glutamic acid level was adjusted to make all amino acid diets isonitrogenous. Glucose level was adjusted correspondingly.

*Experiments 1 and 2.* Reference diet 1 (10) was used in both experiments. The α-hydroxy analogue of isoleucine contained all 4 possible isomers so that 4 times the level of analogue was supplied in diet 2 (1.84% of the diet) as the amount of L-isoleucine supplied in diet 1 (0.46% of the diet). The α-hydroxy analogue of threonine ($H_2O_2$-$OsO_4$ preparation)[3] was supplied as the DL-mixture so that twice the level of analogue was supplied in diet 3 (1.02% of the diet) as the amount of L-threonine in diet 1 (0.51% of the diet). The threonine analogue was dissolved in distilled water (1 part analogue: 3 parts distilled water) for mixing into the diets because of the non-crystalline, syrupy nature of the analogue. After mixing the diet, it was spread in a thin layer and allowed to dry at room temperature. The α-keto analogue of tryptophan was added to diet 4 at the same level as the amount of L-tryptophan supplied in diet 1 (0.10% of the diet). In experiment 2, one-half of the rats on each treatment were equipped with tail cups to prevent coprophagy (12). At the end of each experiment all rats were killed by ether anesthesia, the livers and kidneys were removed, blotted free of blood and weighed.

*Experiment 3.* The α-hydroxy analogue of isoleucine (containing all 4 isomers) and the α-hydroxy analogue of threonine were tested. Two preparations of the latter compound were used, one derived by use of $H_2O_2$-$OsO_4$, the other by $KMnO_4$. They were incorporated into the diets as in experiments 1 and 2. The analogues replaced, by weight, the corresponding amino acid in the reference diet. The level of D-allo, L-isoleucine (hereafter referred to as DL-isoleucine) was 3.00% of the reference diet and 2.50, 2.00 and 1.00% of the remaining diets in the isoleucine series in addition to a diet devoid of isoleucine. The level of DL-threonine was 1.50% of the reference diet and 1.25, 1.00 and 0.50% of the remaining diets in

[2] All amino acid analogues were provided by and prepared in the laboratories of Esso Research and Engineering Company, Summit, New Jersey. The authors gratefully acknowledge Dr. V. L. Hughes and Dr. L. P. Reiff, Esso Research and Engineering Company, Chemicals Research Division, Linden, New Jersey, for providing the compounds and the following description of their preparation and purification.

The α-hydroxy analogue of isoleucine (α-hydroxy-β-methyl valeric acid) was a mixture of the 4 possible isomers prepared by the addition of HCN to 2-methyl butyraldehyde to give a cyanohydrin, which was subsequently hydrolyzed to the analogue. The analogue was isolated by vacuum distillation and purified by recrystallization from petroleum ether. The exact proportions of the 4 isomers were not determined. The observed melting point was 51 to 52° (literature, mp 51 to 52°); neutralization equivalent (N.E.) 132.1; C, 54.39%; H, 9.45% (calculated N.E., 132.2; C, 54.53%; H, 9.16%). The α-hydroxy analogue of lysine (DL-α-hydroxy-Σ-amino caproic acid) was prepared from caprolactam via 6-benzoyl-amino caproic acid. The final product was recrystallized from ethanol. The observed melting point was 223 to 225° (literature, mp 220°); C, 48.72%; H, 8.74%; N. 9.35% (calculated C, 48.94%; H, 8.90%; N, 9.52%). The α-keto analogue of tryptophan (3-indolepyruvic acid) was prepared by condensing indole-3-aldehyde and hydantoin to form indolal-hydantoin and subsequently hydrolyzing this product to form the analogue. It was recrystallized from acetone/acetic acid. The observed melting point was 220 to 223° (literature, mp 190 to 210°); C, 64.95%; H, 4.30%; N, 7.00% (calculated C, 65.01%; H, 4.46%; N, 6.88%). To prepare the α-hydroxy analogue of tryptophan (DL-3-indole lactic acid), 3-indolepyruvic acid obtained by the preceding procedure was reduced. The observed melting point was 145 to 147° (literature, mp 146–147°); C, 64.48%; H, 5.50%; N. 6.69% (calculated C, 64.37%; H, 5.40%; N, 6.82%). The α-hydroxy analogue of threonine was prepared by *cis*-hydroxylation of crotonic acid with $H_2O_2$-$OsO_4$ in one preparation and $KMnO_4$ in the other. It was assumed that the D- and L-isomers were present in exactly equal proportions in each preparation, but no quantitative data are available. Both preparations resisted attempts to induce crystallization. Following repeated extractions with benzene to remove osmium a negative color test for osmium was obtained. The observed neutralization equivalent was 122.

[3] See footnote 2.

TABLE 1

*Composition of basal diets (exps. 1–5)*

| | Reference diet 1 | Reference diet 2 | Casein + methionine diets | |
|---|---|---|---|---|
| | Experiments 1 and 2 | Experiments 3–5 | Experiments 1 and 2 | Experiments 3–5 |
| | *g* | *g* | *g* | *g* |
| Dextrin | 43.61 | — | — | — |
| Sucrose | — | 54.25 | 75.62 | 65.52 |
| Glucose | 14.74 | — | — | — |
| Amino acids [1] | 18.87 | 23.85 | — | — |
| Casein [2] | — | — | 14.00 | 14.00 |
| Cellulose [3] | 2.00 | — | — | — |
| Hydrogenated vegetable oil [4] | — | 14.00 | 5.00 | 14.00 |
| DL-Methionine | — | — | 0.18 | 0.18 |
| Vitamin mixture [5] | 2.20 | 2.20 | 2.20 | 2.20 |
| Salt mixture [6] | 4.00 | 4.00 | 3.00 | 4.00 |
| Antibiotic supplement [7] | — | 0.10 | — | 0.10 |
| Sodium bicarbonate | 0.58 | 1.60 | — | — |
| Total [8] | 100.00 | 100.00 | 100.00 | 100.00 |

[1] For composition, see table 2.
[2] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland.
[3] Solka Floc, Brown Company, Berlin, New Hampshire.
[4] Crisco, Procter and Gamble, Cincinnati.
[5] Vitamin mixture contained: (mg/100 g of diet) vitamin A conc (200,000 units/g), 9.9; vitamin D conc (400,000 units/g), 0.55; α-tocopherol, 11.0; ascorbic acid, 99.0; inositol, 11.0; choline chloride, 165.0; menadione, 4.95; *p*-aminobenzoic acid, 11.0; niacin, 9.9; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; biotin, 0.044; folic acid, 0.198; vitamin $B_{12}$, 0.003 (Vitamin Diet Fortification Mixture in Dextrose, obtained from Nutritional Biochemicals Corporation, Cleveland).
[6] Jones, J. H., and C. Foster, J. Nutrition, *24:* 245, 1942, except casein plus methionine diet, Experiments 1 and 2, Warner, R. G., The Laboratory Rat. National Research Council, Committee on Animal Nutrition, pub. 990. National Academy of Sciences — National Research Council, Washington, D. C., 1962.
[7] Chlortetracycline (Aureomycin, American Cyanamid Company, Princeton, New Jersey) in glucose to supply 100 mg of antibiotic/kg of diet.
[8] All diets contained 0.0125% ethoxyquin (Santoquin, Monsanto Company, St. Louis).

the threonine series, in addition to a diet devoid of threonine.

*Experiment 4.* The DL-α-hydroxy and α-keto analogues of tryptophan were tested. The analogues replaced by weight the tryptophan in the reference diet. The levels of DL-tryptophan in the other diets were 0.40, 0.30, 0.20 and 0.10% and zero.

*Experiment 5.* The DL-α-hydroxy analogue of lysine was tested. The analogue replaced by weight the L-lysine·HCl in the reference diet (1.85%). The levels of L-lysine·HCl in the other diets were 1.40, 1.00, 0.60, and 0.20% and zero.

## RESULTS AND DISCUSSION

*Experiment 1.* The results are summarized in table 3. The performance of rats fed casein plus methionine (diet 5) was superior to that with all other treatments in all criteria. This observation is in agreement with other reports (13–15). The rate of gain of rats consumnig the complete amino acid mixture (reference diet 1) was greater than that of rats fed the tryptophan analogue, but not significantly different from that of rats fed the isoleucine analogue. The response to the tryptophan analogue was less than that which might have been expected in view of the earlier reports in which acid hydrolyzed casein supplied the amino acids.

The analogue of threonine failed to support growth. Rats consuming this diet (diet 3) lost weight steadily throughout the experimental period (4 rats died, one on day 16, 3 on day 20). The lack of utilization of the threonine analogue for growth was accompanied by enlarged kidneys compared with those of rats fed other diets. Whereas adrenal weights were not taken, gross inspection indicated that they were considerably larger among rats fed the threonine analogue than in other groups. The apparent hypertrophy of these glands indicates manifestation of stress associated with metabolism of the analogue or the presence of toxic impurities, or both.

TABLE 2

*Composition of basal amino acid mixture (exps. 1–5)* [1]

| Amino acid [1] | Reference diet 1 [2] | Reference diet 2 [3] |
|---|---|---|
| | Experiments 1 and 2 | Experiments 3–5 |
| | *g* | *g* |
| DL-Alanine | — | 0.60 |
| L-Arginine·HCl | 0.93 | 0.80 |
| L-Aspartic acid | — | 0.60 |
| L-Asparagine·$H_2O$ | — | 0.60 |
| L-Cystine | 0.20 | 0.30 |
| L-Glutamic acid | 12.39 | 4.00 |
| Glycine | — | 0.40 |
| L-Histidine·HCl·$H_2O$ | 0.38 | — |
| L-Histidine·HCl | — | 1.00 |
| L-Iosleucine (allo-free) | 0.46 | — |
| DL-Isoleucine | — | 3.00 |
| L-Leucine | 0.85 | 2.00 |
| L-Lysine·HCl | 1.25 | 1.85 |
| L-Methionine | 0.22 | — |
| DL-Methionine | — | 0.80 |
| L-Phenylalanine | 0.48 | — |
| DL-Phenylalanine | — | 1.30 |
| L-Proline | — | 0.50 |
| DL-Serine | — | 0.50 |
| L-Threonine | 0.51 | — |
| DL-Threonine | — | 1.50 |
| L-Tryptophan | 0.10 | — |
| DL-Tryptophan | — | 0.50 |
| L-Tyrosine | 0.38 | 0.80 |
| L-Valine | 0.72 | — |
| DL-Valine | — | 2.80 |
| Total | 18.87 | 23.85 |

[1] When levels of an individual amino acid were changed, the amount of glutamic acid was altered to keep all diets isonitrogenous. All diet changes were made at the expense of sucrose. All amino acids were obtained from General Biochemicals Inc., Chagrin Falls, Ohio.
[2] Diet of Rechcigl et al. (10).
[3] Diet G of Sauberlich (11).

*Experiment* 2. The results are summarized in table 3. The similarity in performance to that obtained in experiment 1 is striking. The isoleucine analogue again promoted performance similar to that obtained with the complete amino acid mixture (average daily gains of 2.42 vs. 1.89 g were not significantly different). Although the tryptophan analogue again promoted small weight gains, it was inferior to the above diets in both average daily gain and gain-to-feed ratio. Steady weight loss was again observed among those fed the threonine analogue. Four rats died (one on day 17, one on day 18 and two on day 20). In addition, hemoglobinuria was noted in 4 rats on this diet beginning midway through the experiment.

A comparison of performance of rats equipped with tail cups to prevent coprophagy with that of rats allowed to practice coprophagy indicates that this phenomenon did not contribute directly to the growth of rats fed amino acid analogues, in contrast with the response obtained with certain vitamins (16). There were no statistically significant differences due to the use of tail cups in any of the criteria.

*Experiment* 3. The results are summarized in table 4. The α-hydroxy analogue of isoleucine supported growth, although inferior to that obtained with isoleucine. A level of 1.00% DL-isoleucine was superior to 3.00% of the analogue in promoting growth, feed consumption and feed utilization. However, because the analogue preparation contained all 4 isomers and the isoleucine contained 50% L- and 50% D-allo isomers, the potentially metabolically active level of analogue was probably less than half that of the isoleucine used.

Performance of rats fed 2.0% DL-isoleucine was equal to that of rats fed 2.5 or 3.0% DL-isoleucine, indicating that the level of isoleucine in the reference diet was approximately 50% higher than necessary for maximal performance. Rats fed the isoleucine-free diet lost weight continuously and three of five had died by day 20. No specific deficiency symptoms were noted except emaciation and rough hair coats.

When either of the threonine analogues replaced DL-threonine there was a steady weight loss throughout the experiment with death beginning on day 18. Six of the 10 rats fed the analogue prepared with $KMnO_4$ and two of the ten fed the analogue prepared with $H_2O_2$-$OsO_4$ had died by the end of 21 days. Rats fed the threonine-free diet lost weight steadily throughout the experiment and were similar in general appearance to those fed the analogue with no specific deficiency symptoms except emaciation and rough hair coats.

Performance of rats fed 1.25% DL-threonine was equal to that of rats fed 1.50% DL-threonine, demonstrating that the level of threonine in the reference diet was approximately 20% higher than necessary for maximal performance.

TABLE 3

*Effect of α-hydroxy analogues of isoleucine and threonine and α-keto analogue of tryptophan on performance of weanling rats (exps. 1 and 2)*

| | Amino acids,[1] diet no. | | | | | | | | Casein + methionine,[1] diet no. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | | 5 | |
| | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| L-Isoleucine (allo-free), % | 0.46 | 0.46 | — | — | 0.46 | 0.46 | 0.46 | 0.46 | — | — |
| α-Hydroxy analogue of isoleucine (4 isomers), % | 0 | 0 | 1.84 | 1.84 | 0 | 0 | 0 | 0 | — | — |
| L-Threonine, % | 0.51 | 0.51 | 0.51 | 0.51 | 0 | 0 | 0.51 | 0.51 | — | — |
| α-Hydroxy analogue of threonine (2 isomers), % | 0 | 0 | 0 | 0 | 1.02 | 1.02 | 0 | 0 | — | — |
| L-Tryptophan, % | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | — | — | — | — |
| α-Keto analogue of tryptophan, % | 0 | 0 | 0 | 0 | 0 | 0 | 0.10 | 0.10 | — | — |
| No. of rats [2,3] | 9 | 10 | 9 | 10 | 9 | 10 | 9 | 10 | 9 | 10 |
| Daily gain/rat, g | 2.43 | 2.42 | 1.81 | 1.89 | −1.13 | −0.87 | 0.42 | 0.36 | 6.28 | 4.33 |
| Daily feed/rat, g | 9.90 | 8.92 | 8.96 | 7.78 | 4.17 | 3.57 | 6.47 | 5.77 | 14.20 | 10.90 |
| Gain/g feed, g | 0.24 | 0.27 | 0.20 | 0.24 | — | — | 9.06 | 0.04 | 0.44 | 0.39 |
| Liver wt/rat, % of live wt | 5.43 | 4.76 | 5.36 | 4.81 | 6.01 | 5.04 | 6.55 | 4.71 | 7.07 | 5.72 |
| Kidney wt/rat, % of live wt | 0.96 | 0.94 | 1.06 | 1.06 | 2.08 | 1.54 | 1.17 | 1.19 | 1.01 | 0.90 |

[1] For composition, see table 1. Amino acid reference diet (diet 1) is that of Rechcigl et al. (10).

[2] Average initial weight, 56 g in experiment 1 and 59 g in experiment 2. Each experiment 21 days.

[3] In experiment 2, one-half of the rats in each group (5/group) were equipped with tail cups to prevent coprophagy. Since there was no effect of coprophagy prevention on any of the criteria the data for all 10 rats/group were combined.

TABLE 4

*Effect of α-hydroxy analogue of isoleucine and α-hydroxy analogue of threonine and of level of isoleucine and threonine on performance of weanling rats (exp. 3)*

| | Amino acids,[1] diet no. | | | | | | | | | | | | Casein + methionine,[1] diet no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| DL-Isoleucine, % | 3.00 | 2.50 | 2.00 | 1.00 | 0 | 0 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | — |
| α-OH analogue of isoleucine, % | 0 | 0 | 0 | 0 | 0 | 3.00 | 0 | 0 | 0 | 0 | 0 | 0 | — |
| DL-Threonine, % | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 1.25 | 1.00 | 0.50 | 0 | 0 | 0 | — |
| α-OH analogue of threonine, % [2] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.50 | 0 | — |
| α-OH analogue of threonine, % [3] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.50 | — |
| No. of rats [4] | 10 | 10 | 10 | 10 | 5 [5] | 6 | 10 | 10 | 10 | 5 | 10 [6] | 10 [7] | 10 |
| Daily gain/rat, g | 5.17 | 5.29 | 5.26 | 3.51 | −1.07 | 2.70 | 5.20 | 4.49 | 1.00 | −0.90 | −0.77 | −0.73 | 5.76 |
| Daily feed/rat, g | 11.68 | 11.94 | 12.21 | 9.91 | 3.10 | 8.15 | 11.54 | 11.33 | 6.31 | 3.25 | 3.49 | 3.35 | 12.42 |
| Gain/g feed, g | 0.44 | 0.45 | 0.43 | 0.36 | — | 0.33 | 0.45 | 0.40 | 0.16 | — | — | — | 0.47 |

[1] For composition, see table 1. Amino acid reference diet (diet 1) is that of Sauberlich (11).
[2] Prepared with $KMnO_4$.
[3] Prepared with $H_2O_2$–$OsO_4$.
[4] Twenty-one-day experimental period, average initial weight, 51 g.
[5] Three rats died, days 18, 20 and 20 (performance includes rats that died).
[6] Six rats died, days 18, 18, 19, 19, 19, 20 (performance includes rats that died).
[7] Two rats died, day 19 (performance includes rats that died).

*Experiment 4.* The results are summarized in table 5. The α-hydroxy analogue of tryptophan promoted performance equal to that obtained with tryptophan, whereas the α-keto analogue was approximately 80% as effective. The 0.40% level of DL-tryptophan was approximately as effective as 0.50% in all criteria, indicating that the level present in the reference diet may have been appreciably greater than necessary for maximal performance. Lower levels (0.30, 0.20 and 0.10%) tended to be inferior. Interpolation based on the performance of rats fed the α-keto analogue as compared with that of rats fed the graded levels of tryptophan indicates that 0.50% of the analogue is equal in growth-promoting ability to 0.15 to 0.20% DL-tryptophan in the amino acid diet used. The usefulness of these analogues as supplements to intact protein diets low in tryptophan should be studied.

Rats fed the tryptophan-free diet lost weight steadily and exhibited nervous disorders within 14 to 16 days, including hyperexcitability, jerky aimless movements and convulsions followed by complete exhaustion. Other symptoms included emaciation, porphyrin exudate around the mouth and nose and death in 4 to 6 weeks. The former symptoms are unlike those observed during deprivation of other amino acids and undoubtedly represent some specific metabolic disorder. Brain levels of serotonin 10 times normal in rabbits administered 5-hydoxy tryptophan were associated with marked central nervous disturbance (17), similar to the symptoms observed here, but a more plausible occurrence would be a metabolic deficiency of serotonin in rats fed a diet devoid of tryptophan. Brain serotonin levels of rats fed acid-hydrolyzed casein have been shown to be drastically reduced (18).

*Experiment 5.* The results are summarized in table 6. The α-hydroxy-analogue of lysine failed to support growth. This is in agreement with the results of McGinty et al. (2) with rats fed gliadin as the basal protein. Weight losses of the rats fed the analogue and those fed the lysine-free diet were similar. No specific deficiency symptoms were noted except emaciation and rough hair coats.

Performance of rats fed 1.00% L-lysine·HCl was equal to that of rats fed 1.40 or 1.85%, suggesting that the level of lysine in the reference mixture was considerably higher than necessary for maximal performance. Rats fed 0.20 and 0.60% L-lysine·HCl were inferior in performance, but since no other increments were included the minimal level of lysine compatible with maximal performance cannot be stated.

Statistical analyses of the data from all 5 experiments were performed according to the methods of Snedecor (19) and the analyses of variance are presented in tables 7 and 8. Highly significant differences ($P < 0.01$) among treatments were found in all experiments for all criteria.

TABLE 5

*Effect of α-hydroxy and α-keto analogues of tryptophan and of level of tryptophan on performance of weanling rats (exp. 4)*

| | Amino acids,[1] diet no. | | | | | | | | Casein + methionine,[1] diet no. |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| DL-Tryptophan, % | 0.50 | 0.40 | 0.30 | 0.20 | 0.10 | 0 | 0 | 0 | — |
| α-OH analogue of tryptophan, % | 0 | 0 | 0 | 0 | 0 | 0 | 0.50 | 0 | — |
| α-Keto analogue of tryptophan, % | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.50 | — |
| No. of rats [2] | 9 | 10 | 10 | 8 | 9 | 7 | 10 | 9 | 10 |
| Daily gain/rat, g | 4.05 | 3.99 | 3.52 | 3.55 | 2.11 | −0.57 | 4.83 | 3.19 | 5.32 |
| Daily feed/rat, g | 10.79 | 10.55 | 9.80 | 9.78 | 8.38 | 4.39 | 11.65 | 9.38 | 12.29 |
| Gain/g feed, g | 0.37 | 0.37 | 0.35 | 0.36 | 0.25 | — | 0.41 | 0.34 | 0.43 |

[1] Diet composition the same as in experiment 3 (see table 1).
[2] Twenty-one-day experimental period, average initial weight, 53 g, 10 rats/group initially, rats that died in treatments 1, 4, 5 and 8 were unthrifty and had respiratory infection, believed not to be associated with diet. The 3 rats that died in treatment 6 showed extreme emaciation along with muscular incoordination and nervousness believed to be a manifestation of tryptophan deficiency.

TABLE 6

*Effect of α-hydroxy analogue of lysine and of level of lysine on performance of weanling rats (exp. 5)*

| | Amino acids,[1] diet no. | | | | | | | Casein + methionine,[1] diet no. |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| L-Lysine·HCl, % | 1.85 | 1.40 | 1.00 | 0.60 | 0.20 | 0 | 0 | — |
| α-OH analogue of lysine, % | 0 | 0 | 0 | 0 | 0 | 0 | 1.85 | — |
| No. of rats [2] | 10 | 10 | 10 | 10 | 10 | 5 | 10 | 10 |
| Daily gain/rat, g | 5.87 | 5.78 | 5.88 | 4.10 | 0.64 | −0.46 | −0.51 | 6.24 |
| Daily feed/rat, g | 13.01 | 13.17 | 13.05 | 11.25 | 6.68 | 4.17 | 4.40 | 13.64 |
| Gain/g feed, g | 0.45 | 0.44 | 0.45 | 0.36 | 0.10 | — | — | 0.46 |

[1] Diet composition the same as in experiment 3 (see table 1).
[2] Twenty-one-day experimental period, average initial weight, 50 g.

TABLE 7

*Analyses of variance of performance and gland weight data (exps. 1 and 2)*

| Source of variation | Daily gain | | Daily feed | | Gain/feed | | Kidney wt | | Liver wt | |
|---|---|---|---|---|---|---|---|---|---|---|
| | df | Mean square | df | Mean square | df | Mean square | df | Mean square | df | Mean square |
| | | | | Experiment 1 | | | | | | |
| Treatment [1] | 3 | 56.68** | 4 | 128.99** | 3 | 0.225** | 4 | 1.81** | 4 | 4.85** |
| Outcome group | 8 | 0.26 | 8 | 2.97 | 8 | 0.004 | 8 | 0.02 | 8 | 0.56 |
| Residual | 24 | 0.19 | 32 | 1.27 | 24 | 0.001 | 33 | 0.02 | 32 | 0.69 |
| | | | | Experiment 2 | | | | | | |
| Treatment [1] | 3 | 26.87** | 4 | 80.01** | 3 | 0.210** | 4 | 0.66** | 4 | 0.17** |
| Outcome group | 9 | 0.44 | 9 | 0.97 | 9 | 0.002 | 9 | 0.02 | 9 | 0.04 |
| Residual | 27 | 0.46 | 36 | 1.03 | 27 | 0.002 | 36 | 0.03 | 34 [2] | 0.03 |
| | Amino acid diet (reference diet) vs. casein + methionine diet | | | | | | | | | |
| | | | | Experiments 1 and 2 combined | | | | | | |
| Treatment | 1 | 76.25** | 1 | 90.24** | 1 | 0.243** | 1 | 15.73** | 1 | 0.001 |
| Error | 36 | 0.95 | 36 | 2.95 | 36 | 0.002 | 36 | 0.52 | 36 | 0.006 |

[1] Does not include treatments that did not promote body weight gains, except in the case of the average daily feed, kidney weight and liver weight criteria.
[2] Data lost on 2 rats.
** Significant differences ($P < 0.01$).

Inspection of the residual mean squares reveals rather small standard errors throughout.

The superiority of casein over amino acids using either the amino acid diet of Rechcigl et al. (10) or of Sauberlich (11) in promoting growth and feed consumption in all 5 experiments indicates a remaining gap in our knowledge of amino acid and protein nutrition. Recent work (11, 20) indicates that performance equal to that obtained with intact protein can be achieved if amino acids are properly balanced. The factors involved in this work that are responsible for the inequity in response to the amino acid versus the casein diet are under study in this laboratory.

LITERATURE CITED

1. Jackson, R. W., and J. P. Chandler 1939 Metabolism of proteins and amino acids. Ann. Rev. Biochem., *8:* 249.
2. McGinty, D. A., H. B. Lewis and C. S. Marvel 1924–25 Amino acid synthesis in the animal organism. The availability of some caproic acid derivatives for the synthesis of lysine. J. Biol. Chem., 62*:* 75.
3. Cahill, W. M., and G. G. Rudolph 1942 The replaceability of *dl*-methionine in the diet of the rat with its α-keto acid analog. J. Biol. Chem., *145:* 201.
4. Wood, J. L., S. L. Cooley and J. M. Kelley 1950 The non-utilization of acetyldehydroamino acids for growth by the rat. J. Biol. Chem., *186:* 641.
5. Bubl, E. C., and J. S. Butts 1949 The utilization of some phenylpyruvic acids for growth in the rat. J. Biol. Chem., *180:* 839.
6. Armstrong, M. D., and J. D. Lewis 1950 Rat growth experiments with some deriva-

TABLE 8

*Analyses of variance of performance data (exps. 3–5)*

| Source of variation | Daily gain | | Daily feed | | Gain/feed | |
|---|---|---|---|---|---|---|
| | df | Mean square | df | Mean square | df | Mean square |
| Experiment 3, α-OH analogues of isoleucine and threonine ($H_2O_2$-$OsO_4$ and $KMnO_4$) | | | | | | |
| Treatment [1] | 8 | 23.3453** | 12 | 128.3536** | 8 | 0.0941** |
| Outcome group | 9 | 0.2530 | 9 | 2.2341** | 9 | 0.0023** |
| Residual | 68 | 0.1370 | 94 | 0.3525 | 68 | 0.0005 |
| Experiment 4, α-OH and α-keto analogue of tryptophan | | | | | | |
| Treatment [1] | 8 | 8.0615** | 8 | 25.1914** | 7 | 0.0283** |
| Outcome group | 9 | 1.0290 | 9 | 3.8099 | 9 | 0.0054** |
| Residual | 57 | 0.7687 | 64 | 4.1514 | 58 | 0.0019 |
| Experiment 5, α-OH analogue of lysine | | | | | | |
| Treatment [1] | 5 | 46.2847** | 7 | 145.5860** | 5 | 0.2033** |
| Outcome group | 9 | 0.1502 | 9 | 1.1423* | 9 | 0.0003 |
| Residual | 45 | 0.1526 | 58 | 0.5285 | 45 | 0.0003 |
| Amino acid diet vs. casein diet (exps. 3–5 combined) | | | | | | |
| Treatment | 1 | 9.4069** | 1 | 15.1501** | 1 | 0.0149** |
| Residual | 77 | 0.5127 | 77 | 1.2472 | 77 | 0.0012 |

[1] Does not include treatments that did not promote body weight gains, except in the case of the average daily feed criterion.
* Significant differences among treatments ($P < 0.05$).
** Significant differences among treatments ($P < 0.01$).

tives of phenylalanine. J. Biol. Chem., *186:* 849.

7. Meister, A., and J. White 1951 Growth response of the rat to the keto analogs of leucine and isoleucine. J. Biol. Chem., *191:* 211.
8. Winitz, M., J. P. Greenstein and S. M. Birnbaum 1957 Quantitative nutritional studies with water-soluble chemically defined diets. V. Role of the isomeric arginines in growth. Arch. Biochem. Biophys., *72:* 448.
9. Wood, J. L., and S. L. Cooley 1954 Substitution of α-keto acids for five amino acids essential for growth of the rat. Proc. Soc. Exp. Biol. Med., *85:* 409.
10. Rechcigl, M., Jr., J. K. Loosli and H. H. Williams 1957 The net utilization of nonspecific nitrogen sources for the synthesis of non-essential amino acids. I. Growth and nitrogen utilization. J. Nutrition, *63:* 177.
11. Sauberlich, H. E. 1961 Growth of rats fed protein-free diets supplemented with purified amino acid mixtures. J. Nutrition, *74:* 298.
12. Barnes, R. H., G. Fiala, B. McGehee and A. Brown 1957 Prevention of coprophagy in the rat. J. Nutrition, *63:* 489.
13. Rose, W. C., and M. Womack 1946 The utilization of the optical isomers of phenylalanine, and the phenylalanine requirement for growth. J. Biol. Chem., *166:* 103.
14. Howe, E. E., E. W. Gilfillin and J. B. Allison 1960 Efficacy of the FAO amino acid reference standard for growth of the weanling rat. J. Nutrition, *70:* 385.
15. Rama Rao, P. B., V. C. Metta and B. C. Johnson 1960 The amino acid composition and the nutritive value of proteins. II. Amino acid mixtures as a dietary source of nitrogen for growth. J. Nutrition, *71:* 327.
16. Barnes, R. H. 1962 Nutritional implications of coprophagy. Nutrition Rev., *20:* 289.
17. Udenfriend, S., H. Weisbach and D. F. Bognanski 1957 Increase in tissue serotonin following administration of its precursor 5-hydroxy tryptophan. J. Biol. Chem., *224:* 803.
18. Culley, W. J., R. N. Saunders, E. T. Mertz and D. H. Jolly 1963 Effect of a tryptophan deficient diet on brain serotonin and plasma tryptophan level. Proc. Soc. Exp. Biol. Med., *113:* 645.
19. Snedecor, G. W. 1956 Statistical Methods. Iowa State College Press, Ames.
20. Bosshardt, D. K., E. Brand and R. H. Barnes 1961 Nutritional equivalence of β-lactoglobulin and its corresponding amino acids. Proc. Soc. Exp. Biol. Med., *107:* 979.

# Effect of Diet and Digestive Processes on Proteolytic Enzymes

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*ABSTRACT* The effect of digestive processes on proteolytic enzymes was determined by comparing the properties of trypsin and chymotrypsin in pancreatic extracts and in intestinal contents of rats fed protein-free or 15% casein diets. Results of studies involving acidification, dialysis, and chromatography of the enzyme solutions demonstrated that the structural properties of trypsin and chymotrypsin were altered in the small intestine. The presence of dietary protein in upper intestinal contents protected the enzymes; hence, changes in their properties must have been a result of proteolysis of the enzyme molecules concurrent with a retention of activity during the early stages of their digestion. The protective effect of dietary protein also demonstrated that exogenous protein was the preferred substrate, and thus was digested more rapidly than endogenous protein.

Approximately 90% of the protein secreted into the gastrointestinal tract during digestion is broken down and reabsorbed (1). Experimental evidence indicates that digestive enzymes are inactivated and hydrolyzed along with other endogenous proteins. Borgström et al. (2) observed in humans that the concentration of pancreatic enzymes decreased slowly in a distal direction despite a progressive increase in concentration in the same direction of intestinal contents. Wohlman et al. (3) noted that 30-minute incubation in aqueous solution (pH 7.5) resulted in losses of 21% for chymotrypsin and 31% for trypsin. Pelot and Grossman (4) observed in rats that when pancreatic juice was introduced into the small intestine by tube, only 66 to 74% of the trypsin, 18 to 44% of the chymotrypsin and 8 to 18% of the lipase could be recovered after 10 minutes. The inactivation phenomenon must result in part from proteolytic breakdown of the enzymes because the presence of dietary protein in the small intestine retards inactivation (5).

The influence of digestive processes on proteolytic enzymes was determined by comparing the properties of trypsin and chymotrypsin in pancreatic extracts and in intestinal contents of rats fed casein and protein-free diets.

## EXPERIMENTAL

*Fractionation of pancreatic extracts.* Partially purified rat pancreatic enzymes were used to develop the fractionation techniques used in studies of trypsin and chymotrypsin. Rat pancreatic glands were homogenized and extracted with 0.25 N $H_2SO_4$. The extract was then filtered through cheesecloth and partially purified by ammonium sulfate fractionation. That fraction of the pancreatic filtrate precipitating between 20 and 75% ammonium sulfate saturation was redissolved at pH 8.1 in 0.4 M Tris buffer. The zymogens were activated with an aliquot of rat intestinal contents (containing about 0.2 mg of trypsin) in the presence of 0.05 M $CaCl_2$. Subsequently, the activated enzyme solution was prepared for chromatography by adjusting the pH to 4.1 with 0.1 M HCl. The solution was then centrifuged to remove the light precipitate that formed after acidification. The supernatant solution was dialyzed against a starting buffer of 0.005 M acetate, pH 4.1, and fractionated on a carboxymethyl (CM) cellulose column (1.2 × 22 cm). Both stepwise and gradient elution techniques were utilized for chromatography. Elution was accomplished by passing 0.2 followed by 0.3 M NaCl solutions containing 0.1 M acetate buffer, pH 4.1, through a 250-ml mixing chamber originally containing only the buffer solution. When the total molarity of the eluent reached 0.35, three stepwise elutions were carried out with 0.4 M NaCl and 0.1 M

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buffer (acetate or borate) at pH 5.5, 7.0, and 9.0, respectively. All steps were carried out at 4°C.

In later experiments with intestinal contents the acid-precipitate was found to be of importance. Therefore, it was characterized in pancreatic studies by redissolving at pH 7.5, dialyzing against a starting buffer of 0.005 M phosphate, pH 7.5, and fractionating on diethylaminoethyl (DEAE) cellulose columns of the dimensions 1.2 × 25 cm. Gradient elution was accomplished by passing 0.02 M phosphate buffer, pH 7.5, and 0.3 M NaCl through a mixing chamber containing starting buffer. Protein adhering to the column after this treatment was eluted with 0.02 M NaOH.

*Treatment of intestinal contents.* Intestinal contents of male Sprague-Dawley rats, fed protein-free or 15% casein diets,[2] were then fractionated and studied. The rats were trained to eat during one-hour intervals spaced 12 hours apart. After 5 days of experimental feeding, groups of 6 rats from each ration were killed at various times during the digestive period. The contents of the small intestines from each group of 6 were pooled and centrifuged to remove cellular debris. The fractionation of intestinal contents proceeded as described above for pancreatic extracts beginning with the step involving acidification to pH 4.1. The earlier steps were omitted because the enzymes were already active. Pooled contents from the upper two-thirds and the lower one-third of the small intestines were fractionated and assayed before and after acidification to determine whether the enzymes' properties changed in the lower part of the intestine. The samples were recombined before chromatography so that sufficient enzyme activity could be introduced on the ion-exchange columns.

*Enzyme assays.* Spectrophotometric assays for trypsin and chymotrypsin using N-benzoyl-L-arginine ethyl ester (BAEE) and N-acetyl-L-arginine ethyl ester (ATEE), respectively, as substrates were described in a previous paper (5). The specific activity of trypsin or chymotrypsin was calculated as micromoles of substrate hydrolyzed per minute per milligram of enzyme protein. Total proteolytic activity was determined by measuring the solubility of casein in 5% trichloroacetic acid after incubation for 20 minutes with enzyme. The specific proteolytic activity represents the milligrams of casein digested in 20 minutes per milligram of protein.

Miller's modification (6) of the Lowry method was used to measure protein.

## RESULTS

*Acidification studies.* The behavior of trypsin and chymotrypsin during the acidification procedure is shown in table 1. The results shown for the intestinal contents are averages of all values (%) obtained for the various enzyme fractions at all time intervals studied during the digestive period.

In contrast with pancreatic extracts, acidification of intestinal contents resulted in the precipitation of large amounts of material.

Total proteolytic and tryptic activities remained constant in solutions of pancreatic extracts acidified to pH 4.1, whereas chymotryptic activity decreased 13%. Only 10% of the chymotryptic activity was irreversibly inactivated. Acidification of intestinal contents resulted in large losses of all 3 enzyme activities. As much as 92.5% of the chymotryptic activity and 78.7% of the tryptic activity were lost from the contents of rats fed a protein-free diet. Chymotrypsin was less stable than trypsin in solution and appeared to be less stable in intestinal contents. Up to 33.1% of the chymotrypsin and 54.2% of the trypsin were recovered when the acid-insoluble enzymes were redissolved at pH 7.5, but large proportions apparently were irreversibly denatured. Recoveries of enzymes in the acid-soluble fraction of the upper intestinal contents increased 25 to 35% when casein was fed to the rats. The increase in acid-soluble proteolytic activity was significant at the 5% level, whereas the increase in chymotryptic activity was significant at the 1% level. Little change was observed in values for the contents of the lower small intestine. The extent of acid inactivation was lowered when protein was fed to the rats. The reduction of inactivation of chymotrypsin in upper intestinal contents was highly significant (1% level).

[2] The detailed composition of the diet is presented elsewhere (5).

TABLE 1

*Percentage of enzyme activities affected by acidification of intestinal contents and activated pancreatic extracts*

| Enzyme fraction | Pancreatic extracts | Small intestinal contents | |
|---|---|---|---|
| | | 0% Protein | 15% Casein |
| | % | % | % |
| Acid-soluble | | | |
| Proteolytic | 100 | 19.6 ± 4.7 [1] | 54.5 ± 8.5 |
| | | 12.9 ± 1.9 [2] | 12.6 ± 1.1 |
| Tryptic | 100 | 31.0 ± 2.8 | 56.0 ± 17.7 |
| | | 21.3 ± 3.2 | 31.0 ± 4.0 |
| Chymotryptic | 87 | 7.5 ± 1.4 | 31.2 ± 3.1 |
| | | 15.3 ± 4.8 | 13.6 ± 3.1 |
| Reactivated, acid-insoluble | | | |
| Proteolytic | 6 | 68.3 ± 6.1 | 51.8 ± 7.0 |
| | | 66.7 ± 2.9 | 58.8 ± 5.7 |
| Tryptic | 8 | 54.2 ± 3.7 | 41.0 ± 9.5 |
| | | 47.3 ± 6.2 | 48.1 ± 5.0 |
| Chymotryptic | 3 | 33.1 ± 3.1 | 31.5 ± 4.0 |
| | | 21.6 ± 2.8 | 31.3 ± 2.8 |
| Acid-inactivated | | | |
| Proteolytic | 0 | 13.5 ± 10.5 | 6.3 ± 13.0 |
| | | 20.8 ± 1.5 | 28.5 ± 5.2 |
| Tryptic | 0 | 17.2 ± 9.5 | 11.5 ± 14.2 |
| | | 31.2 ± 4.2 | 20.8 ± 3.6 |
| Chymotryptic | 10 | 59.3 ± 1.9 | 37.3 ± 1.9 |
| | | 64.9 ± 6.2 | 55.1 ± 3.9 |

[1] Upper two-thirds of the small intestine.
[2] Lower one-third of the small intestine.
[3] Recovery in acid-soluble and acid-insoluble fractions averaged 106.3%.

*Dialysis studies.* In previous experiments (5), dialysis of intestinal contents resulted in a loss of enzyme activity that was greater than the loss observed when the contents were allowed to stand, without dialysis, for an equal amount of time. This loss was assumed to be almost entirely due to an increased rate of enzyme inactivation after peptides of dietary origin had been dialyzed away from the intestinal contents. However, the possibility of active enzyme fragments in the dialyzate was not excluded. Hence, to test whether partially digested, yet active, enzymes are small enough to pass through the dialysis membrane, the dialyzate from contents of rats fed a casein diet and killed 2.5 hours after feeding, was assayed for total proteolytic activity. The intestinal contents lost 11 and 31%, respectively, of their activity after standing and after dialysis against 0.005 M phosphate buffer, pH 7.5. The data of table 2 show that weak proteolytic activity was recovered in the dialyzate. Weak tryptic activity was also recovered. Neither activity was typical of semipurified rat trypsin since the rate of reaction was very slow. Less than 5% of the activity lost from intestinal contents during dialysis was recovered in the dialyzate.

TABLE 2

*Proteolytic enzyme activity recovered in the dialyzate after 12 hours of dialysis* [1]

| Length of incubation | Optical [2] density |
|---|---|
| *hours* | |
| 1/3 | 0.017 |
| 1 | 0.017 |
| 2 | 0.050 |
| 4 | 0.084 |
| 7 | 0.118 |

[1] Dialysis of intestinal contents of rats fed 15% casein and killed 2.5 hours after feeding.
[2] One milliliter of dialyzate was incubated with 0.5% casein solution. Increasing solubility in trichloroacetic acid was tested by the Miller (6) method using 1 ml of TCA filtrate as sample.

*Chromatographic studies.* Results of chromatography of pancreatic extracts and intestinal contents are summarized in tables 3 and 4. Representative chromatograms are shown in figures 1–4. The specific enzyme activity reported in the tables for each fraction was that of the most active test tube of effluent collected in that fraction. In the intestinal studies the en-

TABLE 3

*Specific activity [1] of enzymes eluted from carboxymethyl-cellulose columns*

| Fraction | Molarity of effluent | Enzyme | Pancreatic extracts | Small intestinal contents | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Protein-free diet | | | | Casein diet | | | |
| | | | | 1 [2] | 5 | 8 | 12 | 1 | 2.5 | 8 | 12 |
| Sample | 0.005 | P [3] | 2055 | — | 86 | 47 | — | 131 | 88 | 125 | 76 |
| | (pH 4.1) | T | 174 | 19 | 16 | 9 | 8 | 35 | 19 | 27 | 6 |
| | | C | 1615 | 24 | 12 | 6 | 16 | 25 | 15 | 24 | 15 |
| 1 | 0.005 | P | 0 | — | 63 | 13 | 69 | 48 | 50 | 17 | 32 |
| | (pH 4.1) | T | 0 | 21 | 7 | 1 | 17 | 4 | 10 | 1 | 7 |
| | | C | 0 | 39 | 18 | 4 | 37 | 10 | 14 | 2 | 0 |
| 2 | 0.15–0.18 | P | 0 | — | 7 | 9 | 4 | — | 0 | 0 | 20 |
| | (pH 4.1) | T | 0 | — | — | 50 | 68 | — | 53 | 187 | 46 |
| | | C | 0 | — | — | 0 | — | — | 0 | — | — |
| 3 | 0.18–0.25 | P | 1690 | — | — | — | 41 | 367 | 179 | 738 | 355 |
| | (pH 4.1) | T | 0 | — | — | 4 | 0 | 0 | 21 | 50 | 14 |
| | | C | 4280 | — | — | 23 | 11 | 106 | 67 | 282 | 109 |
| 4 | 0.25–0.35 | P | 2490 | — | — | — | 0 | 105 | 46 | 290 | 524 |
| | (pH 4.1) | T | 449 | — | — | 23 | 0 | 12 | 8 | 29 | 20 |
| | | C | 0 | — | — | — | — | — | 0 | — | 0 |
| 5 | 0.40 | P | 578 | — | 183 | — | 16 | 106 | 16 | 100 | 90 |
| | (pH 5.5) | T | 653 | — | 38 | — | 0 | 10 | 10 | 9 | 4 |
| | | C | 0 | — | 323 | — | — | 10 | 0 | — | 6 |
| — | 0.40 | P | 480 | — | — | 48 | 161 | 97 | 170 | 263 | 61 |
| | (pH 7.0) | T | 0 | — | — | 6 | 0 | 0 | 0 | 12 | 0 |
| | | C | 0 | — | — | 0 | — | 0 | 0 | 0 | 0 |
| — | 0.40 | P | 0 | — | 385 | — | 0 | 290 | 0 | 125 | 0 |
| | (pH 9.0) | T | 0 | — | 74 | 0 | — | 0 | 0 | 0 | 0 |
| | | C | 0 | — | 0 | — | 0 | — | 0 | — | 0 |

[1] See experimental section for definition of specific activity.
[2] Hour after the initiation of the feeding period.
[3] The letters P, T, and C stand for proteolytic, tryptic and chymotryptic activities, respectively. The values given above represent 10 × the specific activity of the enzyme.

TABLE 4

*Specific activity [1] of enzymes eluted from diethylaminoethyl-cellulose columns*

| Fraction | Molarity of effluent | Enzyme | Pancreatic extracts | Small intestinal contents | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Protein-free diet | | | | Casein diet | | | |
| | | | | 1 [2] | 5 | 8 | 12 | 1 | 2.5 | 8 | 12 |
| Sample | 0.005 | P [3] | 583 | — | 277 | 318 | — | 280 | 137 | 286 | 166 |
| | | T | 68 | 34 | 58 | 14 | 51 | 25 | 32 | 16 | 4 |
| | | C | 287 | 18 | 32 | 31 | 50 | 74 | 20 | 26 | 17 |
| 6 | 0.005 | P | 0 | — | 54 | 20 | 3 | 46 | 63 | 22 | 89 |
| | | T | 0 | 10 | 2 | 6 | 0 | 6 | 8 | 2 | 1 |
| | | C | 0 | 7 | 12 | 5 | 0 | 19 | 10 | 5 | 13 |
| 7a | 0.005–0.15 | P | 0 | — | 26 | 12 | 9 | 18 | 26 | 31 | 136 |
| | | T | 0 | 2 | 2 | 7 | — | 0 | 2 | 3 | 1 |
| | | C | 0 | — | 0 | 5 | 0 | 0 | 7 | — | 83 |
| 7b | 0.15–0.20 | P | 6 | — | 84 | 95 | 65 | 122 | 182 | 205 | 266 |
| | | T | 0 | — | 7 | 14 | — | 0 | 28 | 6 | 16 |
| | | C | 22 | — | 6 | 11 | — | — | 0 | — | 83 |
| 7c | 0.20–0.28 | P | 0 | — | 141 | 333 | 450 | 220 | 182 | 333 | 709 |
| | | T | 0 | — | 65 | 85 | 77 | 32 | 56 | 27 | 66 |
| | | C | 8 | — | — | 41 | 0 | 0 | 21 | 0 | 17 |
| 8 | 0.02 | P | 2 | — | — | — | 40 | 67 | 75 | — | 77 |
| | (NaOH) | T | 0 | 6 | 29 | — | 0 | 7 | 8 | — | 10 |
| | | C | 2 | — | — | — | 9 | 29 | 0 | — | 35 |
| 9 | 0.02 | P | 0 | — | — | 28 | 43 | 91 | 133 | 27 | 100 |
| | (NaOH) | T | 0 | 0 | 0 | 18 | 0 | 0 | 14 | 3 | 0 |
| | | C | 6 | — | — | 25 | 44 | 117 | 189 | 14 | 29 |

[1] See experimental section for definition of specific activity.
[2] Hour after the initiation of the feeding period.
[3] The letters P, T, and C stand for proteolytic, tryptic and chymotryptic activities, respectively. The values given above represent 10 × the specific activity of the enzyme.

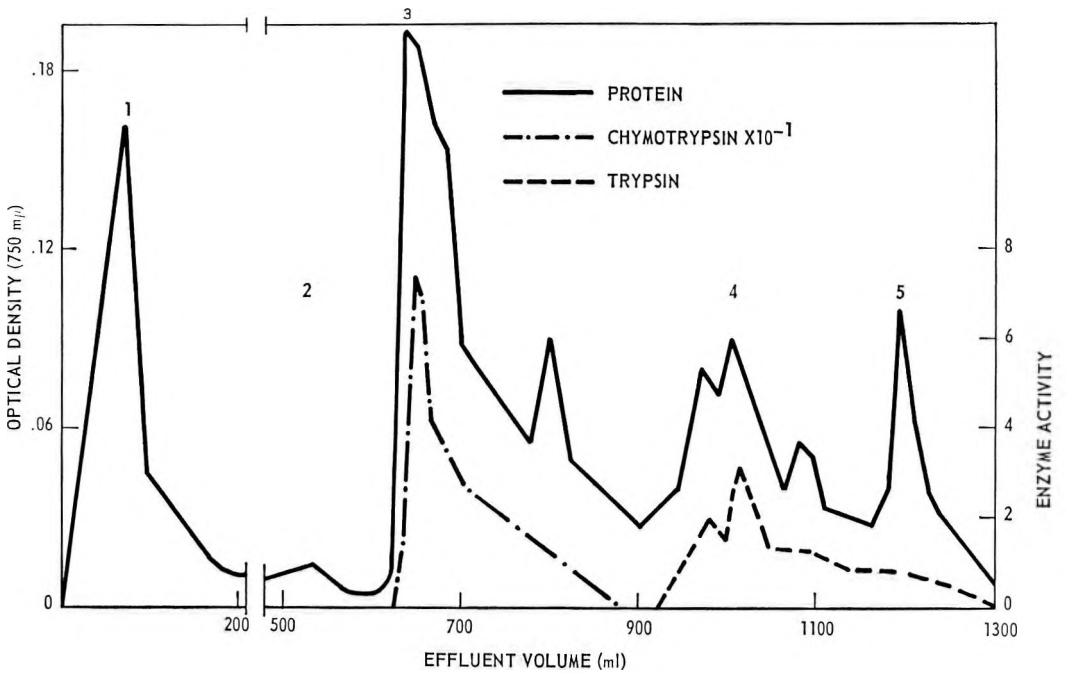


Fig. 1 Chromatography of activated pancreatic extracts on CM-cellulose.

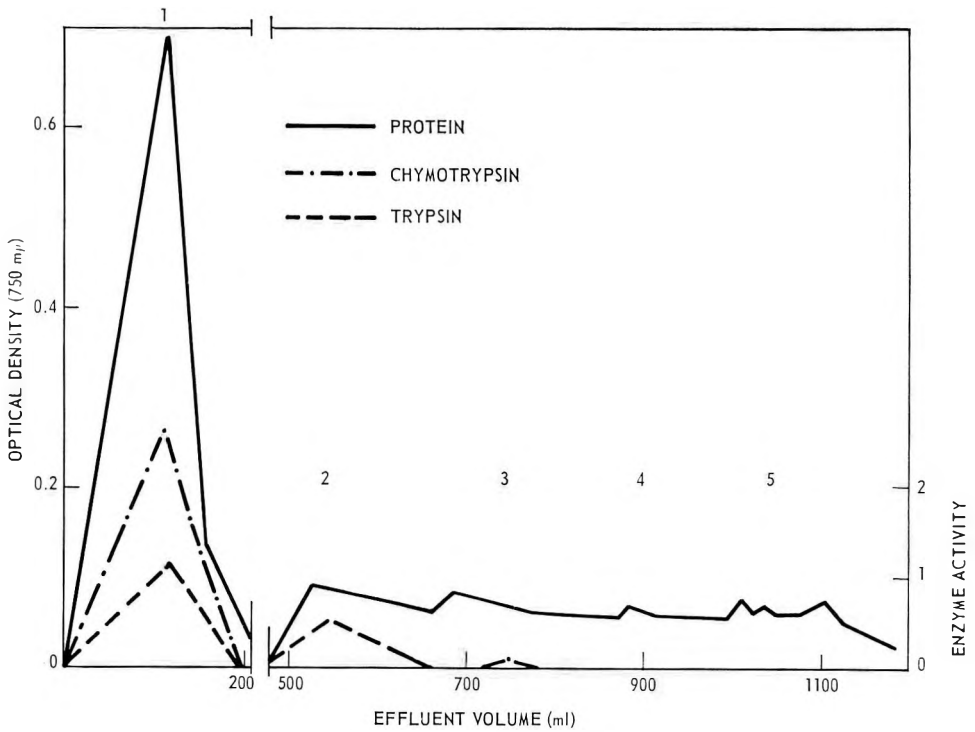


Fig. 2 Chromatography of intestinal contents on CM-cellulose. Rats were fed a protein-free diet.

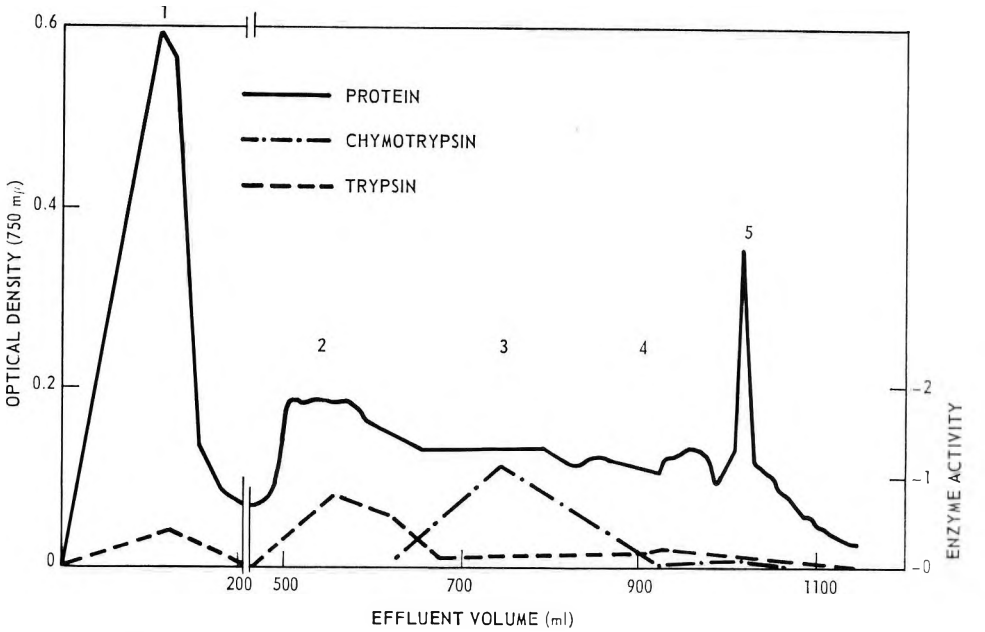


Fig. 3 Chromatography of intestinal contents on CM-cellulose. Rats were fed 15% casein.

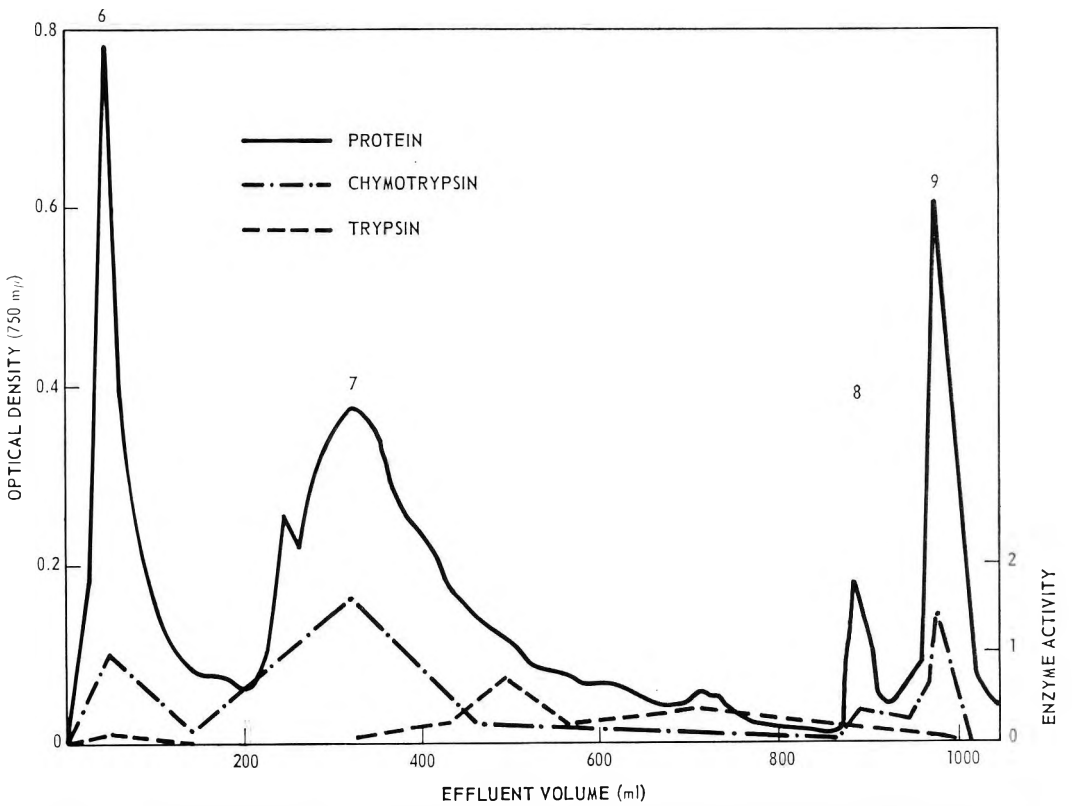


Fig. 4 Chromatography of intestinal contents on DEAE-cellulose. Rats were fed 15% casein.

zyme data were not complete for all fractions. When a fraction contained little or no protein, it generally was not assayed for enzyme activity. However, data were obtained in sufficient detail to permit certain generalizations to be made.

Pancreatic trypsin and chymotrypsin were satisfactorily separated on CM-cellulose (table 3, fig. 1). Chymotrypsin was eluted as a discrete peak in fraction 3. Trypsin appeared to be more heterogeneous and was eluted in fractions 4 and 5. Chromatographic recoveries were as follows: protein, 93%; tryptic activity, 43%; chymotryptic activity, 75%; and proteolytic activity, 46%. Incomplete recoveries of enzyme activity were probably due to inactivation on the column and in the effluent.

The chromatographic properties of trypsin and chymotrypsin were drastically altered in the intestinal contents of rats fed a protein-free ration (table 3, fig. 2). Only small amounts of chymotrypsin were recovered in fraction 3 in contrast with the chromatographic recoveries obtained with activated pancreatic extracts. Weak chymotryptic activtiy appeared at 8 and 12 hours after feeding. Very little protein was recovered in fraction 3 at 1 and 5 hours after feeding and hence the fraction was not assayed for chymotryptic activity. The presence of casein in the rats' diet improved the recoveries of chymotrypsin in fraction 3 throughout the digestive period (table 3, fig. 3).

Similarly, the amount of tryptic activity eluted in fractions 4 and 5 was low when intestinal contents of rats fed a protein-free diet were chromatographed on CM-cellulose. Tryptic activity was recovered more consistently in fractions 4 and 5 when rats were fed casein. In no instance did the recoveries approach those obtained with the partially purified trypsin from the pancreatic extract.

Two important peaks of enzyme activity, not appearing in the chromatogram from the pancreatic extract, were fractions 1 and 2. Considerable amounts of enzyme activity did not absorb onto the CM-cellulose columns and were recovered in fraction 1, the breakthrough peak. This observation indicates that the structures of the enzymes were altered. The enzymes may not have absorbed because they were of the same net charge as the absorbent. They may have had a net negative charge, indicative of an isoelectric point below pH 4.1. Possibly, charge relationships on the enzyme molecule did not favor absorption. Tryptic and proteolytic activities were usually higher in fraction 1 when rats were fed the protein-free diet. Chymotryptic activity was consistently higher. In fact, 1, 5 and 12 hours after feeding the protein-free diet, the specific activity of chymotrypsin was higher in fraction 1 than in the sample of acidic intestinal contents. Thus, fraction 1 was one of the major chromatographic fractions obtained from intestinal contents of rats fed the protein-free diet. Between 40 and 70% of the acid-soluble enzyme activity was recovered in this fraction as compared with 5 to 20% when rats were fed casein.

According to measurements made with the model substrate (BAEE), fraction 3 contained the most active tryptic moiety eluted from CM-celluose. However, only weak proteolytic activity was recovered concomitant with the esterolytic activity. Either this fraction contained a prominent but heretofore unidentified enzyme with an affinity for BAEE or the esterase and protease activities of trypsin were separated during digestion. A similar phenomenon was noted by Ryan and Balls (7) who isolated a powerful inhibitor of chymotrypsin from white potatoes. This inhibitor also complexed with trypsin, inhibiting proteolytic but not esterolytic activity. The authors suggest that one explanation of this result might be the existence of 2 catalytic centers in trypsin, one for esterolysis alone. The separation of 2 active centers during digestion would explain the existence of the fraction 3 moiety. On the other hand, trypsin may have lost its ability to bind protein but not BAEE.

Unidentified proteases of intestinal or pancreatic origin may have been responsible for the proteolytic activity eluted from CM-cellulose at pH 7 and pH 9 since esterase activity was not consistently recovered in these fractions.

Results of chromatographies of the redissolved, acid precipitates of pancreatic extracts and intestinal contents are shown

in table 4 and figure 4. Negligible amounts of trypsin and chymotrypsin were recovered during elution of the pancreatic extract from DEAE-cellulose. Several very active enzyme fractions were eluted during chromatography of intestinal contents.

Fraction 6 contained the tryptic and chymotryptic activities that did not absorb on the column. An important, anionic (at pH 7.5) tryptic moiety, with proteolytic and esterolytic activity resembling that of purified trypsin, was eluted in fractions 7b and 7c. These fractions were not observed in the pancreatic study.

Fraction 9 contained large amounts of chymotryptic activity. Considering total chymotryptic activity, fraction 9 was as important as fraction 3. With the possible exception of fraction 6, all enzyme moieties eluted from DEAE-cellulose appeared to have isoelectric points below those of their pancreatic counterparts.

Preliminary studies with maltase indicated that non-proteolytic enzymes were also altered within the small intestinal contents.

## DISCUSSION

The results of the fractionation studies of proteolytic enzymes isolated from intestinal and from pancreatic extracts differed significantly. Several parameters indicated that the properties of the enzymes were altered within the small intestine.

First, the solubility of trypsin and chymotrypsin in acid solution was decreased within the intestinal contents. Changes in the structural properties of the enzymes may have increased their sensitivity to acid denaturation. The revelation of new acidic groups on the protein molecules (by unfolding) accompanied by shifts in the isoelectric points of the enzymes may have caused the precipitation observed during acidification. The structural alterations must have been a result of proteolysis because the presence of a competing substrate — dietary protein — in upper intestinal contents increased the enzymes' solubility in acid.

The results of the dialysis experiment also supported the hypothesis that the structures of trypsin and chymotrypsin were altered within the small intestine. In principle, rat trypsin and chymotrypsin, which probably have molecular weights in the range of bovine trypsin and chymotrypsin (about 20,000) should not be dialyzable under the conditions used in the experiments reported herein. The fact that weak proteolytic activity and tryptic activity were observed in the dialyzate indicated that the enzyme molecule was split but still remained active. Bresler et al. (8) and Viswanatha et al. (9) also noted that dialyzable digests of trypsin retained some proteolytic activity. Considerable evidence (10) has accumulated indicating that digestion of certain enzymes can occur with retention of at least some activity.

The chromatographic data best demonstrated that the properties of trypsin and chymotrypsin changed within the small intestine. The heterogeneity, the unusual acidity, the loss of ability to absorb on either DEAE-cellulose or CM-cellulose all indicated that trypsin and chymotrypsin underwent structural changes. A complete description of the structural fate of proteolytic enzymes within the intestine during digestion would be impossible to give at this time. The following sequence of events might be postulated, however: trypsinogen and chymotrypsinogen enter the intestine and are activated. Within an indeterminant period certain forces contributing to their molecular structure become disrupted and nonessential parts of the enzymes begin to unfold, thereby revealing more acidic groups. The molecules begin to undergo proteolytic cleavage. When the essential structure of their active center is disrupted they lose all activity. Eventually, they are completely digested and reabsorbed.

Nutritionally, these processes are significant for several reasons. They enable the animal to recover what would otherwise represent a considerable loss of nitrogen in the endogenous protein secretions. On the other hand, the digestive enzymes, which must digest the diet, are able to function actively during the early stages of their breakdown. Dietary protein retards this breakdown in upper intestinal contents by competing with the enzymes as a substrate for proteolysis. Exogenous rather than endogenous protein is probably the preferred substrate and thereby its own rapid digestion is insured, especially since it has already been subjected to gastric digestion.

## LITERATURE CITED


1. Twombly, J., and J. H. Meyer 1961 Endogenous nitrogen secretion into the digestive tract. J. Nutrition, *74:* 453.
2. Borgström, B., A. Dahlquist, G. Lundh and V. Sjövall 1957 Intestinal digestion and absorption in the human. J. Clin. Invest., *36:* 1521.
3. Wohlman, A. B., B. L. Kabacoff and S. Avakian 1962 Comparative stability of trypsin and chymotrypsin in human intestinal juice. Proc. Soc. Exp. Biol. Med., *109:* 26.
4. Pelot, D., and M. I. Grossman 1962 Distribution and fate of pancreatic enzymes in small intestine of the rat. Am. J. Physiol., *202:* 285.
5. Snook, J. T., and J. H. Meyer 1964 Response of digestive enzymes to dietary protein. J. Nutrition, *82:* 409.
6. Miller, G. L. 1959 Protein determination for large numbers of samples. Anal. Chem., *31:* 964.
7. Ryan, C. A., and A. K. Balls 1962 An inhibitor of chymotrypsin from solanum tuberosum and its behavior toward trypsin. Proc. Nat. Acad. Sci., *48:* 1839.
8. Bresler, S. E., M. Champagne and S. Ya Frenkel 1961 A study of enzymatically active trypsin fragments. Biokhimiia, *26:* 909.
9. Viswanatha, T., R. C. Wong and I. E. Liener 1958 The peptic activation of acetyltrypsinogen. Biochem. Biophys. Acta, *29:* 174.
10. Koshland, D. E., Jr. 1960 The active site and enzyme action. Adv. Enzymol., *22:* 45.

# Glycerol Metabolism in Choline-deficient Rats [1]

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*ABSTRACT* The metabolism of glycerol-1,3-$C^{14}$ by liver slices of choline-deficient and normal liver was compared. The uptake of glycerol and its conversion to glycogen was markedly decreased in the choline-deficient liver, whereas glycerol conversion to glucose and $CO_2$ was normal. The incorporation of glycerol into the glycerol moiety of liver phospholipids decreased in the choline-deficient tissue, whereas incorporation of inorganic $P^{32}$ into the phospholipid remained normal or increased.

The gross effects of choline deficiency in weanling rats, fatty infiltration of the liver, and kidney and ocular hemorrhagic degeneration are well known (1–4). However, the mechanism of action of choline in preventing such conditions is still unknown after a half century of investigation (5). One area of these investigations has been an attempt to establish a relationship between the turnover of the phospholipids and their lipotropic function by following the incorporation of inorganic $P^{32}$ into the phospholipid fractions (6). The incorporation of inorganic $P^{32}$ into the nitrogen-containing phospholipids is a reflection, however, of the turnover of the phosphoryl moiety, rather than the entire phospholipid molecule and may represent even more closely the incorporation of $P^{32}$ into the ATP utilized for the synthesis of this moiety.

For these reasons it seemed worthwhile to investigate the possibility of determining the turnover of the liver phospholipids in normal and choline-deficient rats by following the incorporation of glycerol-1, 3-$C^{14}$ into the phospholipids. In order for such data to be useful, the effect of choline deficiency on other pathways of glycerol metabolism in liver was investigated.

The data presented here indicate that liver slices from choline-deficient rats show decreased glycerol uptake, whereas the percentage of that taken up which is converted to $CO_2$ and glucose is the same as for normal liver tissue. However, the percentage of glycerol converted to glycogen is markedly lower in the choline-deficient tissue. The incorporation of glycerol into the liver phospholipids is decreased in the choline-deficient liver, whereas the incorporation of inorganic $P^{32}$ is nearly normal.

## EXPERIMENTAL

Sprague-Dawley male rats, 20 days of age at the beginning of experimental feeding, were fed a basal low choline diet containing 18% casein (7) for either 7 or 10 days. Control rats of the same age received for this period the basal diet to which had been added 1.0 mg choline chloride/g of diet.

Incubation of liver slices was carried out in media containing as cations, K, 110; Mg, 20 and Ca, 2.5 mmoles/liter and as anions, $HCO_3$, 20 and Cl, 135 mmoles/liter. These media were gassed with an appropriate $CO_2$-$O_2$ mixture to give a pH of 7.40. All experiments were carried out in the above media which contained as substrate, 10 mmoles glycerol and either glycerol-1,3-$C^{14}$ (3 μc/flask) or $Na_2HP^{32}O_4$ (8 μc/flask).[2] Handling and slicing of the liver tissue, incubation procedure, all assays and $C^{14}$ counting procedures were performed as previously reported in detail (8).

After isolation of the total lipid from the liver slices incubated with inorganic $P^{32}$, the lipid fraction was washed to remove contaminating $P^{32}$ (9). Determination of the specific activities of the phospholipids labeled with $P^{32}$ was performed by liquid

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[2] Glycerol-1,3-$C^{14}$ obtained from New England Nuclear, Inc., Boston 18, and $Na_2HP^{32}O_4$ obtained from Nuclear Consultants, Inc., Pittsburgh.

scintillation counting and phosphorus determination (10) of aliquots of the lipid fraction.

## RESULTS

At the time of killing the rats, the livers of all those receiving the choline-deficient diet were markedly fatty by gross observation and a high percentage of the rats, especially those fed the diet for 10 days, had gross kidney hemorrhagic degeneration. Livers and kidneys of rats fed diets supplemented with choline for similar lengths of time appeared normal in size and color.

A comparison of glycerol uptake and its conversion to glucose, glycogen and $CO_2$ in choline-deficient and normal liver slices is presented in table 1. All values represent averages of duplicate or triplicate tissue incubations. Glycerol uptake was only 41% of normal in the choline-deficient liver. The percentage of the glycerol taken up that was converted to glucose was the same in both the choline-deficient and normal liver. However, the percentage of glycerol taken up that was converted to glycogen by choline-deficient liver was only 36% of that so converted by normal liver. Because of a decreased uptake of glycerol, plus a decreased conversion of glycerol to glycogen, glycogen synthesis from glycerol in the livers of choline-deficient animals was only approximately 15% of that observed in normal livers. The percentage of glycerol taken up that was metabolized to $CO_2$ by the choline-deficient livers was not significantly different from that observed in normal livers.

In addition to the data presented in table 1, the incorporation of glycerol-1,3-$C^{14}$ into the phospholipids of the liver slices was measured in the same 4 experiments. Four experiments were also performed in an identical manner to those with labeled glycerol except that unlabeled glycerol was used as substrate and tracer amounts of $NaHP^{32}O_4$ were added. The incorporation of inorganic $P^{32}$ activity into the liver phospholipids, isolated as before, was then measured. These data on the effect of choline deficiency on glycerol and inorganic $P^{32}$ incorporation into liver phospholipids are shown in table 2. The results indicate that the incorporation of glycerol into phospholipids of choline-deficient liver was decreased to an average value of 60% of that in normal liver, whereas glycerol uptake was down to 41% of normal in the choline-deficient livers. In the experiments in which $P^{32}$ incorporation was measured, glycerol uptake was decreased to 68% of

TABLE 1

*Glycerol-$C^{14}$ metabolism by liver of normal and choline-deficient weanling rats, in vitro* [1]

| | | Glycerol uptake | Glycerol converted to: | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Glucose | | Glycogen | | $CO_2$ | |
| | | *µmole g × 90 min* | *µmole g × 90 min* | *% of uptake* | *µmole g × 90 min* | *% of uptake* | *µmole g × 90 min* | *% of uptake* |
| 1 | Choline-deficient | 37.9 | 26 | 69 | 0.56 | 1.5 | 1.7 | 4.5 |
| | Normal | 81.1 | 59 | 73 | 2.35 | 2.9 | 5.3 | 6.5 |
| 2 | Choline-deficient | 32.0 | 23 | 72 | 0.78 | 2.4 | 1.7 | 5.3 |
| | Normal | 86.0 | 53 | 62 | 3.15 | 3.7 | 5.3 | 6.2 |
| 3 | Choline-deficient | 32.1 | 23 | 72 | 0.29 | 0.9 | 3.1 | 9.6 |
| | Normal | 79.2 | 56 | 71 | 3.93 | 5.0 | 6.3 | 7.9 |
| 4 | Choline-deficient | 33.6 | 21 | 62 | 0.20 | 0.6 | 2.5 | 7.4 |
| | Normal | 81.5 | 54 | 65 | 3.20 | 3.9 | 10.0 | 12.3 |
| Average, choline-deficient | | 33.9 | 23 | 69 | 0.46 | 1.4 | 2.3 | 6.7 |
| Average, normal | | 81.9 | 55 | 68 | 3.16 | 3.9 | 6.7 | 8.2 |
| Ratio: $\frac{\text{choline-deficient}}{\text{normal}}$ | | 0.41 | 0.42 | 1.00 | 0.15 | 0.36 | 0.34 | 0.82 |
| Average range of duplicates and triplicates | | 10.6 | 5.0 | — | 0.4 | — | 0.7 | — |

[1] Conditions of experiment: pH = 7.40; $[HCO_3^-]$ = 19.7 mmoles; glycerol = 10 mmoles; 90-minute incubation.

TABLE 2

*Glycerol-$C^{14}$ and inorganic $P^{32}$ incorporation into liver of normal and choline-deficient weanling rats, in vitro* [1]

| | Glycerol-$C^{14}$ incorporation | | | Inorganic $P^{32}$ incorporation | | |
|---|---|---|---|---|---|---|
| | Exp. no. | Glycerol uptake | Specific activity of phospholipid-glycerol-$C^{14}$ | Exp. no. | Glycerol uptake | Specific activity of phospholipid-$P^{32}$ |
| | | *μmole g × 90 min* | *count/min mmole C ×10⁴* | | *μmole g × 90 min* | *count/min mmole P × 10⁴* |
| Choline-deficient | 1 | 37.9 | 4.5 | 5 | 84.6 | 161.0 |
| Normal | | 81.1 | 8.5 | | 100.7 | 55.8 |
| Choline-deficient | 2 | 32.0 | 5.1 | 6 | 30.3 | 89.8 |
| Normal | | 86.0 | 8.0 | | 71.5 | 46.4 |
| Choline-deficient | 3 | 32.1 | 8.6 | 7 | 63.4 | 9.9 |
| Normal | | 79.2 | 12.1 | | 88.9 | 9.9 |
| Choline-deficient | 4 | 33.6 | 6.9 | 8 | 61.3 | 30.6 |
| Normal | | 81.5 | 13.3 | | 94.7 | 31.0 |
| Average, choline-deficient | | 33.9 | 6.3 | | 60.4 | 72.8 |
| Average, normal | | 81.9 | 10.5 | | 89.5 | 35.8 |
| Ratio: $\frac{\text{choline-deficient}}{\text{normal}}$ | | 0.41 | 0.60 | | 0.68 | 2.0 |
| Average range of duplicates and triplicates | | 10.6 | 1.3 | | 11.8 | 2.1 |

[1] Conditions of experiment: pH = 7.40; $[HCO_3^-]$ = 19.7 mmoles; glycerol = 10 mmoles; 90-minute incubation.

normal, but $P^{32}$ incorporation into phospholipids was not decreased. Actually, it was unchanged in 2 experiments and increased above normal in two.

## DISCUSSION

The purpose of these experiments was to determine whether choline deficiency changed the incorporation of glycerol into the glycerol moiety of liver phospholipids in vitro. From such incorporation data, it was hoped that information of phospholipid turnover might be obtained. These experiments indicate that in choline-deficient liver the rate of glycerol uptake decreases as does its conversion to glycogen and its incorporation into phospholipids. In view of these results and studies which have shown a decreased content of ATP in choline-deficient liver, it appears likely that less labeled glycerol phosphate is available for use within the cell (11). The decreased incorporation of glycerol into the phospholipid of choline-deficient liver may result from either a decreased turnover of the glycerol moiety of the phospholipid or synthesis of the phospholipid from a glycerol phosphate pool which has a lower specific activity than that of the normal control tissue. Knowledge of the specific activity of the glycerol phosphate would be necessary to settle this point. The normal or greater-than-normal incorporation of $P^{32}$ into the liver phospholipid of choline-deficient animals is in agreement with in vivo studies of Zilversmit and DiLuzio (6).

It was observed that the incorporation of the glycerol moiety into the liver phospholipids was greatly depressed in choline-deficient livers, whereas that of phosphate was not.

## LITERATURE CITED

1. Best, C. H., J. M. Hershey and M. E. Huntsman 1932 The effect of lecithine on fat deposition in the liver of the normal rat. J. Physiol., 75: 56.
2. Beeston, A. W., H. J. Channon, J. V. Loach and H. Wilkinson 1936 Further observations on the effect of dietary caseinogen in the prevention of fatty livers. Biochem. J., *30:* 1040.
3. Griffith, W. H., and N. J. Wade 1939 Some effects of low choline diets. Proc. Soc. Exp. Biol. Med., *41:* 188.
4. Griffith, W. H. 1958 The renal lesions in choline deficiency. Am. J. Clin. Nutrition, *6:* 263.

5. Artom, C. 1960 Mechanism of action of choline. Am. J. Clin. Nutrition, *8:* 303.
6. Zilversmit, D. B., and N. R. Diluzio 1958 The role of choline in the turnover of phospholipids. Am. J. Clin. Nutrition, *6:* 235.
7. Mulford, D. J., and W. H. Griffith 1942 Choline metabolism. VIII. The relation of cystine and of methionine to the requirement of choline in young rats. J. Nutrition, *23:* 91.
8. Longmore, W. J., A. B. Hastings and T. A. Mahoward 1964 Effect of environmental $CO_2$ and pH on glycerol metabolism by rat liver, *in vitro.* J. Biol. Chem., in press.
9. Karnovsky, M. L., and D. F. Wallach 1961 The metabolic basis of phagocytosis. III. Incorporation of inorganic phosphate into various classes of phosphatides during phagocytosis. J. Biol. Chem., *236:* 1895.
10. Bartlett, G. R. 1959 Phosphorus assay in column chromatography. J. Biol. Chem., *234:* 466.
11. Dianzani, M. U. 1957 The contents of adenosine polyphosphates in fatty livers. Biochem. J., *65:* 116.

# Mercury and Silver Interrelationships with Copper [1,2]

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*ABSTRACT* Studies were conducted to determine whether both mercury and silver were antagonistic to copper in the nutrition of the chick. Although there was a statistical interaction between mercury and copper, it was not one of antagonism. Mercury did not reduce growth or increase mortality of copper-deficient chicks, but had both these effects when the diet was supplemented with copper. Silver was antagonistic to copper in that all the symptoms of copper deficiency were accentuated in the presence of silver and absence of copper. In the presence of copper, silver had no effect. These effects are explained on the basis of the similarities between the $Ag^{++}$ ion and the $Cu^{++}$ ion and the dissimilarities between the $Hg^{++}$ ion and either $Cu^{+}$ or $Cu^{++}$ ions.

Both zinc and cadmium have been found to be antagonistic to copper in the chick (1, 2). When either of these elements is added to a copper deficient diet, the severity of the manifestations of copper deficiency, such as retarded growth, decreased aortic elastin, and anemia is heightened. These effects are not observed when the diet is supplemented with copper. The ions of these 3 elements have several common chemical parameters, namely, the valence shells are isoelectronic, they usually have a coordination number of four, and form tetrahedral complexes.

The purpose of the experiments presented in this report was to explore further the phenomenon of antagonism between other elements and copper, by determining whether mercury and silver were also antagonistic to copper. The valence shell of both $Hg^{++}$ and $Ag^{++}$ ions are isoelectronic with the $Cu^{+}$ ion. As far as is known, however, the $Hg^{++}$ ion generally forms linear complexes and has a coordination number of two. Although the $Ag^{+}$ ion forms linear complexes, silver can also exist as the $Ag^{++}$ ion. In the latter instance the ion forms square planar complexes and is isoelectronic with the valence shell of the $Cu^{++}$ ion which also forms square planar complexes. These considerations led to the speculation that silver, but not mercury, would act as a copper antagonist. The experiments reported herein were designed to test this hypothesis.

## EXPERIMENTAL

White Plymouth Rock chicks, obtained from a commercial hatchery, were used in these studies. They were housed in conventional battery brooders with raised wire floors. The basal diet used was based on dried skim milk, glucose, vitamins, minerals, and amino acids as described previously (3). It was found by analysis to contain approximately 1 ppm copper. Iron was added to the diet at 100 ppm by the use of $FeSO_4 \cdot 7H_2O$. Mineral supplements to the basal diet were made by the use of reagent graded $Ag_2SO_4$, $HgSO_4$, ZnO, $CdSO_4 \cdot H_2O$, and $CuSO_4 \cdot 5H_2O$.

In the experiment designed to investigate the possible role of mercury as a copper antagonist, consideration was given to the possibility that mercury, like cadmium, might also be antagonistic to zinc, and, in addition, might show some interaction with cadmium. Accordingly, a factorial experiment was designed in which the interplay of all 4 elements, copper, mercury, zinc, and cadmium could be examined. In this experiment, 20 chicks/treatment were fed the experimental diets from the day of hatching. The chicks were weighed at weekly intervals and mortality was recorded daily. Since the growth rate and mortality have been found to be the most sensitive measurements of zinc-induced copper deficiency (2), these 2 criteria were used to evaluate a possible mercury-copper interaction.

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[2] Published with the approval of the Director of the Experiment Station as Paper no. 1729 of the journal series.

TABLE 1

*Interactions between copper, mercury, zinc, and cadmium* [1]

| | | Avg wt at 2 weeks of age | | | |
|---|---|---|---|---|---|
| | | Cu,0 | | Cu, 25 ppm | |
| | | Hg, 0 | Hg, 400 ppm | Hg, 0 | Hg, 400 ppm |
| | | *g* | *g* | *g* | *g* |
| Cd, 0 | Zn, 0 | 144 | 123 | 160 | 138 |
| | Zn, 400 ppm | 87 | 108 | 147 | 138 |
| Cd, 100 ppm | Zn, 0 | 82 | 81 | 100 | 78 |
| | Zn, 400 ppm | 87 | 100 | 115 | 91 |

[1] Twenty chicks/treatment.

In the experiment designed to evaluate the role of silver as a copper antagonist, 20 chicks also were used per treatment. Body weights and mortality were measured as in the previous experiment. Hemoglobin was determined as the oxyhemoglobin. The elastin content of the aorta was measured by the method of McGavock and Kao (4) as modified by Starcher et al. (5).

## RESULTS AND DISCUSSION

The results of the study of the interaction of mercury with copper, zinc, and cadmium on the body weights of the chicks at 2 weeks of age are presented in table 1. The analysis of variance (table 2) revealed that among other effects, there was a significant interaction between copper and mercury. The nature of this interaction is presented in table 3. The growth of the chicks was not depressed by the addition of mercury to the copper-deficient diet. On the other hand, when copper was added to the diet, mercury did depress growth rate. This is in contrast with interaction of zinc and copper (table 3) which has been observed before (1). Zinc depressed growth in the absence of copper but not in its presence.

TABLE 2

*Analysis of variance of chick weights at two weeks*

| Source | M.S. | *F* value |
|---|---|---|
| Hg | 264 | 13.6** |
| Cu | 1502 | 77.1** |
| Zn | 68 | 3.5 |
| Cd | 6045 | 310.5** |
| Hg × Cu | 495 | 25.4** |
| Hg × Zn | 281 | 14.4** |
| Hg × Cd | 1 | 0.05 |
| Cu × Zn | 248 | 12.7** |
| Cu × Cd | 473 | 24.3** |
| Zn × Cd | 1173 | 60.2** |
| Hg × Cu × Zn | 126 | 6.5** |
| Hg × Zn × Cd | 115 | 5.9** |
| Cu × Zn × Cd | 189 | 9.7** |
| Hg × Cu × Zn × Cd | 56 | 2.9 |
| Error | 19.7 | |

** Highly significant ($P \leq 0.01$).

TABLE 3

*Interactions of copper with mercury and zinc*

| | | Cu, ppm | |
|---|---|---|---|
| | | 0 | 25 |
| | | Body wt, 2 weeks | |
| | | *g* | *g* |
| Mercury, ppm | 0 | 100 | 130 |
| | 400 | 104 | 111 |
| Zinc, ppm | 0 | 107 | 119 |
| | 400 | 95 | 123 |

As the experiment progressed and the mortality pattern emerged, much the same relationships were apparent. The mortality at 4 weeks of age, at the end of the experiment, is shown in table 4. In the absence of copper, mercury, in contrast with cadmium and zinc, did not cause mortality. However, when copper was present there was mortality among the mercury-fed chicks. By these 2 criteria, growth rate and mortality, there is an interrelationship between copper and mercury. This interrelationship, however, is not one of antagonism, since, when the copper content of the diet is low and the antagonism could be expressed most fully, mercury had no effect.

An explanation for the kind of interrelationship observed between mercury and copper is not readily apparent from the

TABLE 4

*Effect of mercury, zinc, and cadmium on mortality of chicks*

| Supplement | Cu, ppm | |
|---|---|---|
| | 0 | 25 |
| | No. dead of 20 started | |
| None | 3 | 0 |
| Zn, 400 ppm | 19 | 3 |
| Cd, 100 ppm | 19 | 5 |
| Hg, 400 ppm | 1 | 7 |
| Zn, 400 + Cd, 100 ppm | 20 | 2 |
| Cd, 100 + Hg, 400 ppm | 20 | 12 |
| Zn, 400 + Hg, 400 ppm | 14 | 3 |
| Hg, 400 + Cd,100 + Zn, 400 ppm | 19 | 8 |

data obtained in this study. Presumably, it could be the result of a "total heavy metal ion effect," namely, 400 ppm $Hg^{++}$ 25 ppm $Cu^{++}$, or of a greater accumulation of mercury in vital organs in the presence of supplementary copper.

The results of the experiment on the possible antagonism of silver for copper are presented in table 5. The criteria used were body weight, mortality, hemoglobin concentration, and elastin content of the aorta. In the absence of copper, 100 ppm silver retarded the growth of the chicks and 200 ppm resulted in an even greater depression. Although there was some variation, this retardation was virtually prevented by the addition of copper to the diet. Mortality was increased by levels of silver of 50 ppm and more among those chicks receiving the copper-deficient diet. This was also prevented by the addition of copper. As the concentration of silver was increased, the hemoglobin levels decreased at all levels of supplementation in the copper-deficient chicks, but not in the copper-supplemented chicks. The elastin content of the aortas of the copper-deficient chicks was depressed by the addition of silver to the diet. This too, was prevented by the addition of copper to the diet as evidenced by its effect on these 4 criteria, growth, mortality, hemoglobin concentration, and elastin content of the aorta. Silver acts as a copper antagonist in a manner analogous to zinc and cadmium.

It is presumed that the sulfate salt of the univalent cation of silver which was added to the diet was converted to or in equilibrium with the divalent ion species of silver by the redox systems of the intestinal tract and of the body (6). Based on available chemical evidence (7) the divalent ion species of mercury appears to be the effective ion species prevailing in this study because only the mercuric ion exists in the monomer form, whereas the univalent ion form of mercury is known to exist only as a dimer.

It is known that copper exists in functional cytochrome oxidase as both $Cu^{+}$ and $Cu^{++}$ and, indeed, its electron transport functions depends on this variable valency property. Presumably, therefore, metal ions that can attain either 4 coordination-tetrahedral (cf. $Cu^{+}$) or 4 coordination-square planar complexes (cf. $Cu^{++}$) would qualify as copper antagonists. From this line of reasoning $Zn^{++}$ and $Cd^{++}$ would be expected to be copper antagonists because these ions can potentially attain the

TABLE 5

*In vivo interaction between silver and copper*

| Ag | Cu, ppm | | | Cu, ppm | | | Cu, ppm | | | Cu, ppm | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 10 | 25 | 0 | 10 | 25 | 0 | 10 | 25 | 0 | 10 | 25 |
| | 3-week body wt [1] | | | Mortality, 4 weeks [1] | | | Hb [2] | | | Aortic elastin [3] | | |
| *ppm* | *g* | *g* | *g* | | | | *g/100 cm³* | | | *% wet wt* | | |
| 0 | 145 | 196 | 232 | 5 | 2 | 3 | 7.45 | 7.17 | 7.72 | 7.58 | 11.63 | 12.12 |
| 10 | 125 | 190 | 229 | 5 | 1 | 0 | 6.37 | 7.32 | 9.00 | 5.39 | 11.68 | 10.46 |
| 25 | 139 | 199 | 244 | 3 | 0 | 2 | 6.05 | 7.35 | 8.22 | 4.92 | 14.58 | 12.45 |
| 50 | 126 | 240 | 243 | 10 | 2 | 0 | 5.02 | 7.40 | 7.30 | 6.67 | 12.05 | 11.15 |
| 100 | 110 | 174 | 240 | 12 | 1 | 4 | 4.62 | 7.00 | 7.60 | 5.26 | 11.03 | 11.33 |
| 200 | 96 | 187 | 207 | 13 | 0 | 0 | 3.52 | 7.75 | 8.32 | 4.78 | 11.59 | 12.46 |

[1] Twenty chicks started in each treatment.

[2] Mean of 4 determinations in each treatment.

[3] Mean of 5 determinations in each treatment.

configuration of $Cu^{+}$. $Ag^{++}$ would also be expected to be a copper antagonist because it has the potential of attaining the configuration of $Cu^{++}$ complexes. Reasoning along the same lines, $Hg^{++}$ would not be expected to be a copper antagonist because neither its favored coordination number, 2, nor its favored configuration, linear, is like those of the 2 copper ions. The biological results obtained in this study substantiate this reasoning.

The results of these 2 studies extend those reported previously (1, 2), and lend further evidence to the hypothesis that for an element to be antagonistic to copper, the ions of the element must be similar to copper in the electronic configuration of the valence shell, in its favored coordination number and in the type of complexes it forms.

LITERATURE CITED

1. Hill, C. H., and G. Matrone 1962 A study of copper and zinc interrelationships. Proc. Twelfth World Poultry Congress, p. 219.
2. Hill, C. H., G. Matrone, W. L. Payne and C. W. Barber 1963 In vivo interactions of cadmium with copper, zinc and iron. J. Nutrition, *80:* 227.
3. Hill, C. H., and G. Matrone 1961 Studies on copper and iron deficiencies in growing chickens. J. Nutrition, *73:* 425.
4. McGavock, T. H., and K. Y. T. Kao 1960 The influence of age and sex on the soluble collagen, insoluble collagen and elastin of rat tissues. Exp. Med. Surg., *18:* 104.
5. Starcher, B., C. H. Hill and G. Matrone 1964 Importance of dietary copper in the formation of aortic elastin. J. Nutrition, *82:* 318.
6. Cotzias, G. C. 1962 Manganese. In Mineral Metabolism, eds., C. L. Comar and F. Bronner. Academic Press, Inc., New York, p. 403.
7. Wells, A. F. 1950 Structural Inorganic Chemistry, ed. 2. Clarendon Press, Oxford, p. 631.

# Influence of Dietary Phytol, Isophytol, and Squalene on the Tocopherol Content of Liver Tissue [1,2]

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*ABSTRACT* Phytol, isophytol, and squalene were fed to 18-day-old vitamin E-depleted chicks for either 48 or 72 hours at levels ranging up to 1% of the diet, and the effect of these compounds on the absorption of *d*, α-tocopherol was examined. No appreciable effect was noted on the tocopherol content of liver tissue when phytol was fed at levels of 0.01, 0.04, 0.125, 0.16, and 0.25% of the diet. However, at 0.50 and 1.0% of the diet, approximately a twofold and a threefold increase, respectively, was observed. Isophytol had the opposite effect, showing approximately a 40% decrease in the liver tocopherol values. Squalene also had an adverse effect, but not to the same extent as did isophytol. An anomalous situation occurred with plasma tocopherols and phytol intake. Increasing levels of phytol resulted in decreases in plasma tocopherol concentrations for both the tocopherol-supplemented and non-supplemented diets. Liver tissue was practically devoid of tocopherol when vitamin A acetate was fed at a level of 0.16% of the diet. When the level was increased to 0.48%, the birds failed to consume a sufficient amount of the diet to give meaningful data.

Reports from this laboratory (1, 2) have shown that the tocopherol in an alfalfa lipid extract is not completely available to the chick, and studies are being conducted in attempts to characterize the lipid material(s) involved. Phytol and isophytol, both decomposition products of chlorophyll, and squalene, a constituent of vegetable oils and leaves (3), have interested the authors following the observation of High and Day (4) of a decreased vitamin A deposition in the liver of rats when phytol, α-tocopheryl acetate, and squalene were fed along with β-carotene. Since carotene and tocopherol are fat-soluble substances having much in common, and phytol, isophytol, and squalene are known to be present per se or as decomposition products of plant pigments, the present study was undertaken to examine the possible effect these 3 compounds might have on the availability to the chick of the tocopherol associated with alfalfa lipids.

## EXPERIMENTAL

One-day-old White Plymouth Rock male chicks were fed a diet low in vitamin E for 18 days. The chicks were then distributed into groups of comparable weight, each containing 4 chicks, which were randomly assigned to treatment for 72 hours on a restricted feed intake basis. Other details, such as composition of the diet and methods for the determination of tocopherol, were described previously (5). The basal diet, by analysis, contained approximately 12 mg of total tocopherols per kg of diet. Synthetic phytol[3] and isophytol,[4] squalene,[5] crystalline vitamin A acetate ,[6] and *d*,α-tocopherol [7] were dissolved in purified Skellysolve B and pipetted into the appropriate amount of either olive oil (USP), or a vegetable oil[8] prior to mixing into the diet. The diluting oil was added at 2% of the diet, olive oil being used for the first experiment and the vitamin E-low coconut oil in the second experiment.

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[4] See footnote 3.

[5] Eastman Organic Chemicals, no. 6966, Rochester 3, New York.

[6] See footnote 3.

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

[8] This is a refined, bleached and deodorized, tocopherol-low and sterol-free coconut oil, Product 1223, of the Drew Chemical Corporation, New York 10, New York.

## RESULTS

During initial exploratory work, incorporation of 0.33 and 1.0% of phytol into the diet of tocopherol "depleted" chicks with 40 mg of *d*,α-tocopherol/kg of diet for a period of 48 hours resulted in approximately a 30% increase of the liver tocopherol above that of chicks receiving a similar diet, but unsupplemented with phytol. In contrast, when isophytol and squalene were fed at the same dietary levels as phytol, decreases in liver tocopherol of approximately 75% and 40%, respectively, occurred.

In the first experiment reported here, graded levels of phytol, isophytol, squalene, and vitamin A acetate were fed. The latter was included since at high dietary levels it had been reported (6, 7) to decrease tocopherol deposition. The results (table 1) essentially substantiate the data from the preliminary experiment in that phytol, at a level of 0.48% of the diet, resulted in an increase, whereas isophytol and squalene at the same level resulted in a decrease of liver tocopherol. Vitamin A acetate caused a decrease in liver tocopherol from an average of 16.5 μg/g of liver at the zero level of added vitamin A to 1.7 μg/g at the 0.48% level. In the case of vitamin A acetate, as indicated in table 1, feed consumption was only 88% of that offered when vitamin A was fed at 0.16% of the diet and only 30% at the 0.48% level of vitamin A.

TABLE 1

*Effect of phytol, isophytol, squalene, and vitamin A acetate on liver tocopherol* [1]

| % in diet | 0 | 0.01 | 0.04 | 0.16 | 0.48 |
|---|---|---|---|---|---|
| | *μg tocopherol/g liver* | | | | |
| Basal | 2.1 | | | | |
| Basal + phytol | | | | 2.1 | 3.8 |
| Basal + phytol + vitamin E [2] | 15.7 [3] | 17.6 | 14.9 | 16.0 | 32.4 |
| Basal + isophytol + vitamin E | 15.3 [3] | 15.4 | 14.7 | 18.2 | 11.8 |
| Basal + squalene + vitamin E | 18.5 [3] | 17.8 | 20.1 | 17.9 | 12.6 |
| Basal + vitamin A + vitamin E | | 11.3 | 10.2 | 3.2 [4] | 1.7 [5] |

[1] Feed consumption was limited to 300 g/group per 72-hour period.
[2] E = 40 mg *d*,α-tocopherol/kg of diet.
[3] The average liver tocopherol value for the 3 positive controls is 16.5 μg.
[4] Feed consumption was 265 g.
[5] Feed consumption was 90 g.

TABLE 2

*Variations in liver tocopherol content due to phytol, isophytol, and squalene with and without added d,α-tocopherol* [1]

| % in diet | 0 | 0.125 | 0.25 | 0.50 | 1.0 |
|---|---|---|---|---|---|
| | | *μg tocopherol/g liver* | | | |
| Basal | 1.1 [2] (69) [3] | | | | |
| Basal + vitamin E [4] | 18.3, 15.6 (1071, 1136) | | | | |
| Basal + phytol | | 1.7 (76) | 1.4 (72) | 2.0 (56) | 3.4 (29) |
| Basal + phytol + vitamin E | | 14.5 (954) | 16.2 (862) | 28.2 (517) | 43.1 (500) |
| Basal + isophytol | | 1.3 | 1.4 | 1.2 | 0.9 |
| Basal + isophytol + vitamin E | | 14.9 | 14.3 | 12.1 | 6.7 |
| Basal + squalene | | 1.4 | 1.5 | 1.1 | 1.4 |
| Basal + squalene + vitamin E | | 15.6 | 15.8 | 14.1 | 11.3 |

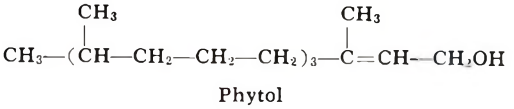
[1] Feed consumption was limited to 300 g/group per 72-hour period.
[2] The standard error of the mean of 13 liver tocopherol values for this basal diet obtained from previous experiments is 1.6 ± 0.07.
[3] Figures in parentheses are micrograms of total tocopherol/100 ml of plasma.
[4] E = 40 mg *d*,α-tocopherol/kg of diet.

In a second experiment, reported in table 2, which included groups receiving no added tocopherol, chicks fed the basal diet with 1.0% of phytol and with no added tocopherol had a significantly higher liver tocopherol than those fed the basal diet alone. Phytol at 0.5 and 1.0% of the diet plus 40 mg of supplemental *d,α*-tocopherol/kg of diet resulted in approximately a twofold and threefold increase, respectively, in liver tocopherol deposition when compared with that of chicks receiving the basal diets supplemented with *d,α*-tocopherol but no phytol. Isophytol, at 0.50 and 1.0% of the diet containing supplemental tocopherol, lowered the liver tocopherol content by approximately 25 and 65%, respectively, when compared with the tocopherol-supplemented basal diets with no added isophytol. The decrease registered by squalene at 1% of the diet was lower than that of the tocopherol control by approximately 30%.

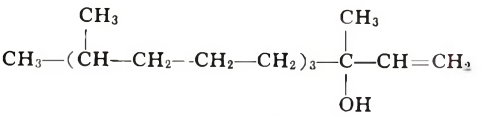
A peculiar situation occurred with plasma tocopherol, as evidenced by the data in parentheses in table 2. In contrast with liver tocopherol deposition, phytol at increasing levels of intake resulted in decreases in plasma tocopherol concentrations for both the tocopherol-supplemented and nonsupplemented diets. The reasons for this anomaly, higher liver tocopherol values but lower plasma values, cannot be explained without further study.

Whether phytol or isophytol interfered in the Emmerie-Engel color reaction used for estimation of tocopherol was explored. Both compounds at a level of 8.4 mg in an Evelyn colorimeter tube showed no Emmerie-Engel color reaction, and recoveries of 40 μg of *d,α*-tocopherol were quantitative from Evelyn colorimetric tubes containing each of the compounds. The tocopherol isolated from livers of chicks that received the 1.0% phytol diet with added tocopherol was spotted on plates of silica gel and developed with 10% diethyl ether in petroleum ether. The large reducing spot, which separated out, had an $R_f$ identical to that of a standard preparation of pure *d,α*-tocopherol and strongly indicated that the isolated compound was tocopherol and not an artifact.

The only difference between the phytol and isophytol molecules, both of which are used in tocopherol synthesis, is in the arrangement of the terminal hydroxy and olefinic groups.



Phytol



Isophytol

It is striking that such a small structural change should result in opposite effects on the tocopherol content of the liver. Since synthetic isophytol has three and phytol has two racemic centers (8, 9) which are not present in the natural compounds, it would be worthwhile to determine whether this stereoisomeric difference would result in any changes in tocopherol absorption. No balance studies were conducted in these experiments. However, on the basis of previous work from this laboratory (3) in which approximately 30% of the dietary tocopherol was excreted by the chick fed a basal diet with added tocopherol and up to 60% was excreted when alfalfa was used as the source of tocopherol, one might speculate that it is the absorption of tocopherol from the intestine that is enhanced by phytol and decreased by isophytol, squalene, and vitamin A.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. 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.
2. Pudelkiewicz, W. J., and L. D. Matterson 1960 A fat-soluble material in alfalfa that reduces the biological availability of tocopherol. J. Nutrition, 71: 143.
3. Alam, A. Q., J. Brossard and G. Mackinney 1962 Detection and estimation of squalene in leaves. Nature, 194: 479.
4. High, E. G., and H. G. Day 1951 Effects of different amounts of lutein, squalene, phytol, and related substances on the utilization of carotene and vitamin A for storage and growth in the rat. J. Nutrition, 43: 245.

5. 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. Nutrition, *71:* 115.
6. Dicks, M. W., J. E. Rousseau, Jr., H. D. Eaton, R. Teichman, A. P. Grifo, Jr., and H. A. Kemmerer, Jr. 1959 Some interrelationships between vitamin E and vitamin A in Holstein calves. J. Dairy Sci., *42:* 501.
7. Edwin, E. E., J. Bunyan, J. Green and A. T. Diplock 1962 The effect of vitamin A on ubiquinone and ubichromenol in the rat, and its relation to the effect of vitamin E. Brit. J. Nutrition, *16:* 135.
8. Isler, O., P. Schudel, H. Mayer, J. Wursch and R. Ruegg 1962 Chemistry of vitamin E. Vitamins and Hormones, *20:* 389.
9. Weiser, H., G. Brubacher and O. Wiss 1963 *l,α*-tocopheryl acetate: Biological activity. Science, *140:* 80.

# Amino Acid Requirements of Children: Nitrogen Balance at the Minimal Level of Essential Amino Acids

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*ABSTRACT* Minimal requirements of 8 essential amino acids previously determined separately, were fed in combination, along with the nonessential amino acids, to five 12-year-old boys for 20 days. Amino acid mixtures furnished 12.25 g nitrogen in the first 10 days, and 10.23 g in the last 10 days. Nitrogen balance and urinary excretion of creatine, creatinine, riboflavin and N-MNA were determined. All children maintained body weight at an almost constant level and a positive nitrogen balance throughout the experiment. Urinary excretion of creatinine remained almost constant, but that of creatine tended to increase during the feeding of the amino acid mixture. No significant trend was observed in urinary excretion of riboflavin and N-MNA.

In previous papers (1–5), the minimal requirement of each essential amino acid for school children was determined separately, and the present investigation was undertaken to ascertain the adequacy of the determined minimal amounts of the combined essential amino acids over an extended period of time.

## EXPERIMENTAL PROCEDURE AND RESULTS

Five healthy 12-year-old boys served as experimental subjects for 23 days (table 1). For the first 3 days, these subjects consumed a normal diet having about the same nitrogen and caloric levels as in the experimental basal diet. This period was used to accustom the subjects to living in a laboratory and also to minimize prior dietary effects. The basal diet contained amino acid mixtures, cornstarch, corn oil, butter fat, and mineral and vitamin mixtures (1, 3, 4). The composition and daily intake of essential and nonessential amino acid mixtures are shown in table 2.

In the first study of this series (1), total nitrogen was given at a level of 12 g, and this level was used in all experiments thereafter. However, after the establishment of the minimal requirements for essential amino acids, it was questioned whether 12 g of nitrogen is necessary for boys of this age group, when the present pattern of minimal amounts of essential amino acids is used. In the experiment to be reported here, the amount of total nitrogen was also studied at the 10-g level. The 20-day experiment with the amino acid mixture was divided into 4 equal periods: in the first 2 periods the amino acid mixtures provided 12.25 g of nitrogen, and in the last 2 periods, 10.23 g of nitrogen. However, the actual intake of total nitrogen was increased by a small quantity, due to the nitrogen in the basal diet (not including the amino acid mixture) and in the cucumber which was added in the last 3 periods to improve appetite and reduce the monotony of the diet.

The nitrogen balance method was used, and riboflavin, $N^1$-methylnicotinamide (N-MNA), creatine, and creatinine in the urine were also determined. The experimental method used has been described in detail in the previous reports (1–5). The results obtained are shown in table 3.

All children remained healthy, and maintained body weight at an almost constant level with some showing slight increases. All children maintained a positive nitrogen balance throughout the experimental period with minimal amounts of the 8 essential amino acids, at both the

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TABLE 1

*Age, height, weight, basal metabolism and energy intake of subjects*

| Subject | Age | Body height | Body wt | Basal metabolism | Avg daily energy intake [1] |
|---|---|---|---|---|---|
| | *years, months* | *cm* | *kg* | *kcal/kg/day* | *kcal/kg* |
| U.N. | 11 7 | 137.8 | 30.3 | 30 | 61 |
| U.D. | 12 1 | 151.9 | 36.5 | 34 | 53 |
| T.U. | 12 0 | 157.6 | 40.3 | 27 | 53 |
| H.S. | 11 11 | 150.3 | 37.2 | 27 | 62 |
| H.N. | 12 3 | 133.2 | 22.1 | 37 | 73 |

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

TABLE 2

*Composition and daily intake of amino acid mixtures*

| Component | Daily intake | | Nitrogen content | |
|---|---|---|---|---|
| | *g* | | *g* | |
| Essential amino acids | Periods 1–4 | | Periods 1–4 | |
| L-Isoleucine | 1.00 | | 0.107 | |
| L-Leucine | 1.50 | | 0.160 | |
| L-Lysine | 1.60 | | 0.307 | |
| L-Methionine | 0.80 | | 0.075 | |
| L-Phenylalanine | 0.80 | | 0.068 | |
| L-Threonine | 1.00 | | 0.118 | |
| L-Tryptophan | 0.12 | | 0.017 | |
| L-Valine | 0.90 | | 0.108 | |
| Nonessential amino acids | Periods 1–2 | Periods 3–4 | Periods 1–2 | Periods 3–4 |
| L-Alanine | 9.22 | 7.57 | 1.449 | 1.190 |
| L-Arginine | 10.81 | 8.87 | 3.476 | 2.853 |
| L-Aspartic acid | 10.76 | 8.84 | 1.132 | 0.930 |
| L-Glutamic acid | 10.76 | 8.84 | 1.024 | 0.842 |
| L-Sodium glutamate | 12.50 | 10.26 | 1.035 | 0.850 |
| Glycine | 7.65 | 6.01 | 1.420 | 1.116 |
| L-Histidine | 1.00 | 1.00 | 0.271 | 0.271 |
| L-Proline | 4.61 | 3.79 | 0.561 | 0.461 |
| L-Serine | 6.92 | 5.68 | 0.922 | 0.757 |
| Total (essential + nonessential) | | | 12.250 | 10.230 |

12-g and the 10-g levels of total nitrogen. Average daily retention of nitrogen by the subjects was as follows: (in grams) U.N., 0.76; U. D., 0.68; T. U., 0.78; H. S., 0.81; and H. N., 1.36.

The urinary excretion of creatinine remained at an almost constant level, but that of creatine tended to increase after children were fed the amino acid mixture, except in the case of subject T. U.

A significant change was not observed in urinary excretion of riboflavin and N-MNA throughout the experimental periods, but for subject H. N. the difference in excretion of N-MNA between the first half and the second half of the experimental period was significant at the 5% level.

## DISCUSSION

All children maintained positive nitrogen balance throughout the experimental period with the minimal requirements of the 8 essential amino acids. In children, it is necessary not only to allow for maintenance of positive nitrogen balance, but also to provide for growth and development. However, it is difficult to define a zone for allowance of retention. If it is assumed that the bodies of 10- to 12-year-old boys contain nitrogen at a level of 3% and that the boys gain 3 kg of body weight during a year,[1] nitrogen retention would increase 90 g in a year, or 0.25 g a day.

[1] Cited from the Report of the National Nutrition Survey in Japan, 1962.

TABLE 3

*Nitrogen balance and urinary excretion of creatinine, creatine, riboflavin and N-MNA with minimal levels of essential amino acids*

| Subjects | Body wt at 5-day periods | Daily N intake | Avg daily N output | | Avg daily N balance | Avg daily urinary excretion | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Urine | Feces | | Creatinine | Creatine | Riboflavin | N-MNA |
| | *kg* | *g* | *g* | *g* | *g* | *mg* | *mg* | *μg* | *mg* |
| U. N. | 30.3 [1] | | | | | 849 [2] | 0 [2] | | |
| | 31.3 | 12.34 | 11.09 | 0.60 | +0.65 | 748 | 107 | 444 | 4.8 |
| | 31.1 | 12.56 | 11.00 | 0.70 | +0.86 | 806 | 134 | 668 | 3.8 |
| | 31.0 | 10.81 | 9.59 | 0.61 | +0.61 | 821 | 118 | 670 | 5.3 |
| | 31.2 | 11.04 | 9.34 | 0.79 | +0.91 | 818 | 153 | 739 | 5.2 |
| U. D. | 36.5 [1] | | | | | 1072 [2] | 16 [2] | | |
| | 38.0 | 12.35 | 11.16 | 0.63 | +0.56 | 1003 | 21 | 408 | 6.2 |
| | 38.4 | 12.58 | 10.73 | 0.70 | +1.15 | 963 | 16 | 582 | 5.1 |
| | 37.4 | 11.05 | 10.12 | 0.57 | +0.36 | 953 | 105 | 511 | 5.2 |
| | 37.3 | 11.05 | 9.67 | 0.75 | +0.63 | 1039 | 141 | 432 | 5.9 |
| T. U. | 40.3 [1] | | | | | 1127 [2] | 122 [2] | | |
| | 41.2 | 12.34 | 11.25 | 0.83 | +0.26 | 1078 | 94 | 689 | 6.3 |
| | 41.5 | 12.55 | 11.00 | 0.64 | +0.91 | 1034 | 16 | 622 | 6.5 |
| | 41.6 | 11.02 | 9.48 | 0.52 | +1.02 | 1039 | 42 | 445 | 5.9 |
| | 41.4 | 11.02 | 9.40 | 0.70 | +0.92 | 1077 | 40 | 430 | 4.4 |
| H. S. | 37.2 [1] | | | | | 849 [2] | 167 [2] | | |
| | 38.8 | 12.35 | 10.98 | 1.05 | +0.32 | 905 | 147 | 616 | 6.0 |
| | 38.7 | 12.57 | 10.83 | 0.84 | +0.90 | 939 | 210 | 702 | 5.9 |
| | 38.5 | 11.05 | 9.44 | 0.60 | +1.01 | 877 | 329 | 430 | 4.4 |
| | 38.7 | 11.06 | 9.28 | 0.76 | +1.02 | 910 | 274 | 529 | 4.9 |
| H. N. | 22.1 [1] | | | | | 658 [2] | 0 [2] | | |
| | 23.6 | 12.35 | 9.44 | 0.72 | +2.19 | 585 | 120 | 523 | 5.6 |
| | 23.8 | 12.58 | 10.47 | 0.94 | +1.17 | 622 | 97 | 835 | 5.6 |
| | 23.3 | 11.03 | 9.46 | 0.68 | +0.89 | 604 | 131 | 778 | 4.3 |
| | 23.4 | 11.05 | 9.05 | 0.82 | +1.18 | 588 | 134 | 545 | 4.7 |

[1] Initial body weight.

[2] Average of urinary excretion for 3 days, when consuming a normal diet.

By adding a margin of 50%, 0.38 g of nitrogen thus may be considered a safe daily level of retention consistent with the demands for growth in this age group.

In previous papers (2–5), a tendency was noted for urinary riboflavin and N-MNA excretion to increase with a negative balance of nitrogen, without consideration of the particular amino acids being tested. In the present experiment in which the nitrogen balance remained positive, the urinary excretion of riboflavin and N-MNA did not tend to increase.

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LITERATURE CITED

1. Nakagawa, I., T. Takahashi and T. Suzuki 1960 Amino acid requirements of children. J. Nutrition, *71:* 176.
2. Nakagawa, I., T. Takahashi and T. Suzuki 1961 Amino acid requirements of children: Isoleucine and leucine. J. Nutrition, *73:* 186.
3. Nakagawa, I., T. Takahashi and T. Suzuki 1961 Amino acid requirements of children: Minimal needs of lysine and methionine based on nitrogen balance method. J. Nutrition, *74:* 401.
4. Nakagawa, I., T. Takahashi, T. Suzuki and K. Kobayashi 1962 Amino acid requirements of children: Minimal needs of threonine, valine and phenylalanine based on nitrogen balance method. J. Nutrition, *77:* 61.
5. Nakagawa, I., T. Takahashi, T. Suzuki and K. Kobayashi 1963 Amino acid requirements of children: Minimal needs of tryptophan, arginine and histidine based on nitrogen balance method. J. Nutrition, *80:* 305.

# Magnitude of the Hypocholesterolemic Effect of Dietary Sitosterol in Man [1,2]

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*ABSTRACT* Ninety-two university students (73 men and 19 women) consumed for 7 days a homogenized formula ration providing 45% of calories as butter fat. The subjects were then divided into 10 groups and continued for 8 days on the same regimen supplemented by a commercial preparation of β-sitosterol in amounts of 50, 100, 200, 300, 400, 600, 800, 1600, 3200, and 6400 mg/950 kcal. Eighty-five subjects (69 men and 16 women) completed the experiment. Starting with the 300-mg level, the β-sitosterol increments caused progressively larger decreases in plasma cholesterol concentrations which were significant in all instances. The implications of these results are discussed in the light of the large amounts of plant sterols present in corn oil and it is concluded that these compounds are important factors in accounting in large part for its hypochloesterolemic property.

The original report by Peterson in 1951 of the hypocholesterolemic action of plant sterols in chicks (1) and the subsequent confirmation of this action in man by Pollak (2) gave rise to numerous clinical trials of these compounds in both normo- and hypercholesterolemic subjects (2–9).[4] Although there were some inconsistencies in the results reported, the majority of these trials confirmed Pollak's observation.

Under the well-controlled conditions provided through the feeding of homogeneous formula rations to large numbers of young male normocholesterolemic subjects, work in this laboratory showed that incorporation of plant sterols at the level of 7 g/950 kcal into a diet high in butter (60% of calories) effected highly significant decreases in plasma cholesterol levels (10). This ration was just as hypocholesterolemic as one providing 60% of calories from corn oil. The striking magnitude and consistency of these responses were ascribed to the uniform distribution of the material throughout the homogeneous formula ration, a mode of administration which was different from that adhered to in the clinical trials hitherto reported in which the plant sterols were given usually in 3 doses per day at meal time.

Although 7 g of plant sterol/950 kcal provided an amount still far in excess of that supplied by corn oil at the level of 60% of calories, the profound hypocholesterolemic responses obtained prompted the use of several feeding trials to determine the extent to which the plant sterols present in this vegetable fat might account for its hypocholesterolemic property. It was found that of a number of molecular distillates of corn oil with comparable concentrations of unsaturated fatty acid residues, the one richest in unsaponifiable matter and hence in plant sterols yielded the greatest hypocholesterolemic response (10). Furthermore, decreases in plasma cholesterol observed as a result of the introduction of corn oil into a fat-free diet at the 60% level for an equicaloric amount of carbohydrate, could be effected similarly by incorporation into the fat-free ration of an equivalent amount of plant sterols alone, namely, 0.63 g sitosterol/950 kcal (11). Less than one-half this amount, equivalent to that supplied by corn oil at the level of 25% of calories, sufficed to depress significantly plasma cholesterol concentrations in subjects subsisting on formula rations containing 35% of calories in the form of butter fat (11). The present experiment extends this work to a study of the effect of supplementing a

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[1] This investigation was financed by a grant from the Ontario Heart Foundation, Toronto, Ontario.
[2] This work was reported in abbreviated form at the Fifth International Congress of Biochemistry, Moscow, U.S.S.R., August, 1961.
[3] Present address: National Institutes of Health, Bethesda, Maryland.
[4] Peterson, D. W., C. W. Nichols, Jr., N. F. Peek and I. L. Chaikoff 1956 Depression of plasma cholesterol in human subjects consuming butter containing soy sterols. Federation Proc., *15*: 569 (abstract).

TABLE 1

*Composition of basal diet;[1] amounts required to make a 950-kcal sample*

| | Amount | Protein | Fat | Carbohydrate |
|---|---|---|---|---|
| | *g* | *g* | *g* | *g* |
| Skim milk powder [2] | 28.1 | 10.0 | 0.1 | 14.6 |
| Calcium caseinate [3] | 28.4 | 25.0 | 0.57 | — |
| Sucrose | 20.0 | — | — | 20.0 |
| Maltose and dextrins [4] | 62.2 | — | — | 61.0 |
| Butter | 55.7 | — | 46.8 | — |
| Total | | 35.0 | 47.5 | 95.6 |
| Kilocalories | 950.03 | 140.0 | 427.5 | 382.5 |
| Calories, % | | 14.7 | 45.0 | 40.3 |

[1] Two grams of iodized salt were added per 950-kcal batch. A vitamin mixture was also added to supply the following: (in mg/950 kcal) thiamine, 0.6; riboflavin, 0.6; niacin, 5.0; pyridoxine, 5.0; Ca pantothenate, 5.0; ascorbic acid, 25.
[2] Mil-ko, Mil-ko Products Limited, Hamilton, Ontario.
[3] Casec, Mead Johnson of Canada Limited, Belleville, Ontario.
[4] Dextri-Maltose, Mead Johnson of Canada Limited, Belleville, Ontario.

diet with progressively increasing amounts of β-sitosterol.

## EXPERIMENTAL

Ninety-two university students (73 men and 19 women) subsisted for 7 days on a homogenized formula ration providing 45% of calories in the form of butter fat (table 1). The subjects were then divided into 10 groups and continued for 8 days on the same regimen modified by incorporation of a commercial preparation of β-sitosterol[5] in amounts of 50, 100, 200, 300, 400, 600, 800, 1600, 3200, and 6400 mg/950 kcal. These supplements were dissolved in the melted butterfat moiety prior to homogenization of the dietary ingredients with water. The diets were dispensed in portions of 950 kcal and stored in cardboard containers in a deep-freeze unit. The subjects were instructed to ingest sufficient of the ration to maintain their body weight constant. No other items were permitted except for water, clear tea or coffee. Fasting plasma cholesterol levels were determined on days zero, 4, 7, 11, and 15 by the method of Abell et al. (13). Eighty-five subjects (69 men and 16 women) completed the experiment. The average change in body weight was − 0.46 kg.

## RESULTS AND DISCUSSION

Transition of the subjects from their free-choice, ad libitum diets to the butterfat regimen unsupplemented by sitosterol did not materially affect their plasma cholesterol levels, since the average values obtained on days zero, 4, and 7 were essentially the same. Table 2 lists the statistical data on the absolute changes in plasma cholesterol expressed as the difference in milligrams per 100 ml between days 7 and 15. Values for the average daily intake of sitosterol for each of the 10 groups are also shown. These were computed from the daily records on dietary consumption submitted by the volunteers.

Although the level of plasma cholesterol was not affected by increments of sitosterol up to, and including, 200 mg/950 kcal, supplementation by as little as 300 mg/950 kcal caused a significant decrease amounting to 12.7 mg of cholesterol/100 ml of plasma ($P = 0.05$–$0.02$). Progressively greater decreases occurred in response to increasing supplementation with sitosterol ($P < 0.01$, in all instances). These culminated in a difference between days 7 and 15 of as much as 35.4 mg of cholesterol/100 ml of plasma when the supplement was incorporated into the ration in amounts of 6400 mg/950 kcal.

In figure 1, these data are plotted in the form of a dose-response curve as the average percentage change in plasma cholesterol per group on day 15 relative to day 7. A decrease of about 20% occurred at the highest level of plant sterol supplementation. The smooth course of the curve ob-

[5] Donated by the Eli Lilly Company, Indianapolis, Indiana, through the courtesy of Dr. R. E. Shipley. By gas-liquid chromatography, 70.9% of this preparation was made up of β-sitosterol; the remainder represented C-28 sterol. A trace of stigmasterol was also present.

tained suggests an exponential decline with which only the plot for the group receiving the supplement at the 800-mg level does not coincide. We are not able to explain this aberrant result.

As determined by various criteria in this laboratory (14), the sterol content of refined corn oil has consistently been found to be about 1%. Although this figure is in the lower range of values reported in the literature (15), it still greatly exceeds those given for other edible oils. It is pertinent to add that corn oil has been shown in this and other (16, 17) laboratories to be the most hypocholesterolemic. Thus, with a 3000-kcal diet, an intake of 45% of calories in the form of corn oil would provide about 1500 mg/day of plant

TABLE 2

*Significance of absolute changes in plasma cholesterol values induced by supplementing a ration containing 45% of calories as butter fat with increasing amounts of β-sitosterol*

| Group no. | No. of subjects [1] | Supplement of plant sterol | Estimated daily intake of plant sterol | Intake plant sterol / Intake cholesterol | Mean difference between days 7 and 15 | SE of difference | *t* | P |
|---|---|---|---|---|---|---|---|---|
| | | *mg/950 kcal* | *mg* | *molar ratio* | *mg cholesterol/ 100 ml plasma* | | | |
| 1 | 9 | 50 | 162 | 0.37 | − 2.72 | 4.91 | 0.55 | > 0.5 |
| 2 | 9 | 100 | 302 | 0.75 | + 1.22 | 4.12 | 0.30 | > 0.5 |
| 3 | 8 | 200 | 592 | 1.49 | 0 | 3.91 | 0 | 1.0 |
| 4 | 9 | 300 | 870 | 2.24 | −12.72 | 4.43 | 2.87 | 0.05–0.02 |
| 5 | 9 | 400 | 1,292 | 2.98 | −15.72 | 4.86 | 3.23 | 0.02–0.01 |
| 6 | 7 | 600 | 1,860 | 4.48 | −18.85 | 3.90 | 4.83 | < 0.01 |
| 7 | 9 | 800 | 2,304 | 5.97 | −12.66 | 2.86 | 4.43 | < 0.01 |
| 8 | 9 | 1600 | 5,456 | 11.93 | −24.77 | 3.79 | 6.54 | < 0.01 |
| 9 | 8 | 3200 | 8,000 | 23.87 | −28.87 | 5.11 | 5.65 | < 0.01 |
| 10 | 8 | 6400 | 20,416 | 47.75 | −35.43 | 5.41 | 6.55 | < 0.01 |

[1] There were 2 female volunteers in groups 1–5 and 7, and one female in groups 6, and 8–10.

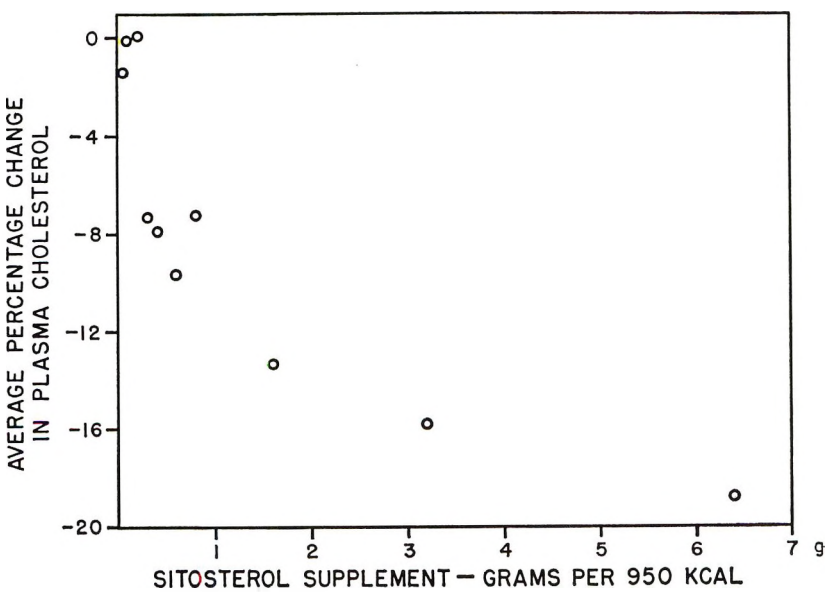


Fig. 1 Magnitude of hypocholesterolemic response in university students to dietary increments of β-sitosterol incorporated into a formula ration providing 45% of calories as butterfat.

sterol calculated as sitosterol. Gas liquid chromatographic analyses performed in our laboratory[6] show that β-sitosterol accounts for about 85% of the total plant sterols present in corn oil. The possibility also must be entertained that there may be present in the unsaponifiable matter substances other than β-sitosterol that have a hypocholesterolemic action. The average sitosterol intake at the lowest level of 300 mg/950 kcal, which in the present experiment evoked a significant hypocholesterolemic response, was only about 1 g/day.

The thesis that the degree of unsaturation of corn oil, and presumably other similar vegetable oils, is wholly responsible for the observed hypocholesterolemic effect cannot be regarded as proved. The data presented in the experiment described here, together with previous observations by others (16–18) and in our laboratory (11), indicate that a factor or factors other than polyunsaturated fatty acids must be involved and that undoubtedly sitosterol is one of these. Our recent demonstration of a close relationship between the level of dietary cholesterol and plasma cholesterol in man over the range of daily intake of zero to about 800 mg (19), together with the well-established property of sitosterol of decreasing the intestinal absorption of cholesterol (12), makes for an entirely logical picture. Our work indicates (cf. table 2) that the molar ratio of dietary sitosterol to cholesterol, over the ranges studied, must be at least 2:1 before a significant hypocholesterolemic effect can be detected. Presumably the endogenously produced cholesterol influences these results.

## LITERATURE CITED


1. Peterson, D. W. 1951 Effect of soybean sterols in the diet on plasma and liver cholesterol in chicks. Proc. Soc. Exp. Biol. Med., *78:* 143.
2. Pollak, O. J. 1953 Reduction of blood cholesterol in man. Circulation, *7:* 702.
3. Barber, J. M., and A. P. Grant 1955 The serum cholesterol and other lipids after administration of sitosterol. Brit. Heart J., *17:* 296.
4. Best, M. M., C. H. Duncan, E. J. Van Loon and J. D. Wathen 1954 Lowering of serum cholesterol by the administration of a plant sterol. Circulation, *10:* 201.
5. Shipley, R. E. 1955 The effects of sitosterol ingestion on serum cholesterol concentration. Trans. N. Y. Acad. Sci., *18:* 111.
6. Le Sesne, J. M., C. W. Castor and S. W. Hoobler 1955 Prolonged reduction in human blood cholesterol levels induced by plant sterols. Univ. Michigan Med. Bull., *21:* 13.
7. Farquhar, J. W., R. E. Smith and M. E. Dempsey 1956 The effect of beta-sitosterol on the serum lipids of young men with arteriosclerotic heart disease. Circulation, *14:* 77.
8. Wilkinson, C. F., Jr., E. Boyle, R. S. Jackson and M. R. Benjamin 1955 Effect of varying the intake of dietary fat and the ingestion of sitosterol on lipid and lipoprotein fractions of human serum. Metabolism, *4:* 302.
9. Levere, A. H., R. C. Bozian, G. Craft, R. S. Jackson and C. F. Wilkinson, Jr. 1958 The "sitosterols": Variability of serum cholesterol levels and difficulty of evaluating decholesterolizing agents. Metabolism, *7:* 338.
10. Beveridge, J. M. R., W. F. Connell and G. A. Mayer 1957 The nature of the substances in dietary fat affecting the level of plasma cholesterol in humans. Canad. J. Biochem. Physiol., *35:* 257.
11. Beveridge, J. M. R., W. F. Connell, G. A. Mayer, H. L. Haust and M. White 1958 Plant sterols, degree of unsaturation, and hypocholesterolemic action of certain fats. Canad. J. Biochem. Physiol., *36:* 895.
12. Blomstrand, R., and E. H. Ahrens, Jr. 1958 Absorption of fats studied in a patient with chyluria. III. Cholesterol. J. Biol. Chem., *233:* 327.
13. Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1958 Standard Methods of Clinical Chemistry, vol. 2. Academic Press, Inc., New York, p. 26.
14. Haust, H. L., and J. M. R. Beveridge 1963 Type and quantity of 3 β-hydroxysterols excreted by subjects subsisting on formula rations high in corn oil. J. Nutrition, *81:* 13.
15. Kritchevsky, D., and S. A. Tepper 1961 The free and ester sterol content of various foodstuffs. J. Nutrition, *74:* 441.
16. Ahrens, E. H., Jr., J. Hirsch, W. Insull, Jr., T. T. Tsaltas, R. Blomstrand and M. L. Peterson 1957 The influence of dietary fats on serum lipid levels in man. Lancet, *1:* 943.
17. Armstrong, W. D., J. Van Pilsum, A. Keys, F. Grande, J. T. Anderson and L. Tobian 1957 Alteration of serum cholesterol by dietary fats. Proc. Soc. Exp. Biol. Med., *96:* 302.
18. Grande, F., J. T. Anderson and A. Keys 1958 Serum cholesterol in man and the unsaponifiable fraction of corn oil in the diet. Proc. Soc. Exp. Biol. Med., *98:* 436.
19. Beveridge, J. M. R., W. F. Connell, G. A. Mayer and H. L. Haust 1960 The response of man to dietary cholesterol. J. Nutrition, *71:* 61.


[6] Kuksis, A. 1961 Gas liquid chromatographic fractionation of plant sterol mixtures. Proc. Canad. Federation Biol. Soc., *4:* 38 (abstract).

# Comparative Effects of Certain Antioxidants on Gestational Performance and Teratogeny in Vitamin E-deficient Rats [1,2]

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*ABSTRACT* In an effort to elucidate the mechanism of function of vitamin E, many substitutes with antioxidant activities have been used. Many of these non-tocopherol substances, when fed to vitamin E-deficient animals, acted like a tocopherol in preventing, ameliorating or relieving deficiency symptoms. Three of these substances, N, N'-diphenyl-*p*-phenylenediamine (DPPD), N-propyl gallate (NPG), and 1, 2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (EMQ) were used to test their effects on the incidence of congenital malformations in vitamin E-deficient rats. It was found that all 3 antioxidants reduced the incidence of congenital abnormalities by promoting the production of normal young. Judged by the criteria of increase in the incidence of normal young with concurrent decrease in the incidence of resorption and the non-occurrence of congenital abnormalities in the groups fed the antioxidants in the rations at the 0.025% level in the absence of vitamin E, DPPD showed the most antioxidant potency. EMQ was the next, and NPG was the least potent of all. However, the gestational performances of the groups receiving either 2 or 10 mg of vitamin E in addition to the antioxidants at this level appears to indicate that EMQ was superior to DPPD. At 0.05% level, DPPD was shown to be superior to EMQ, as evidenced by the gestational performances of all groups of rats.

Thomas and Cheng (1) first reported congenital abnormalities associated with maternal vitamin E deficiency. Later experiments showed that normal young were produced after administration of a single dose of *d, l*-α-tocopheryl acetate to vitamin E-deficient rats on the fourth through the eighth day of gestation. When identical therapy was given on either the ninth, tenth, eleventh, or twelfth day of gestation, a considerable number of young showed a multiplicity of congenital malformations (2). A dose of vitamin on the tenth day of gestation produced the highest level of teratogeny. No noticeable shift of this level was observed, however, by increasing the therapeutic dosage of the vitamin from 1 to 4 mg,[3] but administration after the twelfth day resulted in resorption only.

Preliminary observations were made on the histological differences between the normal- and abnormal-term fetuses.[4] The distribution of glycogen, polymucosaccharides[5] and lipids[6] in the developing embryos was briefly reported. In addition, gross observations of abnormal embryos from the eleventh day of gestation onward were published (3). Arrested development characterized the 11-day-old embryos from the experimental group that had received a single dose of the vitamin on the tenth day of gestation. By the thirteenth day of gestation, exencephalus, when present, was clearly evident. Yet the incidence of congenital malformation was either reduced or eliminated by the administration of 4 mg progesterone or 2 μ estrone, continuously or by a single dose (4). The chemical determinations of the vitamin E content of a portion of the maternal and fetal tissues from the vitamin E-sufficient, and vitamin E-deficient groups, and the

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[1] Supported by grant no. RG-4993, U. S. Public Health Service.

[2] Presented in part at the First Annual Meeting of the Teratology Society at Cincinnati, Ohio, 1961.

[3] Cheng, D. W. 1954 A study of the occurrence of teratogeny in vitamin E-deficient rats and associated abnormalities in blood and tissues. Iowa State College J. Sci., *30:* 340 (abstract).

[4] Cheng, D. W., and B. H. Thomas 1955 Histological changes in the abnormal rat fetuses induced by maternal vitamin E deficiency. Anat. Rec., *121:* 274 (abstract).

[5] Cheng, D. W., and J. Scranton 1959 Distribution of glycogen in the developing vitamin E-sufficient, vitamin E-deficient and congenitally deformed embryos. Anat. Rec., *133:* 367 (abstract).

[6] Cheng, D. W., and L. F. Chang 1960 Distribution of lipid in the early vitamin E-sufficient, vitamin E-deficient and congenitally abnormal embryos and placentas. Anat. Rec., *136:* 312 (abstract).

TABLE 1
*Composition of rations*

| Ration | Dextrin 41% | Lard 22% | Vitamin-free casein [1] 18% | Brewer's yeast [2] 5% | Salt mixture [3] 4% | Cod liver oil 2% | DPPD [4] | | | NPG [5] | | | EMQ [6] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | % 0.025 | % 0.050 | % 0.075 | % 0.025 | % 0.10 | % 0.40 | % 0.0625 | % 0.0125 | % 0.025 | % 0.050 |
| Basal | + | + | + | + | + | + | | | | | | | | | | |
| A | + | + | + | + | + | + | + | | | | | | | | | |
| B | + | + | + | + | + | + | | + | | | | | | | | |
| C | + | + | + | + | + | + | | | + | | | | | | | |
| D | + | + | + | + | + | + | | | | + | | | | | | |
| E | + | + | + | + | + | + | | | | | + | | | | | |
| F | + | + | + | + | + | + | | | | | | + | | | | |
| L | + | + | + | + | + | + | | | | | | | + | | | |
| J | + | + | + | + | + | + | | | | | | | | + | | |
| I | + | + | + | + | + | + | | | | | | | | | + | |
| K | + | + | + | + | + | + | | | | | | | | | | + |

[1] From Nutritional Biochemicals Corporation, Cleveland.
[2] Obtained from Pabst Brewing Company, Milwaukee, Wisconsin.
[3] USP XIV.
[4] DPPD indicates N,N'-diphenyl-*p*-phenylenediamine.
[5] NPG indicates N-propyl gallate.
[6] EMQ indicates 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin).

groups marginally deficient in vitamin E, were reported (5). Also, sites of cholinesterase activity were briefly described.[7]

The data to be presented here deal with the effect of some chemical antioxidants, namely, N, N'-diphenyl-*p*-phenylenediamine (DPPD) N-propyl gallate (NPG) and 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (EMQ, also called ethoxyquin) on the gestational performance and teratogeny of vitamin E-deficient female rats. The investigation was, in a way, a further study of the effect of variation of rations on the incidence of teratogeny as reported previously by Cheng et al. (6).

## MATERIALS AND METHODS

For this investigation weanling female albino rats (Holtzman) weighing about 40 g were used. From the time of arrival until termination of gestation, these rats were fed one of the 11 rations used in this series of experiments (table 1). The chemical antioxidant, DPPD or NPG, was added in 3 percentage levels to the basal ration. Four levels were used when EMQ was employed. The chemical was mixed with melted lard before being incorporated with the other dry ingredients of the ration. The ration was prepared 2 weeks in advance of use and stored in a cold room. A supply sufficient for 2 weeks was prepared each time.

The females, at maturity (175 g), were mated with proven males in a special mating cage. The presence of sperm in the vaginal smear was taken as evidence of mating. The day of this event was labeled as the zero day of gestation. After mating, the females were randomly assigned to one of the 3 subgroups. Those in the negative control subgroup received no additional supplement of vitamin E to their diet during gestation. Those in the positive control subgroup received 10 mg of *d,l*-α-tocopheryl acetate in corn oil [8] administered by stomach tube at the rate of 2 mg/day for the first 5 days of gestation. Previous experiments demonstrated that this supplement provided an adequate positive control group (3). Those in the third experimental subgroup were given 2 mg of

[7] King, D. W., and M. Overturf 1963 Sites of cholinesterase activity in developing rat embryos. Anat. Rec., *145*: 330 (abstract).
[8] Mazola, Corn Products Company, Argo, Illinois.

vitamin E on the tenth day of gestation. This supplement to the basal diet produced female rats that were marginally deficient in vitamin E, and were thus capable of giving birth to some abnormal young. Nine groups of rats each received DPPD and NPG, and 12 groups received EMQ. In addition, female rats fed the basal vitamin E-deficient ration were similarly divided into 3 subgroups, the negative control, the positive control, and the experimental group. The gestational performance of these 3 groups of rats served as reference standards for the corresponding groups fed other rations. Altogether, 485 rats were used in the series of experiments reported here.

On the twenty-first day of gestation the females were killed by etherization. While the rat was still breathing, the complete uterus was removed and incised. The dead and resorbed fetuses were noted, and the live ones were examined under the dissecting binocular microscope for the presence of congenital abnormalities. The term, "dead" fetus, denotes a fetus that was completely intact and of the same size as the live, term fetus, but was dead. The "resorbed" fetuses varied considerably in size, but the common denominator was the partial or complete resorption.

The criteria of a good gestational performance were the high incidence of normal young, the absence of abnormal young, and the low incidence of dead young, or resportion, or of both.

The data were treated by either Student's *t* or the chi-square tests to ascertain the significance of the difference.

## RESULTS

1. *Experiments with DPPD.* When the rats fed the basal diet were examined, the negative controls showed 100% resorption, whereas the positive controls produced only normal young (table 2). The rats in the experimental group yielded normal young, abnormal young, dead fetuses and resorptions at 8.5, 15, 2 and 74.5%, respectively.

The gestational performances of the 3 groups of rats fed the basal ration containing 0.025% DPPD were unusual in many respects. The group receiving 0.025% DPPD and no additional vitamin E gave the best results, with 93.4% of normal young and 6.6% of resorption. In the group receiving 0.025% DPPD and 10 mg of vitamin E, the percentage of live normal young was reduced to 84, with a concomitant increase in the percentage of resorption to 16. The rats in the group

TABLE 2

*Effects of N,N'-diphenyl-p-phenylenediamine (DPPD) on gestational performance of vitamin E-deficient rats*

| Supplement to basal vitamin E-deficient ration | | Vitamin E given during gestation | No. rats bred | Live fetuses | | | Dead fetuses | |
|---|---|---|---|---|---|---|---|---|
| | | | | Normal | Abnormal | | Dead | Resorbed |
| | % | *mg* | | % [1] | % [1] | % [2] | % [1] | % [1] |
| DPPD | 0.025 | 0 | 17 | 93.4 | 0 | 0 | 0 | 6.6 |
| DPPD | 0.025 | 2 | 20 | 62.9 | 1.3 | 2.1 | 0 | 35.8 |
| DPPD | 0.025 | 10 | 12 | 84.0 | 0 | 0 | 0 | 16.0 |
| DPPD | 0.050 | 0 | 10 | 86.8 | 2.9 | 3.2 | 5.9 | 4.4 |
| DPPD | 0.050 | 2 | 21 | 86.3 | 0 | 0 | 1.9 | 11.8 |
| DPPD | 0.050 | 10 | 12 | 87.1 | 1.2 | 1.3 | 0 | 11.7 |
| DPPD | 0.075 | 0 | 9 | 80.0 | 0 | 0 | 0 | 20.0 |
| DPPD | 0.075 | 2 | 20 | 88.5 | 1.6 | 1.8 | 0.8 | 9.1 |
| DPPD | 0.075 | 10 | 9 | 86.3 | 0 | 0 | 0 | 13.7 |
| None | | 0 [3] | 24 | 0 | 0 | 0 | 0 | 100.0 |
| None | | 2 [4] | 20 | 8.5 | 15.0 | 63.8 | 2.0 | 74.5 |
| None | | 10 [5] | 19 | 100.0 | 0 | 0 | 0 | 0 |

[1] In terms of total number of implantation sites.
[2] In terms of total number of viable young.
[3] Negative control with basal E-deficient diet.
[4] Experimental group with basal E-deficient diet.
[5] Positive control with basal E-deficient diet.

receiving 0.025% DPPD and 2 mg of vitamin E gave birth to some abnormal young (1.3%), and yielded the lowest percentage of normal young (62.9%) with the highest incidence of resorption (35.8%). When these 3 groups were compared with one another, except for the significant difference between the negative control and the experimental groups concerning resorption, no other significant difference was observed between any 2 groups with respect to either normal young or resorption.

From the standpoint of teratogeny, the results from the 3 groups of rats fed the basal ration containing 0.05% DPPD were unexpected in that some of the rats from both the positive and the negative controls produced abnormal young, whereas the rats of the experimental group yielded none. The percentages of normal young were about the same in all 3 groups. Again, as in the rats fed the ration with 0.025% DPPD, the incidence of resorption was lowest in the negative group when compared with the other 2 groups. There was no significant difference between any 2 groups as far as the normal young and resorption were concerned.

The rats in the group receiving 0.075% DPPD in the absence of vitamin E gave the lowest percentage of normal young (80%) and the highest incidence of resorption (20%), compared with similar groups fed rations containing 0.025 and 0.05% DPPD. When vitamin E was given at both the 2- and 10-mg levels, its beneficial effect was demonstrated by the increased percentages of normal young (to 88.5 and 86.3%, respectively), with a concurrent decrease in resorption (to 9.1 and 13.7%, respectively). However, none of the differences were significant. The abnormal young occurred in the experimental group only.

Compared with the negative group fed the basal ration, those groups that received rations containing 0.025, 0.05 and 0.075% DPPD and no added vitamin E showed a significant increase in the incidence of normal young with concomitant reduction in the incidence of resorption. On the whole, the experiments with DPPD showed that the percentage of abnormal young in the experimental groups was significantly reduced because of the great increases in the incidence of normal young.

2. *Experiments with NPG.* As in rats receiving 0.05% DPPD without added vitamin E, the occurrence of congenital abnormalities was observed in the offspring of the rats receiving 0.025% NPG and no vitamin E (table 3). With supplementation of vitamin E, at either 2- or 10-mg levels, to rats fed the ration containing 0.025% NPG, there was a significant increase in the percentage of normal young

TABLE 3

*Effects of N-propyl gallate (NPG) on gestational performance of vitamin E-deficient female rats*

| Supplement to basal vitamin E-deficient ration | | Vitamin E given during gestation | No. rats bred | Live fetuses | | | Dead fetuses | |
|---|---|---|---|---|---|---|---|---|
| | | | | Normal | Abnormal | | Dead | Resorbed |
| | % | *mg* | | % [1] | % [1] | % [2] | % [1] | % [1] |
| N-propyl gallate | 0.025 | 0 | 10 | 0 | 1.3 | 100 | 0 | 98.7 |
| N-propyl gallate | 0.025 | 2 | 22 | 18.7 | 0 | 0 | 2.4 | 78.9 |
| N-propyl gallate | 0.025 | 10 | 10 | 97.5 | 0 | 0 | 0 | 2.5 |
| N-propyl gallate | 0.10 | 0 | 10 | 13.6 | 0 | 0 | 1.2 | 85.2 |
| N-propyl gallate | 0.10 | 2 | 20 | 20.9 | 5.7 | 21.4 | 2.5 | 70.9 |
| N-propyl gallate | 0.10 | 10 | 10 | 90.5 | 0 | 0 | 0 | 9.5 |
| N-propyl gallate | 0.4 | 0 | 11 | 1.2 | 0 | 0 | 1.2 | 97.7 |
| N-propyl gallate | 0.4 | 2 | 21 | 49.3 | 4.2 | 7.8 | 0 | 46.5 |
| N-propyl gallate | 0.4 | 10 | 10 | 89.9 | 0 | 0 | 0 | 10.1 |
| None | | 0 | 24 | 0 | 0 | 0 | 0 | 100.0 |
| None | | 2 | 20 | 8.5 | 15.0 | 63.8 | 2.0 | 74.5 |
| None | | 10 | 19 | 100.0 | 0 | 0 | 0 | 0 |

[1] In terms of total number of implantation sites.
[2] In terms of total number of viable young.

(from 0 to 18.7 or 97.5%, respectively), with a concurrent significant decrease in the percentage of resorption (from 98.7 to 78.9 or 2.5%, respectively). Also, in the group receiving 0.025% NPG and 10 mg vitamin E, the incidence of normal young was approximately the same as that of the corresponding group fed the basal ration.

On the whole, the supplementation of vitamin E to the rats receiving the ration containing 0.10% NPG gave similar results as those obtained with rats fed the ration with 0.025% NPG. Congenital abnormalities occurred only in the offspring of rats receiving the 2-mg vitamin E supplementation. The incidence of anomalies, however, was approximately one-third of that observed in corresponding rats fed the basal ration. With respect to the incidences of normal young and resorption, the differences between the positive and the negative controls, and the positive controls and the experimental group were statistically significant.

Similarly, the rats fed the ration containing 0.40% NPG and no added vitamin E gave a gestational performance corresponding to that of rats fed the basal ration, except for the minimal production of normal young. The group receiving 0.40% NPG and 10 mg of vitamin E gave the same degree of performance as the corresponding group reciving 0.10% NPG. Also, rats receiving 0.40% NPG responded to the supplementation of 2 mg of vitamin E differently from those fed the ration with 0.10% NPG. Here, besides the occurrence of congenital malformations (4.2%), there was a significant increase in the percentage of normal young (from 20.9 to 49.3%), with a concomitant significant lowering of the incidence of resorption (from 70.9 to 46.5%). Thus the differences in the incidence of normal young and resorption between the positive and negative controls, positive control and experimental group, and the negative control and experimental group fed this ration with 0.40% NPG were all significant statistically.

When the groups receiving the 3 levels of NPG without added vitamin E were compared with the corresponding group fed the basal ration, the beneficial effect of the 0.10% NPG in the ration became evident, since there was a production of 13.6% normal young instead of 0%, with a concurrent decrease in the incidence of resorption (from 100 to 85.2%, table 3). Comparing all the experimental groups fed the rations containing NPG with the corresponding group fed the basal ration, insofar as the normal live young were concerned, NPG at the levels of 0.025 and 0.10% improved the yield more than twofold, whereas at the level of 0.40%, it improved the yield nearly sixfold. Comparing all the positive controls that received NPG, there was a reduction in the incidence of normal young, together with an increase in the incidence of resorption when the level of NPG was increased from 0.025 to either 0.10 or 0.40%.

3. *Experiments with EMQ (ethoxyquin).* When the gestational performance of the 3 groups of rats fed the ration containing 0.00625% EMQ was examined, there was a slight increase in the percentage of the normal young from a value of 64.6% in the negative controls to 68.2% in the experimental group (table 4). A further increase to 91.8% was observed in the positive controls. However, no significant difference was found between any 2 groups fed this ration as far as the incidence of normal young was concerned. Yet, 0.8% abnormal young was born to rats in the negative controls, but no abnormal young were born to rats in the experimental group. The percentage, then, of resorption was non-significantly reduced in the experimental group, but was significantly decreased in the positive controls when compared with the negative controls. The difference in resorption between the positive controls and the experimental group was also statistically significant.

From the standpoint of the increase in the incidence of normal young and the decrease in the incidence of resorption, the gestational performance of the negative controls and the experimental group fed the ration containing 0.0125% EMQ was slightly better than that of rats that received the ration with 0.00625% EMQ. However, the positive controls fed the ration with 0.0125% EMQ did not do as well as those fed the ration with 0.00625% EMQ, due to the higher incidence of resorption. Also, abnormal young were produced by the positive controls. As far as

TABLE 4

*Effect of ethoxyquin (EMQ) on gestational performance of vitamin E-deficient rats*

| Supplement to basal vitamin E-deficient ration | | Vitamin E given during gestation | No. rats bred | Live fetuses | | | Dead fetuses | |
|---|---|---|---|---|---|---|---|---|
| | | | | Normal | Abnormal | | Dead | Resorbed |
| | % | mg | | % [1] | % [1] | % [2] | % [1] | % [1] |
| EMQ | 0.00625 | 0 | 14 | 64.4 | 0.8 | 1.3 | 1.7 | 33.1 |
| EMQ | 0.00625 | 2 | 13 | 68.2 | 0 | 0 | 1.0 | 30.8 |
| EMQ | 0.00625 | 10 | 11 | 91.8 | 0 | 0 | 2.3 | 5.9 |
| EMQ | 0.0125 | 0 | 18 | 70.3 | 0 | 0 | 0.5 | 29.2 |
| EMQ | 0.0125 | 2 | 20 | 82.6 | 0.5 | 0.6 | 0 | 16.9 |
| EMQ | 0.0125 | 10 | 17 | 88.1 | 0.7 | 0.7 | 0 | 11.2 |
| EMQ | 0.025 | 0 | 15 | 65.0 | 1.4 | 2.2 | 2.1 | 31.5 |
| EMQ | 0.025 | 2 | 14 | 80.2 | 0.8 | 1.0 | 1.7 | 17.3 |
| EMQ | 0.025 | 10 | 13 | 97.0 | 0 | 0 | 1.4 | 1.6 |
| EMQ | 0.050 | 0 | 10 | 78.9 | 2.2 | 2.7 | 4.4 | 14.5 |
| EMQ | 0.050 | 2 | 13 | 75.5 | 0.9 | 1.2 | 0 | 23.6 |
| EMQ | 0.050 | 10 | 10 | 78.8 | 1.2 | 2.6 | 0 | 20.0 |
| None | | 0 | 24 | 0 | 0 | 0 | 0 | 100.0 |
| None | | 2 | 20 | 8.5 | 15.0 | 63.8 | 2.0 | 74.5 |
| None | | 10 | 19 | 100.0 | 0 | 0 | 0 | 0 |

[1] In terms of total number of implantation sites.
[2] In terms of total number of viable young.

the normal young were concerned, no significant difference between any 2 groups fed the ration with 0.0125% EMQ was observed. Nevertheless, with respect to resorption, the difference between the positive and the negative controls was significant statistically.

The 3 groups of rats fed the ration containing 0.025% EMQ showed the best gestational performance, in that there was a large increase in the percentage of normal young in the positive controls. The differences, then, in the incidence of normal young between the positive and negative controls and the positive controls and the experimental group were both significant. The only inconsistency was the appearance of abnormal young in the negative controls fed this ration, as was observed in the corresponding group fed the ration with 0.00625% EMQ.

The results from the 3 groups of rats receiving 0.05% EMQ were unique. First, the incidence of normal young was approximately the same, regardless of whether vitamin E was administered during gestation. Second, abnormal young were found in all 3 groups. Third, the incidence of resorption of both the positive controls and the experimental group was non-significantly higher than that of the negative controls.

Furthermore, when all the negative controls that received rations containing EMQ were compared both with each other and with the group receiving the basal ration, the rats receiving 0.05% EMQ showed the highest incidence of normal young and the lowest incidence of resorption. When the absence of abnormal young was used as the criterion of best gestational performance, then the group receiving 0.0125% EMQ and no added vitamin E showed the best results. When all the positive controls were examined, the rats fed the ration with 0.025% EMQ presented the best performance by having the highest incidence of normal young and the lowest incidence of resorption. From the standpoint of non-occurrence of abnormal young, then, the positive controls fed the rations containing 0.00625% and 0.025% EMQ exhibited the best results. When all the experimental groups were compared from the standpoint of absence of congenital malformations in the young, the group receiving 0.00625% EMQ produced the best results.

4. *Comparison of the efficacy of DPPD, NPG and EMQ as antioxidants.* A. At

TABLE 5

*Comparative efficacy of N,N'-diphenyl-p-phenylenediamine (DPPD), N-propyl gallate (NPG) and ethoxyquin (EMQ) as antioxidants*

| Supplement to basal vitamin E-deficient ration | | Vitamin E given during gestation | No. rats bred | Live fetuses | | | Dead fetuses | |
|---|---|---|---|---|---|---|---|---|
| | | | | Normal | Abnormal | | Dead | Resorbed |
| | % | mg | | % [1] | % [1] | % [2] | % [1] | % [1] |
| DPPD | 0.025 | 0 | 17 | 93.4 | 0 | 0 | 0 | 6.6 |
| NPG | 0.025 | 0 | 10 | 0 | 1.3 | 100.0 | 0 | 98.7 |
| EMQ | 0.025 | 0 | 15 | 65.0 | 1.4 | 2.2 | 2.1 | 31.5 |
| DPPD | 0.025 | 2 | 20 | 62.9 | 1.3 | 2.1 | 0 | 35.8 |
| NPG | 0.025 | 2 | 22 | 18.7 | 0 | 0 | 2.4 | 78.9 |
| EMQ | 0.025 | 2 | 14 | 80.2 | 0.8 | 1.0 | 1.7 | 17.3 |
| DPPD | 0.025 | 10 | 12 | 84.0 | 0 | 0 | 0 | 16.0 |
| NPG | 0.025 | 10 | 10 | 97.5 | 0 | 0 | 0 | 2.5 |
| EMQ | 0.025 | 10 | 13 | 97.0 | 0 | 0 | 1.4 | 1.6 |
| DPPD | 0.05 | 0 | 10 | 86.8 | 2.9 | 3.2 | 5.9 | 4.4 |
| EMQ | 0.05 | 0 | 10 | 78.9 | 2.2 | 2.7 | 4.4 | 14.5 |
| DPPD | 0.05 | 2 | 21 | 86.3 | 0 | 0 | 1.9 | 11.8 |
| EMQ | 0.05 | 2 | 13 | 75.5 | 0.9 | 1.2 | 0 | 23.6 |
| DPPD | 0.05 | 10 | 12 | 87.1 | 1.2 | 1.3 | 0 | 11.7 |
| EMQ | 0.05 | 10 | 10 | 78.8 | 1.2 | 2.6 | 0 | 20.0 |

[1] In terms of total number of implantation sites.
[2] In terms of total number of viable young.

0.025% level. When the gestational performance of the negative controls was examined, rats that received DPPD displayed the best results, giving the highest incidence of normal young and the lowest incidence of resorption (table 5). Those receiving EMQ ranked second, whereas those given NPG were the poorest. For this reason, much higher levels of NPG were tested in succeeding experiments (table 3). When the experimental groups were observed, the group receiving EMQ was found to be the best, that receiving DPPD was second, whereas that given NPG was last. Furthermore, when the positive controls were compared, the groups receiving EMQ and NPG both showed better performance than that given DPPD, in terms of higher incidence of normal young and lower incidence of resorption.

B. At 0.05% level. At this level only DPPD and EMQ could be compared (table 5). The gestational performance of all groups of rats indicated that DPPD was better than EMQ insofar as the increase in the incidence of normal young, the reduction in the incidence of resorption and the non-occurrence of the abnormal young in the experimental groups were concerned. However, the positive and the negative controls fed both rations produced abnormal young.

## DISCUSSION

The biochemistry of vitamin E has been reviewed by Vasington et al. (7), and Pitt and Morton (8), and more recent observations have been reported by Wiss et al. (9). It has been known for some time that vitamin E plays a role as an antioxidant (10–13), and the effects of chemical antioxidants on vitamin E deficiency have been reviewed by Dam (14). Experiments by many investigators (15–32) have shown that DPPD can act as a partial substitute for vitamin E. Likewise, Shull et al. (22,23), Mertz and Schwarz (33), Machlin et al. (34), Schwarz (25), Crider et al. (31), Bunyan et al. (32) and Wiss et al. (9) have noted that EMQ can replace vitamin E to a certain extent. Few people, however, have tested NPG as a substitute for vitamin E, except Schwarz and his group (25, 33), and Telford et al. (35). No definite theory regarding the relationship between tocopherol and a large num-

ber of synthetic chemical antioxidants has been established. However, the generally accepted theory has been the sparing action of the antioxidants on vitamin E, which might still be either in the deficient diet or tenaciously held in the body tissues (36), although this theory has been challenged lately (37, 38).

Recently, Wiss et al. (9), in an attempt to learn the differences in biological activity between vitamin E and EMQ, performed experiments to determine the amounts of both substances in the various organs at various time intervals following the administration of $C^{14}$-labeled *d,l*-α-tocopheryl acetate and EMQ. The results showed that vitamin E was absorbed rather slowly, whereas EMQ was absorbed rapidly. However, high levels of vitamin E remained for longer periods in the tissues, whereas EMQ was eliminated rapidly from the body (39). The bulk of the tocopherol was located in the mitochondria and microsome fractions of the liver, but EMQ was located primarily in the supernatant solution rather than the structural parts of the cells.

Our experiments were started in 1959 and lasted for more than 2 years. The deleterious effect of higher levels of DPPD (21) appears to be borne out by our results which showed a reduction in the percentage of normal young in rats fed the vitamin E-deficient ration containing 0.075% DPPD in the absence of added vitamin E, when compared with the corresponding groups fed rations containing 0.025 and 0.05% DPPD (table 2). The beneficial effect of vitamin E supplementation, as judged by the increase in the incidence of normal young and the decrease in the incidence of resorption, was shown only in the rats receiving this high level of DPPD (0.075% ). The increase in the incidence of resorption, in the rats fed diets supplemented with 10 mg of vitamin E, over those of the groups receiving no vitamin E supplement in rations containing 0.025 and 0.05% DPPD, might indicate not only a non-sparing action of DPPD but also an antagonistic action between DPPD and vitamin E under the circumstances. This premise may also explain the "non-consistent" responses, observed by Bunnell et al. (16), of lower values for liver and plasma tocopherol, live weight, and liver lipid in chicks receiving both DPPD and vitamin E than in those in the vitamin E-deficient chicks receiving DPPD alone.

The rats in the group receiving 0.025% DPPD in the absence of vitamin E produced the most live normal young compared with all the other groups fed DPPD (table 2). This indicates that this level was the best to be incorporated into the basal ration as a replacement for vitamin E under the conditions of the experiments.

The reason for the occurrence of abnormal young in the offspring of rats fed the rations containing 0.05% DPPD, 0.025% NPG, and 0.00625, 0.025 and 0.05% EMQ and no added vitamin E was probably that, because of their sparing action on vitamin E, the incorporation of DPPD, NPG and EMQ into the vitamin E-deficient rations at the specified levels increased the vitamin E content of the body tissues of the rats to the marginal level where teratogenesis was possible. The appearance of some abnormal young in the groups receiving rations containing 0.05% DPPD, 0.0125, and 0.05% EMQ plus 10 mg of vitamin E might also be another manifestation of the antagonism between DPPD, EMQ and vitamin E under these conditions.

The results of our experiments with vitamin E-deficient rations containing different levels of DPPD, NPG and EMQ generally indicated a reduction of teratogeny due to maternal vitamin E deficiency. The reductions in these cases, however, were due to increases in the number of normal young rather than to increases in the number of resorptions, as were observed in our previous experiments with estrone and progesterone (4) and γ-tocopherol.[9]

Our data from the groups receiving DPPD, NPG and EMQ in the absence of vitamin E also substantiated the observations of Schwarz (25) concerning the relative potency of these 3 chemical antioxidants as substitutes for vitamin E. In general, DPPD was most potent, EMQ was intermediate, and NPG was the least potent of all.

[9] Cheng, D. W., and S. Subbammal 1959 Effect of gamma-tocopherol on the incidence of teratogeny due to maternal vitamin E deficiency. J. Animal Sci., *18*: 1532 (abstract).

Vitamin E and the other antioxidants function by donating hydrogen to a lipid peroxyl radical and breaking the chain reaction of peroxidation (40). They protect the animals from the damage to the cellular and subcellular membranes and widespread metabolic derangements. It would be worthwhile to study the changes in the liver mitochondria, microsomes, and lysosomes of the different groups of rats used in this series of experiments, in order to correlate these results with teratogenesis.

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LITERATURE CITED

1. Thomas, B. H., and D. W. Cheng 1952 Congenital abnormalities associated with vitamin E malnutrition. Proc. Iowa Acad. Sci., *59:* 218.
2. Cheng, D. W., and B. H. Thomas 1953 Relationship of time of therapy to teratogeny in maternal avitaminosis E. Proc. Iowa Acad. Sci., *60:* 290.
3. Cheng, D. W., L. F. Chang and T. A. Bairnson 1957 Gross observations on developing abnormal embryos induced by maternal vitamin E deficiency. Anat. Rec., *129:* 167.
4. Cheng, D. W. 1959 Effect of progesterone and estrone on the incidence of congenital malformations due to maternal vitamin E deficiency. Endocrinology, *64:* 270.
5. Cheng, D. W., K. G. Braun, B. J. Braun and K. H. Udani 1961 Tocopherol content of maternal and fetal rat tissues as related to vitamin E intake during gestation. J. Nutrition, *74:* 111.
6. Cheng, D. W., T. A. Bairnson, A. M. Rao and S. Subbammal 1960 Effect of variations of rations on the incidence of teratogeny in vitamin E deficient rats. J. Nutrition, *71:* 54.
7. Vasington, F. D., S. M. Reichard and A. Nason 1960 Biochemistry of vitamin E. Vitamins and Hormones, *18:* 43.
8. Pitt, G. A. J., and R. A. Morton 1962 Fat-soluble vitamins. Ann. Rev. Biochem., *31:* 491.
9. Wiss, O., R. H. Bunnell and U. Gloor 1962 Absorption and distribution of vitamin E in the tissues. Vitamins and Hormones, *20:* 441.
10. Hove, R. L., and Z. Hove 1944 The effect of temperature on the relative antioxidant activity of alpha-, beta- and gamma-tocopherols and of gossypol. J. Biol. Chem., *156:* 623.
11. Dam, H. 1953 Vitamin E as an in vivo antioxidant. Experientia (Supp.), *1:* 195.
12. Bro-Rasmussen, F., and W. Hjarde 1961 Fat-soluble vitamins. Ann. Rev. Biochem., *30:* 447.
13. Horwitt, M. K. 1961 Vitamin E in human nutrition — an interpretative review. Borden's Rev. Nutrition Res., *22:* 1.
14. Dam, H. 1957 Influences of antioxidants and redox substances on signs of vitamin E deficiency. Pharmacol. Rev., *9:* 1.
15. Bunnell, R. H., L. D. Matterson, E. P. Singsen, L. M. Potter, A. Kozeff and E. L. Jungherr 1955 Studies on encephalomalacia in the chick. 3. The influence of feeding or injecting various tocopherols or other antioxidants on the incidence of encephalomalacia. Poultry Sci., *33:* 1068.
16. Bunnell, R. H., L. D. Matterson, E. P. Singsen and H. D. Eaton 1956 Studies on encephalomalacia in the chick. 5. The effect of fish oil and diphenyl-*p*-phenylenediamine on the vitamin E metabolism of the chick. Poultry Sci., *35:* 436.
17. Scott, M. L., F. W. Hill, L. C. Norris, D. C. Dobson and T. S. Nelson 1955 Studies on vitamin E in poultry nutrition. J. Nutrition, *56:* 387.
18. Singsen, E. P., R. H. Bunnell, L. D. Matterson, A. Kozeff and E. L. Jungherr 1955 Studies on encephalomalacia in the chick. 2. The protective action of diphenyl-*p*-phenylenediamine against encephalomalacia. Poultry Sci., *34:* 262.
19. Ames, S. R., M. I. Ludwig, W. J. Swanson and P. L. Harris 1956 Effect of DPPD, MB, BHT and hydroquinone on reproductive process in the rat. Proc. Soc. Exp. Biol. Med., *93:* 39.
20. Jensen, L. S., J. S. Carver and J. McGinnis 1956 Effects of vitamin E, N, N'-diphenyl-*p*-phenylenediamine and fish liver oil on reproduction in turkeys. Proc. Soc. Exp. Biol. Med., *91:* 386.
21. Draper, H. H., S. Goodyear, K. D. Barbee and B. C. Johnson 1958 A study of the nutritional role of antioxidants in the diet of the rat. Brit. J. Nutrition, *12:* 89.
22. Shull, R., R. B. Alfin-Slater, H. J. Deuel, Jr. and B. H. Ershoff 1957 Comparative effects of alpha-tocopherol, DPPD and other antioxidants on muscular dystrophy in guinea pig. Proc. Soc. Exp. Biol. Med., *95:* 263.
23. Shull, R., B. H. Ershoff and R. B. Alfin-Slater 1958 Effects of antioxidants on muscle and plasma lipids of vitamin E-deficient guinea pigs. Proc. Soc. Exp. Biol. Med., *98:* 364.
24. Tucker, R. G., S. M. Greenberg, A. E.Heming and J. K. Mathues 1957 Iron absorption and metabolism. II. Substitution of N, N'-diphenyl-*p*-phenylenediamine (DPPD) for vitamin E. J. Nutrition, *63:* 33.
25. Schwarz, K. 1958 Effect of antioxidants on dietary necrotic liver degeneration. Proc. Soc. Exp. Biol. Med., *99:* 20.
26. Sharman, I. M., and T. Moore 1958 The partial vitamin E activity of N, N'-diphenyl-*p*-phenylenediamine in the rat. Biochem. J., *69:* 61P.

27. Corwin, L. M., and K. Schwarz 1960 Prevention of decline of alpha-ketoglutarate and succinate oxidation in vitamin E deficient rat liver homogenates. J. Biol. Chem., 235: 3387.
28. Draper, H. H., and A. S. Csallany 1958 Action of N, N'-diphenyl-*p*-phenylenediamine in tocopherol deficiency diseases. J. Biol. Chem., *99:* 739.
29. Draper, H. H. 1959 Biopotency of non-tocopheral compounds in vitamin E deficiency diseases. Proc. Soc. Exp. Biol. Med., *102:* 737.
30. Csallany, A. S., and H. H. Draper 1961 On the occurrence of vitamin E in the liver of dystrophic and antioxidant-fed rabbits. Arch. Biochem. Biophys., 92: 462.
31. Crider, Q., P. Alaupovic and B. C. Johnson 1961 On the function and metabolism of vitamin E. III. Vitamin E and antioxidants in the nutrition of the rats. J. Nutrition, *73:* 64.
32. Bunyan, J., A. T. Diplock, E. E. Edwin and J. Green 1962 Exudative diathesis and lipid peroxidation in the chick. Brit. J. Nutrition, *16:* 519.
33. Mertz, W., and K. Schwarz 1958 Reversal of respiratory decline in necrotic liver degeneration by intraportal antioxidants. Proc. Exp. Biol. Med., *98:* 808.
34. Machlin, L. J., R. S. Gordon and K. H. Meisky 1959 The effect of antioxidants on vitamin E deficiency symptoms and production of liver "peroxide" in the chicken. J. Nutrition, 67: 333.
35. Telford, I. R., C. S. Woodruff and R. H. Linford 1962 Fetal resorption in the rat as influenced by certain antioxidants. Am. J. Anat., *110:* 29.
36. Harris, P. L., and K. E. Mason 1955 Vitamin E and metabolic processes in vitamin E. Atti del Terzo Congresso Internazionale, Venezia, p. 1.
37. Schwarz, K. 1961 A possible site of action for vitamin E in intermediary metabolism. Am. J. Clin. Nutrition, *9:* 71.
38. Diplock, A. T., E. E. Edwin, J. Bunyan and J. Green 1961 The effect of tocopherol, vitamin A, selenium and antioxidants on ubiquinone in the rat. Brit. J. Nutrition, *15:* 425.
39. Wilson, R. H., J. O. Thomas, C. R. Thompson, H. F. Launer and G. O. Kohler 1959 Absorption, metabolism and excretion of antioxidant 6-ethoxyl-1,2-dihydro-2, 2,4-trimethylquinoline. Agr. Food Chem., 7: 206.
40. Tappel, A. L. 1962 Vitamin E as the biological lipid antioxidant. Vitamins and Hormones, *20:* 493.

# Effect of Vitamins $D_2$ and $D_3$ on Serum Calcium and Phosphorus in Rachitic Chicks [1]

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*ABSTRACT* The experimental schedule presented in this paper provides 3 independent quantitative criteria by which antirachitic effectiveness can be evaluated. Crystalline vitamins $D_2$ and $D_3$ were given orally to chicks fed a rachitogenic diet, and a variety of parameters were evaluated after 28 days. Chicks fed the rachitogenic diet exhibited low serum calcium concentrations and percentage bone ash, but had significantly higher serum phosphorus levels than chicks fed a standard chick diet or treated adequately with either form of vitamin D. Their serum phosphorus response was therefore unlike that reported for most previously studied experimental animals. Dose-response curves based on the 3 parameters indicated a flatter response to vitamin $D_2$ than to vitamin $D_3$ and therefore any efficacy ratio calculated from data of this nature would be a function of the degree of healing taken as an endpoint. When based on $CD_{50}$ (curative dose giving a response midway between animals on rachitogenic and standard chick diets), the vitamin $D_3$-to-$D_2$ efficacy ratio was estimated at about 8:1-to-11:1. Pronounced subperiosteal rarefaction was observed in the microradiographs of tibia from chicks fed the rachitogenic diet.

Many different criteria have been used in the biological assay of the antirachitic effectiveness of the various vitamin D substances (1). These include assays utilizing subjective endpoints (namely, the line test in rats), as well as those based on experimentally measured dynamic units, as in the determination of bone deposition by percentage ash weight or $P^{32}$ uptake.

Although bone ash weight has been firmly established for the assay of vitamin D in chicks, there is a paucity of data concerning changes in serum calcium and phosphorus during such a bioassay. Some of the data have been rather puzzling; they have indicated, for example, practically no increase in serum calcium of chicks treated with cod liver oil in comparison with rachitic controls at a time when bone ash was back to normal, and in the same experiment, a significant serum calcium and phosphorus increase in chicks treated with irradiated ergosterol which showed minimal bone ash changes (2).

It is such seemingly contradictory evidence as this which prompted us to reevaluate the effects of vitamins $D_2$ and $D_3$ in chicks, with special attention to their serum calcium and phosphorus levels, and also to analysis of bone ash, body weights and the histological, radiographic and microradiographic appearance of their bones. A primary purpose of this study was to evaluate the feasibility of assessing the antirachitic effectiveness of various preparations containing vitamin D in relatively small groups of animals by several independent parameters. In these experiments, crystalline vitamins $D_2$ and $D_3$ were administered to chicks fed a rachitogenic diet, by daily oral intubation to achieve precise dosing on a "preventive" basis.

## METHODS

One-day-old male White Leghorn chicks (Mt. Hope Queens) were placed in wire cages in groups of five or six. Feeding with the rachitogenic diet and distilled water ad libitum was instituted immediately. The temperature of the brooder was set at 30°C for the first week, 27°C the second week and 24°C thereafter.

Extreme care was taken to exclude all irradiation that might possibly promote production of antirachitic substances in the chick. Windows and the interior lighting of the animal quarters were completely covered by a red cellophane material

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(which was analyzed on the Beckman DK-2 spectrophotometer to have less than 1% transmission between 220–290 mμ).

Except for a group maintained with a standard chick diet,[2] all birds were fed a commercial "rachitogenic chick test diet."[3] The composition of this diet was originally formulated by Olsson and is given in the publication by Jones and Elliot (3). 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).

Crystalline vitamin $D_2$ and vitamin $D_3$[4] were dissolved in sesame oil in quantities such that each daily dose was contained in a volume of 0.1 ml.

Purity of the vitamins was checked by determining the molar extinction at 265 mμ and checking the ultraviolet absorption spectra of fractions eluted from Fluoropak 80 chromatographic (4) columns. No impurities were detected by these methods. Each chick was intubated daily with 0.1 ml of oil administered into the stomach through a blunt tipped no. 18-gauge needle. The vitamin solutions were stored at 0°C between intubation periods.

At the end of 28 days, each chick was decapitated and its blood collected from the neck. One milliliter of serum and 9 ml of 10% trichloroacetic acid were mixed and the mixture was centrifuged. The trichloroacetic acid filtrate was analyzed for calcium by titration with EDTA in the presence of calcein following oxalate precipitation and ignition to calcium oxide (5), and for inorganic phosphorus by reduction of phosphomolybdate by ascorbic acid (6).

Both legs were removed, and one from each bird was X-rayed immediately. The tibia of each leg was then dissected and a section of one tibia was fixed in ethanol and subsequently imbedded in methacrylate for microradiographic examination. A proximal end was split and fixed in neutral formalin for histological preparation. The other tibia from each bird was split and the bones from each group placed together in a Soxhlet thimble, extracted for 48 hours with absolute ethanol and for an equal period with diethyl ether. The bones were dried in platinum evaporating dishes at 100°C and ashed in a muffle furnace at 750°C for 16 hours. The percentage of ash weight was determined for each group of tibiae.

## RESULTS

By 28 days, severe rickets was visibly obvious in all control chicks fed the rachitogenic diet. The chicks were debilitated in appearance and could barely stand, preferring to squat rather than use their legs for support.

*Histological sections and X-ray appearance.* Stained slides of the tibia of each chick and radiographs clearly indicated severe rickets in control animals and various stages of healing or a normal appearance in vitamin D-treated birds or those fed the standard diet.

*Body weight.* No differences in growth rate as measured by body weight could be observed between birds fed the standard diet compared with those fed the rachitogenic diet until a period of 14 days had elapsed. Growth of the chicks fed the rachitogenic diet was essentially linear from 14 to 28 days, whereas with adequate vitamin D, body weights were significantly greater at 28 days than weights of deficient animals. Growth data for the various groups of chicks are shown in table 1.

*Microradiographic appearance.* Microradiographs of a cross section through the tibia are illustrated in figure 1. In rachitic chick bone, rarefaction was typically most pronounced in the outer cortex just under the periosteum with a much greater degree of mineralization in the endosteal inner portion of the cortex. This appearance, as far as we are aware, has never been previously reported for rachitic bone.

*Bone ash.* The percentage bone ash for the various groups of chicks is plotted against the log dose of vitamin D in figure 2A. Excellent differentiation can be observed between rachitic control chicks (31.8% bone ash) and those fed the standard chick diet (46.8% bone ash).

[2] Purina Chick Growena, Ralston Purina Company, St. Louis.
[3] General Biochemicals Inc., Chagrin Falls, Ohio.
[4] Manufactured by Philips-Duphar, Weesp, Netherlands.

TABLE 1

*Chick growth data*

| Group | Body weight | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 |
| | *g* | *g* | *g* | *g* | *g* |
| Standard chick diet, control | 39.6 ± 0.51 [1] | 63.0 ± 1.78 | 113.8 ± 4.16 | 198.6 ± 8.55 | 287.5 ± 9.74 |
| Rachitogenic diet, control | 38.6 ± 0.40 | 62.0 ± 1.30 | 106.6 ± 2.25 | 149.4 ± 2.50 | 174.8 ± 6.48 |
| Rachitogenic diet + vitamin $D_2$ | | | | | |
| +30 IU/day | 39.8 ± 0.65 | 61.5 ± 2.32 | 108.5 ± 5.60 | 179.1 ± 8.65 | 236.3 ± 4.00 |
| +60 IU/day | 41.6 ± 3.53 | 62.8 ± 2.15 | 103.2 ± 4.78 | 167.0 ± 9.16 | 234.6 ± 7.35 |
| +300 IU/day | 40.4 ± 2.56 | 66.0 ± 3.56 | 116.0 ± 6.41 | 187.0 ± 10.29 | 257.6 ± 8.60 |
| Rachitogenic diet + vitamin $D_3$ | | | | | |
| +1 IU/day | 38.7 ± 1.20 | 63.8 ± 1.66 | 105.5 ± 2.40 | 150.7 ± 5.91 | 176.0 ± 4.00 |
| +3 IU/day | 42.0 ± 1.58 | 60.2 ± 6.52 | 121.5 ± 7.13 | 182.5 ± 11.81 | 224.0 ± 12.6 |
| +6 IU/day | 37.4 ± 1.75 | 60.2 ± 0.97 | 106.6 ± 3.82 | 177.2 ± 4.87 | 263.6 ± 4.36 |

[1] Mean ± SE.

All values for bone ash of the vitamin D-treated birds fell between these values.

*Serum calcium.* Using the same abscissa as in the above mentioned figure and graphed directly below the percentage bone ash are shown the average serum calcium values, with the curves being drawn through mean values. Rachitic control chicks exhibited extremely low serum calcium concentrations averaging only 4.8 mg/100 ml, whereas chicks fed the standard diet showed a mean of 11.2 mg/100 ml. The serum calcium responded to either vitamin $D_2$ or $D_3$ and the increases qualitatively paralleled the increase in percentage bone ash.

*Serum phosphorus.* In rachitic chicks the serum phosphorus levels were much higher than in normal birds, averaging 10.9 mg/100 ml as compared with 8.2 mg/100 ml for the chicks fed the standard diet. Here the effect of vitamin $D_2$ or $D_3$ was to bring the high phosphorus levels down to normal, as shown in figure 2C, and the results form a smooth dose-response curve.

## DISCUSSION

In this study, each of the experimentally measured criteria illustrated in figure 2 responded in a quantitatively significant fashion according to the dosage and form of vitamin D administered. Bone ash was predictably increased, and the dramatic effect on serum calcium, while not unexpected, was great in magnitude compared with previously reported results (2, 7–9). Unexpected however, was the exceedingly high serum phosphorus level in the rachitic chicks and its reduction by vitamin D treatment.

The high serum phosphorus levels of the young rachitic chicks in the present study contrasts sharply with the subnormal concentrations previously reported in mammals, namely, rachitic infants (10), chicks (8) or older hens (9). Either vitamin $D_2$ or $D_3$, if administered orally in sufficient dosage, was capable of lowering these serum phosphorus values to levels considered normal and represented here by response to the standard diet. Conversely, the serum calcium levels were extremely low in our rachitic birds with values that would probably give rise to tetany in a mammal. The dramatic increases in the serum calcium wrought by vitamin $D_2$ or $D_3$ mirrored the serum phosphorus lowering.

These reciprocal changes in serum calcium and phosphorus were unexpected, for clinical experience suggests that parathyroid compensation comes into effect in vitamin D deficiency, tending to lower serum phosphorus and keep up the serum calcium. The results of Crawford et al. (11) and of Harrison and associates (12) are pertinent to this point, indicating that despite parathyroid hyperplasia, the serum calcium was low in vitamin D-deficient rats and that injected parathyroid extract was relatively ineffective. Such rats, al-

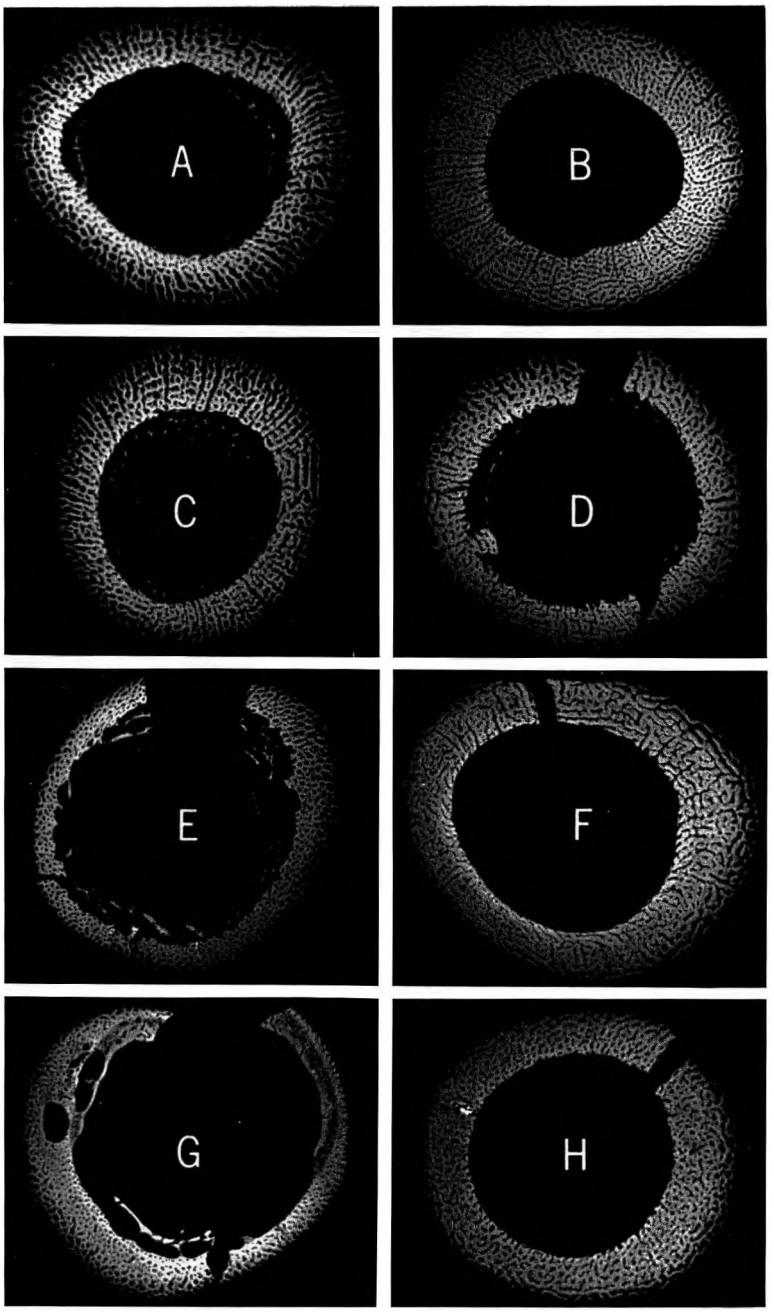


Fig. 1 Microradiographic appearance of tibial cross sections.

A. Rachitogenic diet
B. Standard diet
C. Vitamin $D_3$, 1 IU/day
D. Vitamin $D_2$, 30 IU/day
E. Vitamin $D_3$, 3 IU/day
F. Vitamin $D_2$, 60 IU/day
G. Vitamin $D_3$, 6 IU/day
H. Vitamin $D_2$, 300 IU/day

though exhibiting significant changes in serum calcium and citrate in response to treatment showed practically no bone or serum phosphorus changes. It is precisely these latter points which render the rat a somewhat peculiar experimental animal in studying mineral metabolism and thus make difficult extrapolation from this species to another species.

If the theory is correct that vitamin D possesses a "permissive role" in allowing parathyroid hormone to function properly, then it would be expected that in a severe deficiency of vitamin D there could be little parathyroid compensation of serum calcium or phosphorus levels. Although our results are consistent with the presence of such a severe deficiency in these chicks, they could equally well be explained by a deficiency in parathyroid secretion for one reason or another. However, it is not known what the biochemical response (especially in serum phosphorus) of the chick to its own parathyroid hormone would be. An increase in serum calcium upon injection with bovine parathyroid extract has been reported by Polin et al. (13).

Although it was not possible for us to determine concentrations of ionic calcium or ionic phosphorus, there is no evidence to indicate that the young chick behaves differently from man with respect to its serum electrolytes. It can be assumed as a first approximation that in such young birds, the serum phosphorus was essentially all diffusible and the fraction of diffusible calcium remained constant. The calcifying ability of the serum should therefore be proportional to the calcium $\times$ phosphorus product. We have calculated from the average values for total calcium and phosphorus the products of Ca $\times$ P. The products were smaller in rachitic chicks and chicks given low doses of vitamin $D_2$ and $D_3$ than in birds given the highest doses of the vitamins or fed the standard diet as shown in table 2.

All 3 quantitative criteria of vitamin D effectiveness (percentage bone ash, serum calcium, serum phosphorus) bear out the same qualitative difference between vitamins $D_2$ and $D_3$, namely, that a relatively greater quantity of vitamin $D_2$ than $D_3$ was necessary to effect the higher degrees of healing. Graphically this shows up as a flatter response curve for vitamin $D_2$ when the log dose was plotted against response (see fig. 2) and resulted in a variable "efficacy" ratio, namely, if complete healing was the criterion for relative potency, the ratio would be much higher than if only partial healing was chosen. Since the estimation of a threshold "curative or complete healing" dose cannot be made with

TABLE 2

*Ca $\times$ P product in chick serum*

| | |
|---|---|
| Standard chick diet | 91.3 [1] |
| Rachitogenic diet | 52.6 |
| Vitamin $D_3$, 1 IU/day | 61.2 |
| 3 IU/day | 64.5 |
| 6 IU/day | 89.0 |
| Vitamin $D_2$, 30 IU/day | 62.6 |
| 60 IU/day | 78.7 |
| 300 IU/day | 83.8 |

[1] Product of calcium and phosphorus concentrations expressed in mg/100 ml.

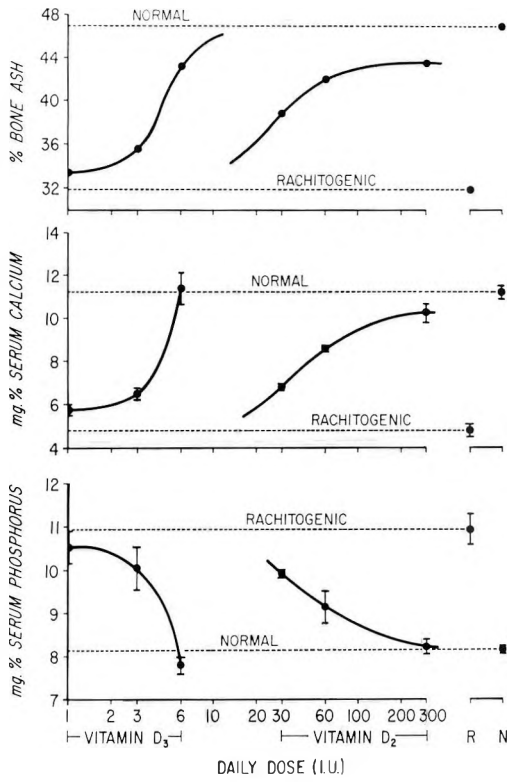


Fig. 2 Dose (log scale) of vitamins $D_2$ and $D_3$ vs. percentage of bone ash, serum calcium and serum phosphorus levels; R = chicks fed rachitogenic diet; N = chicks fed normal diet; means $\pm$ SE.

as great an accuracy as estimation of doses giving intermediate degrees of healing, the use of $CD_{50}$ (i.e., curative dose-50, a dose which produces an effect midway between severe rickets and complete healing, postulated here to be the response of chicks fed the standard diet) is suggested. This would correspond on our figure 2 to a dose giving responses of 39.2% bone ash, 8.0 mg/100 ml serum calcium and 9.5 mg/100 ml serum phosphorus. The use of such an intermediate biological effect was in fact suggested long ago by Massengale and Bills (14) who first pointed out (using ergosterol and cod liver oil) that the vitamin D in ergosterol ($D_2$) did not produce a dose response curve of the same shape as that in cod liver oil ($D_3$). Our results using the crystalline vitamins and daily oral intubation indicate a vitamin $D_3$-to-$D_2$ efficacy ratio of between 8:1 and 11:1 for a $CD_{50}$, which is a figure smaller than most previously reported values (2, 7, 14–19). Except for the study by Remp and Marshall (20), however, other workers have not specified that they used the pure crystalline vitamins.

The responses of the birds fed the standard chick diet were greater than those in any of the groups treated with vitamin $D_2$ or $D_3$ in certain parameters. One possibility for this phenomenon is a deficiency of other essential nutritional factors in the rachitogenic diet. For example Bruce et al. (21) observed perosis in chicks fed Olsson's diet and report a manganese content (20 to 30 ppm) lower than the suggested requirement (45 to 50 ppm). Another consideration is that our highest doses of vitamin $D_2$ and $D_3$ may not have been sufficient to give a maximal therapeutic response.

From a practical standpoint, however, the results indicate that the experimental schedule described provides a technique by which antirachitic effectiveness can be readily evaluated using 3 independent quantitative parameters. In assessing the physiological and pharmacological response to vitamin D or its metabolic products, it is expected that these parameters may not always parallel each other for every type of compound studied.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Bliss, C. I., and P. György 1951 Vitamin Methods, vol. 2, Academic Press Inc., New York, p. 97.
2. Bills, C. E., O. N. Massengale, G. G. McDonald and A. M. Wirich 1935 The action of activated ergosterol in the chicken. III. Evidence of the existence of only one provitamin D in crude ergosterol. J. Biol. Chem., *108:* 323.
3. Jones, J. I. M., and J. F. Elliot 1943 The biological assay of vitamin $D_3$. Biochem. J., *37:* 209.
4. Chen, P. S., Jr., A. R. Terepka and N. Remsen 1963 Column partition chromatography of vitamins A and D on Fluoropak 80. Anal. Chem., *35:* 2030.
5. Toribara, T. Y., and L. Koval 1961 Determination of calcium in biological material. Talanta, *7:* 248.
6. Chen, P. S., Jr., T. Y. Toribara and H. Warner 1956 Microdetermination of phosphorus. Anal. Chem., *28:* 1756.
7. Massengale, O. N., and M. Nussmeier 1930 The action of activated ergosterol in the chicken. I. The effect of the calcium and inorganic phosphorus of the blood serum. II. The prevention of leg weakness. J. Biol. Chem., *87:* 415.
8. 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. Nutrition, *26:* 81.
9. Steenbock, H., E. B. Hart, J. H. Jones and A. Black 1923–24 Fat soluble vitamins. XIV. The inorganic phosphorus and calcium of the blood used as criteria in the demonstration of the existence of a specific antirachitic vitamin. J. Biol. Chem., *58:* 59.
10. Howland, J. and B. Kramer 1921 Calcium and phosphorus in the serum in relation to rickets. Am. J. Dis. Child., *22:* 105.
11. Crawford, J. D., D. Gribetz, W. C. Diner, P. Hurst and B. Castleman 1957 The influence of vitamin D on parathyroid activity and the metabolism of calcium and citrate during calcium deprivation. Endocrinology, *61:* 59.
12. Harrison, H. C., H. E. Harrison and E. A. Park 1958 Vitamin D and citrate metabolism, effect of vitamin D in rats fed diets adequate in both calcium and phosphorus. Am. J. Physiol., *192:* 432.
13. Polin, D., P. D. Sturkie and W. Hunsaker 1957 The blood calcium response of the chicken to parathyroid extracts. Endocrinology, *60:* 1.
14. Massengale, O. N., and C. E. Bills 1936 A quantitative method for the assay of vitamin D with chickens. J. Nutrition, *12:* 429.

15. Steenbock, H. S., W. F. Kletzein and J. G. Halpin 1932 The reaction of the chicken to irradiated ergosterol and irradiated yeast as contrasted with the natural vitamin D of fish liver oils. J. Biol. Chem., *97:* 249.
16. Bethke, R. M., P. R. Record and D. C. Kennard 1953 The comparative antirachitic efficiency of irradiated ergosterol, irradiated yeast and cod liver oil for the chicken. J. Nutrition, 6: 413.
17. Russell, W. C., M. W. Taylor and D. E. Wilcox 1934 The fate of the antirachitic factor in the chicken. II. The effective levels and the distribution of the factor from cod liver oil and from irradiated ergosterol in certain tissues of the chicken. J. Biol. Chem., *107:* 735.
18. Haman, R. W, and H. Steenbock 1936 The antirachitic effectiveness of vitamin D from various sources. J. Biol. Chem., *114:* 505.
19. Grab, W. 1936 Die Auswertung der antirachitischen Wirksamkeit neuer Sterinderivate im Versuch an Ratten und Kuhen. Zeitschr. Physiol. Chem., *243:* 63.
20. Remp, D. G., and I. H. Marshall 1938 The antirachitic activity of various forms of vitamin D in the chick. J. Nutrition, *15:* 525.
21. Bruce, H. M., S. K. Kon, J. V. Watson, E. Cotchin and E. G. White 1946 Observations on perosis occurring in chicks used for vitamin $D_3$ assay. J. Comp. Path. Therap., *56:* 53.

# Vitamin $D_2$ Requirement of the Baby Pig [1,2]

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*ABSTRACT* The vitamin D requirement of baby pigs receiving a purified diet containing 0.8% of Ca, 0.6% of P and 350 ppm of Mg was studied in three 5-week trials using levels of vitamin $D_2$ from zero to 10,000 IU/kg of diet. All pigs receiving no dietary vitamin $D_2$ exhibited symptoms of either acute magnesium deficiency or rickets. Increasing the dietary Mg prevented tetany but not rickets in vitamin D-deficient pigs. All pigs receiving 100 IU or more of vitamin $D_2$/kg of diet exhibited optimal rates of growth and economy of diet utilization together with normal levels of serum Ca, P, Mg and alkaline phosphatase and adequate skeletal development with the absence of rachitic pathology. Under the conditions of this study the minimal vitamin $D_2$ requirement of the baby pig is not greater than 100 IU/kg of diet.

The rachitic syndrome which appears in swine that are deprived of dietary vitamin D and direct sunlight has been described previously (1–7). Wahlstrom and Stolte (8) were unable to produce rachitic symptoms unless dietary calcium concentration was also lowered. Johnson and Palmer (5) observed that vitamin D supplied in the ration of weanling pigs by sun-cured alfalfa hay at a level of about 72 IU/kg was sufficient to cure gross symptoms of rickets and to correct serum calcium and phosphorus values. Bethke et al. (7) concluded that the minimal practical vitamin D requirement of growing-finishing pigs was 200 IU/kg of ration and that the forms of vitamin D in irradiated yeast and cod liver oil were equally effective for swine. Lucas and Lodge (9) have chosen as preferred estimates of dietary vitamin D requirement 220 IU/kg for the 4.5-, 9- or 13.6-kg animal although no studies have been made of the vitamin D requirement of the pre-weanling pig.

The present study was conducted to assay the vitamin $D_2$ requirement of the baby pig reared with a purified diet, using measures similar to those made in previous mineral studies (10,11) as criteria.

## MATERIALS AND METHODS

Three trials were conducted using 45 Yorkshire-Hampshire crossbred pigs of either sex. Pigs were taken from the sow at one to three days of age and bottle-fed homogenized nonfortified cow's milk 4 times daily during the first few days. A purified diet (table 1) in the form of a dry meal was also placed in small feeders before the pig and intake was encouraged by placing small amounts of the dry meal into the animal's mouth after liquid consumption. The pigs were consuming the dry purified diet very well before one week of age and liquid milk feeding was stopped. At one week of age the animals were assigned to experimental levels of dietary vitamin $D_2$. Bases of treatment assignment and environmental conditions were similar to those described by Miller et al. (10, 11) and all windows were covered with heavy opaque paper to eliminate entrance of sunlight. Constant levels of dietary calcium (0.8%), phosphorus (0.6%) and magnesium (350 ppm) were maintained throughout the experimental period (5 weeks) except in the third trial in which 2 levels of dietary magnesium (350 and 750 ppm) were used (table 1). Casein constituted 30% of the diet during the first 3 weeks of each trial and was reduced to 20% during the final 2 weeks. This necessitated adjustment of the mineral mixture as shown in table 1. Experimental levels of irradiated ergosterol[3] were zero,

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[2] Presented in part at the Sixth International Congress of Nutrition, Edinburgh, August 1963 and the Midwest Section of the American Society of Animal Science, Chicago, November, 1963.

[3] Viosterol, Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

*Composition of basal purified diet*

| | % |
|---|---|
| Casein [1] | 30 (20) [2] |
| Lard | 5 |
| α-Cellulose [3] | 5 |
| Glucose [4] | 53 (63) [2] |
| Mineral mixture | 6 |
| Corn oil [5] | 1 |
| Vitamin mixture | + |
| **Mineral mixture** | |
| KCl | 10.0 |
| KI | 0.002 |
| $FeSO_4 \cdot 2H_2O$ | 0.7 |
| $CuSO_4$ | 0.1 |
| $CoCO_3$ | 0.1 |
| $MnSO_4 \cdot H_2O$ | 0.1 |
| $ZnSO_4 \cdot H_2O$ | 0.4 |
| $MgSO_4 \cdot 7H_2O$ | 5.5 (12.2) [6] |
| $NaHCO_3$ | 25.0 |
| $CaHPO_4$ | 28.5 (33.5) [2] |
| $CaCO_3$ | 12.5 (8.8) [2] |
| Glucose [4] | 17.1 (15.8) [2] (10.4 and 9.1) [6] |
| **Vitamin mixture [7]** | |
| | *mg/kg diet* |
| Thiamine·mononitrate | 3 |
| Riboflavin | 6 |
| Nicotinamide | 40 |
| Calcium pantothenate | 30 |
| Pyridoxine·hydrochloride | 2 |
| *p*-Aminobenzoic acid | 13 |
| Ascorbic acid | 80 |
| α-Tocopheryl acetate | 10 |
| Inositol | 130 |
| Choline chloride | 1300 |
| | *μg/kg diet* |
| Pteroylglutamic acid | 260 |
| Biotin | 50 |
| Cyanocobalamin | 100 |
| 2-Methyl-1,4-naphthoquinone | 40 |
| Vitamin A palmitate | 1500 |

[1] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.
[2] Changed to this value during final 2 weeks of each trial.
[3] Solka Floc, Brown Company, Boston.
[4] Cerelose, Corn Products Company, Argo, Illinois.
[5] Geo. Miesel and Son Company, Detroit.
[6] Two lots in trial 3 received increased magnesium level.
[7] Generously donated by Merck and Company, Inc., Rahway, New Jersey.

100, 1000 and 10,000 IU/kg of diet in trial 1; zero, 100, 500 and 1000 IU/kg in trial 2 and zero and 500 IU/kg in trial 3. Potency of the vitamin $D_2$ was verified by rat line test (12) comparison with USP vitamin $D_3$ reference standard.[4] Diet and tap water were made available for individual ad libitum consumption.

All animals (4 pigs per level of vitamin $D_2$ per trial except that only one pig received the 10,000 IU/kg level) were weighed weekly. Blood was withdrawn on 3 occasions (initial, 3 weeks and final) during each trial from the anterior vena cava for the determination of levels of serum calcium, inorganic phosphorus, magnesium and alkaline phosphatase by the methods of Mori (13), Gomorri (14), Orange and Rhein (15) and Bessey et al. (16), respectively. Determinations of bone composition and strength were made by methods used in previous studies (10, 11). Statistical analyses of data were performed with application of the multiple range test of Duncan (17).

## RESULTS AND DISCUSSION

The results of growth, serum analyses and sketetal measures of pigs in the 3 trials are summarized in tables 2, 3 and 4.

*Growth and food consumption.* Growth and food consumption were depressed only in pigs receiving no vitamin D in the diet. All pigs receiving levels of dietary vitamin $D_2$ of 100 IU/kg or greater ate and gained normally in each trial (0.26 to 0.29 kg daily gain, 0.40 to 0.44 kg daily food intake and 0.60 to 0.65% efficiency of utilization of food for body weight gain). Pigs receiving no vitamin D in the diet ate and gained normally during the first 2 or 3 weeks of each trial but during the final 2 weeks of each trial growth rate was greatly depressed. During the fourth week of each trial some of these pigs exhibited symptoms of magnesium deficiency[5] including the stepping syndrome, tetany and sudden mortality. Other pigs receiving no vitamin D did not exhibit these symptoms and survived to the end of the trial, exhibiting classical symptoms of rickets, including deformed limbs and great difficulty in walking (fig. 1). Appearance of these rachitic pigs was more typically that observed in calcium deficiency (10) rather than that in phosphorus deficiency (11).

## SERUM STUDIES

*Serum calcium.* Three weeks after the initiation of each trial, serum calcium concentration was significantly depressed in pigs receiving no dietary vitamin D.

[4] U.S.P. Reference Standards, New York.
[5] Miller, E. R., D. E. Ullrey, C. L. Zutaut, B. V. Baltzer, B. H. Vincent, D. A. Schmidt, J. A. Hoefer and R. W. Luecke 1962. Magnesium requirement of the baby pig. J. Animal Sci., *21*: 1006 (abstract).

TABLE 2

*Growth, serum analyses and skeletal development of baby pigs fed different levels of vitamin $D_2$ (trial 1)*

| | Dietary vitamin $D_2$ concentration, IU/kg | | | |
|---|---|---|---|---|
| | 0 | 100 | 1,000 | 10,000 |
| No. of pigs | 4 | 4 | 4 | 1 |
| Initial weight, kg | 1.9 ±0.1 [1] | 1.9 ±0.2 | 1.9 ±0.2 | 1.9 |
| Daily gain, kg | 0.18 ±0.02 | 0.28 ±0.01[aa] | 0.29 ±0.02[aa] | 0.29[aa] |
| Daily food intake, kg | 0.32 ±0.05 | 0.42 ±0.02[a] | 0.44 ±0.02[a] | 0.44[a] |
| Food/gain | 1.80 ±0.11 | 1.52 ±0.02[a] | 1.52 ±0.02[a] | 1.52[a] |
| Mortality, % | 50 | 0 | 0 | 0 |
| Serum Ca, mg/100 ml | | | | |
| Initial | 11.1 ±0.2 | 11.8 ±0.4 | 11.7 ±0.3 | 11.2 |
| 3 Weeks | 9.2 ±1.0 | 11.6 ±0.2[a] | 11.9 ±0.6[a] | 12.2[a] |
| 5 Weeks | 7.6 ±0.5 | 12.0 ±0.5[aa] | 12.4 ±1.1[aa] | 11.3[aa] |
| Serm inorganic P, mg/100 ml | | | | |
| Initial | 6.2 ±0.4 | 7.2 ±0.4 | 6.2 ±0.4 | 7.3 |
| 3 Weeks | 7.5 ±0.3 | 7.9 ±0.3 | 8.1 ±0.4 | 9.2 |
| 5 Weeks | 6.2 ±1.3 | 7.7 ±0.4 | 7.5 ±0.4 | 7.3 |
| Serum Mg, mg/100 ml | | | | |
| Initial | 3.4 ±0.3 | 2.8 ±0.7 | 3.1 ±0.2 | 3.0 |
| 3 Weeks | 2.2 ±0.3 | 2.1 ±0.2 | 2.2 ±0.2 | 2.7 |
| 5 Weeks | 1.7 ±0.2 | 2.4 ±0.1[aa] | 2.3 ±0.1[aa] | 2.5[aa] |
| Serum alkaline phosphatase, Bessey-Lowry units | | | | |
| Initial | 12.1 ±0.4 | 12.4 ±1.4 | 9.3 ±0.4 | 12.5 |
| 3 Weeks | 18.8 ±1.8 | 8.7 ±0.8[aa] | 8.5 ±0.5[aa] | 9.6[aa] |
| 5 Weeks | 39.6 ±4.4 | 9.3 ±0.3[aa] | 7.7 ±0.7[aa] | 6.8[aa] |
| Humeral analyses, dry, fat-free basis | | | | |
| Ash, % | 39.5 ±0.1 | 50.1 ±0.5[aa] | 49.8 ±0.4[aa] | 48.7[aa] |
| Ca, % | 13.8 ±0.1 | 18.1 ±0.3[aa] | 17.8 ±0.3[aa] | 17.6[aa] |
| P, % | 7.4 ±0.0 | 9.5 ±0.1[aa] | 9.4 ±0.1[aa] | 9.2[aa] |
| Ca/P | 1.86 ±0.02 | 1.90 ±0.01 | 1.89 ±0.01 | 1.91 |
| Specific gravity | | | | |
| Femur | 1.14 ±0.00 | 1.20 ±0.01[aa] | 1.20 ±0.01[aa] | 1.20[aa] |
| 8th rib | 1.18 ±0.02 | 1.26 ±0.01[aa] | 1.26 ±0.01[aa] | 1.25[aa] |
| Weight, g | | | | |
| Femur | 48.9 ±1.4 | 63.5 ±1.8[aa] | 66.2 ±2.4[aa] | 68.8[aa] |
| 8th rib | 4.02 ±0.05 | 6.12 ±0.40[aa] | 5.90 ±0.94[aa] | 5.92[aa] |
| Femur strength [2] | | | | |
| Breaking load, kg | 56 ±5 | 90 ±6[aa] | 95 ±4[aa] | 94[aa] |
| Bending moment, kg-cm | 108 ±9 | 210 ±47[aa] | 218 ±19[aa] | 228[aa] |
| Moment of inertia, $cm^4$ | 0.15 ±0.01 | 0.14 ±0.01 | 0.16 ±0.01 | 0.18 |
| Breaking stress, $kg/cm^2$ | 475 ±44 | 1011 ±61[aa] | 929 ±44[aa] | 908[aa] |

[1] SE.
[a] Significantly different from unsupplemented lot mean value ($P < 0.05$); [aa] $P < 0.01$.
[2] For formulae, see Miller et al. (10).

Deficient animals that survived throughout the entire trial had serum calcium concentrations averaging 6 mg/100 ml (4.8 to 7.9) at the end of the trial. All animals receiving 100 IU or more of vitamin $D_2$/kg of diet had essentially normal serum calcium concentrations throughout the studies.

*Serum inorganic phosphorus.* Serum inorganic phosphorus concentration did not become significantly depressed in vitamin D-deficient pigs in trial 1; however, in trials 2 and 3 serum inorganic phosphorus concentration was significantly depressed in those animals that survived to the end of either trial. In the latter 2 trials, however, this measure did not become significantly lowered until 2 weeks after a significant depression in serum calcium had occurred, indicating that the reduced con-

TABLE 3

*Growth, serum analyses and skeletal development of baby pigs fed different levels of vitamin $D_2$ (trial 2)*

| | Dietary vitamin $D_2$ concentration, IU/kg | | | |
|---|---|---|---|---|
| | 0 | 100 | 500 | 1000 |
| No. of pigs | 4 | 4 | 4 | 4 |
| Initial weight, kg | 3.3 ±0.5 [1] | 3.3 ±0.5 | 3.4 ±0.6 | 3.5 ±0.8 |
| Daily gain, kg | 0.22 ± 0.02 | 0.28 ± 0.02[a] | 0.28 ± 0.02[a] | 0.28 ± 0.04[a] |
| Daily food intake, kg | 0.42 ± 0.02 | 0.44 ± 0.02 | 0.44 ± 0.02 | 0.44 ± 0.03 |
| Food/gain | 1.99 ± 0.12 | 1.62 ± 0.09[a] | 1.64 ± 0.11[a] | 1.63 ± 0.12[a] |
| Mortality, % | 50 | 0 | 0 | 0 |
| Serum Ca, mg/100 ml | | | | |
| Initial | 11.7 ±0.3 | 11.5 ±0.4 | 11.7 ±0.2 | 11.1 ±0.2 |
| 3 Weeks | 7.0 ±0.5 | 9.7 ±0.4[aa] | 10.6 ±0.1[aa] | 10.8 ±0.1[aa,b] |
| 5 Weeks | 5.2 ±0.4 | 12.3 ±0.1[aa] | 11.5 ±0.7[aa] | 11.9 ±0.4[aa] |
| Serum inorganic P, mg/100 ml | | | | |
| Initial | 7.5 ±0.9 | 7.7 ±0.7 | 7.2 ±0.5 | 6.8 ±0.3 |
| 3 Weeks | 8.4 ±0.5 | 8.1 ±0.4 | 8.7 ±0.3 | 8.7 ±0.2 |
| 5 Weeks | 6.1 ±0.3 | 7.6 ±0.4[aa] | 7.6 ±0.7[aa] | 7.4 ±0.5[aa] |
| Serum Mg, mg/100 ml | | | | |
| Initial | 3.2 ±0.2 | 3.0 ±0.1 | 2.8 ±0.3 | 2.7 ±01. |
| 3 Weeks | 0.6 ±0.2 | 2.2 ±0.1[aa] | 1.9 ±0.1[aa] | 2.0 ±0.1[aa] |
| 5 Weeks | 1.9 ±0.2 | 2.4 ±0.1[a] | 2.3 ±0.1[a] | 2.4 ±0.1[a] |
| Serum alkaline phosphatase, Bessey-Lowry units | | | | |
| Initial | 11.8 ±1.9 | 14.6 ±3.0 | 9.5 ±1.0 | 9.5 ±1.4 |
| 3 Weeks | 39.1 ±6.9 | 18.2 ±1.0[aa] | 14.3 ±1.7[aa] | 13.9 ±2.5[aa] |
| 5 Weeks | 31.8 ±1.5 | 10.2 ±1.9[aa] | 7.1 ±0.3[aa] | 5.7 ±1.1[aa,b] |
| Humeral analyses, dry, fat-free basis | | | | |
| Ash, % | 43.1 ±1.4 | 48.0 ±1.2[a] | 47.2 ±0.8[a] | 46.8 ±1.7[a] |
| Ca, % | 15.2 ±0.7 | 17.4 ±0.4[a] | 17.1 ±0.4[a] | 17.2 ±0.1[a] |
| P, % | 8.1 ±0.3 | 9.0 ±0.3 | 8.9 ±0.2 | 8.7 ±0.3 |
| Ca/P | 1.88 ± 0.02 | 1.96 ± 0.02[a] | 1.95 ± 0.01[a] | 1.97 ± 0.00[a] |
| Specific gravity | | | | |
| Femur | 1.14 ± 0.00 | 1.19 ± 0.01[aa] | 1.20 ± 0.00[aa] | 1.19 ± 0.01[aa] |
| 8th rib | 1.18 ± 0.00 | 1.24 ± 0.01[aa] | 1.24 ± 0.00[aa] | 1.25 ± 0.00[aa] |
| Weight, g | | | | |
| Femur | 42.1 ±1.5 | 53.9 ±4.2[aa] | 58.3 ±1.1[aa] | 44.2 ±3.7 |
| 8th rib | 3.97 ± 0.90 | 6.06 ± 0.42 | 5.54 ± 0.28 | 5.00 ± 0.12 |
| Femur strength [2] | | | | |
| Breaking load, kg | 86 ± 10 | 114 ± 11 | 100 ± 12 | 82 ± 4 |
| Bending moment, kg-cm | 150 ± 18 | 232 ± 24 | 207 ± 32 | 155 ± 9 |
| Moment of inertia, $cm^4$ | 0.12 ± 0.01 | 0.13 ± 0.03 | 0.16 ± 0.03 | 0.09 ± 0.01 |
| Breaking stress, $kg/cm^2$ | 776 ± 44 | 1180 ± 70[aa] | 892 ± 9 | 1025 ± 25[aa] |

[1] SE.
[a] Significantly different from unsupplemented lot mean value ($P < 0.05$); [aa] $P < 0.01$.
[b] Significantly different from mean value of lot receiving 100 IU/kg of dietary vitamin $D_2$ ($P < 0.05$).
[2] For formulae, see Miller et al. (10).

centration of serum inorganic phosphorus may have resulted from poor phosphorus utilization by the animal in a state of calcium deficiency. This has been frequently observed and is further indicated by balance studies.[6] All pigs receiving vitamin $D_2$ in the diet had normal levels of serum inorganic phosphorus.

*Serum magnesium.* Serum magnesium levels were depressed in a majority of the pigs receiving no dietary vitamin D, particularly in those animals exhibiting the

[6] Miller, E. R., D. E. Ullrey, C. L. Zutaut, J. A. Hoefer and R. W. Luecke 1963 Effects of dietary vitamin $D_2$ and magnesium upon calcium, phosphorus and magnesium utilization by the baby pig. Federation Proc., 22: 491 (abstract).

TABLE 4

*Growth, serum analyses and skeletal development of pigs fed different levels of vitamin $D_2$ and magnesium (trial 3)*

| Dietary vitamin $D_2$, IU/kg | 0 | 0 | 500 | 500 |
|---|---|---|---|---|
| Dietary Mg, ppm | 350 | 750 | 350 | 750 |
| No. of pigs | 4 | 4 | 4 | 4 |
| Initial weight, kg | 2.6 ±0.2 [1] | 2.6 ±0.3 | 2.5 ±0.2 | 2.6 ±0.3 |
| Daily gain, kg | 0.17±0.01 | 0.15±0.01 | 0.26±0.01[a] | 0.27±0.01[a] |
| Daily food intake, kg | 0.34±0.01 | 0.31±0.01 | 0.40±0.01[a] | 0.41±0.01[a] |
| Food/gain | 1.95±0.15 | 2.10±0.12 | 1.57±0.10[a] | 1.53±0.07[a] |
| Mortality, % | 75 | 0 | 0 | 0 |
| Serum Ca, mg/100 ml | | | | |
| Initial | 10.9 ±0.6 | 10.4 ±0.2 | 10.2 ±0.2 | 10.8 ±0.3 |
| 3 Weeks | 8.4 ±0.6 | 7.6 ±0.2 | 10.6 ±0.1[a] | 11.3 ±0.2[a] |
| 5 Weeks | 5.2 | 5.8 ±0.3 | 10.1 ±0.2[a] | 10.9 ±0.2[a] |
| Serm inorganic P, mg/100 ml | | | | |
| Initial | 7.6 ±0.7 | 8.1 ±0.5 | 8.7 ±0.2 | 8.6 ±0.6 |
| 3 Weeks | 7.0 ±0.9 | 8.3 ±0.1 | 9.3 ±0.8 | 8.9 ±1.6 |
| 5 Weeks | 5.1 | 4.8 ±0.3 | 7.4 ±0.2[a] | 7.3 ±0.1[a] |
| Serum Mg, mg/100 ml | | | | |
| Initial | 3.0 ±0.1 | 3.0 ±0.1 | 3.0 ±0.1 | 3.0 ±0.3 |
| 3 Weeks | 2.6 ±0.4 | 2.6 ±0.1 | 2.6 ±0.2 | 2.7 ±0.1 |
| 5 Weeks | 2.6 | 2.0 ±0.2 | 2.7 ±0.2[b] | 2.6 ±0.0 |
| Serum alkaline phosphatase, Bessey-Lowry units | | | | |
| Initial | 11.4 ±0.4 | 12.4 ±2.4 | 10.1 ±1.3 | 9.7 ±1.1 |
| 3 Weeks | 33.9 ±3.0 | 31.9 ±4.4 | 12.8 ±0.5[a] | 12.3 ±1.4[a] |
| 5 Weeks | 28.8 | 28.1 ±2.8 | 9.9 ±1.6[a] | 9.3 ±1.4[a] |
| Humeral analyses, dry, fat-free basis | | | | |
| Ash, % | 38.5 ±2.8 | 36.1 ±1.3 | 50.0 ±0.6[a] | 50.1 ±0.9[a] |
| Ca, % | 13.3 ±1.0 | 12.4 ±0.5 | 17.8 ±0.3[a] | 17.7 ±0.4[a] |
| P, % | 7.2 ±0.6 | 6.7 ±0.3 | 9.4 ±0.1[a] | 9.4 ±0.2[a] |
| Mg, % | 0.33±0.01 | 0.33±0.01 | 0.45±0.03[a] | 0.52±0.01[c] |
| Specific gravity | | | | |
| Femur | 1.12±0.01 | 1.14±0.01 | 1.20±0.01[a] | 1.21±0.01[a] |
| 8th rib | 1.18±0.01 | 1.16±0.01 | 1.24±0.01[a] | 1.24±0.01[a] |
| Weight, g | | | | |
| Femur | 32.5 ±7.3 | 37.6 ±5.5 | 63.0 ±1.4[a] | 59.0 ±4.7[a] |
| Humerus | 29.3 ±5.0 | 35.0 ±0.8 | 53.3 ±1.2[a] | 51.9 ±2.6[a] |
| 8th rib | 3.3 ±0.5 | 4.0 ±0.3 | 6.3 ±0.3[a] | 6.6 ±0.6[a] |
| Femur strength [2] | | | | |
| Breaking load, kg | 47±3 | 50±1 | 100±5[a] | 110±9[a] |
| Bending moment, kg-cm | 73±4 | 78±1 | 196±11[a] | 217±20[a] |
| Moment of inertia, $cm^4$ | 0.06±0.01 | 0.07±0.01 | 0.14±0.01[a] | 0.16±0.02[a] |
| Breaking stress, $kg/cm^2$ | 669±75 | 752±62 | 971±61[a] | 963±68[a] |

[1] SE.
[a] Significantly different from mean value of either lot receiving no vitamin D ($P < 0.01$).
[b] Significantly greater than least value ($P < 0.05$).
[c] Significantly greater than all other values ($P < 0.01$).
[2] For formulae, see Miller et al. (10).

stepping syndrome and tetany. These pigs either succumbed suddenly in tetany or their serum magnesium values returned to near normal and symptoms of severe rickets appeared. Balance studies [7] revealed poor magnesium absorption by the vitamin D-deficient pigs. Meintzer and Steenbock (18) improved intestinal absorption of magnesium in rats with dietary vitamin D; however, magnesium retention was not improved. Worker and Migicovsky (19) obtained similar results with chicks. Bechtel et al. (20) obtained reduced plasma magnesium levels in some rachitic dairy

[7] See footnote 6.

Fig. 1 Vitamin D-deficient pig exhibiting short weakened limbs typical of advanced rickets. Walking was done with great difficulty. This pig (3–4) from trial 1 received a purified diet containing no vitamin D, 0.8% of Ca, 0.6% of P and 350 ppm of Mg.

calves. Some of the vitamin D-deficient pigs in the present study did not have reduced levels of serum magnesium and did not exhibit symptoms of magnesium deficiency. Huffman and Duncan (21) have shown the vitamin D-sparing action of magnesium in the diets of dairy cattle and von Euler and Rydbom (22) observed similar results in rats. In the third trial higher levels of magnesium (750 ppm) were supplied to the diets of two of the experimental groups of pigs to supply some information on its vitamin D sparing action in the pig. Data presented in table 4 do not indicate an increase in serum magnesium levels as a result of increased dietary magnesium. However, external symptoms of magnesium deficiency were delayed in vitamin D-deficient pigs receiving the additional dietary magnesium and no deaths occurred in this group, whereas 3 out of 4 pigs in the vitamin D-deficient group receiving the requirement level of magnesium (350 ppm)[8] died before the end of the experimental period.

*Serum alkaline phosphatase.* Levels of serum alkaline phosphatase have been shown to increase in dietary deficiencies of calcium (10) and phosphorus (11) but not magnesium[9] in the baby pig. In the present study vitamin D-deficient pigs had greatly elevated levels of serum alkaline phosphatase. Increasing the level of dietary magnesium in trial 3 was without effect in preventing this elevation. Dietary vitamin $D_2$ at a level of 100 IU/kg resulted in a normal serum alkaline phosphatase concentration, and higher levels of vitamin $D_2$ did not cause a further reduction.

## BONE STUDIES

*Humeral analyses.* The humeri of baby pigs receiving no dietary vitamin D contained significantly reduced levels of ash,

[8] See footnote 5.
[9] See footnote 5.

calcium and phosphorus. In general the calcium concentration was more severly affected than phosphorus concentration, resulting in a reduced calcium-to-phosphorus ratio in the bone ash. Humeral magnesium levels were determined in the third trial and were significantly lower in the vitamin D-deficient pigs. Additional dietary magnesium did not significantly alter the magnesium level of humeri in the vitamin D-deficient pigs; however, it resulted in a significant increase with pigs receiving adequate dietary vitamin D. A dietary vitamin $D_2$ level of 100 IU/kg appears adequate to provide for optimal bone ash composition.

*Specific gravity and weight.* Absence of vitamin D in the diet resulted in significant reductions of rib and femur weights and specific gravity. The degree of reduction of these measures in vitamin D-deficient pigs was similar to that observed in previous studies of animals receiving no dietary calcium (10) or 0.2% of dietary phosphorus (11).

*Femur strength.* Vitamin D deficiency resulted generally in an impairment of strength characteristics of the femur. Breaking load is primarily dependent upon the diameter of the femur and the thickness of the compact layer of the diaphysis. This is apparent by comparing the values of breaking load and moment of inertia in tables 2, 3 and 4. Bending moment is a more accurate term than breaking load for indicating femur strength because it considers femur length. Breaking stress is perhaps the measure that best indicates the degree of compact layer development because it is a measure of femur strength per unit of cross-sectional area. This is illustrated by rather unusual data obtained for breaking load and moment of inertia of femurs of pigs receiving 1000 IU of vitamin $D_2$/kg in trial 2 (table 3). For some unexplained reason the femurs of these

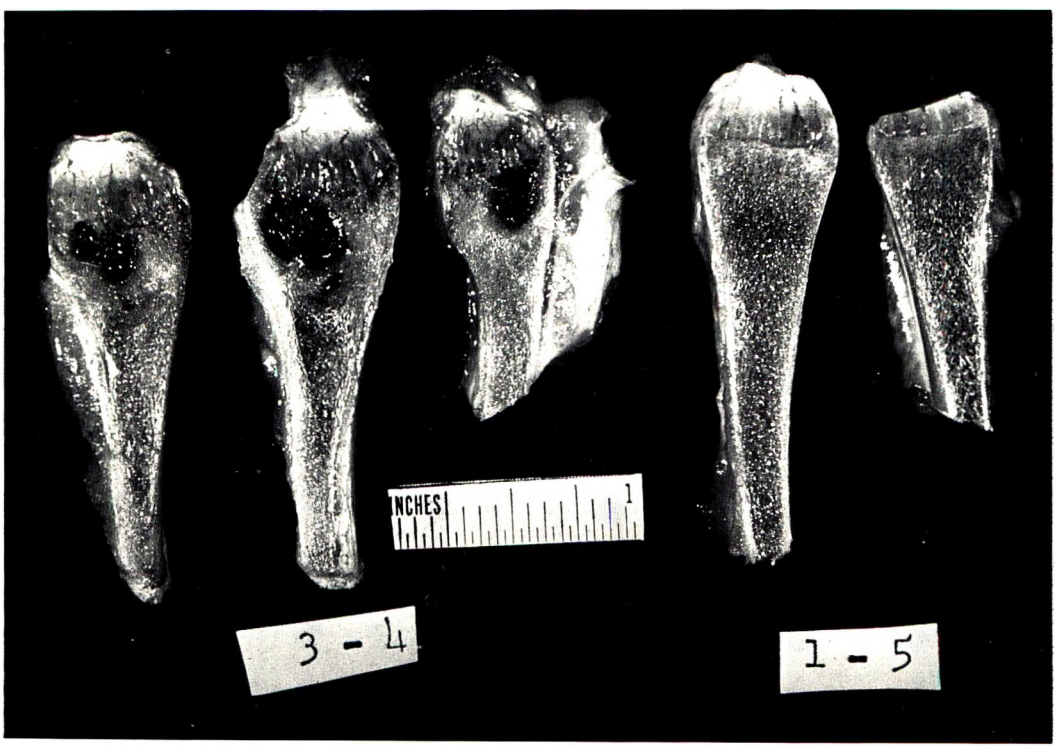


Fig. 2 Longitudinal sections of eighth ribs from pigs receiving purified diets containing no vitamin D (3–4) or 100 IU vitamin $D_2$/kg (1–5). Note the wide and irregular zone of proliferating cartilage and the chaotic condition of the line of ossification and the bony matrix in the rib of the vitamin D-deficient pig.

animals were smaller in diameter than normal, resulting in reduced values of breaking load and moment of inertia. These femurs were not shortened, and hence bending moment values were also low. These femurs, however, had normal breaking stress values because of the high degree of development of compact layer in the diaphysis. Femurs of pigs receiving no dietary vitamin D were similar to osteoporotic femurs observed in calcium-deficient pigs (10).

*Pathology.* Examination at necropsy revealed only rachitic lesions in the vitamin D-deficient animals. Fewer fractured ribs were noted in these animals than in calcium-deficient (10) or phosphorus-deficient (11) pigs. There was some beading of the costochondral junctions but it was not as exaggerated as in phosphorus-deficient pigs (11). Histopathological findings were similar to those observed in calcium (10) or phosphorus (11) deficiency with a more chaotic condition of the bony trabeculae (fig. 2) similar to that observed in vitamin D-deficient calves (20, 21). All of the ribs from animals receiving dietary vitamin $D_2$ supplementation were essentially normal. There was a tendency for the zone of proliferating cartilage to be somewhat wider in pigs receiving 100 IU of vitamin $D_2$/kg of diet than in pigs receiving higher levels of vitamin $D_2$.

The serum and skeletal data from the 3 trials, together with the growth and pathological observations, demonstrate that vitamin D deficiency symptoms develop very rapidly in baby pigs deprived of dietary vitamin $D_2$ and direct sunlight. The data indicate that the minimal dietary vitamin $D_2$ requirement of the baby pig under the experimental conditions of this study does not exceed 100 IU/kg. Recent studies[10,11] indicate that the dietary vitaman D requirement may be increased by type and level of dietary protein. This consideration is under study.

## LITERATURE CITED


1. Sheehy, E. J. 1932 Cod liver oil in pig feeding. J. Dept. Agr. (Irish Free State), 32: 202.
2. Senior, B. J. 1941 The requirements of the pig for vitamin A and D. Sci. Proc. Royal Dublin Soc., 22: 229.
3. Senior, B. J. 1941 The vitamin D requirements of the growing pig. Sci. Proc. Royal Dublin Soc., 22: 379.
4. Johnson, D. W., and L. S. Palmer 1939 Individual and breed variations in pigs on rations devoid of vitamin D. J. Agr. Res., 58: 929.
5. Johnson, D. W., and L. S. Palmer 1941 Meeting the vitamin D requirement of pigs with alfalfa hay and winter sunshine. J. Agr. Res., 63: 639.
6. Dunlop, G. 1935 The calcium, phosphorus and vitamin D requirement of swine. J. Agr. Sci., 25: 22.
7. Bethke, R. M., W. Burroughs, O. H. M. Wilder, B. H. Edington and W. L. Robison 1946 The comparative efficacy of vitamin D from irradiated yeast and cod liver oil for growing pigs, with observations on their vitamin D requirements. Ohio Agr. Exp. Sta. Bull. 667, Columbus, Ohio.
8. Wahlstrom, R. C., and D. E. Stolte 1958 The effect of supplemental vitamin D in rations for pigs fed in the absence of direct sunlight. J. Animal Sci., 17: 699.
9. Lucas, I. A. M., and G. A. Lodge 1961 The nutrition of the young pig. Commonwealth Agricultural Bureaux, Farnham Royal, Slough, Bucks, England.
10. Miller, E. R., D. E. Ullrey, C. L. Zutaut, B. V. Baltzer, D. A. Schmidt, J. A. Hoefer and R. W. Luecke 1962 Calcium requirement of the baby pig. J. Nutrition, 77: 7.
11. Miller, E. R., D. E. Ullrey, C. L. Zutaut, B. V. Baltzer, D. A. Schmidt, J. A. Hoefer and R. W. Luecke 1964 Phosphorus requirement of the baby pig. J. Nutrition, 82: 34.
12. Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D. C.
13. Mori, K. 1959 Direct micro complexometric analysis of calcium in biological material. Arch. Biochem. Biophys., 83: 552.
14. Gomorri, G. 1942 A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med., 27: 955.
15. Orange, M., and H. C. Rhein 1951 Microestimation of magnesium in body fluids. J. Biol. Chem., 189: 379.
16. Bessey, O. A., O. H. Lowry and M. J. Brock 1946 A method for the rapid determination of alkaline phosphatase with 5 cubic millimeters of serum. J. Biol. Chem., 164: 321.
17. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, 11: 1.


[10] McGinnis, J., C. W. Carlson, H. C. Saxena and L. S. Jensen 1963 Growth promoting rachitogenic and antirachitic effects of various soya bean fractions. Proc. Sixth International Congress of Nutrition, 154 (abstract).

[11] Miller, E. R., C. L. Zutaut, D. E. Ullrey, B. V. Baltzer, J. A. Hoefer and R. W. Luecke 1963 Protein source and mineral balance in the baby pig. J. Animal Sci., 22: 1127 (abstract).

18. Meintzer, R. B., and H. Steenbock 1955 Vitamin D and magnesium absorption. J. Nutrition, 56: 285.
19. Worker, N. A., and B. B. Migicovsky 1961 Effect of vitamin D on the utilization of beryllium, magnesium, strontium and barium in the chick. J. Nutrition, 74: 490.
20. Bechtel, H. E., E. T. Hallman, C. F. Huffman and C. W. Duncan 1936 Pathology of rickets in dairy calves. Tech. Bull. 150, Michigan Agr. Exp. Sta., East Lansing.
21. Huffman, C. F., and C. W. Duncan 1935 Vitamin D studies in cattle. II. The vitamin D sparing action of magnesium in the ration of dairy cattle. J. Dairy Sci., *18:* 605.
22. von Euler, H., and M. Rydbom 1931 Über den Einfluss von Magnesiumsalzen auf Knockenbildung und Rachitis. Biochem. Ztschr., 241: 14.

# Effect of Dietary Protein Level on Cholesterolemia, Thrombosis, Atherosclerosis and Hypertension in the Rat [1]

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*ABSTRACT* The thrombotic syndrome induced in the rat by a hyperlipemic diet containing laboratory chow, sodium cholate, butter and cholesterol was prevented by increasing the protein content of the diet, without affecting the cholesterolemia. With a purified diet, decreasing the protein level increased the cholesterolemic response; thrombosis could be produced under these conditions, provided that the dietary casein was decreased to approximately one-half of its normal level for the rat. With purified diets, the thrombi were located at various sites: 1) in small vessels; 2) in large coronary arteries with massive infarction ensuing; or 3) in the cardiac cavities. Aortic fatty streaks and even atherosclerotic plaques were consistently observed within 7 months with hyperlipemic purified diets, irrespective of the dietary level of protein. Although malignant hypertension increased the dietary-induced lipemia, as an additional factor it did not alter the thrombotic response of the rat to the protein level, but induced the formation of severe coronary atherosclerosis. Under our experimental conditions, hypercholesterolemia appears to be a prerequisite for both thrombosis and atherosclerosis, but the incidence or the severity of the lesions do not necessarily depend on the magnitude of the lipemic changes. The dietary level of protein appears to markedly influence the experimental production of thrombosis, atherosclerosis and hypertension in the rat, although not in the same direction.

A thrombotic phenomenon of high incidence was produced in the rat by feeding a simple hyperlipemic diet containing a commercial laboratory chow[2] supplemented with cholic acid, cholesterol and butter (1, 2). Under our conditions as well as those of other workers (3), thrombosis could not be reproduced by utilizing a purified base instead of the commercial laboratory chow in the thrombogenic diet, the other elements (cholesterol, cholic acid, and butter) being the same. It appeared, therefore, that something in the purified diet exerted an anti-thrombogenic effect. In subsequent experiments it was shown that this effect was not due to vitamins (4). As it was known that the dietary protein level markedly affected the cholesterolemic response in various species (5–8), the influence of this factor was investigated in rats, using in the diets either laboratory chow or a purified base. With the thrombogenic diet containing laboratory chow, even if hyperlipemia was maintained for several months, early lesions of atherosclerosis were rarely observed (1) unless the animals were rendered hypertensive (9). With the purified diets used in the present experiment, aortic fatty streaks were always observed, even in normotensive animals. The influence of the dietary protein level on the hyperlipemic-hypertensive response of rats submitted to meta-corticoid hypertension was also investigated. Finally, since it was found that, in hooded rats, feeding the hyperlipemic diet containing laboratory chow induced lesions more severe than those observed in albino rats (10), the effects of 2 purified diets differing only in their casein level were studied in this animal. The purpose of the present paper is to report the influence, in the rat, of the dietary protein (casein) level on the production of cholesterolemia, thrombosis, hypertension and early atherosclerotic lesions and on the effects that these conditions exert on each other.

## EXPERIMENTAL

A total of 108 male albino and 24 male hooded rats[3] with an initial body weight of

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[2] Purina Laboratory Chow, Ralston Purina Company of Canada.

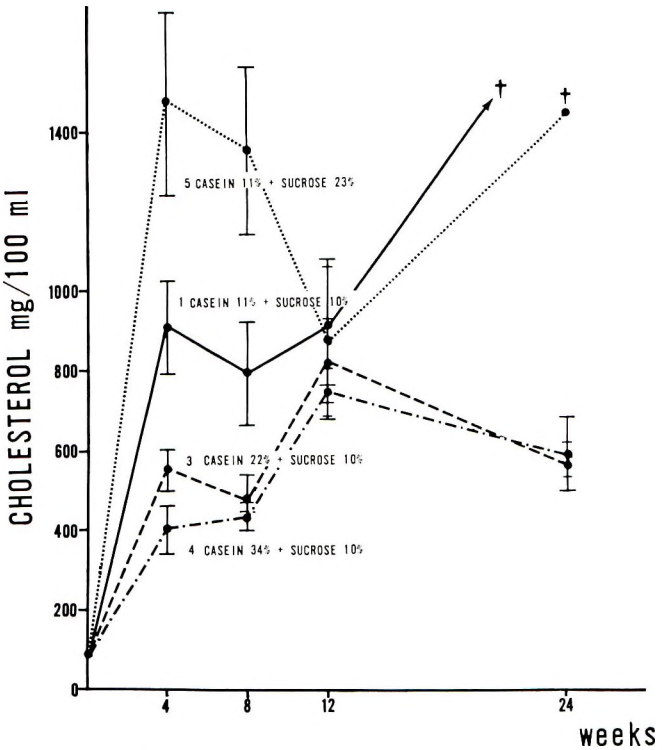



Fig. 1 Influence of dietary casein and sucrose on the total serum cholesterol, (in exp. 2). Results are expressed as mean and standard error. Dagger (†) indicates death with thrombosis.

130 to 140 g (exps. 1, 2 and 4) and 96 male albino rats weighing 100 to 110 g (exp. 3) were used for this study. At the beginning of the 4 experiments reported here, each group comprised 12 rats. In the course of these studies, several animals were eliminated for various reasons; the number of animals kept in each group is listed in the corresponding table. Parts of the experiment were repeated under the same conditions, namely, on groups 1 and 4 of experiment 2 and groups 1 to 4 of experiment 4. The results listed in the 2 tables constitute the data obtained from both sets of observations, where applicable.

The rats were housed 6/cage and given, ad libitum, tap water alone (exp. 4) or tap water to which was added 187.5 mg/1000 ml of oxytetracycline[4] to prevent pulmonary infection (exps. 1 to 3). However, from the first to the tenth week, 1% sodium chloride was added to the tap water for groups 2 and 4 of experiment 3. The diets, also given ad libitum, all contained cholesterol 5%, sodium cholate 2%, and salted butter 40%, and in addition, at a level indicated in the tables, the following constituents: casein,[5] cellulose,[6] sucrose, vitamin mixture[7] and salt mixture.[8]

[3] Quebec Breeding Farm, Saint-Eustache, P. Qué., Canada.

[4] In the form of Liquamycin, kindly furnished by Pfizer Company of Canada.

[5] Casein (purified) and cellulose (Alphacel) were obtained from Nutritional Biochemicals Corporation, Cleveland.

[6] See footnote 5.

[7] Vitamin diet fortification mixture (Nutritional Biochemicals Corporation, Cleveland), contained/kg: vitamin A, 900,000 units; vitamin D, 100,000 units; and (in g) α-tocopherol, 5; ascorbic acid, 45; inositol, 5; choline chloride, 75; menadione, 2.25; *p*-aminobenzoic acid, 5; niacin, 4.5; riboflavin, 1; pyridoxine·HCl, 1; thiamine·HCl, 1; Ca pantothenate, 3; and (in mg) biotin, 20; folic acid, 90; vitamin $B_{12}$, 1.35; and dextrose to make 1 kg.

[8] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents, Science, 75: 339. Composition/100 g: Ca carbonate, 21.000; copper sulfate ($5H_2O$), 0.039; ferric phosphate, 1.470; manganous sulfate (anhydrous), 0.020; magnesium sulfate (anhydrous), 9.000; potassium aluminum sulfate, 0.009; potassium chloride, 12.000; potassium dihydrogen phosphate, 31.000; potassium iodide, 0.005; sodium chloride, 10.500; sodium fluoride, 0.057; tricalcium phosphate, 14.900. (Purchased from Nutritional Biochemicals Corporation.)

In experiment 3 the hyperlipemic diet was not initiated until the beginning of the eighth week of the experiment; for the first 7 weeks the animals were fed laboratory chow, as in previous studies (9). In addition, groups 2 and 4 were also given 2 mg of desoxycorticosterone acetate (DCA),[9] 5 days a week, for the first 7 weeks, by subcutaneous injection. Systolic blood pressure was recorded regularly under ether anesthesia by the microphonic method. At given periods (see fig. 1 and tables 1–4), 2 ml of blood were removed from the jugular vein after 18 hours' fasting, under ether anesthesia. To assess the degree of lipemia the total serum cholesterol was determined on each rat by the Trinder method (11), whereas the lipoproteins were estimated by electrophoresis in paper (12) on the pooled sera of 5 animals (exp. 1). All determinations were carried out in duplicate, on fresh serum.

The duration of each experiment is listed in the corresponding table. At the autopsy time, the survivors were killed with chloroform and all organs were examined under a stereoscopic microscope, including the aorta, after being opened longitudinally. All hearts, aortae, pancreas and kidneys were examined histologically. The hearts were stained by the periodic-acid-Schiff technique and the other organs with hematoxylin-phloxine-saffron. Each heart was sectioned at 3 levels; a positive reading for thrombosis indicates that thrombi could be found in each section if they were small and multiple, or at least in one if a thrombus was present in a large coronary artery or in a cardiac cavity. The aortic fatty streaks were arbitrarily graded in terms of a scale of zero to 3, by examination under stereoscopic microscope. These lesions were then verified histologically. Hair loss was recorded, at its maximal severity, at 12 weeks.

## RESULTS

*Experiment 1, (table 1).* Feeding male albino rats the usual hyperlipemic diet containing laboratory chow (group 1), of which the protein content is approximately 11%, resulted in a 70% mortality rate and an incidence of thrombosis also of

[9] Generously supplied by Ciba Company of Canada.

TABLE 1

*Influence of casein on the production of thrombosis and lipemic changes in rats fed a chow-containing diet* [1]

| Group | Diet [2] | Cholesterol | | | α-Lipoproteins | | | Loss of hair | Thrombosis, incidence | Fatty streaks, incidence | Mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Week | | | Week | | | | | | |
| | | 8 | 14 | 30 | 8 | 14 | 30 | | | | |
| | | *mg/100 ml* | *mg/100 ml* | *mg/100 ml* | % | % | % | | % | % | % |
| 1 | Chow, 53%<br>Casein, 0% | 266 ± 38 [3] | 277 ± 30 | — | 3 | 15 | — | +++ | 70 | 0 | 70 |
| 2 | Chow, 40%<br>Casein, 13% | 245 ± 18 | 371 ± 45 | 521 ± 64 | 6 | 14 | 12 | 0 | 0 | 0 | 0 |

[1] Duration of experiment, 30 weeks; 10 animals/group.
[2] In addition to the elements listed in the table, the diets contained cholesterol, 5%; cholic acid, 2%; and butter, 40%.
[3] Mean ± SE.

70% that occurred in the small heart vessels and was of the type previously reported (1). After some weeks of the dietary feeding, the animals of group 1 also showed loss of hair at various sites of their body. Addition of 13% casein at the expense of chow (group 2) prevented the occurrence of the syndrome, since after 30 weeks there was no mortality or thrombosis and in addition, at no time did loss of hair occur in any of these animals. However, there was no significant difference in the serum cholesterol or in the percentage of α-lipoproteins at the eighth- or fourteenth-week period between these 2 groups. In none of these animals were aortic fatty streaks observed, and only the valves showed some atherosclerotic involvement.

*Experiment 2 (table 2).* With a purified diet, mortality occurred only in groups 1, 2 and 5, in which the casein level was 12% or less. It was also only in these groups that a certain incidence of thrombosis occurred, the highest being in group 1, which received 11% of casein and 10% sucrose. The thrombi, instead of always being located in the small cardiac vessels, as was the case with the diet containing chow (group 1, table 1), were sometimes seen in the large coronary vessels (fig. 2) and sometimes in the cardiac cavities (ventricles and even auricles). When located in the large coronary vessels, thrombi were responsible for large infarcts, peripherally delimited by inflammatory cells, mono- and polynuclears.

Only in groups 1, 2 and 5 did a loss of hair similar to that described in the first experiment occur. In contrast with the first experiment, fatty streaks were detected, particularly in the abdominal part of the aorta, in some animals of all groups. Sometimes, also, these fatty streaks were found in the aortic arch or along the entire length of the aorta. Histologically, the fatty streaks were usually composed of several layers of foam cells covered by endothelial cells, with little fibrosis. However, in the ascending aorta in the vicinity of the aortic valves and also on the mitral valve of animals of group 4, large atherosclerotic plaques were frequently observed (fig. 3). In the superficial layers, these plaques were composed of fibrous tissue; more deeply in the intima, they consisted of a lipidic mass containing cholesterol crystals. It is difficult to make an exact comparison between the different diets with respect to their atherogenicity, since survival was not the same from group to group and since all the animals presented a certain degree of atherosclerosis. As a compromise measure, when one-half of the rats in group 1 had died, one-half of the rats of groups 3 and 4 were killed and autopsied. Using this protocol, it appears that group 4 with the highest level of dietary casein also had the most severe and the highest incidence of atherosclerotic lesions. As shown in figure 1, increasing the level of casein at the expense of cellulose from 11 to 34% decreased the level of cholesterol, particularly until the twelfth week. After the 12-week period, cholesterol was not analyzed in group 1, because from then on there were enormous variations from one animal to another and the

TABLE 2

*Effect of dietary level of casein and sucrose on the production of thrombosis and aortic fatty streaks in rat* [1]

| Group no. | Diet [2] | No. of rats | Loss of hair | Thrombosis, (Incidence) | Aortic fatty streaks | | Mortality |
|---|---|---|---|---|---|---|---|
| | | | | | Incidence | Intensity (scale 0–3) | |
| | | | | % | % | | % |
| 1 | Casein, 11% + sucrose, 10% | 18 | ++ | 50 | 50 | 1.3 | 83 |
| 2 | Casein, 12% + sucrose, 10% | 10 | + | 40 | 40 | 1.1 | 70 |
| 3 | Casein, 22% + sucrose, 10% | 10 | 0 | 0 | 60 | 0.7 | 0 |
| 4 | Casein, 34% + sucrose, 10% | 22 | 0 | 0 | 81 | 1.7 | 0 |
| 5 | Casein, 11% + sucrose, 23% | 9 | +++ | 30 | 44 | 1.4 | 66 |

[1] Total duration of experiment, 28 weeks.
[2] In addition to the elements listed in the table, the diets contained: (in per cent) butter, 40; cholesterol, 5; cholic acid, 2; vitamin mixture, 2; salt mixture, 4; and cellulose to make 100.

results became difficult to interpret (1). If the sucrose instead of the casein level was increased (group 5), the serum cholesterol increased instead of decreasing.

*Experiment 3 (table 3).* The hyperlipemic diets either at 11% (groups 1 and 2) or 34% (groups 3 and 4) casein level, increased the blood pressure of normotensive (groups 1 and 3) and hypertensive (groups 2 and 4) animals. In addition, the hypertensive rats presented more severe hypercholesterolemia (groups 2 and 4) than did the normotensive (groups 1 and 3). In groups fed the 34% casein diet (groups 3 and 4), this difference was particularly striking at the 12- and 18-week period.

Comparison of groups 1 and 3 shows the effect of the dietary casein level on the cholesterolemic response. As noted in experiment 2, an increase in the casein level resulted in a decrease in the cholesterolemia. This effect was no more evident when the animals were rendered hypertensive, since there was no significant difference in the cholesterolemia between groups 2 and 4 at the 12- and the 19-week period. However, hypertension appeared to be more severe in group 4, with a high casein level, than in group 2, with a low casein level.

At a low casein level, hypertension slightly increased the mortality rate and the incidence of cardiac thrombosis but markedly increased the incidence of fatty streaks (group 1 as compared with group 2). At the high casein level, hypertension (group 4) caused 40% mortality as compared with none in group 3. Cardiac thrombosis was not observed in normo- or hypertensive animals fed at the high casein level.

Histologically the thrombi in the heart were located, as in experiment 2, in the cardiac cavities or in large or small coronary vessels. They were not multiple and disseminated as observed with the chow-containing diet (exp. 1). The most striking results were obtained with combined hypertension-hyperlipemia (groups 2 and 4). Although the fatty streaks of the aorta were similar to those already reported in experiment 2, severe atherosclerotic lesions could be seen in the main coronary arteries or their large branches of

TABLE 3

*Effect of dietary casein level and hypertension on the production of hypercholesterolemia, thrombosis and aortic fatty streaks in rats* [1]

| Group no. | Casein level of diet [2] | Treatment | Blood pressure | | Serum cholesterol | | | Cardiac thrombosis, incidence | Fatty streaks, incidence | Mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Week 8 | Week 12 | Week 8 | Week 12 | Week 18 | | | |
| | % | | *mm Hg* | *mm Hg* | *mg/100 ml* | *mg/100 ml* | *mg/100 ml* | % | % | % |
| 1 | 11 | None | 128 ± 3 [3] | 141 ± 3 | 83 ± 4 | 408 ± 33 | 704 ± 51 | 55 | 15 | 85 |
| 2 | 11 | DCA [4] + NaCl | 164 ± 5 | 173 ± 7 | 111 ± 10 | 418 ± 63 | 803 ± 54 | 60 | 60 | 95 |
| 3 | 34 | None | 125 ± 4 | 148 ± 4 | 77 ± 2 | 358 ± 39 | 401 ± 26 | 0 | 65 | 0 |
| 4 | 34 | DCA + NaCl | 162 ± 5 | 194 ± 7 | 105 ± 8 | 517 ± 60 | 756 ± 78 | 0 | 75 | 40 |

[1] Total duration of experiment, 37 weeks; 20 animals/group.
[2] In addition to casein at a level indicated in the table, diets contained: (in per cent) butter, 40; cholesterol, 5; cholic acid, 2; salt mixture, 4; vitamin mixture, 2; sucrose, 10; and cellulose to make 100.
[3] Mean ± SE.
[4] DCA indicates desoxycorticosterone acetate.

hypertensive animals and specially of group 4. They were characterized by a marked thickening of the vessel wall, both the intima and the media being involved, with the presence of foam cells, but also of marked reactive fibrosis. Cholesterol crystals could also be observed in the deeper part of the intima and in the media. These lesions resulted in partial or complete obstruction of the lumen and recanalization (figs. 4 and 5).

The kidneys of some animals of group 2 and of all those of group 4 presented lesions of malignant nephrosclerosis. They consisted of hyalinization of the arterioles, the glomerular tuft capillaries and the interstitial tissue. Cystic dilatation of the tubules with numerous hyaline casts and thrombosis in arterioles and capillaries were also always present. All the animals of groups 1 and 2 presented lesions of nephrosis, as described previously (1).

Lesions of polyarteritis nodosa that usually develop in rats fed laboratory chow and rendered hypertensive with the method used here, were not observed in any animals of group 2 and in only 20% of the animals of group 4.

*Experiment 4 (table 4).* The atherogenic effects of high and low levels of dietary casein were compared more adequately in the following way: 1) by utilizing hooded rats, as these respond to hyperlipemic diet feeding by more constant and more severe lesions than albino rats (10); 2) by killing and autopsying the survivors when only 40% of the animals on the low casein level (10%) (group 1) had died. Under these conditions at least 6 animals of group 1 could be compared exactly with 10 animals of group 2. As a result, but after only 24 weeks, the incidence and the severity of the aortic fatty streaks in group 1 (10% casein) were observed to be approximately twice those of group 2 (34% casein). The cholesterol was also significantly higher in group 1 than in group 2 and the thrombi, which occurred in group 1, were not located in the large coronary vessels or the cardiac cavities, but were seen in the small cardiac vessels and were multiple, as in experiment 1.

## DISCUSSION

The feeding (13, 14) or the intravenous injection (15) of certain natural bile acids and particularly cholic acid, is known to induce hypercholesterolemia in various animal species. Under the present experimental conditions in the rat, the addition of sodium cholate to a diet rich in lipids results in hyperlipemia with serum changes similar to those encountered in essential hyperlipemia: milky serum, increase in total cholesterol and total lipids, decrease in the percentage of alpha lipoproteins and increase in the alpha$_2$ fraction (1). Utilizing this experimental condition, it has been shown here that, provided the dietary protein level is 12% or less, thrombosis can be produced by hyperlipemic diets in albino or hooded rats. On the other hand, by increasing the protein content of the diet, thrombosis can be prevented completely with or without a decrease in serum cholesterol. As a result of an increased dietary level of casein, the total calories of the diet were increased; nevertheless, the hypocholesterolemic ef-

TABLE 4

*Effect of dietary casein level on the production of hypercholesterolemia, thrombosis and aortic fatty streaks in the hooded rat* [1]

| Group no. | Casein level of diet [2] | Serum cholesterol | | Thrombosis, incidence | Fatty streaks | | Mortality |
|---|---|---|---|---|---|---|---|
| | | Week 6 | Week 10 | | Incidence | Intensity (scale 0–3) | |
| | % | *mg/100 ml* | *mg/100 ml* | % | % | | % |
| 1 | 10 | 856 ± 54 [3] | 543 ± 48 | 30 | 90 | 2.2 | 40 |
| 2 | 34 | 419 ± 38 | 321 ± 47 | 0 | 40 | 1.1 | 0 |

[1] Total duration of experiment, 24 weeks; 10 animals/group.

[2] In addition to casein at a level indicated in the table, diets contained: (in per cent) butter, 40; cholesterol, 5; cholic acid, 2; salt mixture, 4; vitamin mixture, 2; sucrose, 10; and cellulose to make 100.

[3] Mean ± SE.

fect (when present) and anti-thrombotic effect of casein could not have been the result of the increase in total calories, since this effect could not be reproduced by increasing the dietary level of sucrose. It also appears that the anti-thrombotic effect of casein was not a consequence of its hypocholesterolemic effect, for the following reasons. In experiment 1, addition of casein to the diet did not influence the serum cholesterol or percentage of $\alpha$-lipoproteins. In experiment 3, the hypertensive animals fed at a high casein level exhibited approximately the same degree of cholesterolemia as animals fed at the low casein level. However in these 2 experiments, thrombosis occurred, in the period of observation, only when diets were fed at a low casein level.

In these diets, other factors in addition to the protein level appear to play a role in the production of hypercholesterolemia and thrombosis. In the present experiment and numerous others the cholesterolemic response is much lower in animals fed a chow-containing diet than a purified diet, even when the protein level is the same. In addition, the incidence of thrombosis is lower when purified diets are fed and thrombosis occurs in the cardiac cavities or large coronary vessels instead of in the small cardiac vessels. However, the fact that thrombosis is not observed in every instance does not necessarily imply that thrombosis was not the cause of death. When thrombi are multiple and disseminated, as when a chow-containing diet is fed, they can be easily recognized on routine histology. If they are larger but small in number, they are more difficult to locate unless serial sections are made for every organ.

The loss of hair that occurs in animals fed the thrombogenic diet was prevented by increasing the dietary level of casein. It is probable that this loss of hair results from a certain amino acid deficiency. However, more experiments are needed to prove this hypothesis and to establish whether there is any relationship between the loss of hair and the occurrence of thrombosis.

Another difference between chow-containing and purified diets is that, within 7 months, aortic fatty streaks and even atherosclerotic plaques were consistently observed in animals fed the purified diets, whereas they never occurred with hyperlipemic diets containing chow, unless the animals were rendered hypertensive (9). This result, which serves to confirm the work of others (3), was probably due to the higher level of cholesterol obtained with purified diets. With respect to the influence of the protein level on the production of atherosclerosis, it appeared that in the albino rat, high levels of casein increased the incidence and the severity of the lesions. However, in hooded rats, the opposite result was obtained. This is probably due to a difference in susceptibility between these 2 strains of rats, since in the 2 experiments there were only minor variations in the experimental conditions used. Conflicting results have also been reported in the literature, according to the experimental procedure and the animal species utilized (16–19).

It is generally recognized that hyperlipemia and malignant hypertension considerably increase the risk of coronary heart disease in man. Here, in hypertensive hyperlipemic animals severe coronary atherosclerotic lesions were observed frequently. Increasing the level of dietary protein appeared to increase the hypertensive response and also the severity of the nephrosclerotic and atherosclerotic lesions. Nevertheless, occlusive thrombosis was obtained solely with a low protein diet.

From these experiments in the rat it can be concluded that even though hypercholesterolemia appears to be a prerequisite for both thrombosis and atherosclerosis, the incidence or severity of the lesions do not necessarily depend on the magnitude of the lipemic changes. The occurrence of thrombosis is dependent on a low dietary protein level rather than on the magnitude of the cholesterolemic response. In contrast with this, atherosclerosis can be produced by diets with both a low or a high protein level. With a high protein level, therefore, thrombosis would not occur and coronary atherosclerosis would have a chance to develop until complete occlusion was reached. A similar result has recently been reported in man (20). The coronary occlusions observed in the Bantu consuming a diet low in animal protein were

predominantly thrombotic. The occlusive lesions of those eating a diet high in animal protein and fat were due mainly to atherosclerosis.

## LITERATURE CITED


1. Renaud, S., and C. Allard 1962 Thrombosis in connection with serum lipidic changes in the rat. Circulation. Res., *11:* 388.
2. Renaud, S. 1962 A thrombotic thrombocytopenia in the rat as compared to human thrombotic thrombocytopenic purpura. Lab. Invest., *11:* 854.
3. O'Neal, R. M., W. J. S. Still and W. S. Hartroft 1961 Experimental atherosclerosis in the rat. J. Path. Bact., 82: 183.
4. Renaud, S., and C. Allard 1963 Dietary fats, vitamins and bile acids in the production of thrombosis and hyperlipemia in rats. Rev. Canad. Biol., 22: 309.
5. Leveille, G. A., and H. Fisher 1958 Plasma cholesterol in growing chicken as influenced by dietary protein and fat. Proc. Soc. Exp. Biol. Med., *98:* 630.
6. 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. Nutrition, *69:* 269.
7. Moyer, A. W., D. Kritchevsky, J. B. Logan and H. R. Cox 1956 Dietary protein and serum cholesterol in rats. Proc. Soc. Exp. Biol. Med., *92:* 736.
8. Ringsdorf, W. M., Jr., and E. Cheraskin 1963 Effect of a relatively high-protein low-refined-carbohydrate diet upon serum cholesterol concentration. J. Am. Geriat. Soc., *11:* 156.
9. Renaud, S., and C. Allard 1963 Hypertension, thrombosis and atherosclerosis in the rat. J. Canad. Med. A., *88:* 1275.
10. Renaud, S. 1964 Influence of sex on the dietary production of thrombosis and fatty streaks in the hooded rat. J. Atheroscler. Res., in press.
11. Trinder, P. 1952 Determination of cholesterol in serum. Analyst., 77: 321.
12. Allard, C., and G. Choquette 1962 Percentage of alpha-lipoproteins, total cholesterol, and atherosclerosis. Canad. J. Biochem., *40:* 1515.
13. Member, S., M. Bruger and E. Oppenheim 1944 Experimental atherosclerosis. VI. Effects of various bile acids on cholesterol levels. Arch. Path., 38: 210.
14. Howe, E. E., D. K. Bosshardt and J. W. Huff 1960 Effect of bile acids on plasma cholesterol in the mouse. J. Nutrition, 72: 379.
15. Friedman, M., and S. O. Byers 1951 Cholic acid: An adequate stimulus for hypercholesteremia in the normal fasting rat. Proc. Soc. Exp. Biol. Med., 78: 528.
16. Clarkson, S., and L. N. Newburgh 1926 The relation between atherosclerosis and ingested cholesterol in the rabbit. J. Exp. Med., 43: 595.
17. Pick, R., J. Stamler and L. N. Katz 1959 Effects of high protein intake on cholesterolemia and atherogenesis in growing and mature chickens fed high-fat, high-cholesterol diet. Circulation Res., 7: 866.
18. Fisher, H., A. Feigenbaum, G. A. Leveille, H. S. Weiss and P. Griminger 1959 Biochemical observations on aortas of chickens. Effect of different fats and varying levels of protein, fat and cholesterol. J. Nutrition, 69: 163.
19. Prichard, R. W., T. B. Clarkson, H. B. Lofland, Jr., H. O. Goodman, C. N. Herndon and M. G. Netsky 1962 Studies on the atherosclerotic pigeon. J.A.M.A., *179:* 49.
20. Seftel, H. C., K. J. Keeley and A. R. P. Walker 1963 Characteristics of South African Bantu who have suffered from myocardial infarction. Am. J. Cardiol., *12:* 148.


## PLATE 1

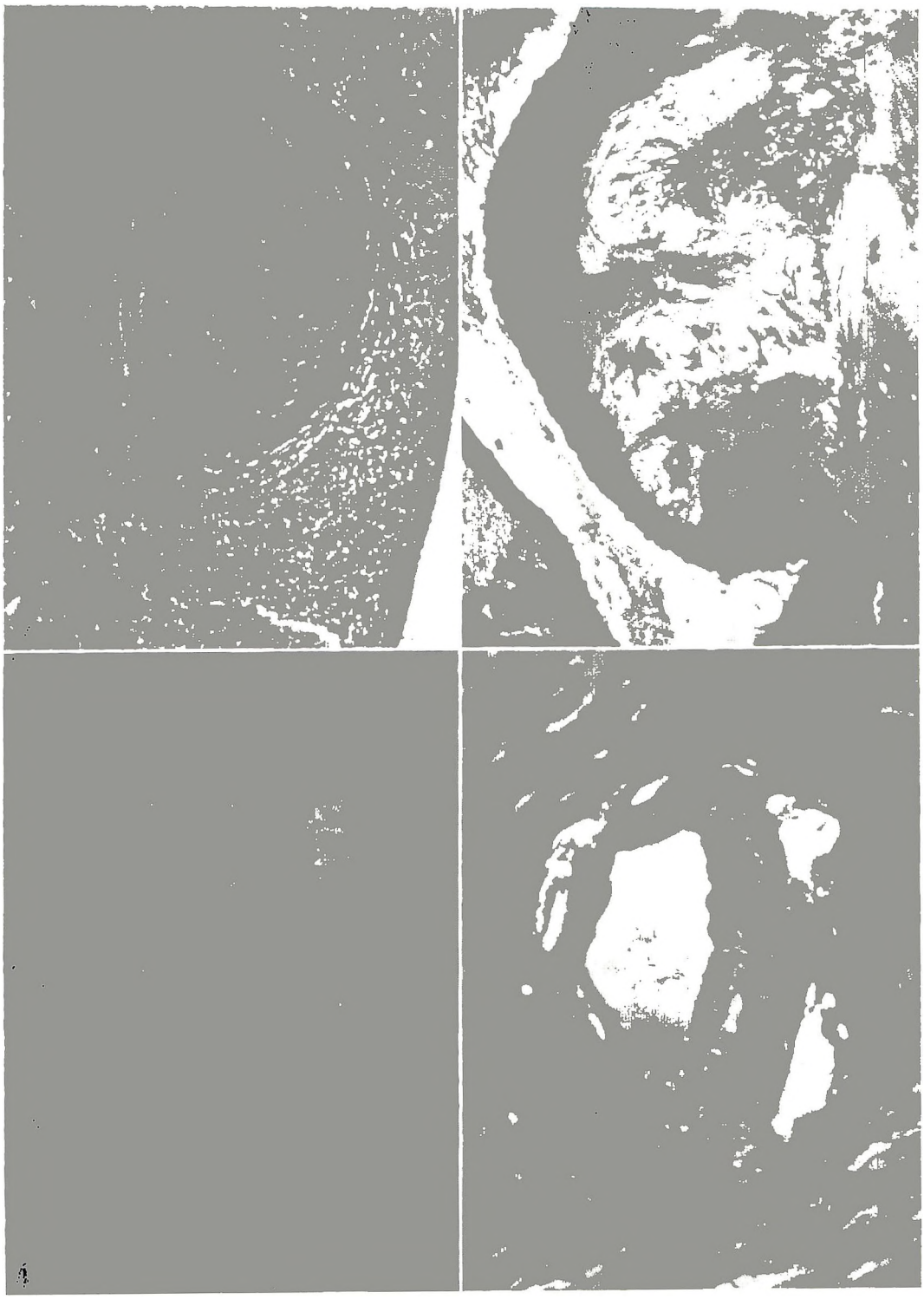
### EXPLANATION OF FIGURES

2 A thrombus completely occluding a large branch of the left coronary artery of a rat of group 1 (exp. 2). The entire portion of the myocardium seen in the figure is infarcted. Periodic-acid-Schiff. × 100.

3 A large atherosclerotic plaque on a mitral valve near its attachment on the atrioventricular annulus of a rat of group 4 (exp. 2). Although the superficial part of the plaque is fibrotic, the deeper part is largely composed of degenerated foam cells. Hematoxylin-phloxine-saffron. × 100.

4 An atherosclerotic lesion in a coronary artery of a hypertensive-hyperlipemic rat. Note the complete rupture of the internal elastic membrane, the presence of cholesterol crystals and of numerous smooth muscle cells in the lesion. Only a few muscle cells of the media are normally located. Hematoxylin-phloxine-saffron. × 250.

5 A partially occluded and recanalized coronary artery of a hypertensive-hyperlipemic rat. Periodic-acid-Schiff. × 150.



# Influence of Gelatin on Growth and Liver Pyridine Nucleotide Concentration of the Rat [1]

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*ABSTRACT* Growth and liver pyridine nucleotide concentrations (NAD-NADP) of rats fed a 6% casein, niacin-deficient basal diet supplemented with either 12% of gelatin or a mixture of indispensable or dispensable amino acids simulating the amino acid composition of 12% of gelatin were determined. Growth was significantly depressed by these supplements below that obtained for rats fed the basal diet. Tryptophan prevented the growth depression caused by gelatin or the mixture of indispensable amino acids, but not that due to the mixture of dispensable amino acids. The liver NAD-NADP of rats fed the diets that were not supplemented with tryptophan were not markedly different. A tryptophan supplement significantly increased the liver NAD-NADP of rats fed the basal diet or the diet containing the mixture of indispensable amino acids, but produced only small increases in rats fed the diets containing gelatin or the mixture of dispensable amino acids. By feeding rats diets containing various combinations of the dispensable amino acids in the quantities in which they occur in 12% of gelatin, glycine or L-4-hydroxyproline were shown to be responsible for both the growth depression, and the decreased synthesis of liver NAD-NADP in response to a supplement of tryptophan. Rats fed diets containing gelatin did not show a decreased ability to synthesize NAD-NADP when large amounts of tryptophan were injected, nor did gelatin appear to interfere with the induction of tryptophan pyrrolase.

Supplements of tryptophan-deficient proteins, such as gelatin and zein, when added to diets lacking in niacin and low in protein, cause retardation of growth and development of niacin deficiency in rats (1). Additions of various amino acids to such diets also cause growth depressions and deficiency signs analogous to those induced by a supplement of gelatin (2). Morrison and Harper (3) studied in some detail the effects of additions of single L-indispensable amino acids and mixtures of L-indispensable amino acids in quantities simulating the composition of 12% of gelatin on the growth of rats fed a low-protein diet lacking niacin. They found that only when L-threonine was present in the amino acid mixture was there a marked depression in the growth rate.

Morrison et al. (4) observed that a supplement of tryptophan increased both growth and liver pyridine nucleotide concentrations of rats fed a 9% casein, niacin-deficient diet supplemented with threonine. Tryptophan also stimulated the growth of rats fed a similar diet supplemented with gelatin but did not increase their liver pyridine nucleotide concentration significantly. These results agreed with earlier observations of Rosen and Perlzweig (5) and supported their suggestion that gelatin affected in some manner the ability of the rat to utilize tryptophan for the synthesis of liver pyridine nucleotides. The experiments reported in the present paper were undertaken to investigate the effect of dietary additions of various amino acids on liver pyridine nucleotide synthesis in response to administration of tryptophan.

## EXPERIMENTAL

Male weanling rats (Holtzman) weighing 40 to 50 g were housed in individual suspended cages and were fed the basal diet for 2 or 3 days to allow them to adjust to the environment. The percentage composition of the basal diet was as follows: casein, 6; DL-methionine, 0.3; salt mixture,[4] 5; choline chloride, 0.15; corn oil, 5;

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[4] Harper, A. E. 1959 Amino acid balance and imbalance. I. Dietary level of protein and amino acid imbalance. J. Nutrition, 68: 405.

water-soluble vitamin mixture (less niacin) in sucrose,[5] 0.25; and sucrose to make up to 100. Fat-soluble vitamins were included in the corn oil to provide the following concentrations per 100 g of diet: vitamin A, 400 IU; vitamin D, 40 IU; and $\alpha$-tocopherol, 10 mg.

After the adjustment period the animals were separated into groups of either 5 or 6 rats each so that the average weights of the groups did not differ by more than 1 g. The various groups of rats were fed the basal diet plus additions of gelatin, indispensable amino acids or dispensable amino acids and water ad libitum for 14 days. The values for the average amino acid content of gelatin were obtained from tables in Block and Weiss (6) with the exception of glycine. Block and Weiss indicated the glycine content of gelatin to be 15.7 g/16 g of nitrogen. However, Block and Bolling (7) reported the glycine content as 23.6 g/16 g of nitrogen, Tristram (8) reported 26.9 g glycine/16 g of nitrogen and Sahyun (9) reported 25.5 g glycine/16 g of nitrogen. Thus an average of these 3 values, 25.3 g glycine/16 g of nitrogen, was used in these studies as the glycine content of gelatin. Gelatin or the amino acids were included in the basal diet at the expense of sucrose. L-Isomers of the amino acids were used with the exception of DL-methionine. Each diet was fed with and without a supplement of 0.1% L-tryptophan. All animals were weighed 3 times weekly.

At the end of the experimental period the rats were decapitated, their livers removed within one minute, blotted dry and frozen in a mixture of dry ice and acetone to reduce possible destruction of the pyridine nucleotides. One- to two-gram samples were homogenized in 10 ml of 2% trichloroacetic acid; the supernatant fluid was decanted after centrifugation and frozen for later analysis. The total oxidized liver NAD plus NADP was determined by the method of Feigelson et al. (10). For each set of determinations, recoveries using 96% pure nicotinamide adenine dinucleotide[6] ranged from 90 to 100%.

In the experiment in which tryptophan pyrrolase activity was determined, Holtzman male rats weighing approximately 100 g were fed by stomach tube 2 g of gelatin or casein dissolved in 4 ml of distilled water. The rats were injected intraperitoneally with either 4 ml of L-tryptophan solution (20 mg/ml in 0.9% NaCl) or 4 ml of a 0.9% NaCl solution. They were decapitated 5 hours after the injections. The livers were removed immediately, chilled in ice, blotted dry and 1.0- to 1.5-g samples were homogenized in 10 ml of a cold 0.9% KCl solution for approximately 30 seconds. Two milliliters of each homogenate were added to a flask containing 5.0 $\mu$moles of L-tryptophan and 0.05 M $KH_2PO_4$. Glass-distilled water was added to give a final volume of 3 ml. The flasks were stoppered and incubated at 37°C in a shaker for one hour with air as the gas phase. The reaction was stopped by adding 1 ml of a 16% trichloroacetic acid solution to all flasks. All samples were run in duplicate and a zero-time flask was included for each determination. The precipitate was removed by centrifugation and the clear supernatant solution was decanted and frozen for later analysis. This procedure is essentially that described by deCastro et al. (11) with certain modifications as indicated above. The kynurenine formed during the incubation period was determined by the method of Brown and Price (12).

## RESULTS AND DISCUSSION

The results presented in table 1 show that the addition of 12% of gelatin or a mixture of indispensable amino acids based on the composition of 12% of gelatin to a low-protein, niacin-deficient diet produced an amino acid imbalance characterized by marked growth retardation. With both diets a tryptophan supplement prevented the growth depression and enhanced the growth rate. Growth was also depressed significantly ($P < 0.01$) when the basal diet was supplemented with a dispensable amino acid mixture providing each dispensable amino acid in an amount equivalent to that provided by 12% of gelatin. This growth depression was not prevented by the addition of tryptophan.

The liver pyridine nucleotide concentrations of rats fed the diets without the

[5] See footnote 4.
[6] Obtained from Sigma Chemical Company, St. Louis.

TABLE 1

*Effect of tryptophan on the growth and liver pyridine nucleotide concentration of rats fed a low-protein, niacin-deficient diet plus gelation or a mixture of the dispensable or indispensable amino acids simulating gelatin*

| Diet | No. rats | Wt gain | | Liver NAD-NADP | |
|---|---|---|---|---|---|
| | | Without L-tryptophan | 0.1% L-tryptophan | Without L-tryptophan | 0.1% L-tryptophan |
| | | *g/14 days* | *g/14 days* | *μg/g* | *μg/g* |
| Basal diet | 21 | 15.2 ± 0.9 [1] | 14.9 ± 0.9 | 455 ± 15 | 663 ± 18 |
| Basal + 12% gelatin | 24 | 3.0 ± 0.6 | 61.9 ± 2.2 | 493 ± 20 | 559 ± 11 |
| Basal + mixture of L-indispensable amino acids [2] | 15 | 4.3 ± 0.9 | 50.0 ± 2.4 | 525 ± 24 | 845 ± 25 |
| Basal + mixture of L-dispensable amino acids [2] | 16 | 7.8 ± 0.9 | 9.2 ± 0.8 | 409 ± 12 | 541 ± 11 |

[1] Average value of several studies ± SE of mean.
[2] Mixture of L-indispensable and L-dispensable amino acids simulated the amino acid composition of 12% of gelatin.

tryptophan supplement were not markedly different (table 1). A supplement of L-tryptophan increased the pyridine nucleotide concentrations of all groups but the responses were quite different. The increases in the livers of rats fed the basal diet and the basal diet plus the indispensable amino acid mixture were 208 and 320 μg, respectively, wherease the increases in the livers of rats fed the diets containing gelatin or the dispensable amino acid mixture were only 66 and 132 μg, respectively. The results obtained with gelatin in this study are in agreement with those of Morrison et al. (4) and suggested further that one or more of the dispensable amino acids of gelatin might be responsible for the decreased synthesis of pyridine nucleotides.

To ascertain which dispensable amino acid(s) of gelatin was responsible for this effect, rats were fed the basal diet supplemented with dispensable amino acids in the quantities present in 12% of gelatin (table 2, diets 3–10). Neither the addition of L-tyrosine, L-cystine and L-serine together, nor the stepwise additions of L-alanine, L-aspartic acid, L-glutamic acid and L-proline to the basal diet, affected growth significantly ($P > 0.01$) (table 2, diets 3–7). A supplement of tryptophan had no significant effect on the growth of rats fed these diets. However, addition of glycine or L-4-hydoxyproline, alone or together, (table 2, diets 8, 9 and 10) caused a marked depression in growth. A tryptophan supplement prevented the growth depression caused by L-4-hydroxyproline, but not that caused by glycine or glycine and L-4-hydroxyproline together.

Liver pyridine nucleotide concentrations of rats fed the diets without a tryptophan supplement were not markedly different from the value obtained for the group fed the diet containing gelatin. Liver pyridine nucleotide concentrations of rats fed the basal diet increased when the diet was supplemented with tryptophan, but no response to tryptophan was observed in those fed the diet containing gelatin. The addition of L-tyrosine, L-serine, L-cystine and the stepwise addition of L-alanine, L-aspartic acid, L-glutamic acid and L-proline to the basal diet did not significantly ($P > 0.01$) depress the liver pyridine nucleotide concentration below the value obtained for the group receiving the basal diet supplemented with tryptophan. The addition of either glycine or L-4-hydroxyproline, alone or together, depressed pyridine nucleotide concentration to a value just above that of rats receiving gelatin.

The depressing action of glycine and L-4-hydroxyproline on growth and liver pyridine nucleotide concentration was studied further in rats fed the basal diet supplemented with increasing levels of either glycine or L-4-hydroxyproline. As shown in table 3, increasing levels of glycine in the diet caused a depression in growth rate which was not prevented by a supplement of tryptophan. The depression in growth was significant ($P < 0.05$) when glycine constituted 3.08 and 4.62%

of the diet. These are the amounts of glycine that would be present in diets containing 12 and 18% gelatin. In the absence of tryptophan, increasing levels of glycine had no significant effect on liver pyridine nucleotide concentration. However, increasing the level of glycine in diets supplemented with tryptophan depressed pyridine nucleotide concentration below that obtained with rats fed the basal diet plus tryptophan. The depressions observed when glycine constituted 3.08 and 4.62% of the diet were statistically significant ($P < 0.05$).

Increasing the level of L-4-hydroxyproline did not affect growth to any marked extent in the absence of added tryptophan. Supplementation of the various diets with

TABLE 2

*Effect of the stepwise addition of the L-dispensable amino acids as found in 12% of gelatin to a low-protein, niacin-deficient diet on weight gain and liver pyridine nucleotide concentrations*

| Diet no. | Diet | Wt gain | | Liver NAD-NADP | |
|---|---|---|---|---|---|
| | | Without L-tryptophan | 0.1% L-tryptophan | Without L-tryptophan | 0.1% L-tryptophan |
| | | *g/12 days* | *g/12 days* | *μg/g* | *μg/g* |
| 1 | 6% Casein | 12.4 ± 1.1 [1] | 15.2 ± 0.9 | 422 ± 14 | 645 ± 39 |
| 2 | Diet 1 + 12% gelatin | 4.4 ± 2.1 | 61.8 ± 2.5 | 506 ± 13 | 520 ± 13 |
| 3 | Diet 1 + L-tyrosine, L-cystine and L-serine | 10.0 ± 1.1 | 14.8 ± 2.2 | 451 ± 17 | 598 ± 42 |
| 4 | Diet 3 + L-alanine | 12.0 ± 0.8 | 15.8 ± 0.7 | 505 ± 47 | 620 ± 29 |
| 5 | Diet 4 + L-aspartic acid | 10.6 ± 1.5 | 16.8 ± 1.6 | 466 ± 16 | 747 ± 22 |
| 6 | Diet 5 + L-glutamic acid | 12.0 ± 2.5 | 12.2 ± 0.4 | 449 ± 37 | 610 ± 18 |
| 7 | Diet 6 + L-proline | 10.2 ± 1.2 | 12.6 ± 0.6 | 441 ± 12 | 610 ± 23 |
| 8 | Diet 7 + glycine | 7.2 ± 0.4 | 9.2 ± 0.7 | 414 ± 28 | 572 ± 33 |
| 9 | Diet 7 + L-4-hydroxyproline | 6.8 ± 1.2 | 14.9 ± 1.8 | 489 ± 32 | 554 ± 25 |
| 10 | Diet 7 + L-4-hydroxyproline + glycine | 8.1 ± 1.8 | 7.8 ± 1.0 | 415 ± 17 | 540 ± 23 |

[1] Average of 5 animals ± SE of mean.

TABLE 3

*Effect of graded increments of glycine and L-4-hydroxyproline on growth and liver pyridine nucleotide concentration of rats fed a niacin-deficient 6% casein diet with and without a tryptophan supplement*

| Supplement | Wt gain | | Liver NAD-NADP | |
|---|---|---|---|---|
| | Without L-tryptophan | 0.1% L-tryptophan | Without L-tryptophan | 0.1% L-tryptophan |
| | *g/15 days* | *g/15 days* | *μg/g* | *μg/g* |
| None | 17.4 ± 3.4 [1] | 15.8 ± 1.5 | 499 ± 42 | 661 ± 34 |
| 0.77% Glycine | 15.8 ± 2.3 | 14.4 ± 0.5 | 478 ± 16 | 576 ± 37 |
| 1.54% Glycine | 12.2 ± 2.3 | 15.8 ± 1.3 | 500 ± 18 | 589 ± 31 |
| 3.08% Glycine | 7.8 ± 2.2 | 10.4 ± 2.8 | 488 ± 23 | 523 ± 27 [2] |
| 4.62% Glycine | 8.0 ± 1.6 | 8.2 ± 3.0 | 461 ± 21 | 552 ± 16 [2] |
| | *g/10 days* | *g/10 days* | | |
| None | 7.2 ± 1.4 | 7.0 ± 1.1 | 501 ± 33 | 693 ± 22 |
| 0.40% L-4-Hydroxyproline | 7.8 ± 1.8 | 11.6 ± 0.9 | 479 ± 17 | 712 ± 13 |
| 0.80% L-4-Hydroxyproline | 11.4 ± 1.5 | 13.8 ± 1.6 | 484 ± 15 | 632 ± 19 |
| 1.60% L-4-Hydroxyproline | 7.8 ± 0.7 | 12.2 ± 1.7 | 538 ± 33 | 608 ± 22 [3] |
| 2.40% L-4-Hydroxyproline | 8.0 ± 2.4 | 12.8 ± 1.0 | 454 ± 44 | 599 ± 38 [3] |

[1] Average of 5 animals ± SE of mean.
[2] Significantly different ($P < 0.05$) from value obtained for rats fed no glycine supplement.
[3] Significantly different ($P < 0.05$) from value obtained for rats fed no L-4-hydroxyproline supplement.

tryptophan produced significant growth increases ($P < 0.05$) at the 0.4 and 1.6% levels of L-4-hydroxyproline but not at the 0.8 and 2.4% levels. The short duration of this experiment (10 days) may partly explain the variability of these results. Liver pyridine nucleotide concentration was not affected appreciably by increasing the level of L-4-hydroxyproline in the absence of added tryptophan but the pyridine nucleotide concentration was significantly lowered ($P < 0.05$) in the tryptophan-supplemented groups by the addition of 1.6 or 2.4% L-4-hydroxyproline. These are the amounts of L-4-hydroxyproline that would be present in diets containing 12 and 18% of gelatin.

The toxic effect of high dietary levels of glycine is well known (13). The growth-depressing effect of glycine obtained in these studies is essentially in agreement with observations of Hardin and Hove (14). Swenseid et al. (15) reported that a combined supplement of L-tryptophan and L-threonine could prevent a growth depression caused by the addition of 4.9% glycine to a low-protein diet but not that caused by 7.5% glycine. In this study a 0.1% L-tryptophan supplement did not prevent the growth depression caused by either 3.08 or 4.65% glycine in a 6% casein diet deficient in niacin.

Hydroxyproline has been reported to have a depressing effect on growth when fed at levels of 3.4% of the diet (16). The L-4-hydroxproline content of 12% of gelatin is only 1.6%. When this amount of L-4-hydroxyproline was added to the basal diet supplemented with the other dispensable amino acids (exclusive of glycine) growth was depressed (table 2, diet 9). In contrast with these results no growth depressions were observed when either 1.6 or 2.4% of L-4-hydroxyproline was added alone to the basal diet. This suggests that growth in rats can be affected by the balance between the other dispensable amino acids of gelatin (exclusive of glycine) and L-4-hydroxyproline. Since the addition of tryptophan, the most limiting amino acid in the diet, alleviated the growth depression caused by the dispensable amino acid mixture containing L-4-hydroxyproline but not glycine (table 2, diet 8 and 9), there is a resemblance between this situation and that resulting from the amino acid imbalance caused by addition of the indispensable amino acids (table 1).

The effects on growth and liver pyridine nucleotides of adding glycine and L-4-hydroxyproline to the basal diet containing a mixture of the indispensable amino acids simulating the composition of 12% of gelatin are shown in table 4. The addition of tryptophan resulted in a marked growth response in rats fed the diets containing gelatin or the indispensable amino acid mixture. The growth rate of rats fed the indispensable amino acid mixture plus glycine and L-4-hydoxyproline was only 69% of that of rats fed the indispensable amino acid mixture. The liver pyridine nucleotide concentration of rats fed the indispensable amino acid mixture plus glycine and L-4-hydroxyproline in the presence of the tryptophan supplement was

TABLE 4

*Effect of glycine and L-4-hydroxyproline on the growth and liver pyridine nucleotide concentration of rats fed a low-protein, niacin-deficient diet plus a mixture of the indispensable amino acids simulating 12% of gelatin*

| Supplement | Wt gain | | Liver NAD-NADP | |
|---|---|---|---|---|
| | Without L-tryptophan | 0.1% L-tryptophan | Without L-tryptophan | 0.1% L-tryptophan |
| | *g/14 days* | *g/14 days* | *μg/g* | *μg/g* |
| 12% Gelatin | 4.8 ± 0.6 [1] | 54.8 ± 4.3 | 444 ± 22 | 539 ± 13 |
| Mixture of indispensable amino acids | 2.2 ± 1.1 | 51.0 ± 3.8 | 495 ± 43 | 766 ± 25 |
| Mixture of indispensable amino acid + 3.08% glycine and 1.60% L-4-hydroxyproline [2] | −0.2 ± 1.0 | 35.4 ± 3.7 | 522 ± 44 | 575 ± 26 |

[1] Average of 5 animals ± SE of mean.
[2] Amounts of glycine and L-4-hydroxyproline equal to those provided in 12% of gelatin.

depressed below the value obtained for rats fed the indispensable amino acid mixture and was comparable to the value obtained for the rats fed gelatin. This is further evidence that glycine and L-4-hydroxyproline interfere in some way with the synthesis of liver pyridine nucleotides that normally occurs in response to a dietary supplement of tryptophan.

In an attempt to elucidate the mechanism by which gelatin prevents this increase in pyridine nucleotide concentration, rats were fed either casein or gelatin by stomach tube and immediately given an intraperitoneal injection of L-tryptophan (100 mg/100 g body weight). The average tryptophan pyrrolase activities, measured 5 hours following the tryptophan injection, are shown in table 5 and indicate that gelatin under these conditions did not interfere with the induction of tryptophan pyrrolase, and that if a block in the metabolic pathway between tryptophan and pyridine nucleotides occurs it is not at this step.

TABLE 5

*Tryptophan pyrrolase activity 5 hours after injection of L-tryptophan into rats fed casein or gelatin by stomach tube*

| Protein fed | Tryptophan pyrrolase activity [1] | |
|---|---|---|
| | NaCl injected | Tryptophan injected |
| | *μmoles kynurenine/g/hr* | |
| Casein | 0.91 | 11.87 |
| Gelatin | 0.15 | 10.37 |

[1] Average of 5 animals.

Morrison et al. (4) showed that rats maintained for 10 weeks with an 8% casein-6% gelatin diet lacking niacin were still able to utilize injected nicotinamide for the synthesis of liver pyridine nucleotides. Thus, it was of interest to test the ability of rats maintained with a 6% casein-12% gelatin diet for 2 weeks to utilize injected tryptophan for the synthesis of pyridine nucleotides. The liver pyridine nucleotide concentrations 4 hours after an intraperitoneal injection of L-tryptophan (100 mg/100 g body weight), shown in table 6, indicate that the ability of the rats fed the gelatin-containing diet to synthesize pyridine nucleotides from tryptophan was not impaired. The levels

TABLE 6

*Effect of intraperitoneal injection of L-tryptophan on liver pyridine nucleotide concentration of rats fed a diet containing 6% of casein with and without 12% gelatin.*

| Diet | Liver NAD-NADP [1] | |
|---|---|---|
| | NaCl injected | Tryptophan injected |
| | *μg/g liver* | *μg/g liver* |
| 6% Casein | 544 | 856 |
| 6% Casein + 12% gelatin | 606 | 1005 |

[1] Average of 4 animals.

at which tryptophan was injected in these latter 2 studies were considerably higher than the daily dietary intake of tryptophan of rats fed the various experimental diets in the earlier studies and it may be that this excessive amount of tryptophan masked any effect the gelatin might have had on the synthesis of liver pyridine nucleotides.

LITERATURE CITED

1. Krehl, W. A., P. S. Sarma, L. J. Teply and C. A. Elvehjem 1946 Factors affecting the dietary niacin and tryptophan requirement of the growing rat. J. Nutrition, *31:* 85.
2. Lyman, R. L., and C. A. Elvehjem 1951 Further studies on amino acid imbalance produced by gelatin in rats on a niacin-tryptophan low ration. J. Nutrition, *45:* 101.
3. Morrison, M. A., and A. E. Harper 1960 Amino acid balance and imbalance. IV. Specificity of threonine in producing an imbalance in diets deficient in niacin and tryptophan. J. Nutrition, *71:* 296.
4. Morrison, M. A., M. S. Reynolds and A. E. Harper 1960 Amino acid balance and imbalance. V. Effect of an amino acid imbalance involving niacin on liver pyridine nucleotide concentration in the rat. J. Nutrition, *72:* 302.
5. Rosen, F., and W. A. Perlzweig 1949 The effect of gelatin in the transformation of tryptophan to niacin in rats on low casein diets. J. Biol. Chem., *177:* 163.
6. Block, R. J., and A. B. Weiss 1956 Amino Acid Handbook. Charles C Thomas, Springfield, Illinois.
7. Block, R. J., and D. Bolling 1945 The Amino Acid Composition of Proteins and Foods. Charles C Thomas, Springfield, Illinois.
8. Tristram, G. R. 1949 Amino acid composition of purified proteins. Adv. Prot. Chem., *5:* 143.
9. Sahyun, M. 1944 Outline of The Amino Acids and Proteins. Reinhold Publishing Company, New York.

10. Feigelson, P., J. N. Williams, Jr. and C. A. Elvehjem 1950 Spectrophotometric estimation of pyridine nucleotides in animal tissues. J. Biol. Chem., *185:* 741.
11. deCastro, F. T., R. R. Brown and J. M. Price 1957 The intermediary metabolism of tryptophan by cat and rat tissue preparations. J. Biol. Chem., *228:* 777.
12. Brown, R. R., and J. M. Price 1956 Quantitative studies on metabolites of tryptophan in urine of dog, cat, rat and man. J. Biol. Chem., *219:* 985.
13. Arnstein, H. R. V. 1954 The metabolism of glycine. Adv. Prot. Chem., *9:* 1.
14. Hardin, J. O., and E. L. Hove 1951 Prevention of DL-methionine toxicity in rats by vitamins E, $B_{12}$, folacin, glycine and arginine. Proc. Soc. Exp. Biol. Med., *78:* 728.
15. Swendseid, M. E., J. B. Hickson and B. W. Friedrich 1962 Effect of nonessential nitrogen supplements on growth and on the amino acid content in plasma and muscle of weanling rats fed a low-protein diet. J. Nutrition, *78:* 115.
16. Womack, M., and W. C. Rose 1947 The role of proline, hydroxyproline and glutamic acid in growth. J. Biol. Chem., *171:* 37.

# Protein Reserves: Evidence for Their Utilization under Nutritional and Disease Stress Conditions [1]

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*ABSTRACT* A series of experiments with growing chickens was carried out to evaluate the possible significance of increased body N resulting from a super-normal protein intake. The experimental animals were fed during a preliminary conditioning period either a normal (22%) or a super-normal (28%) protein diet and then subjected to 2 types of stress conditions: 1) they were given a low protein diet deficient in an essential amino acid or a second diet which was further imbalanced through the addition of an amino acid mixture devoid of the same limiting amino acid; and 2) after the birds had been fed the normal and super-normal protein diets for different time intervals, they were inoculated with Newcastle Disease virus and mortality recorded. Parental immunity, as determined from hemagglutination-inhibition titers, was also studied. It was consistently observed that chickens prefed the super-normal protein diet had higher carcass N and gained more weight with the low protein, amino acid-imbalanced rations than did the animals prefed the normal protein diets. The former birds also showed greater parental immunity to and lower mortality from Newcastle Disease virus inoculation. It was concluded that beneficial effects may be realized from protein intakes beyond those necessary for optimal body weight gains.

Protein reserves have recently been defined as "a moiety beyond current needs which may be called upon to meet situations of privation or stress" (1). Halac (2) has called attention to the improper use of the term protein reserves in describing "the nitrogenous portion of the healthy body which may be lost during dietary privation when the body is obliged to rely on its own tissues." In a similar vein, many studies have alluded to protein reserves when inadequate dietary protein levels were compared with adequate ones in relation to disease or other forms of stress

The work of Holt and of Halac has been concerned with an evaluation of the usefulness of any nitrogen moiety which might be stored in excess of that laid down in the body on protein intakes adequate for "normal" growth or maintainance. These workers (3) studied survival time of rats fed protein-free diets, having previously received super-normal as compared with normal protein diets. Other rats, similarly fed, were subjected to swimming tests in cold water, to X-ray exposure or to injection with a central nervous system stimulant.[3] The rats that had been fed the super-normal protein diets did not, under any of these stress conditions, survive for a longer period than did those given the normal diet. It was therefore concluded that protein reserves, as defined by Holt (1), do not exist.

We have interpreted these studies differently. Since it is well known that protein catabolism and efficiency of utilization depend on prior dietary intake (4, 5), we believe that total deprivation of protein places animals previously given a super-normal protein diet at a disadvantage. Vaughan et al. (4) showed clearly that a high protein diet interfered with the ability of pigs to cope with the stress of sudden protein deprivation; this resulted in a considerably faster protein catabolism and turnover than occurred with a more normal protein intake. Thus, in the studies by Holt during the early days of total deprivation, rats fed a super-normal protein diet catabolized protein at a greater rate than those previously given a "normal" diet and major differences in survival

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would, therefore, not be expected. The other stresses these workers applied do not appear to be sufficiently specific in terms of protein utilization to permit an unequivocal answer to the problem.

The present studies were initiated to compare the utilization of "normal" compared with super-normal protein intakes under more specific protein stresses. We investigated the immunological response of chicks to Newcastle Disease virus and also their response to dietary stress in the form of diets imbalanced with respect to an essential amino acid. Our results suggest that under these stress conditions protein reserves, as defined by Holt, do have a purposeful function in the protein metabolism of the animal organism.

## EXPERIMENTAL

*General.* Cross-bred male chicks were used in all experiments. They were fed either a 22 or 28% protein diet for 2, 3 or 4 weeks before being subjected to a particular stress. As will be indicated in the tables of results, body weights at the end of each preliminary conditioning period were essentially the same for the chicks maintained with either protein level. The percentage composition of the basal diets was as follows: sesame meal, 43.5 or 54.4; corn oil, 3.0 or 5.0; dicalcium phosphate, 2.0; limestone with trace minerals, 1.0; L-lysine·HCl, 1.1 or 1.3; sodium chloride, 0.5; choline chloride (70% conc), 0.3; vitamins, 0.25; and glucose monohydrate to 100. These diets had similar calorie-to-protein ratios through the addition of extra fat to the super-normal protein (28%) diet.

A number of animals were killed with chloroform at the end of each conditioning period for nitrogen (N) analysis. These animals were starved for 12 hours, weighed and killed. The carcasses were then dried to constant weight at 100°C in a forced-draft oven, finely ground and mixed, and triplicate samples taken for N analyses. The analyses were carried out by semi-micro Kjeldahl digestion, followed by the colorimetric determination of ammonium sulfate on the AutoAnalyzer (6).

*Imbalance experiments.* After an initial feeding period with the 22 or 28% preliminary conditioning diets, chicks were assigned to the experimental regimens. They were given either a control diet which supplied 13% protein and which was inadequate with respect to either the sulfur amino acids or lysine, or the control diet imbalanced by the addition of a 4% amino acid mixture from which the limiting amino acid was omitted. The composition of these diets as well as that of the amino acid mixture was the same as used in previous studies (7). The chicks were given these diets for 1- to 3-week periods and their weight gains recorded.

For the last experiment of this series 5 replicates of 10 chicks each were used per treatment. In the other 3 experiments duplicate groups of 5 or 10 chicks were used per treatment. For the carcass analyses 8 to 10 birds were killed except for the last experiment in which 25 were analyzed from each preliminary conditioning group.

*Immunological studies.* The chicks used in these experiments were progeny from a flock that had been vaccinated periodically against Newcastle Disease virus. These vaccinations confer parental immunity of approximately 3 weeks' duration to the newly hatched chick. In one experiment the immune titer in serum was measured by the hemagglutination-inhibition (HI) reaction using the LaSota strain of Newcastle Disease virus to challenge chicks that had been maintained with either the 22 or the 28% protein diet for a period of 4 weeks.

In another experiment chicks were inoculated with a given dosage of the Horowitz strain of Newcastle Disease virus when they were 4 weeks old and after they had been maintained with either the 22 or 28% preliminary conditioning diets. Following the inoculation they were maintained for a subsequent 2-week period with a 13 or a 26% protein diet. In two other experiments weekly inoculations were made of separate groups of 15 to 20 chicks that had been raised up to that time with either the 22 or the 28% protein diets. The GB strain of Newcastle Disease virus was used (which is more virulent than the Horowitz strain) and the mortality within each age group as a percentage of the number of chicks inoculated was recorded as a criterion of differential immunity between the 2 groups receiving different lev-

els of protein. In nonimmune birds of this age the expected mortality with the GB strain, used intranasally, is between 90 and 100%.

## RESULTS

*Imbalance experiments.* Table 1 gives a summary of 2 experiments in which chicks that had been raised with either a

TABLE 1

*Effect of previous protein intake on the response of chickens to low protein diets deficient in sulfur amino acids*

| Measurement | Preliminary dietary protein level | |
|---|---|---|
| | 22% | 28% |
| Experiment 1 | | |
| Starting weight, 4-weeks, g | 429 ± 6 [1] | 423 ± 2 |
| Carcass N, 4-weeks, % | 9.26 ± 0.29 | 9.61 ± 0.09 |
| 7-Day gain with: | | |
| (A) Low protein, low sulfur amino acid diet,[2] g | 111 ± 8 | 112 ± 7 |
| (B) As (A) plus sulfur amino acid-deficient amino acid mix,[3] g | 99 ± 8 | 114 ± 7 |
| Experiment 2 | | |
| Starting weight, 2-weeks, g | 187 ± 3 | 184 ± 4 |
| Carcass N, 2-weeks, % | 8.64 ± 0.33 | 8.94 ± 0.31 |
| 21-Day gain with: | | |
| (A) Low protein, low sulfur amino acid diet,[2] g | 141 ± 12 | 141 ± 16 |
| (B) As (A) plus sulfur amino acid-deficient amino acid mix,[3] g | 119 ± 14 | 158 ± 12 |

[1] Mean ± SE.
[2] Thirteen per cent isolated soy-peanut protein diet; methionine content 2% of protein; for composition see Fisher and Shapiro (7).
[3] A complete amino acid mixture for growing chicks from which the sulfur amino acids were omitted and added at 4% of diet; for composition of diet and amino acid mixture see Fisher and Shapiro (7).

TABLE 2

*Effect of previous protein intake on the response of chickens to low protein diets deficient in lysine*

| Measurement | Preliminary dietary protein level | |
|---|---|---|
| | 22% | 28% |
| Experiment 1 | | |
| Starting weight, 3-weeks, g | 326 ± 2 [1] | 325 ± 2 |
| Carcass N, 3-weeks, % | 9.71 ± 0.11 | 10.10 ± 0.16 |
| 14-Day gain with: | | |
| (A) Low protein, low lysine diet,[2] g | 136 ± 16 | 164 ± 20 |
| (B) As (A) plus lysine-deficient amino acid mix,[3] g | 96 ± 20 | 129 ± 20 |
| Experiment 2 | | |
| Starting weight, 3-weeks, g | 363 ± 5 | 359 ± 4 |
| N consumed (g/bird/3 weeks) | 19.1 | 22.5 |
| Carcass N, 3 weeks, % | 10.35 ± 0.27 | 10.93 ± 0.10 |
| 21-Day gain with: | | |
| (A) Low protein, low lysine diet,[2] g | 303 ± 10 | 358 ± 13 |
| (B) As (A) plus lysine-deficient amino acid mix,[3] g | 219 ± 9 | 252 ± 10 |

[1] Mean ± SE.
[2] Thirteen per cent sesame protein diet, lysine content 4% of protein; for composition see Fisher and Shapiro (7).
[3] A complete amino acid mixture for growing chicks from which lysine was omitted and added at 4% of diet; for composition of diet and amino acid mixture see Fisher and Shapiro (7).

22 or 28% protein diet were subjected to the stress of a low protein, sulfur amino acid-imbalanced diet. In both experiments the chicks that had been maintained with the normal protein diet (22%) grew more slowly with the imbalanced diet than did the corresponding chicks that had been raised with the super-normal diet (28%). Thus, the chicks raised with the super-normal protein diet were not affected by the imbalance as were those raised with the normal protein diet.

Table 2 gives the results of studies similar to those presented in table 1 except that lysine was the limiting amino acid. Both of the experiments in table 2 show the same trend, with the chicks prefed the 28% protein diet growing faster with the imbalanced as well as with the control diet than those prefed the 22% protein diet. The differences in weight gain are statistically significant at $P < 0.001$ for the groups fed the control diet (A) and at $P < 0.02$ for the groups fed the imbalanced diets (B).

The magnitude of the differences in carcass N was similar — namely, approximately 3% in all experiments (tables 1 and 2) — but significant ($P < 0.05$) only for experiment 2 with lysine.

*Immunological studies.* Table 3 shows the frequency distribution of HI titers for Newcastle Disease virus of chicks raised for 4 weeks with either the 22 or the 28% protein diet. A Chi-square contingency test indicated that the birds raised with the 28% protein diet had a significantly greater immunity ($P < 0.001$) than those previously fed the 22% diet. Table 4 shows the weight gain of chicks pretreated as before and inoculated at 4 weeks with the Horowitz strain of Newcastle Disease virus (mildly neurotropic, sublethal) and maintained for 2 subsequent weeks with a low (13%) and a high (26%) protein diet, both diets properly balanced with respect to amino acids. Chicks fed the low protein diet after having been raised with the 28% protein diet, gained more weight than those from the 22% protein pretreatment. There was no difference in growth response between the chicks receiving the high protein diet following virus inoculation. Figures 1 and 2 show the mortality rate (number that died as a percentage of those inoculated) of chicks receiving either the 22 (lot 1) or the 28% (lot 2) protein diets of which separate groups were inoculated at weekly intervals with the more

TABLE 3

*Serum antibody titers for Newcastle Disease virus of chicks after feeding either a normal (22%) or a super-normal (28%) protein diet for a 4-week period.*

| Dilution | Frequency distribution of HI titers [1] | |
|---|---|---|
| | Preliminary dietary protein level | |
| | 22% | 28% |
| | % | % |
| 40 | 68 | 33 |
| 40–80 | 27 | 33 |
| 80–160 | 5 | 34 |

[1] Forty birds/group; serum hemagglutination-inhibition (HI) titers were determined after the 2 groups had been fed the 22 and 28% protein diets, respectively, for 4 weeks and using the La Sota strain of Newcastle Disease virus to challenge the birds.

TABLE 4

*Weight gain of chicks inoculated with Newcastle Disease virus after being fed a normal (22%) and a super-normal (28%) protein diet for a 4-week period*

| Measurement | Weight gains of Newcastle virus-inoculated birds [1] | |
|---|---|---|
| | Preliminary dietary protein level | |
| | 22% | 28% |
| Starting weight, 4 weeks, g | 501 ± 5 [2] | 512 ± 4 |
| 14-Day gain with: | | |
| Low protein diet [3] | 242 ± 15 | 265 ± 14 |
| High protein diet [4] | 358 ± 10 | 353 ± 17 |

[1] Twenty-five birds/group were inoculated with the Horowitz strain of Newcastle Disease virus after having been fed the normal and super-normal protein diets.
[2] Mean ± SE.
[3] Provided 13% balanced protein from isolated soy, peanut meal and additional methionine plus lysine.
[4] Provided 26% balanced protein from isolated soy, peanut meal and additional methionine plus lysine.

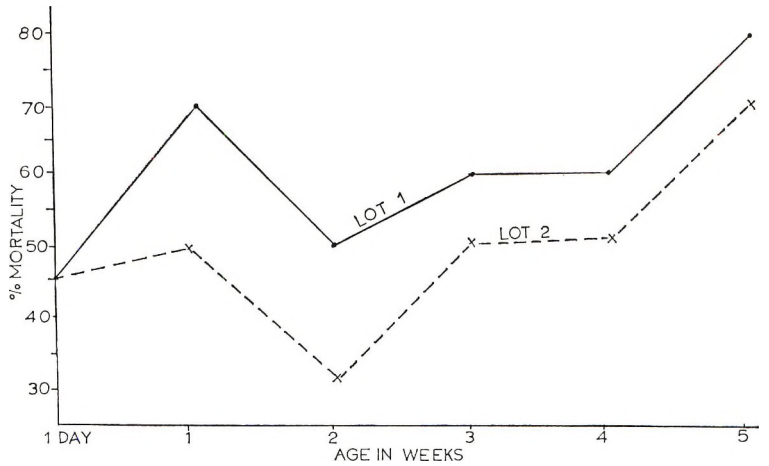


Figure 1

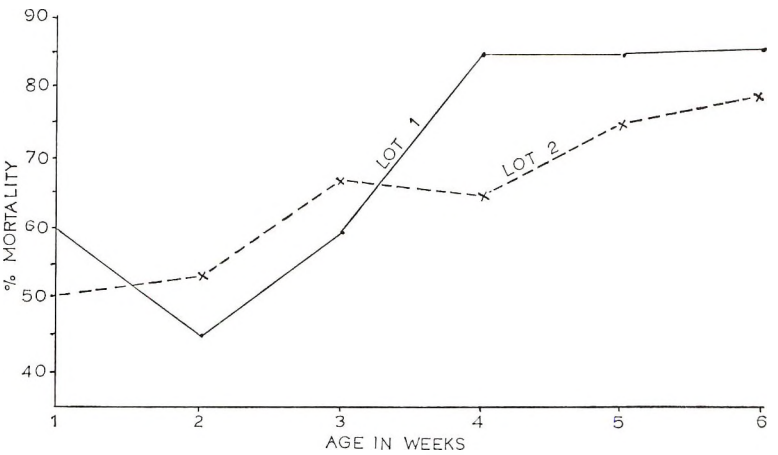


Figure 2

Figs. 1 and 2 Number of chicks that died expressed as a percentage of those inoculated at weekly intervals with the GB strain of Newcastle Disease virus. Until inoculation, the chicks had been fed diets containing either 22% (lot 1) or 28% (lot 2) protein. For the results given in figure 1, the first group of chicks was inoculated immediately after hatching (1-day-old); for figure 2, the first chicks were inoculated after having been fed their respective diets for one week.

neurotropic and lethal GB strain of Newcastle Disease virus. In both studies there was a lower mortality for the chicks that had been fed the 28% protein diet.

## DISCUSSION

The results presented suggest that a protein intake in excess of that required for "normal" growth of chicks can be utilized to advantage when the animals are subjected to certain types of stress such as represented by certain amino acid imbalances and by Newcastle Disease virus. As was stated in the introduction, we are of the opinion that our results are not in conflict with those reported by Holt and Halac (1–3); instead, we feel that the stress imposed in our studies reflects more

specifically upon the protein needs of the animal than did the stress conditions imposed by the New York University group.

The immunological studies presented herein for Newcastle Disease virus are at variance with the observations reported by Boyd and Edwards (8) who showed a greater mortality in chicks given a 30% compared with a 15% protein diet after inoculation with Newcastle Disease virus. This type of study, so commonly made, compares a normal with a suboptimal protein intake. These workers also made no reference to parental immunity, nor to changes in protein quality in shifting from a 15 to the 30% protein diet.

Our disease studies are similar to those of Ritterson and Stauber (9) who showed with an intracellular parasite that excess dietary protein favored the survival of the host. In the nutrition-disease interaction of our studies, parental immunity is a contributing factor in survival. Figures 1 and 2 show clearly that parental immunity declines with age as noted from the increasing mortality with the older groups of chicks, but is modified in the groups fed the excess protein. These observations may be worth considering in Newcastle Disease vaccination, even though the mechanism of the interaction is unknown.

The results in tables 1 and 2 suggest a difference in response between lysine and sulfur amino acid-deficient diets. This difference is probably due to the extent of the amino acid limitation. Thus, lysine was relatively more limiting than the sulfur amino acids and this probably explains the growth differential with the lysine-low control diet. This explanation is supported by the better growth with the sulfur amino acid-limiting diets than with the lysine-deficient diets (compare the 7-day gains, exp. 1, table 1, with those of the 14-day gains, exp. 1, table 2), and also by the greater growth depression with the lysine-deficient diets as a result of adding the amino acid mixture.

It may be questioned whether 22% protein is really a satisfactory level for growing chicks or whether a higher level that permits a higher carcass N should not be considered normal. We believe this to be a question of semantics in much the same way as a discussion of "normal" growth. The important consideration, however, should be the realization that with very high protein intakes growing chickens retain dietary N which may be of value under specific stress conditions, although of little or no import under nonstress conditions.

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LITERATURE CITED

1. Holt, L. E., Jr., E. Halac, Jr. and C. N. Kajdi 1962 The concept of protein stores and its implications in diet. J.A.M.A., *181:* 699.
2. Halac, E., Jr. 1962 Studies of protein reserves. The relation between protein intake and resistance to protein deprivation. Am. J. Clin. Nutrition, *11:* 574.
3. Halac, E., Jr. 1961 Effects of stress on animals fed high protein diets. Am. J. Clin. Nutrition, *9:* 557.
4. Vaughan, O. W., L. J. Filer, Jr. and H. Churella 1962 Influence of prior dietary protein levels on resistance to the stress of protein depletion. Pediatrics, 29: 90.
5. Muramatsu, K., T. Sato and K. Ashida 1963 Dietary protein level and the turnover rate of tissue proteins in rats. J. Nutrition, *81:* 427.
6. Ferrari, A. 1960 Nitrogen determination by a continuous digestion and analysis system. Ann. N. Y. Acad. Sci., *84:* 792.
7. Fisher, H., and R. Shapiro 1961 Amino acid imbalance: Rations low in tryptophan, methionine or lysine and the efficiency of utilization of nitrogen in imbalanced rations. J. Nutrition, 75: 395.
8. Boyd, F. M., and H. M. Edwards, Jr. 1963 The effect of dietary protein on the course of various infections in the chick. J. Infect. Dis., *112:* 53.
9. Ritterson, A. L., and L. A. Stauber 1949 Protein intake and leishmaniasis in the hamster. Proc. Soc. Exp. Biol. Med., 70: 47.

# Effect of Propionate on the Dietary Vitamin $B_{12}$, Biotin and Folic Acid Requirement of the Rat [1]

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*ABSTRACT* Weanling rats fed a control diet free of biotin, folic acid and vitamin $B_{12}$ showed no growth response to dietary additions of these vitamins. However, when 5% sodium propionate was included in the diet marked depressions in growth rate and diet intake were observed. A significant response in growth rate resulted when vitamin $B_{12}$ was included in the propionate diets. A significant response to biotin was observed during the early part of the trial, but this was not sustained. The very small response to folic acid was not statistically significant. In a second experiment, in which zero, 3 and 6% sodium propionate were included in the diet, vitamin $B_{12}$ was again partially effective in preventing depressions in growth rate and diet intake, and was more effective with the 3% than the 6% propionate diet. A level of 25 $\mu$g of vitamin $B_{12}$/kg of diet was fully as effective as higher levels. The possibility of using propionate as a constituent in vitamin $B_{12}$ assay diets is suggested.

The critical role of vitamin $B_{12}$ in propionate metabolism in the sheep has been demonstrated by Marston and co-workers (1). By using propionate tolerance tests with sheep and in vitro studies with liver homogenates of sheep (1) and rats (2) it was clearly indicated that in vitamin $B_{12}$ deficiency the ability of the animal to convert methyl malonate to succinate is seriously impaired. More recently Hartman and Dryden (3) reported that dietary propionate or other odd-carbon short-chain fatty acids, including formate, intensified the depression in weight gains in vitamin $B_{12}$-deficient rats. Methyl malonate had a similar effect, but succinate or even-carbon short-chain fatty acids did not affect growth.

It is normally difficult to demonstrate a growth response to dietary vitamin $B_{12}$ in young rats without prior depletion of the adult female, prevention of coprophagy (4) or the use of thyroid (5), or iodinated casein (6). It therefore seemed worthwhile to learn whether the addition of propionate to the diet of young undepleted weanling rats might be a simple expedient in accomplishing this objective. The participation of biotin in propionyl carboxylase reactions (7, 8) and the observation of Marston et al. (1) that folic acid deficiency occasionally accompanies an induced vitamin $B_{12}$ deficiency in sheep, prompted the present study to determine whether propionate feeding might also influence the dietary need for these 2 vitamins.

## EXPERIMENTAL PROCEDURE

*Experiment 1.* One hundred forty-four weanling (40 to 50 g) female rats (Holtzman) were randomly assigned to 16 dietary treatments (9 rats /treatment) arranged as a $2^4$ factorial and continued on these treatments for 39 days. The experimental variables included 2 basic diets (control and sodium propionate) as shown in table 1, and 2 levels each of vitamin $B_{12}$ (zero, and 50 $\mu$g/kg of diet), biotin (zero, and 0.75 mg/kg of diet), and folic acid (zero, and 3.0 mg/kg of diet). Thus, all combinations of the 4 variables were included. Sodium bicarbonate was added to the control diets to approximately equalize the sodium content. The animals were housed in individual wire cages and allowed the appropriate diet and water ad libitum. They were weighed semi-weekly and feed consumption was recorded. The accuracy of the measurements of feed consumption was affected by a tendency for the diets containing propionate to be hygroscopic. During the first 2 days of the trial an attempt was made to use diets

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TABLE 1

*Diet composition*

| | Experiment 1 | | Experiment 2 | | |
|---|---|---|---|---|---|
| | | | Sodium propionate level | | |
| | Control | Propionate | 0 | 3 | 6 |
| | % | % | % | % | % |
| Casein [1] | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 |
| Sucrose | 67.12 | 66.5 | | | |
| Dextrose | | | 64.0 | 61.0 | 58.0 |
| Corn oil [2] | 2.5 | 2.5 | 5.0 | 5.0 | 5.0 |
| Salt mix [3] | 2.0 | 2.0 | 4.0 | 4.0 | 4.0 |
| Cellulose [4] | 3.0 | 3.0 | 5.0 | 5.0 | 5.0 |
| Vitamin supplement [5] (except vitamin $B_{12}$, folic acid and biotin) | 1.0 | 1.0 | 2.0 [6] | 2.0 [6] | 2.0 [6] |
| Sodium bicarbonate | 4.38 | — | | | |
| Sodium propionate (N.F.) | — | 5.0 | 0.0 | 3.0 | 6.0 |

[1] Vitamin-free Casein, Nutritional Biochemicals Corporation, Cleveland.

[2] Mazola, Corn Products Company, Argo, Illinois.

[3] Phillips-Hart IV (plus $CoCl_2$), Nutritional Biochemicals Corporation, Cleveland; contained: (in per cent) $K_2PO_3$, 32.2; $CaCO_3$, 30.0; NaCl, 16.7; $MgSO_4 \cdot H_2O$, 10.2; $CaHPO_4 \cdot 2H_2O$, 7.5; $FeCaH_5O_7$, 2.75; $MnSO_4$, 0.51; KI, 0.08; $CuSO_4$, 0.03; $ZnCl_2$, 0.025; $CoCl_2$ 0.005.

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

[5] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland: contained (per kg) vitamin A, 900,000 IU; vitamin D, 100,000 IU; α-tocopherol, 5.0 g; ascorbic acid, 45.0 g; inositol, 5.0 g; choline·Cl, 75.0 g; menadione, 2.25 g; *p*-aminobenzoic acid, 5.0 g; niacin, 4.5 g; pyridoxine·HCl, 1.0 g; thiamine·HCl, 1.0 g; Ca pantothenate, 3.0 g; riboflavin, 1.0 mg; remainder, dextrose.

[6] Folic acid and biotin also included.

containing 10% sodium propionate, but acute palatability problems were encountered. Four animals died during the early stages of the experiment, apparently due to their inability to adjust to the propionate diet. Appropriate missing plot data were calculated in these instances.

*Experiment 2.* In a second experiment 120 weanling (40 to 50 g) female rats (Holtzman) were randomly divided into 12 groups of 10 each and fed the experimental diets (table 1). Basic diets were altered somewhat from those used in experiment 1. Treatments consisted of 3 levels of sodium propionate (zero, 3% and 6%) and 4 vitamin $B_{12}$ levels (zero, 25, 50 and 100 μg/kg) arranged factorially. Sodium bicarbonate was not added to the control diet in this experiment. The animals were continued on experiment for 21 days and all experimental procedures were comparable with those of experiment 1.

## RESULTS AND DISCUSSION

In experiment 1 no significant differences in growth rate (table 2) or feed consumption (table 3) were observed when any combination of the 3 vitamins tested was added to the control (bicarbonate) diet. The increase in growth rate and feed consumption attributable to the inclusion of vitamin $B_{12}$ in this diet amounted to only 3.8 and 0.2%, respectively, at 39 days. The inclusion of biotin or folic acid, or both, resulted in even smaller increases at this time. However, when 5% sodium propionate was present in the diet a marked depression in growth rate was observed, along with a lesser depression in diet intake. The presence of supplementary vitamin $B_{12}$ to the propionate diet resulted in a highly significant ($P < 0.01$) growth response (fig. 1) as well as an increase in feed consumption. This demonstrates clearly that a mild vitamin $B_{12}$ deficiency can be induced in the rat by

the addition of sodium propionate to a basal diet which itself does not result in such a deficiency.

The growth curves in figure 2 suggest a similar, but less marked effect of propionate on the biotin requirement during the first 2 to 3 weeks of the trial. At 18 days the rats fed the propionate diet supplemented with biotin were significantly heavier ($P < 0.01$) than their controls. The response to biotin, however, was not sustained to the end of the trial, so that gains for the entire trial were not significantly affected. The very small response to folic acid was not statistically significant. A biotin by folic acid interaction ($P < 0.05$)

TABLE 2

*Effect of added biotin, folic acid and vitamin $B_{12}$ on the growth (body weight) of rats fed diets containing sodium propionate or sodium bicarbonate (exp. 1)*

| Diet | No biotin | | Biotin | | Avg | Increase |
|---|---|---|---|---|---|---|
| | No folic acid | Folic acid | No folic acid | Folic acid | | |
| | *g* | *g* | *g* | *g* | *g* | *%* |
| | Average gain in body weight to 18 days | | | | | |
| Control (bicarbonate) | | | | | | |
| No vitamin $B_{12}$ | 68.1 [1] | 70.0 | 69.2 | 74.6 | 70.4 | |
| Vitamin $B_{12}$ | 74.8 | 67.9 | 74.7 | 71.9 | 72.3 | 2.6 |
| Average | | 70.2 | | 72.6 | | |
| Increase, % | | | | 3.4 | | |
| Propionate | | | | | | |
| No vitamin $B_{12}$ | 39.0 | 38.2 | 47.3 | 50.2 | 43.7 | |
| Vitamin $B_{12}$ | 50.3 | 57.6 | 58.2 | 60.4 | 56.6 | 29.5 [2] |
| Average | | 46.3 | | 54.0 | | |
| Increase, % | | | | 16.6 [2] | | |
| | Average gain in body weight to 39 days | | | | | |
| Control (bicarbonate) | | | | | | |
| No vitamin $B_{12}$ | 133.4 | 132.1 | 125.9 | 139.1 | 132.6 | |
| Vitamin $B_{12}$ | 138.1 | 131.9 | 136.4 | 144.1 | 137.6 | 3.8 |
| Average | | 133.9 | | 136.4 | | |
| Increase, % | | | | 1.2 | | |
| Propionate | | | | | | |
| No vitamin $B_{12}$ | 88.4 | 92.4 | 89.3 | 85.9 | 89.0 | |
| Vitamin $B_{12}$ | 114.7 | 120.4 | 108.7 | 122.0 | 116.4 | 30.8 [2] |
| Average | | 104.0 | | 101.5 | | |
| Increase, % | | | | −2.4 | | |

[1] Represents an average of 9 rats.
[2] Statistically significant ($P < 0.01$).

TABLE 3

*Effect of added biotin, folic acid and vitamin $B_{12}$ on the feed consumption of rats fed diets containing sodium propionate or sodium bicarbonate (exp. 1)*

| Diet | No biotin | | Biotin | | Avg | Increase |
|---|---|---|---|---|---|---|
| | No folic acid | Folic acid | No folic acid | Folic acid | | |
| | *g* | *g* | *g* | *g* | *g* | *%* |
| Control | | | | | | |
| No vitamin $B_{12}$ | 517.0 [1] | 534.0 | 559.4 | 542.4 | 538.2 | |
| Vitamin $B_{12}$ | 542.8 | 544.9 | 531.0 | 538.8 | 539.4 | 0.2 |
| Propionate | | | | | | |
| No vitamin $B_{12}$ | 365.6 | 434.4 | 470.0 | 416.7 | 421.7 [2] | |
| Vitamin $B_{12}$ | 435.0 | 459.4 | 455.6 | 442.2 | 448.1 | 6.3 |

[1] Represents an average of 9 rats for 39 days.
[2] Control > propionate ($P < 0.01$).

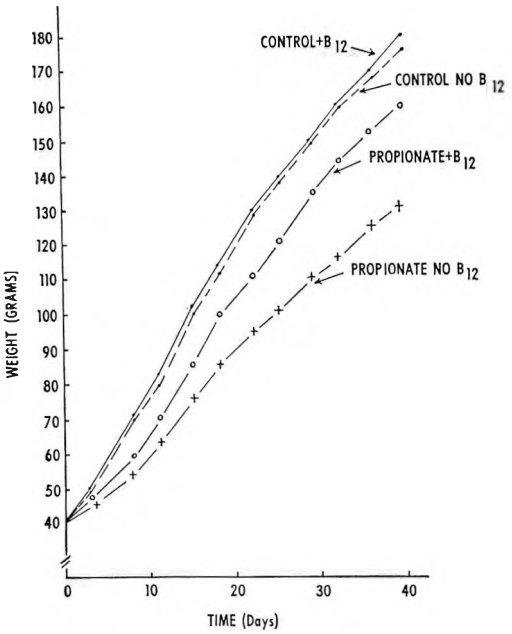


Fig. 1 Growth curves of rats fed control (bicarbonate) or propionate diets with and without additional vitamin $B_{12}$ (trial 1).

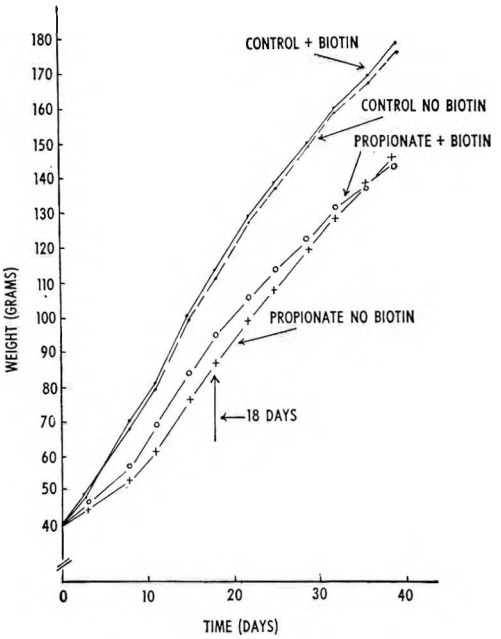


Fig. 2 Growth curves of rats fed control (bicarbonate) or propionate diets with and without additional biotin (trial 1).

TABLE 4

*Effect of varying levels of sodium propionate and vitamin $B_{12}$ in the diet on the growth (body weight) of rats (exp. 2)*

| Sodium propionate | Vitamin $B_{12}$, μg/kg | | | | |
|---|---|---|---|---|---|
| | 0 | 25 | 50 | 100 | Avg |
| % | g | g | g | g | g |
| 0.0 | 95.8 [1] | 98.9 | 97.1 | 91.9 | 95.9 |
| 3.0 | 68.7 | 93.3 | 84.0 | 92.9 | 84.7 |
| 6.0 | 58.1 | 72.4 | 71.0 | 68.8 | 67.6 |
| Average | 74.2 | 88.2 | 84.0 | 84.5 | |

[1] Ten rats/group; average total weight gained/rat in 21 days.

TABLE 5

*Effect of varying levels of sodium propionate and vitamin $B_{12}$ on the feed consumption of rats (exp. 2)*

| Sodium propionate | Vitamin $B_{12}$, μg/kg | | | | |
|---|---|---|---|---|---|
| | 0 | 25 | 50 | 100 | Avg |
| % | g | g | g | g | g |
| 0.0 | 252.6 [1] | 260.7 | 255.0 | 251.5 | 255.0 |
| 3.0 | 192.1 | 239.3 | 220.1 | 241.6 | 223.3 |
| 6.0 | 176.6 | 188.7 | 191.6 | 186.9 | 186.0 |
| Average | 207.1 | 229.6 | 222.2 | 226.7 | |

[1] Ten rats/group; average total diet consumed/rat in 21 days.

was observed in diet consumption. This was apparently due to a slightly greater response in diet intake in the propionate-fed rats due to biotin or folic acid individually than to the combination of both. The biological significance of this interaction may be questioned, however, especially since it was not associated with like changes in growth rate. No other interactions were detected.

In experiment 2, statistically significant ($P < 0.01$) differences in growth rate and feed consumption due to propionate level and vitamin $B_{12}$ were observed (tables 4, 5). The interaction between propionate and vitamin $B_{12}$ levels in both growth rate (table 4) and diet consumption (table 5) were also significant ($P < 0.05$). The propionate effect was quite marked, and greater depressions in both growth rate and diet consumption were observed with the diet containing the higher level of propionate. Again as in experiment 1 no response to vitamin $B_{12}$ was apparent when no propionate was included in the diet, but a significant response to vitamin $B_{12}$ was observed with the propionate diets. Increasing the levels of vitamin $B_{12}$ supplementation failed to increase the growth response, indicating that a level of 25 μg/kg of diet was adequate even at the higher level of propionate fed. Vitamin $B_{12}$ was more effective in stimulating growth (fig. 3) with the diet containing 3.0% sodium propionate (average increase of 21.4 g) than with the diet containing 6.0% sodium propionate (average increase of 12.6 g). This appears to be due in part at least to the tendency toward reduced feed consumption with increasing levels of propionate.

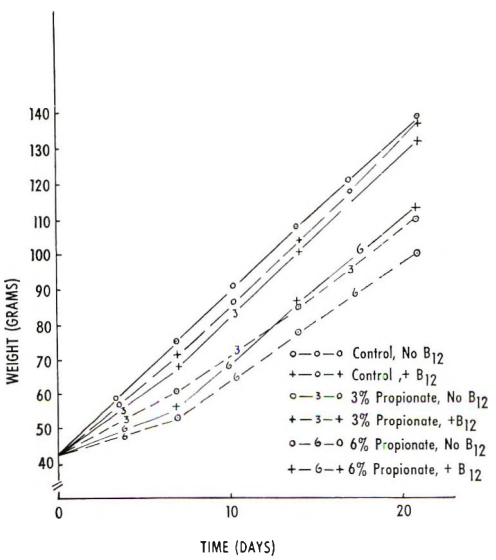


Fig. 3 Growth curves of rats fed diets varying in propionate content with and without additional vitamin $B_{12}$ (trial 2).

Further studies using graded levels of vitamin $B_{12}$ ranging between zero and 25 μg/kg of diet are needed. The data reported herein, however, together with those of Hartman and Dryden (3) suggest the possibility of using propionate as a dietary constituent in vitamin $B_{12}$ assays with weanling rats from undepleted stock.

To the authors' knowledge, an effect of feeding propionate on the dietary biotin requirement of the rat has not previously been reported. The lack of a sustained biotin response may have been due to destruction with time of the supplementary biotin in the diets, or to an adaptation of the rats to the propionate diets in respect to their biotin requirement. Further studies will be required before the effect on biotin can be considered conclusive.

## LITERATURE CITED

1. Marston, H. R., S. H. Allen and R. M. Smith 1961 Primary metabolic defect supervening on vitamin $B_{12}$ deficiency in the sheep. Nature, *190:* 1085.
2. Smith, R. M., and K. J. Monty 1959 Vitamin $B_{12}$ and propionate metabolism. Biochem. Biophys. Res. Comm., *1:* 105.
3. Hartman, A. M., and L. P. Dryden 1962 Vitamin $B_{12}$ and the metabolism of rumen fatty acids. J. Dairy Sci., *45:* 691.
4. Barnes, R. H., and G. Fiala 1958 Effects of the prevention of coprophagy in the rat. II. Vitamin $B_{12}$ requirement. J. Nutrition, *65:* 103.
5. Emerson, G. A. 1949 Growth promoting activity of vitamin $B_{12}$ in rats receiving thyroid substance. Proc. Soc. Exp. Biol. Med., *70:* 392.
6. Frost, D. V., H. H. Fricke and H. C. Spruth 1949 Rat growth assay for vitamin $B_{12}$. Proc. Soc. Exp. Biol. Med., *72:* 102.
7. Halenz, D. R., and M. D. Lane 1960 Properties and purification of mitochondrial propionyl carboxylase. J. Biol. Chem., *235:* 878.
8. Kosow, D. P., and M. D. Lane 1961 Restoration of biotin-deficiency induced depression of propionyl carboxylase activity in vivo and in vitro. Biochem. Biophys. Res. Comm., *4:* 92.