# Influence of Dietary Protein Level and Amino Acids on Plasma Cholesterol of the Growing Chick

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The role of dietary factors in regulating plasma cholesterol has received considerable attention. Low-protein diets have been shown to elevate plasma cholesterol levels in the rat (Moyer et al., '56) and chick (Kokatnur et al., '58; Nishida et al., '58; Leveille and Fisher, '58; Leveille et al., '60, '61), whereas increased dietary protein levels lowered plasma cholesterol. The mechanism(s) by which protein exerts this effect remains obscure, although several hypotheses have been proposed (for review see Portman and Stare, '59).

Several reports support the hypothesis that methionine exerts a specific hypocholesterolemic effect, particularly in low-protein diets, (Mann, '60; Nishida et al., '58; Seidel et al., '60; Johnson et al., '58) which might account for the cholesterol depression noted for high-protein diets. However, the data of Nishida et al. ('58) indicate that methionine cannot account completely for the cholesterol lowering effect of protein.

The present investigation was initiated to study further the influence of methionine, lysine, tryptophan and arginine on the plasma cholesterol level of growing chicks fed high- and low-protein diets.

## EXPERIMENTAL

Male Hy-line chicks were fed a commercial feed for one week. At the end of this period, the chicks were distributed into experimental groups in such a manner as to have essentially identical mean initial weights for all groups. The chicks were then fed the experimental diets for a three-week period.

Chicks were housed in heated cages with raised wire floors. Feed and water were supplied ad libitum and body weight and food consumption were determined at weekly intervals.

The composition of the basal diet used was as follows in gm/100 gm of diet: salt mix,[1] 4.00; vitamin mix,[2] 0.40; choline Cl, 0.20; corn oil, 5.00; glucose to 100. Soybean oil meal (48% protein, N × 6.25) or sesame oil meal (46.8% protein, N × 6.25) were added to the basal diet at the expense of glucose to supply 10 or 25% protein as indicated in the table of results. Amino acids and cholesterol, as indicated in the tables of results, were added at the expense of glucose.

At the end of the three-week experimental period, the chicks were bled by cardiac puncture with a heparinized syringe. The plasma was separated by centrifugation and analyzed for total cholesterol by a modification of the method of Searcy and Bergquist ('60). Two-tenths of a milliliter of plasma were added to a graduated centrifuge tube containing 4 ml of chloroform:methanol (2:1), blank and standard tubes were included; to the blank tube 0.2 ml of chloroform was added and to the standard tube 0.2 ml of a standard cholesterol solution (1 mg per ml). The tubes were heated (50 to 55°C) for 15 minutes; methanol was added to the 5-ml mark and the tubes were centrifuged after mixing. One milliliter of the clear supernatant was transferred to colorimeter tubes and the remainder of the procedure carried out as originally described (Searcy and Bergquist, '60).

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[1] Composition of salt mix, gm/100 gm of mix: $CaCO_3$, 5.65; $Ca_3(PO_4)_2$, 52.77; $K_2HPO_4$, 16.96; $MgSO_4$, 2.36; Fe gluconate, 4.23; $ZnSO_4 \cdot 7H_2O$, 0.083; KI, 0.075; $CuSO_4$, 0.038; $H_3BO_3$, 0.017; $CoSO_4 \cdot 7H_2O$, 0.002; $MnSO_4 \cdot H_2O$, 1.225; NaCl, 16.58.

[2] Composition of vitamin premix, mg/100 gm of mix: thiamine·HCl, 625; riboflavin, 400; Ca pantothenate, 500; pyridoxine·HCl, 150; biotin, 15; folic acid, 100; *p*-aminobenzoic acid, 50; 2-methyl-1,4 naphthoquinone, 125; vitamin $B_{12}$, 0.50; and (in gm) α-tocopheryl acetate, 2.50; inositol, 2.50; ascorbic acid, 6.25; niacin, 3.75; and (in IU) vitamin A acetate, 250,000; vitamin $D_3$, 23,000.

The statistical significance of mean differences between treatments was ascertained by *t* test.

## RESULTS

The influence of supplemental methionine on plasma cholesterol and gain of chicks fed two levels of soybean oil meal is demonstrated by the results presented in table 1. At all levels of methionine supplementation, chicks fed the 25% protein diets showed greater gains ($P < 0.001$) and lower plasma cholesterol levels ($P < 0.005$) than those fed 10% of protein. Supplementation of the low-protein diet with methionine resulted in a highly significant increase in weight gain ($P < 0.001$) and a decrease in plasma cholesterol ($P < 0.01$) except for the animals fed the 1.5% level of methionine ($P < 0.10$). The group fed the 1.5% methionine level still demonstrated a 14% decrease in plasma cholesterol when compared with the unsupplemented control group. Methionine supplementation of the 25% protein diet did not stimulate growth, but did depress plasma cholesterol levels significantly at the 1.0 and 3.0% levels of supplementation ($P < 0.001$) (table 1). At the 1.5 and 2.0% levels of supplementation, the plasma cholesterol levels were 10 and 9% lower than the unsupplemented control, respectively; however, because of the greater standard deviations in these groups, a relatively low degree of significance was observed ($P < 0.10$). The plasma cholesterol level of chicks fed 4% of the protein as supplemental methionine was not significantly different from the unsupplemented control. The results presented in table 1 demonstrate a methionine deficiency in the unsupplemented 10% protein diet, since the addition of this amino acid stimulated growth significantly; further, the growth improvement obtained by methionine supplementation of the 25% protein diet, although not significant, does indicate that methionine was marginal in this diet.

TABLE 1

*Influence of methionine supplementation on weight gain and plasma cholesterol of chicks fed two levels of soybean oil meal protein*

| Dietary protein level | Added DL-methionine | Body weight gain | Plasma cholesterol |
|---|---|---|---|
| *% of diet* | *% of protein* | *gm* | *mg/100 ml* |
| 10 | — | 32 ± 18[1] | 272 ± 36[1] |
| 10 | 1.0 | 98 ± 38 | 218 ± 43 |
| 10 | 1.5 | 106 ± 42 | 233 ± 53 |
| 10 | 2.0 | 135 ± 23 | 208 ± 27 |
| 10 | 3.0 | 138 ± 33 | 221 ± 36 |
| 10 | 5.0 | 127 ± 34 | 208 ± 28 |
| 25 | — | 248 ± 28 | 184 ± 15 |
| 25 | 1.0 | 262 ± 27 | 159 ± 10 |
| 25 | 1.5 | 261 ± 23 | 165 ± 34 |
| 25 | 2.0 | 274 ± 31 | 167 ± 24 |
| 25 | 3.0 | 282 ± 33 | 156 ± 15 |
| 25 | 4.0 | 257 ± 13 | 177 ± 23 |

[1] Mean of 10 animals ± standard deviation.

TABLE 2

*Influence of methionine supplementation on weight gain and plasma cholesterol of chicks fed cholesterol and two levels of soybean oil meal protein*[1]

| Dietary protein level | Added DL-methionine | Body weight gain | Plasma cholesterol |
|---|---|---|---|
| *% of diet* | *% of protein* | *gm* | *mg/100 ml* |
| 10 | — | 36 ± 20[2] | 1148 ± 274[2] |
| 10 | 2.0 | 130 ± 33 | 489 ± 172 |
| 10 | 3.0 | 129 ± 33 | 570 ± 183 |
| 25 | — | 227 ± 25 | 529 ± 101 |
| 25 | 2.0 | 278 ± 21 | 455 ± 98 |
| 25 | 3.0 | 245 ± 29 | 430 ± 130 |

[1] 2.0 and 0.3% of cholesterol added to the 10 and 25% protein containing diets, respectively.
[2] Mean of 10 animals ± standard deviation.

The results presented in table 2 demonstrate the influence of methionine supplementation on the gain and plasma cholesterol levels of chicks fed two levels of protein and cholesterol. These data indicate that supplemental methionine at either level fed, significantly increased gain ($P < 0.001$) and depressed plasma cholesterol levels ($P < 0.001$) of chicks fed 10% protein. At the 25% protein level, the lower level of methionine stimulated growth but not the higher level fed; both levels of methionine depressed plasma cholesterol; however, the difference was not large enough to be statistically significant ($P < 0.10$).

In table 3 are presented the results of an experiment designed to study the influence of lysine supplementation on the plasma cholesterol level of chicks fed a lysine-deficient protein. The influence on plasma cholesterol of tryptophan, arginine and methionine added to diets containing

TABLE 3

*Influence of lysine, methionine, tryptophan and arginine supplementation on weight gain and plasma cholesterol of chicks fed two levels of sesame oil meal protein*

| Amino acid supplement | Dietary protein level | | | |
|---|---|---|---|---|
| | 10% | | 25% | |
| | Weight gain | Plasma cholesterol | Weight gain | Plasma cholesterol |
| *% of protein* | *gm* | *mg/100 ml* | *gm* | *mg/100 ml* |
| None | 3 ± 4[1] | 271 ± 45[1] | 39 ± 8[1] | 225 ± 40[1] |
| L-Lysine, 2.5 | 50 ± 21 | 278 ± 54 | 246 ± 24 | 171 ± 11 |
| L-Lysine, 3.5 | 71 ± 21 | 241 ± 30 | 260 ± 28 | 161 ± 14 |
| L-Lysine, 4.5 | 70 ± 22 | 232 ± 32 | 257 ± 26 | 170 ± 13 |
| L-Lysine, 4.5 + DL-methionine, 2.0 | 70 ± 23 | 263 ± 50 | 262 ± 18 | 164 ± 13 |
| L-Lysine, 4.5 + DL-tryptophan, 1.0 | 70 ± 21 | 243 ± 37 | 244 ± 23 | 163 ± 15 |
| L-Lysine, 4.5 + L-arginine, 2.0 | 67 ± 30 | 236 ± 25 | 255 ± 28 | 181 ± 14 |

[1] Mean of 10 chicks ± standard deviation.

adequate amounts of these amino acids for growth was also studied. These results demonstrate that chicks receiving 25% rather than 10% of protein and the same amino acid supplements had significantly higher weight gains and lower plasma cholesterol levels ($P < 0.001$). At both levels of protein fed, lysine supplementation significantly improved growth ($P < 0.001$); however, methionine, tryptophan and arginine did not influence weight gain. At the 10% level of protein, the groups receiving the 3.5 or 4.5% levels of added lysine had lower plasma cholesterol levels than did the control animals or those fed the 2.5% level of lysine. Arginine, tryptophan and methionine did not significantly influence the plasma cholesterol level of chicks fed 10% protein. However, there is an indication that methionine caused an elevation in plasma cholesterol at the 25% protein level, the addition of lysine significantly depressed the plasma cholesterol level ($P < 0.005$), but methionine, tryptophan and arginine were without effect (table 3).

## DISCUSSION

The results of the present study demonstrate a cholesterol depressing effect in the growing chick for methionine, in diets supplying marginal amounts of this amino acid, and for dietary protein. The plasma cholesterol depressing effect of methionine has been demonstrated to be independent of growth (tables 1 and 2), since methionine supplementation of 25% protein diets depressed cholesterol but did not stimulate growth. These observations are in accord with previous reports for both the rat and chick (Seidel et al., '60; Johnson et al., '58; Nishida et al., '58). The present report further demonstrates that methionine depresses plasma cholesterol in chicks only when the dietary level of methionine is marginal, since the depression of plasma cholesterol noted was not related to the level of supplementary methionine (tables 1 and 2). Furthermore, the addition of methionine to an otherwise complete diet did not significantly alter the plasma cholesterol level of growing chicks (table 3).

Chicks fed high-protein diets had lower plasma cholesterol levels than chicks fed low-protein diets with supplemental methionine to bring the methionine level up to that of the high-protein diets (table 1). This observation demonstrates rather conclusively that protein has an effect in addition and unrelated to that of methionine. This demonstration is in accord with the conclusions of Nishida et al. ('58, '60a).

Lysine, in the present studies, depressed plasma cholesterol only when growth was stimulated, indicating that its effect was probably through the increased availability of protein resulting from lysine supplementation. No evidence of a specific plasma cholesterol depressing effect was noted for lysine, tryptophan or arginine.

The work of Nishida et al. ('60a, b) indicates that the cholesterol depression observed in chicks fed a high-protein diet is the result of (1) depressed hepatic cholesterol synthesis, and (2) increased catabolism of cholesterol to bile acids. Although these mechanisms do not exclude the possibility that others are also in effect, they present a plausible explanation for the hypocholesterolemic action of protein and methionine.

## SUMMARY

Data were presented which demonstrate a hypocholesterolemic effect for methionine, unrelated to growth, in growing chicks fed diets containing marginal levels of this amino acid. Furthermore, the data show a specific plasma cholesterol depressing effect for dietary protein, unrelated to the methionine effect.

Lysine depressed cholesterol levels and stimulated growth only when added to diets deficient in this amino acid. No specific hypocholesterolemic effect was evident for lysine.

The addition of methionine to diets adequate in this amino acid did not alter the plasma cholesterol or gain of growing chicks, demonstrating that the methionine effect is only evident in chicks fed diets containing marginal levels of this amino acid.

Arginine and tryptophan had no effect on the plasma cholesterol of growing chicks.

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# Effect of Vitamin A Intake on Some Biochemical and Physiological Changes in Swine[1,2]

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Early investigations on the effect of vitamin A deficiency in swine have revealed that incoordination, emaciation, loss of weight, paralysis and blindness may result (Hart et al., '16). A change in guttural tone, xerophthalmia and a ruffling of the hair on the forehead was observed by Frape et al. ('59) in 5-week-old pigs that had been fed a vitamin A-deficient diet for 4 weeks. Hentges et al. ('52a) suggested that appetite and growth rate were not affected until paralysis and weakness prevented the pigs from obtaining feed or water. Although these visible signs may be significant they are highly variable depending upon the degree of the deficiency, and the possibility of complication with other nutritional deficiencies cannot be ignored.

In 1940, Moore and Sykes confirmed the theory that increased cerebrospinal fluid pressure occurred in vitamin A-deficient calves. Since that time increased cerebrospinal fluid pressure has been observed in vitamin A-deficient sheep (Eveleth et al., '49), pigs (Hentges et al., '52a, b; Sorensen et al., '54; Frape et al., '59), rabbits (Millen and Dickson, '57) and chicks (Woollam and Millen, '55, '56). These investigators also noted that after plasma vitamin A decreased, an increase in cerebrospinal fluid pressure was the first measurable change to occur in vitamin A deficiency.

The mechanism by which vitamin A influences cerebrospinal fluid pressure has not been elucidated. Woollam and Millen ('56) suggested that over-production of the fluid might be the cause. Millen and Dickson ('57) proposed that overactivity of the choroid plexus was responsible.

Another possibility is that if an increase in cerebrospinal fluid protein occurs, the protein molecules could accumulate against the semipermeable membrane of the arachnoid villa, thus preventing reabsorption (Gardner et al., '54). These investigators demonstrated that increased protein concentrations induced experimentally in the cerebrospinal fluid of dogs, resulted in elevated cerebrospinal fluid pressure. During vitamin A deficiency in cattle, changes in protein concentration have been observed in blood plasma (Madsen and Earle, '47) in serum (Erwin et al., '57, '59) and in serum, cerebrospinal fluid and aqueous humor.[4] Also, an unequal ion concentration on opposite sides of the semipermeable membrane may be responsible. A slight decrease in blood serum calcium and inorganic phosphorus in vitamin A-deficient beef cattle was reported by Madsen and Earle ('47). Although Dehority et al.[5] reported no change in osmotic pressure of blood serum, cerebrospinal fluid and aqueous humor, in a later study (Dehority et al., '60) some changes were observed in the osmotic pressure of aqueous humor and in some constituents of cerebrospinal fluid and aqueous humor of dairy calves as carotene intake was reduced.

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[2] Presented in part before the Annual Meeting of the American Society of Animal Production, Chicago, 1960. See J. Animal Sci., *19:* 1278 (abstract no. 163).

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[4] Dehority, B. A., H. D. Eaton, D. G. Hazzard, A. P. Grifo, Jr. and J. E. Rousseau, Jr. 1959 Biochemical and physiological changes in bovine hypovitaminosis A. Federation Proc., *18:* 522 (abstract).

[5] See footnote 4.

One of the objectives of the present experiment was to determine whether any changes occurred in the ion and protein concentration of blood serum, cerebrospinal fluid and aqueous humor of pigs maintained with different levels of vitamin A, and if so, whether these changes were related to the cerebrospinal fluid pressure or to the vitamin A status of the animal, or both. A second objective was to establish more nearly the vitamin A requirement of the pig on the basis of micrograms of vitamin A per pound of live weight per day.

## EXPERIMENTAL

Thirteen purebred Yorkshire pigs (5 barrows and 8 gilts) 17 to 24 days of age were weaned and placed on concrete in individual feeding stalls measuring approximately 30 by 60 inches. Stalls were cleaned daily but no bedding was used. Prior to the experiment the pigs had been with their dams on pasture. The pigs received a vitamin A depletion basal diet[6] (table 1) and water ad libitum. Particular care was taken to exceed the National Research Council's ('59) minimum requirements for minerals and all vitamins, except vitamin A.

Vitamins were premixed in quantities that would be fed within two weeks and were stored in a refrigerator prior to being incorporated into the basal diet. The basal diet was mixed in a horizontal ribbon mixer in 200- to 500-pound quantities. All dry ingredients were added and allowed to mix completely before the corn oil was added.

Each pig was fed the basal diet until blood plasma vitamin A was determined to be less than 7 μg/100 ml. Shortly thereafter, cerebrospinal fluid (CSF) pressure was measured with a saline manometer. The pigs were anesthetized and placed on their sides; a puncture of the subarachnoid space was then made by passing through the dorsal opening of the atlanto-occipital articulation with a 4-inch 18-gauge spinal needle. For larger pigs a lumbar puncture was made. After depletion and the initial CSF pressure determination the pigs were randomly assigned within sex to one of 4 levels of vitamin A intake: 2, 4, 8 or 16 μg per pound of live weight per day. The 2- and 4-μg levels were chosen to supply what was considered a subminimal level and the 8- and 16-μg levels to supply an adequate amount of vitamin A based on previous data reported by Frape et al. ('59) and Myers et al. ('59). Vitamin A palmitate[7] stabilized in a gelatin beadlet was used. The product was analyzed according to the spectrophotometric method described in the U. S. Pharmacopoeia ('55). The mean and standard deviation of the mean for the concentration of vitamin A was 104,777 ± 1,460 μg per gm. The basal diet was fed at the rate of 4.0% of each pig's body weight per day and was increased 0.3 pound per pig at the end of each 7-day period (Myers et al., '59). As each pig was being fed a different quantity of vitamin A per day the vitamin A supplement was mixed with a portion of the basal diet at the rate of 500 or 2,000 μg per pound

TABLE 1

*Composition of basal diets*

| Age of pigs | 3–6 Weeks | 6–20 Weeks |
|---|---|---|
| | % | % |
| Dried skim milk | 35.00 | 20.00 |
| Soy protein[1] | 5.50 | 8.65 |
| Starch | 51.00 | 61.85 |
| Cellulose[2] | 2.00 | 2.00 |
| Corn oil[3] | 2.00 | 2.00 |
| Vitamin mixture[4] | 2.00 | 2.00 |
| Mineral mixture[5] | 1.00 | 1.00 |
| Dicalcium phosphate | 1.25 | 2.20 |
| Calcium carbonate | 0.25 | 0.30 |
| | 100.00 | 100.00 |

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.

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

[3] Mazola Corn Oil, Corn Products Company, Argo, Illinois.

[4] Vitamin additions per 100 pound of feed were: vitamin D (as calciferol), 50,000 IU; thiamine·HCl, 200 mg; riboflavin, 150 mg; niacin, 1.5 gm; Ca pantothenate, 500 mg; pyridoxine·HCl, 150 mg; choline chloride, 40 gm; cyanocobalamin, 2 mg; α-tocopheryl acetate, 300 mg; menadione, 25 mg; folic acid, 25 mg; ascorbic acid, 1 gm; p-aminobenzoic acid, 500 mg; inositol, 1 gm; Aurofac D, 0.5 pound (containing 5 gm chlortetracycline per pound); soy protein as carrier, 1.4 pound.

[5] Mineral additions per 100 pound of feed were: trace-mineralized salt, 0.5 pound (contained 99% NaCl, 0.2% Mn; 0.16% Fe; 0.033% Cu; 0.007% I; 0.01% Co; 0.005% Zn); $FeSO_4 \cdot H_2O$, 20.0 gm; $MgCO_3$, 50 gm; $ZnCO_3$, 0.5 gm; $CuSO_4 \cdot 5H_2O$, 1.0 gm; $MnSO_4$, 1.0 gm; $CoCl_2$, 0.3 gm; soy protein as carrier, 0.35 pound.

[6] Aurofac D was provided by Mr. J. J. Drain, American Cyanamid Company, Princeton, New Jersey.

[7] Rovimix A-325 kindly supplied by Dr. R. H. Bunnell, Hoffman-LaRoche, Inc., Nutley, New Jersey.

of basal mix. These two concentrations allowed the vitamin A supplement mixture to vary from 0.1 to 1.0 pound per pig per day. To insure consumption, the vitamin A mixture was fed first each morning in an individual feeder. After the vitamin A mixture was completely consumed, the remaining basal allowance was fed. Each pig was weighed to the nearest 0.5 pound every 14 days and the amount of vitamin A mixture to be fed was adjusted to meet the expected growth rate as predicted on the basis of the previous two weeks' growth.

At two-week intervals venous blood samples were drawn into oxalated vials, cooled at 4°C for one hour, centrifuged, and the plasma collected. Blood plasma vitamin A was determined by the method of Kimble ('39). If the vitamin A concentrations were not determined immediately, the plasma samples were frozen and stored at − 20°C.

After 78 ± 1 days at one of the assigned vitamin A levels, terminal CSF pressures were measured and blood samples were taken for terminal vitamin A analysis. Approximately 5 days after terminal CSF pressure determinations the animals were anesthetized with $CO_2$, CSF sampled, blood collected and the animals killed. The eyes were immediately removed and the aqueous humor collected. The livers were removed, frozen at − 20°C, weighed, homogenized and vitamin A and carotene determined by a modification of the method of Gallup and Hoefer (Bunnell et al., '54). Moisture was determined by drying a sample of the liver homogenate at 100°C.

Blood serum and cerebrospinal fluid were analyzed for total protein by the biuret method according to Gornall et al. ('49) and inorganic phosphorus by the method of Fiske and Subbarow ('25). Sodium, potassium and calcium analyses were carried out with a Beckman DU flame photometer. Sodium and potassium concentrations were also determined in the aqueous humor.

The eyes and parotid salivary glands and ducts were collected from each animal. Eyes were examined for papillary edema and the parotid salivary glands and ducts for squamous cell metaplasia. Two sections were taken from each gland. In addition, several sections showing interlobular ducts and three areas from each main parotid duct were taken, one from the proximal end as it emerges from the gland, one midway in the duct and one at the distal end close to the external papillae.

Additional tissues were collected from several animals and examined for changes associated with vitamin A deficiency. These included the ileocecal valve, trachea, aorta, intercostal lymph node at aortic arch, esophagus, auricle, ventricle, kidneys, liver, hepatic lymph node, gall bladder, mesenteric lymph node, spleen, adrenal glands, skin, pancreas, pancreatic lymph node, duodenum, urinary bladder, cerebellum, medulla, cerebrum, pituitary gland, ileum, tongue, spinal cord, thymus, eyelid, thyroid, psoas muscle and uterus.

All tissues were fixed in 10% neutral formalin, embedded in paraffin, sectioned at 5 μ and stained with hematoxylin-eosin. Special stains were used where needed.

## RESULTS

*Animal performance.* The average weight of the 13 pigs was 12.0 pounds (6.5 to 19.5) at the beginning and 44.6 pounds (18.5 to 63.0) at the end of the depletion period. Average depletion time was 40 days ranging from 34 to 47 days. The average feed consumption was 1.75 pounds per pig per day and the pigs made an average daily gain of 0.82 pound per pig per day.

The pigs were fed at one of 4 levels of vitamin A for 78 days (± 1). Performance data are included in table 2. Pigs fed the 2 μg intake level made the most rapid gain and had the highest feed conversion, whereas the 16 μg group required the greatest amount of feed per unit of gain. This may have been a result of restricted feed intake and was probably not related to the vitamin A levels fed.

Although blood plasma vitamin A values in some instances decreased to less than 2 μg/100 ml, at no time during the depletion or repletion periods could any visible changes be noticed in the appearance, general condition, or behavior of the animals. There was a 15-day period of intermittent diarrhea near the fourth and fifth

TABLE 2

*Effect of vitamin A intake on growth rate and feed conversion following depletion*

| | Vitamin A intake | | | | |
|---|---|---|---|---|---|
| | 2[1] | 4 | 8 | 16 | x[2] |
| | *μg/pound live weight/day* | | | | |
| Av. initial weight | 44.6 | 45.2 | 39.7 | 49.0 | 44.6 ±11.8 |
| Av. final weight | 135.4 | 134.2 | 117.7 | 135.5 | 131.0 ±22.4 |
| Av. daily gain | 1.17 | 1.13 | 1.05 | 1.11 | 1.12± 0.14 |
| Av. daily feed[3] | 3.48 | 3.52 | 3.38 | 3.74 | 3.53± 0.54 |
| Feed/pound gain | 3.00 | 3.11 | 3.20 | 3.44 | 3.17± 0.44 |

[1] Three pigs/treatment except at 2-μg level which contained 4 pigs.
[2] Mean and standard deviation of the mean of 13 pigs.
[3] Daily allowance of feed was predetermined by each pig's initial weight as described in experimental section in text.

TABLE 3

*Effect of vitamin A intake on cerebrospinal fluid pressure, plasma vitamin A and liver vitamin A concentration after depletion*

| Vitamin A intake | Pig no. | Plasma vitamin A | | Liver vitamin A | Cerebrospinal fluid pressure | |
|---|---|---|---|---|---|---|
| | | Initial | Terminal | Terminal | Initial | Terminal |
| *μg/pound live wt/day* | | *μg/100 ml* | | *μg/gm dry tissue* | *mm of saline* | |
| 2 | 1 | 2.05 | 8.27 | 2.05 | —[1] | 118 |
| | 2 | 2.29 | 11.80 | 1.81 | 300 | 107 |
| | 3 | 4.24 | 8.43 | 1.35 | 199 | 144 |
| | 4 | 3.88 | 6.32 | 1.10 | 158 | 218 |
| | Average | 3.12 | 8.71 | 1.58 | 219 | 147 |
| 4 | 5 | 5.32 | 12.44 | 6.78 | 158 | 50 |
| | 6 | 5.42 | 14.03 | 6.57 | 280 | 120 |
| | 7 | 3.71 | 11.49 | 7.41 | 145 | 138 |
| | Average | 4.82 | 12.65 | 6.92 | 194 | 103 |
| 8 | 8 | 3.39 | 11.31 | 9.11 | 62 | 137 |
| | 9 | 3.55 | 15.71 | 21.48 | 180 | 95 |
| | 10 | 5.90 | 15.27 | 12.65 | 190 | 124 |
| | Average | 4.28 | 14.10 | 14.41 | 144 | 119 |
| 16 | 11 | 6.65 | 18.24 | 58.18 | 278 | 50 |
| | 12 | 5.57 | 20.95 | 78.71 | 170 | 70 |
| | 13 | 2.76 | 16.31 | 59.46 | —[1] | 77 |
| | Average | 4.99 | 18.50 | 65.45 | 224 | 66 |

[1] Initial value not obtained.

week of repletion. Antibiotics and a kaolin-pectin mixture[8] were used to treat the condition. The cause of the scouring was not determined but at no time could the occurrence or severity be correlated to the vitamin A status of the animals. After alleviation of this condition all animals appeared to be in good health until slaughter.

*Vitamin A concentration in blood plasma and liver.* The blood plasma and liver vitamin A concentrations are shown in table 3. Initial plasma vitamin A represents the end of depletion just prior to allotment to one of 4 levels of vitamin A. Based on blood plasma vitamin A concentration, the pigs were depleted of vitamin A at a fairly constant rate. Terminal plasma vitamin A concentrations increased in a linear manner ($P < 0.01$) with an increase in the logarithm of vitamin A intake. Liver vitamin A concentrations are reported as micrograms of vitamin A per gram of dry tissue. There was very little or no vitamin A storage at the 2 μg level. The logarithm of liver vitamin A

[8] Kaopectate, The Upjohn Company, Kalamazoo, Michigan.

concentration increased in a linear manner ($P < 0.01$) with an increase in the logarithm of vitamin A intake. No carotenoids were noted in the liver from any animal.

*Cerebrospinal fluid pressure.* The initial and terminal cerebrospinal fluid (CSF) pressures are shown in table 3. Initial CSF pressures at the end of the depletion period were much higher than the values after 78 ± 1 days when supplying any of the 4 levels of vitamin A. The 16 μg group, however, had decreased to an average of 66 mm of saline as compared with 147 mm of saline for the 2 μg group. The relationship between CSF pressure and plasma vitamin A concentration is illustrated in figure 1. As plasma vitamin A concentration increased there was a decrease in CSF pressure and at the lower plasma vitamin A values the CSF pressure was much more variable. The effect of vitamin A intake on CSF pressure is illustrated in figure 2 and shows that CSF pressure decreased as vitamin A intake increased.

*Constituents of blood serum, cerebrospinal fluid and aqueous humor.* Levels of the 5 serum constituents measured are shown in table 4. No consistent changes

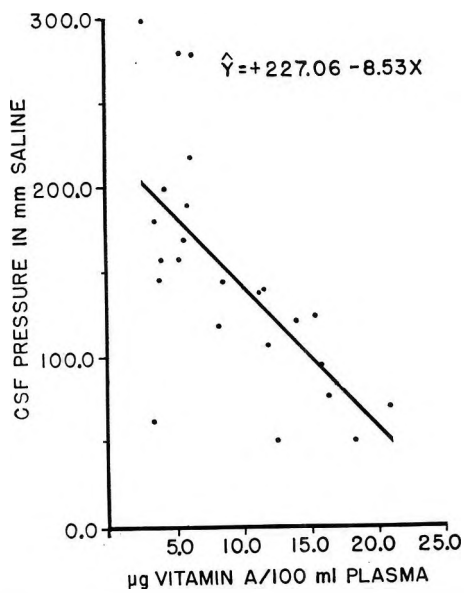


Fig. 1 The relationship between cerebrospinal fluid pressure and plasma vitamin A concentration in the pig.

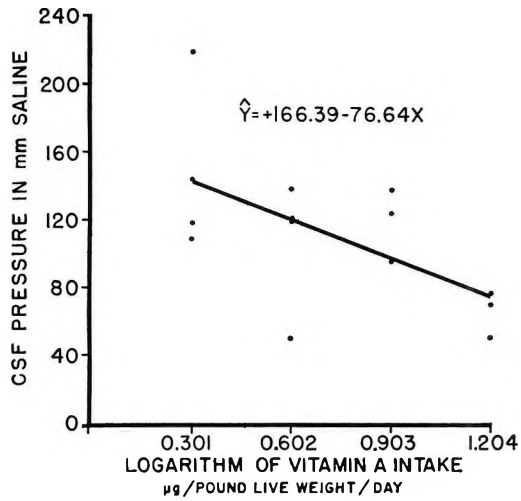


Fig. 2 The effect of vitamin A intake on cerebrospinal fluid pressure in the pig.

or trends were noticeable when compared to vitamin A intake except in the case of serum potassium which increased in a linear manner with an increase in vitamin A intake ($P < 0.05$). But this linear change was erratic and, thus cannot be considered with any degree of certainty to be related to the levels of vitamin A fed.

The mean values of the constituents measured in cerebrospinal fluid and aqueous humor are also shown in table 4. Concentration differences in constituents measured were not significant among the levels of vitamin A intake fed.

*Histopathology.* Histological examination of the various tissues showed no consistent changes relative to vitamin A intake. In sections of the parotid salivary gland, its main duct, many interlobular and intralobular ducts and the accessory parotid glands only one histological change was observed, but this did not appear to be related to vitamin A. In one pig on the 8 μg level, a focus of squamous metaplasia was seen similar to that described in other papers on vitamin A deficiency (Jungherr et al., '50; Madsen et al., '42; Wolbach and Bessey, '42).

The ocular changes usually associated with vitamin A deficiency were not seen either grossly or microscopically in any of the pigs in this study, nor were there any changes in the other tissues examined.

TABLE 4

*Mean concentration of constituents in blood serum, cerebrospinal fluid and aqueous humor*

| | Vitamin A intake (μg/pound live wt/day) | | | | |
|---|---|---|---|---|---|
| | 2 | 4 | 8 | 16 | Mean[1] |
| | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* |
| Serum | | | | | |
| Total protein | 8120 | 7330 | 7410 | 7560 | 7650 ±229 |
| Inorganic phosphorus | 14.4 | 15.8 | 13.7 | 13.8 | 14.4± 0.8 |
| Calcium | 13.8 | 13.7 | 13.7 | 13.5 | 13.7± 0.1 |
| Sodium | 354 | 356 | 351 | 353 | 353 ± 5 |
| Potassium | 29.5 | 24.0 | 35.9 | 24.1 | —[2] |
| Cerebrospinal fluid | | | | | |
| Total protein | 73.1 | 64.2 | 58.9 | 56.0 | 64.3± 10.6 |
| Inorganic phosphorus | 1.7 | 1.6 | 2.0 | 1.7 | 1.7± 0.1 |
| Calcium | 6.8 | 6.3 | 6.8 | 6.6 | 6.6± 0.1 |
| Sodium | 330 | 328 | 348 | 329 | 336 ± 4 |
| Potassium | 14.0 | 13.4 | 14.4 | 13.5 | 13.8± 0.3 |
| Aqueous humor | | | | | |
| Sodium | 380 | 383 | 379 | 380 | 380 ± 1 |
| Potassium | 25.5 | 23.9 | 29.9 | 26.3 | 26.5± 1.2 |

[1] Mean and standard deviation of the mean for 13 pigs.
[2] Significantly affected by vitamin A intake ($P < 0.05$).

## DISCUSSION

Based on the results obtained in this study it appears that an uncomplicated vitamin A deficiency of the pig was produced as evidenced by low plasma and liver vitamin A concentration and increased CSF pressure. The complete absence of the classical signs of night-blindness, incoordination, emaciation and death, so long associated with vitamin A deficiency in the pig (Hart et al., '16), suggests the possibility that previous workers were dealing with complicated vitamin A deficiencies. One cannot overlook the possibility, however, that subjecting the animal to a prolonged period of deficiency might result in the eventual appearance of these physical changes.

In the present study vitamin A was stored in the livers of all pigs, with the 2 μg group showing only a trace. Frape et al. ('59) reported that no liver storage was evident when 7 IU (2.1 μg) of vitamin A per pound of live weight per day were fed and suggested that liver storage was a very precise criterion in establishing vitamin A adequacy. Hentges et al. ('52b) noted that 25 μg of purified carotene per kg of body weight per day was the minimal amount required for restoration of plasma vitamin A levels and some liver storage in pigs that had previously been vitamin A-deficient. In studying the relative values of vitamin A and carotene in pigs, Myers et al. ('59) fed 8, 16, 24 and 32 μg of vitamin A per pound of live weight per day. Even though the 8 μg level was the lowest fed, there was sufficient liver storage to indicate the level was adequate (316 μg vitamin A per gm of fresh liver).

The cerebrospinal fluid pressures of the 4- and 8-μg lots were in the range reported for apparently normal pigs by Sorensen et al. ('54) (80 to 145 mm), Hentges et al. ('52a) (88 to 121 mm) and Frape et al. ('59) (83 to 122 mm of saline). CSF pressures recorded for the 16 μg group were considerably below these reported normal values. Repletion for 11 weeks would seem to be sufficient time to return previously high CSF pressures to normal if the quantity of vitamin A fed was sufficient to overcome the deficiency. It might, therefore, be concluded that if the 16-μg group had low but normal CSF pressures, they were repleted to the extent that no evidence of vitamin A deficiency was apparent. It would then follow that the 2,-4- and 8-μg groups were still showing signs of vitamin A deficiency as evidenced by their higher CSF pressures, although some vitamin A was present in all livers analyzed. The critical amount of vitamin A

required by the pigs in this study to result in normal plasma vitamin A, some liver storage, and low CSF pressure would therefore appear to be between 8 and 16 μg per pound of live weight per day.

Similar results with respect to CSF pressure were reported by Dehority et al. ('60) in which 16, 24, 32 and 40 μg of carotene was fed per pound of live weight per day to 16 male Holstein calves that had been depleted of their vitamin A stores. Although vitamin A and carotene were deposited in the livers of all 4 groups, the terminal CSF pressures were 172, 175, 131 and 44 mm of saline, respectively, thus indicating, that liver storage alone may not be an adequate criterion in establishing the vitamin A adequacy of a diet. These results and those of the present study also indicate a need to further establish a range of minimal intakes of vitamin A or precursors of vitamin A needed to produce normal CSF pressures.

A lack of consistent trends was noted in the concentration of the several constituents measured in blood serum, cerebrospinal fluid (CSF) and aqueous humor. Serum potassium concentrations in the pigs increased slightly in a linear manner with increases in vitamin A intake. Dehority et al. ('60) observed a slight decrease in CSF potassium and an increase in aqueous humor potassium in a linear manner with increased carotene intake in calves. These observations may not be analogous, but in both species potassium concentration seemed to be affected.

The general absence of consistent changes in the constituents analyzed in the three body fluids are not in agreement with previous findings. Madsen et al. ('47) observed a slight decrease in serum calcium and phosphorus in a study of vitamin A deficiency in cattle. Dehority et al. ('60) reported that the changes in serum protein concentrations observed in a vitamin A experiment involving two pairs of Guernsey and two pairs of Holstein calves may have been due to inanition and breed difference. The possibility was not ruled out, however, that a balance between these constituents might be altered in a more severe deficiency.

There were no consistent lesions in the limited number of animals included in this study which could be considered pathognomonic for vitamin A deficiency such as those reported by Helmboldt et al. ('53) for cattle. This may have been owing to a relatively short depletion period, to the 11-week repletion period, or to the possibility that pigs do not develop the same lesions as those seen in other species.

## SUMMARY

Thirteen purebred Yorkshire pigs were weaned at three weeks of age and fed a purified vitamin A-deficient diet ad libitum until blood plasma vitamin A concentrations were less than 7 μg/100 ml. Cerebrospinal fluid pressures were then measured and the pigs were assigned to either 2, 4, 8 or 16 μg of vitamin A per pound of live weight per day. The basal diet was then fed at the rate of 4.0% of each pigs body weight and increased 0.3 pound per pig at the end of each week. At the end of 11 weeks, terminal cerebrospinal fluid pressures and plasma vitamin A concentrations were measured, the animals were killed and the concentrations of some constituents of blood serum, cerebrospinal fluid and aqueous humor determined. In addition, a histological examination was made of various tissues including the eye and the parotid salivary gland and duct.

Growth rate was not affected adversely by vitamin A intake. Cerebrospinal fluid pressure increased with decreased blood plasma vitamin A concentration and vitamin A intake. Blood plasma vitamin A, liver vitamin A and serum potassium increased with vitamin A intake. Histological examinations indicated there were no consistent changes relative to vitamin A intake. The minimal vitamin A requirement for the pigs under the experimental conditions of this study necessary to produce normal plasma vitamin A, some liver storage and low CSF pressure appeared to be between 8 and 16 μg of vitamin A per pound of live weight per day.

## ACKNOWLEDGMENTS

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# Nutrition of the Pale Western Cutworm, *Agrotis orthogonia* Morr. (Lepidoptera:Noctuidae)

## V. UTILIZATION OF PROTEIN LABELED WITH CARBON-14 [1,2]

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Proteolytic enzymes have been found in the gut of most insects that have been studied (Day and Waterhouse, '53; Gilmour, '61). Recently, however, Hocking and Depner ('61) reported that neither third-instar nor mature larvae of the pale western cutworm, *Agrotis orthogonia* Morr., possessed proteolytic enzymes in the gut, and concluded that they utilized nitrogen only in the free amino acid form. When newly hatched larvae of the pale western cutworm were fed different tissues of the wheat plant, striking differences in growth and development were observed (Kasting and McGinnis, '61). In view of the report by Hocking and Depner ('61) it appeared that the inability of the larvae to utilize dietary protein might account, at least in part, for differences in nutritional value of the tissues. No information was available, however, concerning the ability of the very small newly hatched larvae to utilize dietary protein. Moreover, as pointed out by Day and Waterhouse ('53) ". . . conclusions drawn from enzyme studies alone are open to question, since the enzymes may be endoenzymes and play no part in digestion." It was desirable therefore, to study proteolysis in the newly hatched larvae with a sensitive *in vivo* method. The digestive processes of mammals have been investigated with carbon-14 labeled substrates (Nasset and Ju, '61; Conrad et al., '58). Protein utilization would be demonstrated if $C^{14}$-labeled amino acids were present in tissues from larvae that were fed $C^{14}$-labeled protein. Such a technique offers a sensitive means to study proteolysis, *in vivo,* in both very small and large insects. The results of a study in which $C^{14}$-labeled algal protein was fed to both first- and fifth-instar larvae of the pale western cutworm are presented.

### MATERIALS AND METHODS

*Diet.* Carbon-14-labeled algal protein[3] was incorporated into a diet prepared from 10-day-old leaves of Thatcher wheat that had been lyophilized, ground, and made up to 66.7% moisture content (McGinnis and Kasting, '60). Algal protein-U-$C^{14}$ (0.93 mg) containing 100 μc of $C^{14}$ was suspended in a small volume of water and ground thoroughly in a mortar. Appropriate quantities of leaf meal and water were added gradually with continuous grinding until 25 gm of diet were prepared. The diet was divided into about 1-gm portions and each was wrapped separately in aluminum foil. The packets were sterilized in an autoclave at 15 pounds steam pressure for 17 minutes and were then stored at −30°C until used.

*Rearing procedure.* The insects used in this study were from a laboratory-reared population. The first-instar larvae were supplied with $C^{14}$-labeled food immediately upon hatching, whereas the fifth-instar larvae were reared through the first 4 instars with fresh-frozen Thatcher wheat sprouts. Newly hatched larvae were reared in groups of 5 and the fifth-instar larvae individually fed in 60-mm petri dishes. Each day the larvae were transferred to clean dishes and supplied with fresh portions of the $C^{14}$-labeled food. The food remnants and the excreta passed during the previous 24 hours were collected separately and stored in 80% ethanol. Moist blotting paper was placed in the top of each petri dish to decrease moisture loss from the food but did not interfere with

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[1] Contribution from Entomology Section and Chemistry Section.
[2] A preliminary report of some of this work was presented at the Fifth International Congress on Nutrition, Washington, D. C., September, 1960.
[3] Purchased from Atomic Energy of Canada Limited.

the quantitative recovery of either food remnants or excreta.

When the larvae were fed the radioactive food the petri dishes were kept in a closed container equipped to collect the respired $CO_2$. Every 24 hours, just before the larvae were transferred to clean dishes, the air in the container was drawn through an absorption train, consisting of 1.0 M $BaCl_2$ and 0.5 M KOH solutions. Each day the solutions in the absorption train were combined and made to volume; aliquots were assayed for radioactivity.

In the first experiment 15 newly molted fifth-instar larvae were fed the $C^{14}$-labeled food throughout the instar. Each larva was starved for 24 hours after it molted into sixth instar and was then removed from the test. Before the larvae in subsequent experiments were removed from test they were provided with nonlabeled basal medium for at least 48 hours. The larvae were rinsed thoroughly with distilled water and either stored at −30°C or processed immediately.

*Preparation of samples for $C^{14}$ measurements.* The quantity of radioactivity in the diet was determined after hydrolyzing 0.813 gm of the sterile medium with 12 ml 6 N HCl under reflux for 24 hours. The HCl was removed *in vacuo* at about 50°C and the soluble components were dissolved in water and made to volume after removal of the insoluble "humin." The radioactivity was measured in aliquots of the hydrolysate. In addition, aliquots of the hydrolysate were chromatographed in two dimensions and autoradiograms were prepared.

To determine whether free amino acids in the sterile diet contained radioactivity it was extracted with 80% ethanol. Two samples were each extracted 4 times and filtrates from each sample were combined. Aliquots of each of the two final solutions were assayed for radioactivity. An aliquot of one of the ethanol extracts was treated on a Dowex-50 column in the $H^+$ form as described by Plaisted ('58) and both the effluent and eluate were assayed for radioactivity. Subsequently aliquots of the ethanol extract, column effluent, and column eluate were chromatographed in two dimensions and autoradiograms were prepared.

The larvae from the first experiment were extracted three times with boiling 80% ethanol in a glass homogenizer. The extracts were combined, reduced in volume, and passed through a Dowex-50 column (Plaisted, '58). The eluate, which contained the amino acids, was fractionated on a second Dowex-50 column by gradient elution with HCl (Kasting and McGinnis, '60). Aliquots of the eluate from the first column and from every fifth fraction from the second column were assayed for radioactivity; chromatograms and autoradiograms were also prepared.

The larval tissues, insoluble in 80% ethanol, were hydrolyzed with 6 N HCl. The larvae from subsequent experiments were hydrolyzed in 6 N HCl without prior ethanol extraction. All hydrolysates were treated as described above and two dimensional chromatograms and autoradiograms were prepared.

The excreta produced by the 15 larvae in the first experiment were combined daily yielding 6 samples. The residual food was similarly collected and combined. Each sample of excreta and of residual food was extracted 4 times with 80% ethanol in a glass homogenizer. The extracts from each sample were combined and aliquots were assayed for radioactivity. Chromatograms and autoradiograms were also prepared.

*Paper chromatography and autoradiography.* All extracts and hydrolysates were chromatographed in two dimensions on Whatman no. 1 filter paper first with *n*-butanol:acetic acid:water (4:1:5) and then with water-saturated phenol, pH12 (Kasting and McGinnis, '62). Fractions removed from the second Dowex-50 column were chromatographed in one dimension with *n*-butanol:acetic acid:water (4:1:5). Autoradiograms were prepared by exposing Kodak-No-Screen x-ray film to the chromatograms.

*Radioactivity counting procedures.* Radioactivity in all extracts, hydrolysates, and column fractions was measured in aliquots dried on copper planchets under an infrared lamp. Aliquots of solutions used to

absorb $CO_2$ were similarly plated and corrections for self absorption (Calvin et al., '49) were applied. All measurements of radioactivity were made with a windowless gas-flow counter (Tracerlab Incorporated) using a 99% helium-1% isobutane gas mixture.

*Preparation of gut homogenates.* Guts dissected from laboratory-reared fifth-instar larvae were opened and washed free of their contents. The guts were ground in a glass homogenizer with either 0.1 M Sorenson's phosphate buffer, pH7.6, or 0.5% $NaHCO_3$ depending upon the assay method (Charney and Tomarelli, '47; Kunitz, '47).

## RESULTS AND DISCUSSION

It was essential that the $C^{14}$ added to the basal medium be in the protein form because appreciable quantities of radioactivity in free amino acids would invalidate the method. Almost one-half of the radioactivity in the diet was extracted with ethanol (table 1) and nearly half of the ethanol-soluble radioactivity was retained on a Dowex-50 column, which suggested that the free amino acids did contain radioactivity (Plaisted, '58). However, the amino acids which were eluted from the column with $NH_4OH$ *were not radioactive* (compare the chromatogram, figure 1A, with the autoradiogram, figure 1B), whereas the diet after acid hydrolysis, contained radioactive amino acids (compare the chromatogram, figure 1C, with the autoradiogram, figure 1D). Thus the radioactivity in the eluate was not associated with free amino acids in the diet but was in other ethanol-soluble compounds. Autoradiograms of this eluate before and after hydrolysis demonstrated that most of the radioactivity was present in compounds that yielded amino acids.

TABLE 1

*Distribution of radioactivity in diet containing algal protein-U-$C^{14}$*

| | *count/min/mg dry matter* | *%* |
|---|---|---|
| Ethanol extract[1] | $7.9 \times 10^3$ | 48 |
| Column effluent | $3.9 \times 10^3$ | 23 |
| Column eluate | $3.4 \times 10^3$ | 20 |
| Left on column | $0.6 \times 10^3$ | 5 |
| Residue | $8.7 \times 10^3$ | 52 |
| Total | $16.6 \times 10^3$ | 100 |

[1] Ethanol extract of second sample of diet contained $8.0 \times 10^3$ count/min/mg of dry matter.

The remaining one-fourth of the radioactivity in the diet was in the effluent from the Dowex-50 column (table 1). Amino acids were not evident in autoradiograms of the effluent, but radioactivity appeared in components at the front. When the effluent was acid-hydrolyzed, autoradiograms showed a pattern of radioactive amino acids. The results indicate that most of the radioactivity added to the diet was present in protein or at least in compounds that upon acid hydrolysis yielded $C^{14}$-labeled amino acids.

The quantities of radioactivity present in the ethanol extract and the acid-hydrolyzed residue from the 15 larvae in the first experiment were $1.84 \times 10^6$ and $3.21 \times 10^6$ count/min, respectively. It was possible that food in the guts of the larvae may have been a source of radioactivity. However, for all the radioactivity in the hydrolyzed residue to have come from food in the gut, about 80 mg would have had to be present in each gut. Since the larval gut of the cutworm is evacuated before a molt and because the test insects, averaging 200 mg in weight, were not fed after molting, this quantity of food would not be present. Therefore, it is apparent that radioactivity was incorporated into the larval tissues.

Autoradiograms of ethanol extracts of the diet and the larvae showed that the free amino acids from the latter were radioactive whereas those from the former were not. Thus, whether the $C^{14}$-labeled amino acids in the ethanol extract of the larvae were derived from the tissues or from the lumen of the gut, they were the result of enzymatic hydrolysis. Although these data do not indicate quantitatively the importance of protein for this insect, they do show that protein is utilized.

Larvae of the pale western cutworm can synthesize alanine, aspartic acid, glutamic acid, proline, and serine from glucose-U-$C^{14}$ but not phenylalanine, arginine, lysine, histidine, the leucines, and tyrosine (Kasting and McGinnis, '62). In the present study, therefore, the presence of $C^{14}$ in amino acids of the former group might

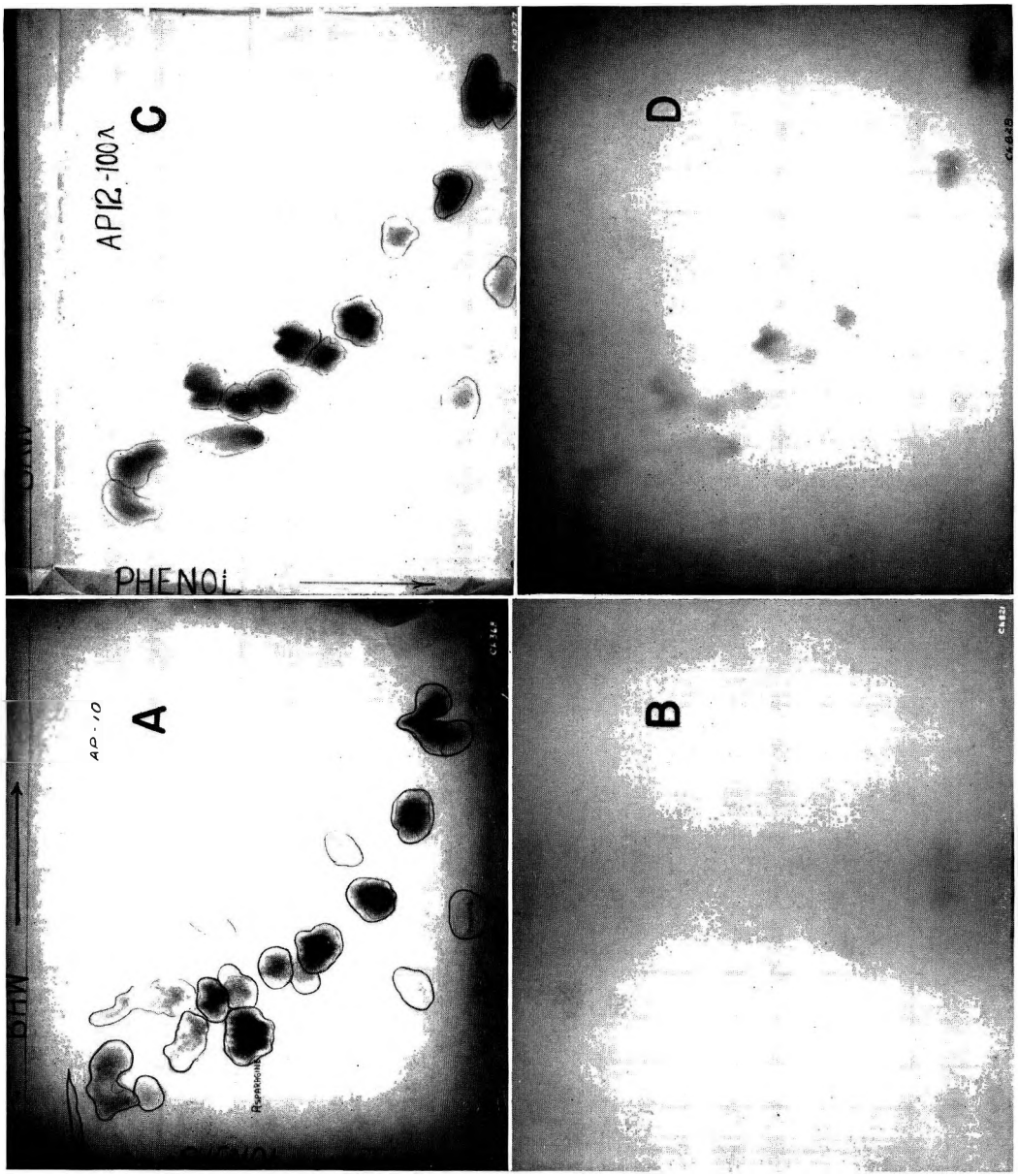


Figure 1

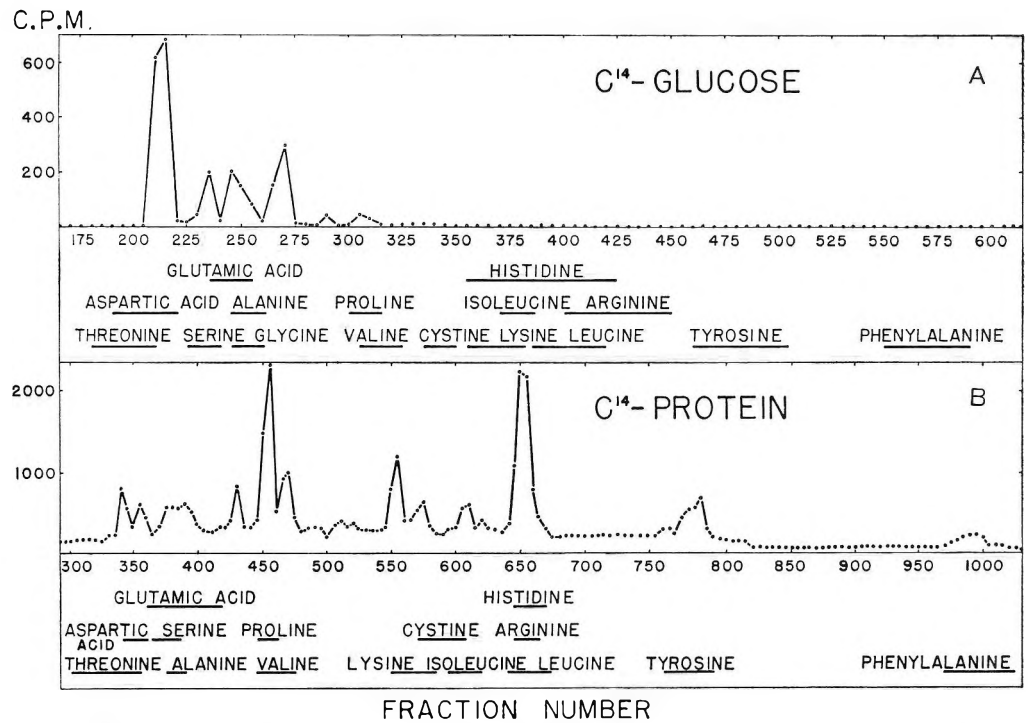


Fig. 2 Distribution of radioactivity in fractions from Dowex-50 columns after gradient elution. A, hydrolysate of pale western cutworms injected with glucose-U-$C^{14}$; B, ethanol extract of pale western cutworms fed diet supplemented with algal protein-U-$C^{14}$.

merely indicate synthesis from $C^{14}$-labeled nonprotein material rather than protein utilization. However, the presence of radioactivity in amino acids that cannot be synthesized would almost certainly indicate ultization of the labeled protein. The elution curves (figs. 2A and 2B) show that amino acids not synthesized from glucose-U-$C^{14}$ were labeled when larvae were fed protein-U-$C^{14}$, which demonstrates utilization of the labeled protein.

The larvae were fed sterile $C^{14}$-labeled diet and reared in sterile petri dishes. Since the larvae were not reared aseptically it was possible that some $C^{14}$-labeled amino acids could have been released from the algal protein by microorganisms. Comparison of the specific activity (quantity of ethanol extractable radioactivity per milligram of dry matter) of the sterile diet (table 1) with that of the food after exposure to the larvae for 24 hours (table 2) indicates no increase in the ethanol extractable $C^{14}$-labeled components. In contrast, the specific activity of excreta was consistently higher than that of the residual food on a daily basis (table 2). Moreover, the differences shown are minimal because a certain amount of mixing of excreta and residual food was unavoidable. These data

Fig. 1 A, Chromatogram of amino acids extracted with 80% ethanol from $C^{14}$-labeled diet; B, autoradiogram of A; C, chromatogram of hydrolysate of $C^{14}$-labeled diet; D, autoradiogram of C.

TABLE 2

*Radioactivity in 80% ethanol extracts of residual food and excreta*

| Day | Residual food | Excreta |
|---|---|---|
| | *count/min/mg dry matter* | *count/min/mg dry matter* |
| 1 | $8.4 \times 10^3$ | $11.9 \times 10^3$ |
| 2 | $6.4 \times 10^3$ | $14.1 \times 10^3$ |
| 3 | $8.5 \times 10^3$ | $12.1 \times 10^3$ |
| 4 | $7.5 \times 10^3$ | $11.9 \times 10^3$ |
| 5 | $6.8 \times 10^3$ | $10.6 \times 10^3$ |
| 6 | — | $12.5 \times 10^3$ |
| Mean | $7.5 \times 10^3$ | $12.2 \times 10^3$ |

indicate that the protein was not hydrolyzed by microorganisms prior to consumption by the larvae, a circumstance that might lead to misinterpretation of the results. The higher specific activity in excreta than in residual food (table 2) presumably resulted from concentration of ethanol-soluble labeled protein by removal of nonprotein substances and enzymatic release of ethanol-soluble radioactive components from the diet. Metabolism of $C^{14}$-labeled components in the diet is also indicated by the fact that about 2,000 count per min per insect per day were recovered in expired $CO_2$.

The evidence for protein utilization by fifth-instar larvae of the pale western cutworm is qualitative. Weak proteolytic activity would yield positive results by the radioactivity method yet the enzymes might have little importance in the overall nitrogen economy of the larvae. Consequently quantitative data were obtained with 6 newly molted fifth-instar larvae fed $C^{14}$-labeled diet for three days. Because one molted on the fourth day it was hydrolyzed separately. The remaining 5 were fed nonlabeled medium until they molted to sixth instar (two days) and were then hydrolyzed. Measurements were made of the amount of $C^{14}$-labeled diet consumed by the larvae and the quantities of excreta passed before and after the nonlabeled medium was supplied.

Fifty per cent of the radioactivity recovered was in the larval bodies and the remainder was in the excreta (table 3). Only about two-thirds of the radioactivity that was consumed was recovered. Nonetheless, these data indicate that about one-half of the protein carbon was retained in the insect tissue, a proportion that agrees well with the nitrogen retention recorded earlier (Kasting and McGinnis, '59). More than one-third of the radioactivity recovered in the excreta was eliminated from the insect bodies after the larvae were returned to the unlabeled medium. This radioactivity originated as either residual $C^{14}$-labeled food from the digestive tracts or $C^{14}$-labeled tissues of the insects. When the rapid growth of the fifth-instar cutworm is considered (McGinnis and Kasting, '59), it is unlikely that the food left in the gut was equivalent to one-third of that consumed during the preceding three days. Thus it is probable that at least some of the radioactivity excreted during the final two days on test was in products of tissue metabolism. The recovery of radioactivity in the larval tissues and in excreta indicate that the utilization of dietary protein was quantitatively significant and in excess of 50%.

TABLE 3

*Distribution of radioactivity from algal protein-U-$C^{14}$ consumed by fifth-instar larvae of the pale western cutworm*

| | *count/min* | % |
|---|---|---|
| Insect hydrolysate | $2.00 \times 10^6$ | 50 |
| Excreta I hydrolysate[1] | $1.25 \times 10^6$ | 31 |
| Excreta II hydrolysate[2] | $0.77 \times 10^6$ | 19 |
| $CO_2$ | $0.02 \times 10^6$ | — |

[1] Excreted while larvae were fed $C^{14}$-labeled diet.
[2] Excreted after larvae were returned to nonradioactive diet.

The qualitative results obtained by the *in vivo* radioactivity procedure were confirmed using gut homogenates by two classical enzyme methods (Charney and Tomarelli, '47; Kunitz, '47). Protein hydrolysis was clearly demonstrated despite differences in method and insect population. The proteolytic activity per larval gut was in the order of $2 \times 10^{-3}$ Kunitz trypsin units.

Because the experiment with fifth-instar larvae established the validity of the radioactivity method the procedure was extended to newly hatched larvae. The small size of the larvae made it desirable to hydrolyze the insects without prior ethanol extraction but it was necessary that the larval guts be entirely free of $C^{14}$-labeled food. Ninety-nine larvae were reared through two instars; during the first instar, they were supplied with the radioactive diet, and throughout the second instar they were fed the unlabeled medium. Thus an average of 4 days and two molts were allowed to clear the radioactive food from the gut. Crowell ('43) showed with fifth- and sixth-instar larvae of the southern army worm, *Prodenia eridania* Cramer, that food was completely eliminated from the alimentary tract within 8 hours of being consumed. At this laboratory,[4] it was shown that the period between consump-

[4] Jacobson, L. A., unpublished data.

tion and excretion is shorter for the pale western cutworm. In the present experiment, therefore, it was assumed that there was sufficient time to permit complete elimination of radioactive food from the gut. The chromatograms and autoradiograms of the hydrolysate of these small insects showed that both essential and nonessential amino acids were radioactive. Thus it is concluded that the first-instar larvae were able to utilize dietary protein. Quantitative utilization of $C^{14}$-labeled protein was estimated from earlier consumption data (Kasting and McGinnis, '59). More than one-fifth of the radioactivity consumed by the larvae ($10^6$ count per min) was recovered in the hydrolysate ($2 \times 10^5$ count per min) and the respired $CO_2$ ($5 \times 10^4$ count per min). During the second instar, no $C^{14}$-labeled diet was provided and, based on results with fifth-instar larvae, one might expect that radioactive products of tissue metabolism were excreted. It appears, therefore, that first-instar larvae utilized more than 20% of the protein consumed.

The studies reported herein show that protein is utilized by both first- and fifth-instar larvae of the pale western cutworm and that it is quantitatively significant in their nitrogen economy. Results from the *in vivo* experiments cannot be considered artifacts of enzyme extraction because the radioactive protein was fed to and metabolized by living larvae. The radioactivity method has not conclusively established the presence of proteolytic enzymes in the gut. This conclusion seems justified, however, because of the general occurrence of proteolytic enzymes in the gut or gut contents of the majority of insects that have been studied (Day and Waterhouse, '53; Gilmour, '61) and our results with classical methods. The larvae used in the present studies were not reared aseptically and it is possible that microorganisms in the gut were responsible for proteolysis. It is also possible that intact proteins were transferred across the epithelial lining of the gut as with young mammals (Roy, '59; Walker, '59) and that proteolysis occurred elsewhere. Despite these possibilities there is no doubt that the larvae utilized protein. This is in direct contrast with the conclusions of Hocking and Depner ('61) who reported that proteolytic enzymes were absent in gut extracts of larvae of the pale western cutworm. The conflicting results are difficult to reconcile but it may be that the methods used by them lacked sensitivity.

## SUMMARY

A diet containing algal protein-U-$C^{14}$ provided a convenient means of studying *in vivo* protein utilization by larvae of the pale western cutworm *Agrotis orthogonia* Morr. Utilization of dietary protein in both first- and fifth-instar larvae was found to be quantitatively significant. This technique avoids the difficulty of enzyme extraction artifacts and has particular application to organisms that are too small to permit investigation by classical methods.

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# Changes in Tissue Lipids in Response to Diet

## I. FATTY ACIDS OF SUBCUTANEOUS, MESENTERIC AND INTERSCAPULAR FAT[1]

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Recent emphasis on the role of the unsaturated fatty acids in the prevention of atherosclerotic lesions makes it desirable to find out more about the extent to which a reserve supply of these acids may be built up in adipose tissue, and the rates at which they change in response to changes in the dietary fat.

Hirsch et al. ('60) have developed a technique for aspiration of biopsy samples of fat from human subjects. Their data indicate a rather constant percentage of linoleate (about 11%) in this adipose tissue which was not much altered by high levels of corn oil fed for 10 weeks. This was in sharp contrast with the increases in serum linoleate in the same subjects. Tove and Smith ('59, '60) studied the kinetics of depletion of linoleic acid from carcass lipids of mice. They believe that linoleic acid is depleted rapidly until a critical level is reached, after which depletion proceeds more slowly. Their data might be explained either by Hirsch's postulation of an easily altered and a stable fraction in adipose tissue or by a homeostatic regulation of essential fatty acid concentration. An equally possible explanation of the data from both investigations might be that changes in fatty acid composition occur at different rates in different types of adipose tissue and that the biopsy samples were not adequately representative.

In a study of the effects of feeding safflower oil on the plasma cholesterol ester in man, it was found that the cholesterol values remained low, and the esterified linoleic acid high, for at least a month after feeding of the linoleate-rich fat was discontinued (Okey et al., '60). This raised the question of possible selective retention of linoleate in some fat depot from which plasma linoleic acid moieties might be replenished. The present paper reports a study of the changes in the fatty acids of three types of adipose tissue in rats — subcutaneous, mesenteric and interscapular fat — following changes in composition of the dietary fat. Data for liver and plasma composition in the same animals are reported in another paper (Okey et al., '62).

### METHODS

*Diets.* The semisynthetic diet formula, containing 10% of fat and 1% of cholesterol, has been described previously (Lis and Okey, '61). Safflower oil, furnishing approximately 75% of linoleate and 12% of oleate, and coconut oil furnishing about 50% of laurate, 18% of myristate and a little less than 3% of linoleate, were chosen as fats of widely different fatty acid composition. Neither was fed at a level excessively high for the rat, and the coconut oil diet was not deficient in essential fatty acid. The cholesterol was added to secure a high enough concentration of liver cholesterol ester to permit measurement of small changes in its fatty acid components.

*Experimental plan.* Weanling rats of the Long-Evans strain were caged individually and given the coconut oil diet (CN) ad libitum. After three weeks, about two-thirds were transferred to the safflower

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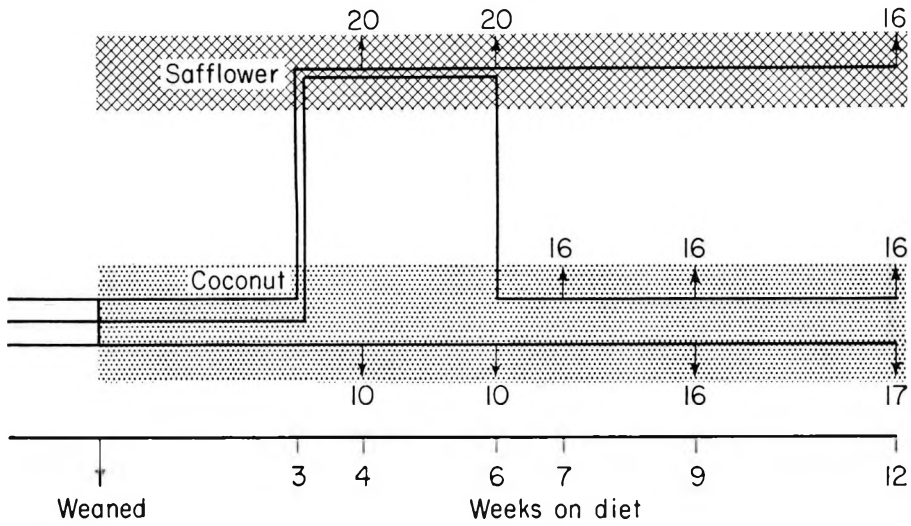


Note: Arrows indicate time of autopsies. Numbers indicate numbers of animals used for each autopsy. There were equal numbers of each sex.

Fig. 1 Design of experiment.

oil diet (Saff). Part of the latter were transferred back to the CN diet after another three weeks. The experimental plan, with the numbers of animals killed at each interval, is shown in figure 1. For a few of the animals fed the diet for the longer periods (namely, the CN 9- and 12-week groups), times of autopsy varied from the averages indicated by one to 4 days.

Food cups were removed 12 to 15 hours before autopsy. Rats were anesthetized with sodium pentothal and bled from the heart. Plasma and liver samples were prepared for analysis as previously described (Lis and Okey, '61). Great care was taken to secure adipose tissue samples that were uniform in amount and position of origin. Subcutaneous fat was taken from the insides of the thigh and foreleg. Mesenteric fat was taken from the region in which the large and small intestines join. Interscapular fat was excised as quantitatively as possible. All visible connective tissue was eliminated.[2]

Adipose tissue was extracted as follows: 300 to 400 mg of interscapular fat or 600 to 800 mg of mesenteric or subcutaneous fat were weighed into 75 ml of 1:1 95% ethanol-acetone. After standing overnight at room temperature, the hardened samples were disintegrated with a glass rod. The solution was decanted through glass wool, and the procedure was repeated with another 75 ml of solvent. The extracted tissue and the glass wool were washed and stored for the nitrogen analysis performed later by a semimicro-Kjeldahl procedure (McKibben, '49). The combined fat extracts were partially evaporated, and the volume was adjusted to 100 ml with ethanol-acetone. Aliquots were used for analyses.

Total lipids were determined by oxidation (Bloor, '28). Preliminary tests indicated that the lipid of the adipose tissue was very nearly all triglyceride. Samples were therefore not fractionated on silicic acid columns. Aliquots of the original extract were saponified with KOH in ethanol. The nonsaponifiable material was extracted from alkaline solution, and the free fatty acids after acidification, both with petroleum ether. Methyl esters of the fatty acids were prepared and analyzed by gas-liquid phase chromatography as previously described (Lis and Okey, '61). The content of total nitrogen and of fatty acid

[2] We did not attempt to study epididymal fat pads or those adjacent to the kidney, partly because we wished to compare fat retention in males and females, and partly because, in the younger animals, pads in the renal area were not sufficiently developed for good sampling.

could be calculated for interscapular fat only, because it was not feasible to determine total amounts of subcutaneous and mesenteric fats.[3] Moisture content of the adipose tissue was estimated roughly on the basis of the difference between the total wet weight and the sum of the total lipids and protein of the tissue.[4] Determination by drying seemed inadvisable because of the differences in probable oxidation of fats containing different proportions of polyunsaturated fatty acids.

## RESULTS

*Effects of age and growth* must be considered in interpretation of the data, since the animals were growing during the period of the experiment. Food intakes and weight gains closely resembled those described by Lis and Okey ('61) for rats fed ad libitum similar diets containing cottonseed or coconut oils. Alterations in food intake and rate of growth due to the change from one diet to the other were not statistically significant, although mean growth rates were slightly higher for animals fed safflower oil than for those fed coconut oil throughout the period of the experiment.

*Gross composition of the different types of adipose tissue* varied considerably. Nitrogen content of interscapular fat varied from 0.25 to 0.40% wet weight (0.75 to 1.35 mg per rat), and that of mesenteric and subcutaneous fats varied between 0.10 and 0.15% wet weight. Rough estimates indicated that water content varied with that of protein (as indicated by nitrogen content). Total fatty acid content, on the other hand, varied inversely with protein (fig. 2). Total fatty acid content of mesenteric and subcutaneous adipose tissues of the rats fed coconut oil and those fed coconut-safflower-coconut oils tended to decrease after the animals were about 12 weeks old (9 weeks on diet). The decrease was much more pronounced in females than in males. Unfortunately, it was impossible to tell whether the safflower-fed animals had high values after being fed the diet for 9 weeks, since none of them were killed at that time.

*Changes in individual fatty acids of adipose tissue.* The linoleate content of all three types of adipose tissue of the coconut-fed rats remained at a fairly constant level, slightly higher than that of the coconut oil itself. This occurred despite the fact that the diets furnished only about 22% of their calories as fat, and some of the adipose tissue fatty acid was probably derived from carbohydrate. When safflower oil was fed, the percentage of linoleate in the fat of the adipose tissue increased rapidly during the first week, and more slowly thereafter (fig. 3a). The zero-week points were obtained by projection of the curves for the coconut-fed controls.

Expressed as milligrams of fatty acid per milligram of nitrogen (fig. 3b), values for interscapular fat were much lower than for mesenteric and subcutaneous fat. This was due to the higher nitrogen content of interscapular fat.

The rats transferred back to coconut oil after safflower oil showed some indication that, in subcutaneous fat, the rate of depletion of linoleate was slower than its rate of entrance (fig. 3b). (The first measurements on the safflower groups were made one week after the change from CN to Saff diet.) The subcutaneous fat of females even showed an increase in linoleate content during the first week after withdrawal of safflower oil. This was associated with an increase in total fatty acid content (fig. 2), which is probably influenced by age. Equilibration of the composition of adipose tissue in response to a change of fat in the diet was reached most rapidly in the interscapular fat, followed by mesenteric and finally, by subcutaneous. This is in keeping with the concept that interscapular fat is not strictly a depot fat. Six weeks after the rats were returned to the coconut oil diet, the linoleate content of the subcutaneous fat of the rats that had been fed safflower oil was still

[3] Areas under the curves were measured either with a compensating planimeter or a mechanical integrator. Since the thermal conductivity cell of the Aerograph has different sensitivities for the methyl esters of different fatty acids, appropriate mixtures of known amounts of standard fatty acid methyl esters were analyzed each day. From these relative sensitivity factors were computed for each fatty acid. Results were calculated both as percentages of the total fatty acid and as milligrams of fatty acid per milligram of nitrogen in the tissues. For example:

$$\frac{\text{mg total lipids}}{\text{mg N}} \times \frac{\%\text{ each fatty acid}}{100} = \frac{\text{mg fatty acid}}{\text{mg N}}.$$

[4] Glycogen was not determined because previous work has shown that its concentration in these adipose tissues is very small, less than 0.1% (Lepkovsky and Ostwald, unpublished results).

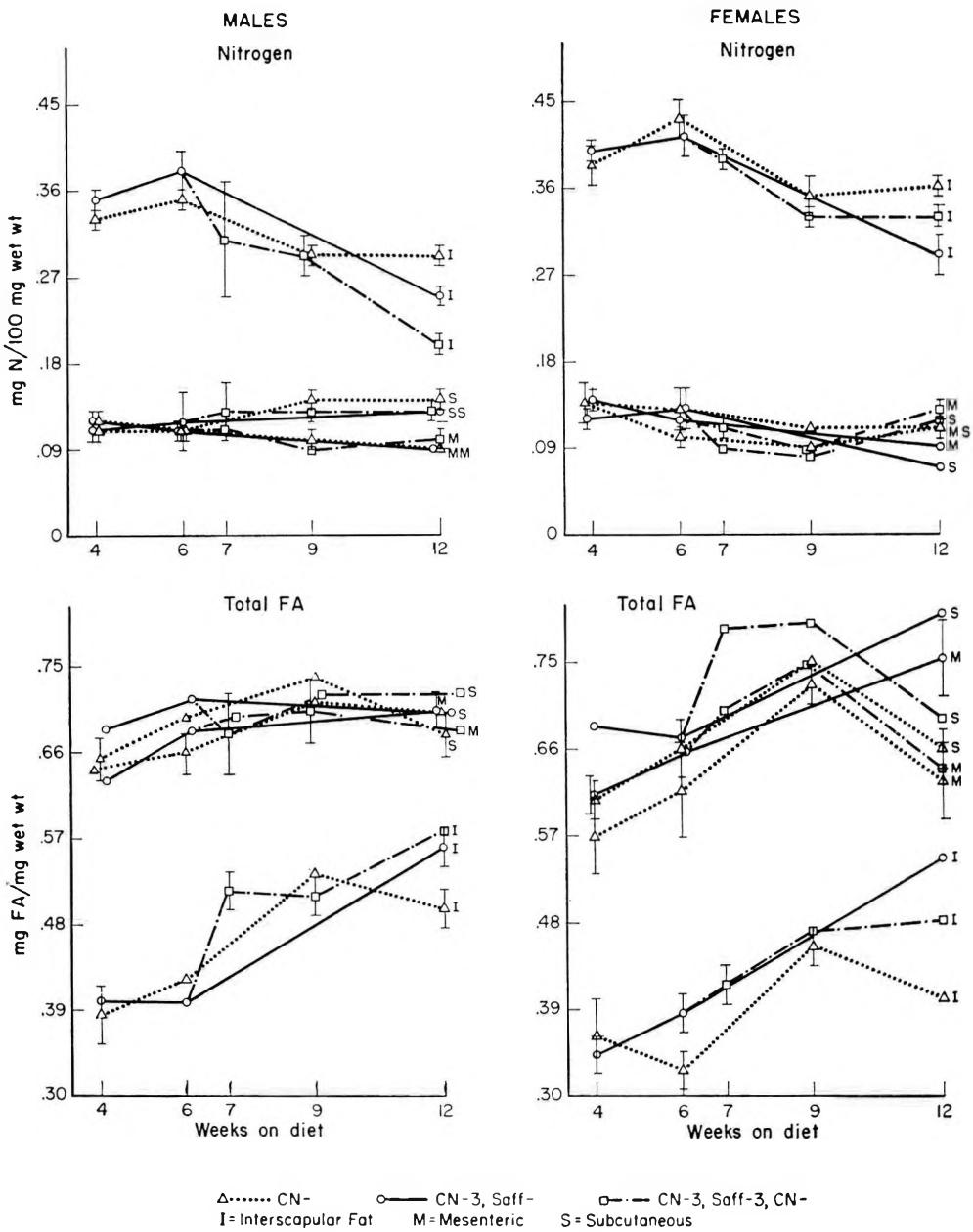


Fig. 2 Total nitrogen and total fatty acid content of adipose tissues. The diet groups are indicated throughout as follows: CN-: animals fed coconut oil as only source of fat; CN-3, Saff-: animals fed safflower oil only, following three weeks of coconut oil; CN-3, Saff-3, CN-: animals fed coconut oil for three weeks followed by safflower oil for three weeks and then returned to coconut oil. Each point represents the mean value for the group. Vertical bars through the points represent standard errors. Standard errors less than 0.01 or smaller than the symbols used have been omitted throughout. The number of animals in each group is indicated on figure 1.

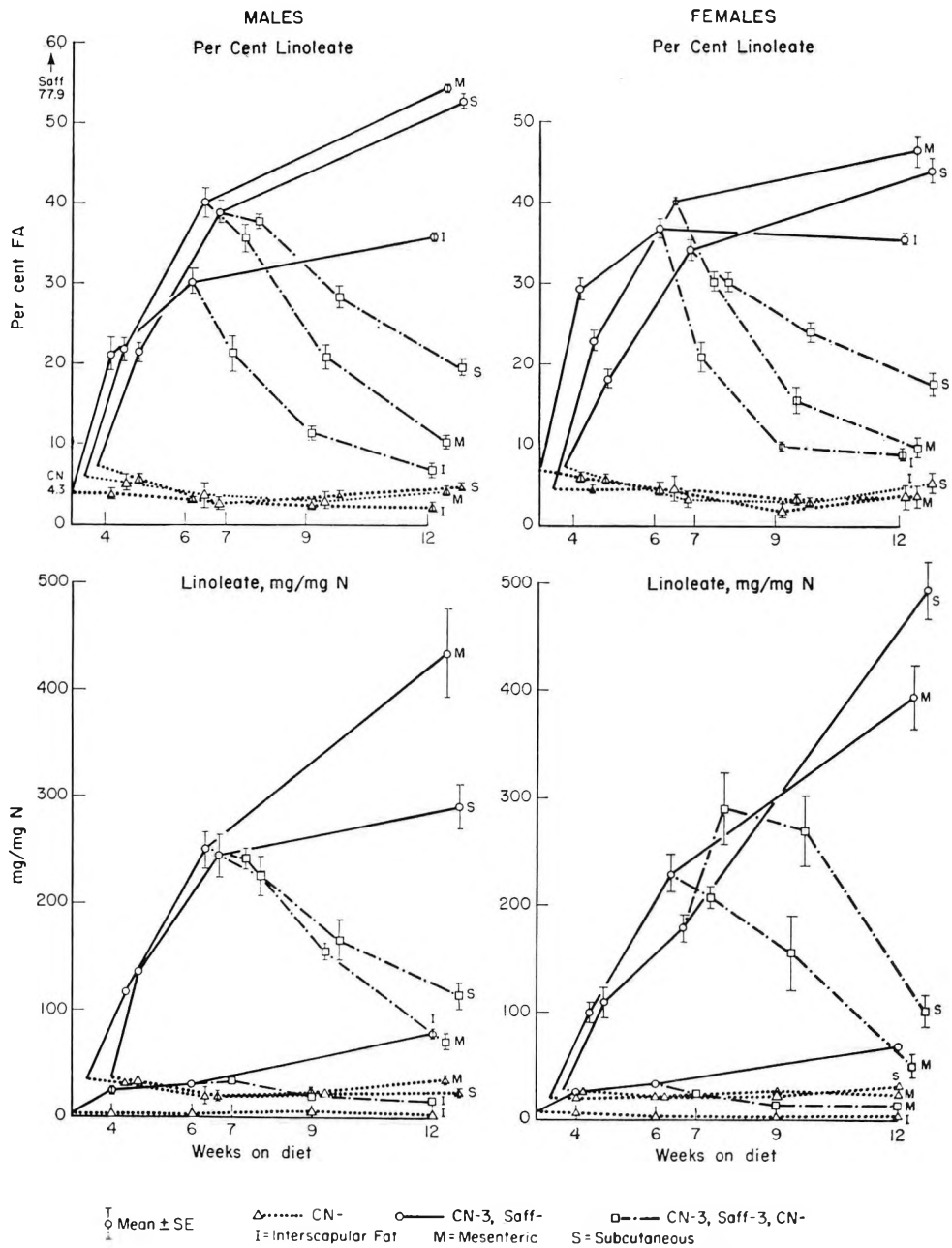


Fig. 3 Linoleic acid content of adipose tissues. The graphs for the three adipose tissues are superimposed on this and the following figures. In the interest of clarity there is a slight shift in respect to time for each tissue. The portions of the curves between three and four weeks are extrapolations. The points represent amounts of the fatty acid in question in the given adipose tissue. Values are expressed as per cent of total fatty acids present or as milligrams of fatty acid per milligram of nitrogen (footnote 3 in text). For explanation of symbols see legend to figure 2.

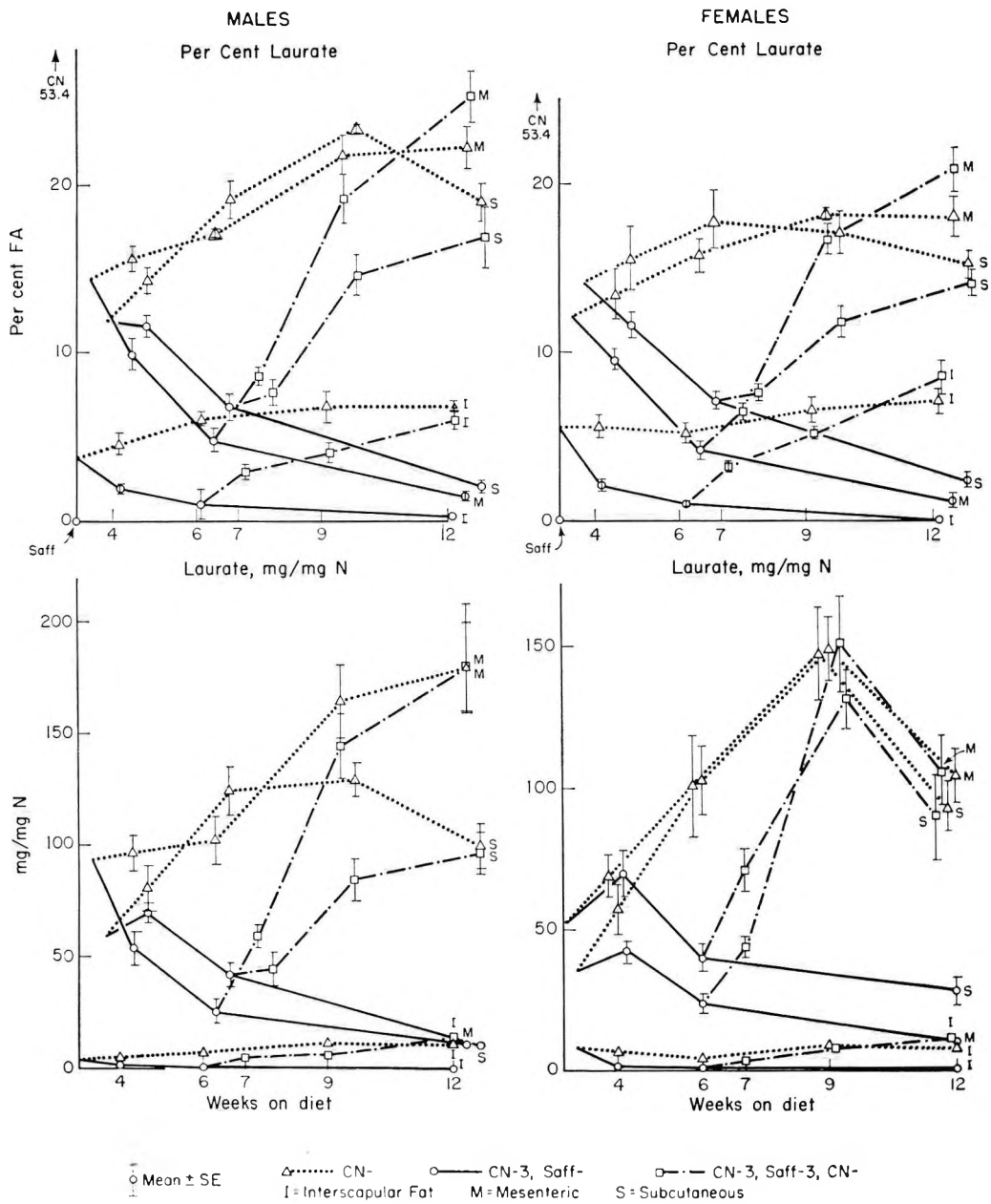


Fig. 4 Lauric acid content of adipose tissues. Data are arranged as in figure 2.

5 to 7 times higher than that of the animals fed coconut oil for the entire period. The linoleate content of their mesenteric fat was also appreciably higher than that of the CN controls, and even interscapular fat retained some extra linoleate.

Percentages of monoenoic acids as well as of palmitate were always higher than those in either of the dietary fats. Palmitate in interscapular fat reached 25% in contrast with about 7% in either safflower or coconut oil. Monoenoic acids reached

concentrations of over 50% in interscapular fat, whereas safflower oil contained 13% and coconut oil 5%. In contrast, there was no evidence of synthesis of laurate in these adipose tissues (fig. 4). Even after coconut oil containing 53% of laurate was fed for 12 weeks, the laurate concentration in mesenteric fat reached only 25%. Conversely, its concentration in the same fat dropped from 15 to 5% after safflower oil, which contains no measurable amount of lauric acid, was fed for three weeks.[5]

Age, sex and growth evidently have some influence on the composition of adipose tissues. The fatty acid concentration in adipose tissues of females fed coconut oil reached a peak for all fatty acids (except linoleic) at 9 weeks on diet (fig. 5). The corresponding curves for the subcutaneous fat of males show a similar, though less pronounced decrease after the diet was fed for 6 weeks. This phenomenon is associated with parallel changes of the total fatty acid content of these tissues (fig. 2).

## DISCUSSION

The rat does not store arachidonic acid in any quantity in adipose tissue. Because of the very high percentage of this acid in circulating cholesterol ester and phospholipid, the storage and turnover rates of its known precursor, linoleic acid, become especially important. Tove and Smith ('59) calculated rates of depletion of linoleic acid in the total body fat of mice. Their animals were returned to an "almost fat-free basal diet" (which, however, contained cornstarch known to furnish essential fatty acids) after three-week feeding periods with diets containing 15%, respectively, of safflower, corn and peanut oils. They estimated dienoic and trienoic acids by alkali isomerization. On the basis of the assumption that depletion of linoleic acid in body fat was a first-order reaction, they estimated that it had a half-life of about 11 days until a concentration of about 12% of the body fat was reached. After that, their data more nearly fitted the slope characteristic of a half-life of 25 days. (Their animals were changed from a diet that furnished about 28.4% of its calories as fat to one essentially fat-free.) Wagner et al. ('58, '61) reported determination of the rate of depletion of linoleic and linolenic acids from a great number of tissues of mature male rats. They returned the animals to an "almost fat-free diet" after a given period (55 days) of feeding 10% of sunflower oil as a source of linoleic acid and of linseed oil as a source of linolenic acid, respectively. Their data indicate depletion half-times of 77 and 65 days, respectively, for linoleic acid in subcutaneous and mesenteric fat. These half-times are considerably longer than our data indicate, but they also show that the rate of depletion is slower in subcutaneous than in mesenteric fat. Our data, in contrast, represent not depletion of a given fat when feeding a "fat-free" diet but, rather, depletion of linoleic acid during a period of change from a linoleate-rich to a linoleate-poor diet with total fat fed at the same level.

On the basis of Tove and Smith's ('59) assumption, our data for the first three weeks after transfer from safflower to coconut oil indicate the following half-time values (days) for the depletion of linoleic acid (fig. 6): interscapular fat — males 15, females 11; mesenteric fat — males 23, females 19; subcutaneous fat — males 51, females 43.

Our data indicate, as do Tove and Smith's, that the depletion slope tends to be steep immediately after a diet transfer and then to flatten. In neither case were data obtained at sufficiently frequent intervals to permit an exact calculation of the kinetics of the process. It is important to keep in mind that our experiment, as well as those of Tove and Smith ('59) and of Hirsch et al. ('60), furnishes data about the rates at which linoleic acid in depot fat is replaced by another fatty acid. Stetten and Grail ('43) measured the rate of disappearance of deuterium from the depot fat of mice, and calculated its half-life to be 5 to 6 days. Thompson and Ballou ('56) measured the retention time of organically bound tritium in rat tissue and calculated the "biological half-life" of perirenal and genital fat to be 70 days for 70% and 17 days for 30% of these tissues.

[5] Detailed data on compositon of the adipose tissue have been omitted. They are available on request.

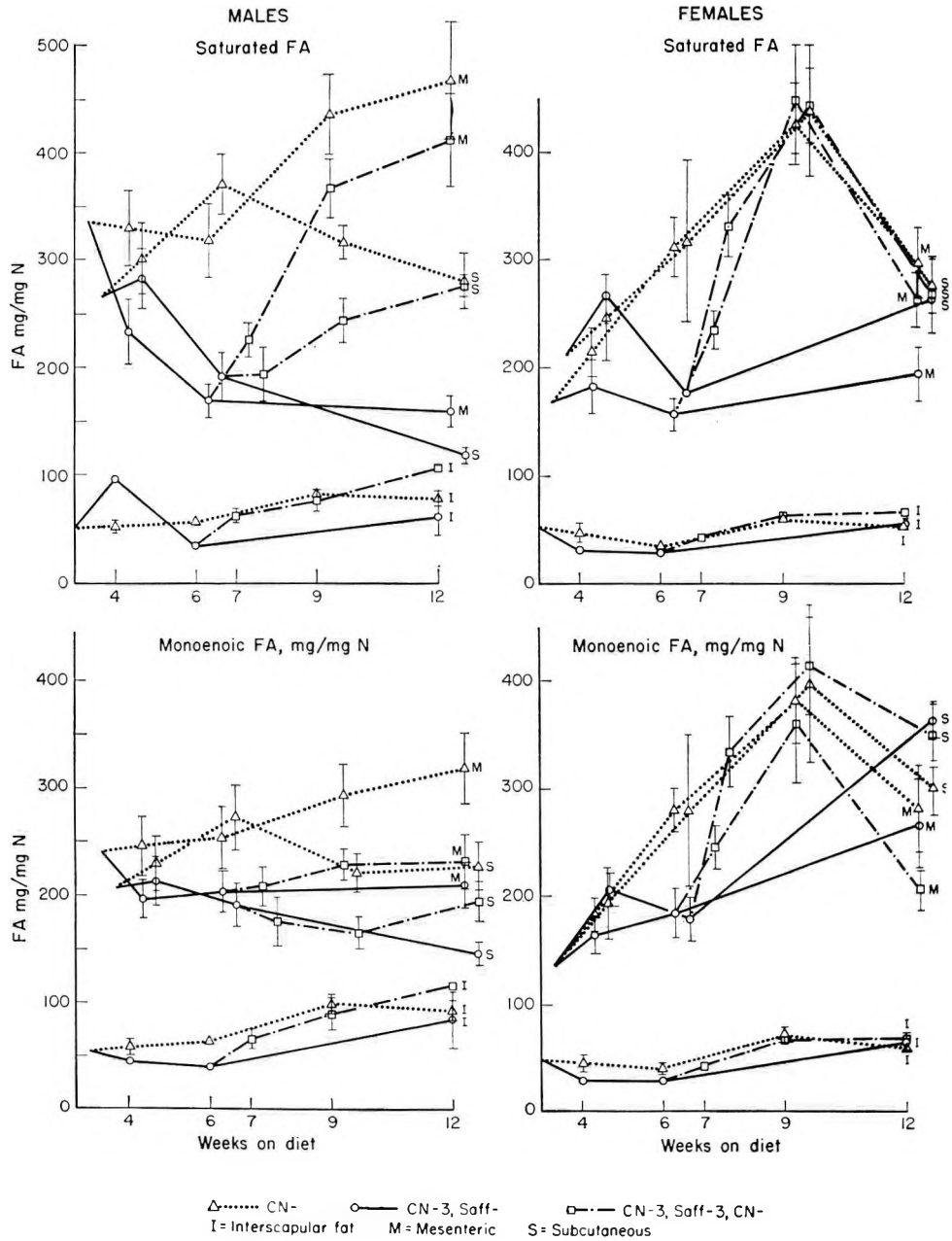


Fig. 5 Saturated and monoenoic fatty acids of adipose tissues. Data are arranged as in figure 2.

Pihl et al. ('50) measured the rise of the $C^{14}$ content of eviscerated-carcass fatty acids of rats, and calculated the half-lives to be 16 to 20 days, based on the rate of synthesis. The rate of replacement of one fatty acid by others in a given tissue is comparable with the rate of turnover of that tissue only if certain assumptions are made, the most important of which is that the average rate of replacement of all fatty acids is the same as that of the fatty acid under consideration. We have no direct

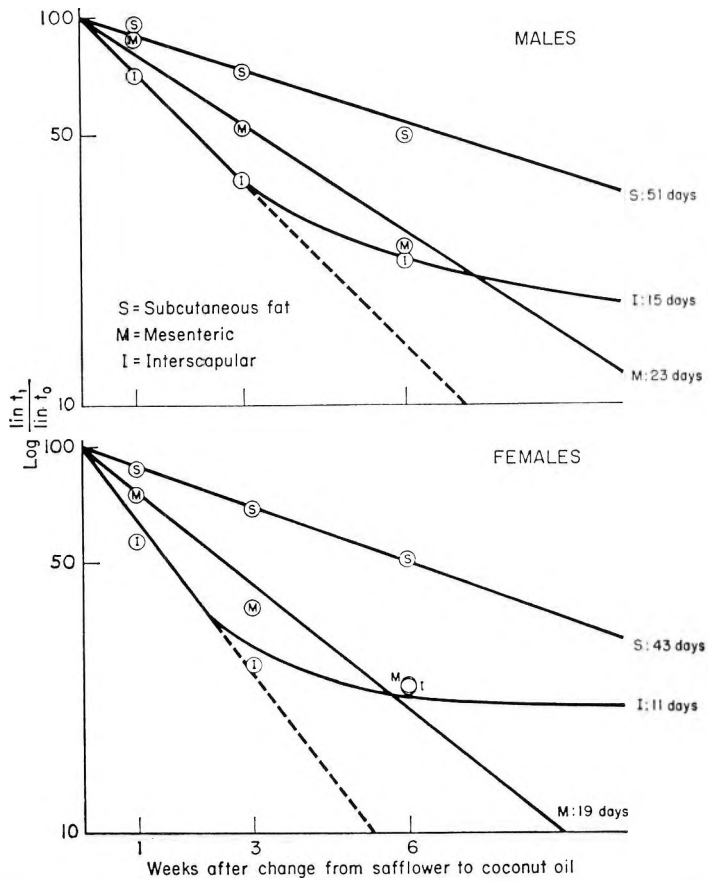


Fig. 6 Depletion of linoleic acid from adipose tissues. This graph represents the decline of the concentration of linoleic acid in the adipose tissues of animals which had been returned to a coconut oil diet after an intermediate period of being fed safflower oil (CN-3, Saff-3, CN-). Each point was calculated from the mean per cent content of linoleic acid of a group of animals at a given time after the change of diet, divided by the mean per cent found at zero time, e.g., the time just before the change of diet:

$$\log \frac{\text{lin}_t}{\text{lin}_0} = \left[\frac{\text{per cent of linoleic acid at time t}}{\text{per cent of linoleic acid at time zero}}\right] \times 100, \text{ plotted on semilog paper.}$$

Numbers at the right of the curves are the "half-times of depletion" of linoleic acid in the given tissues, e.g., the times at which the concentration of linoleic acid had fallen to half of the value observed at zero time.

evidence that this assumption is valid. Nevertheless, the data furnished by the disparate experiments mentioned here are of the same order of magnitude.

Interscapular fat does not constitute a large proportion of body mass, and possibly should be considered an active tissue rather than a storage depot. It is therefore not strange that the rate of depletion of linoleic acid in subcutaneous and mesenteric fats should be slower than in interscapular fat if a supply of linoleic acid is selectively conserved in the fat depots. Scrutiny of the variations in the proportions of the fatty acids in terms of "relative percentages" tempts the conclusion that the animal does not tend to maintain constant ratios of saturated to unsaturated fatty acids. This is shown by the fact that the percentage of oleic acid relative to linoleic increased with an increasing percentage of linoleic acid, whereas the "rela-

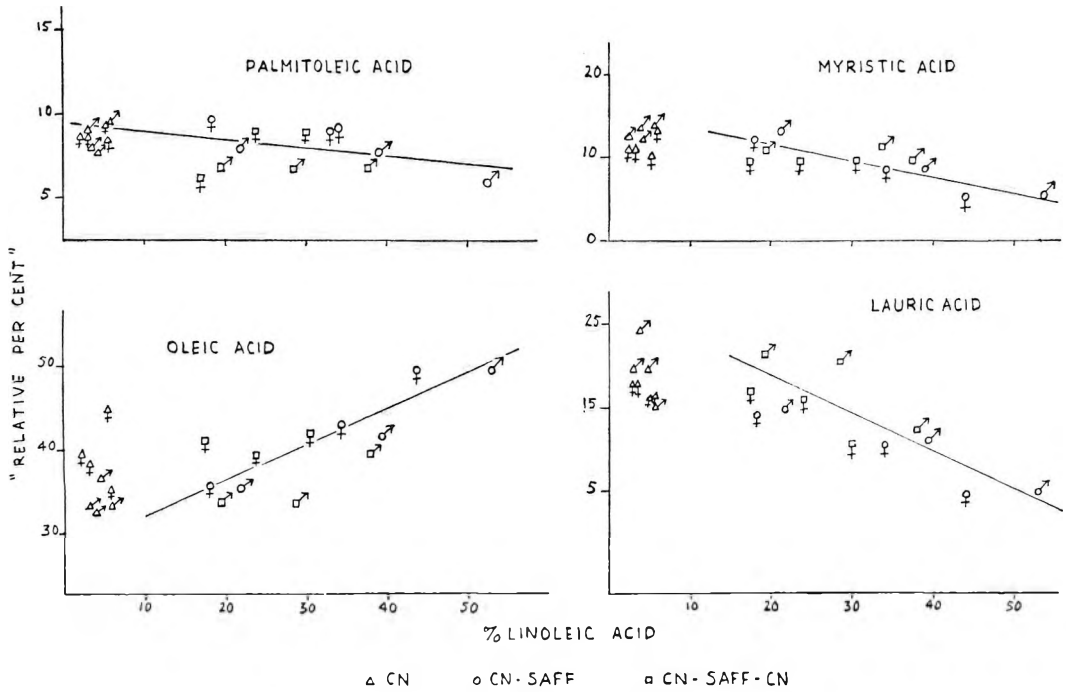


Fig. 7 Relative per cent of fatty acids in subcutaneous fat. "Relative per cent" of a fatty acid in respect to linoleic acid is $\frac{\text{per cent fatty acid}}{100 - \text{per cent linoleic acid}}$. The points represent values computed from the means of each group. The male and female groups are identified by the symbols ♂ and ♀, respectively. The shape of the symbols represents the diet groups as indicated on the figure (see legend to figure 2). The slopes of the regression lines calculated according to Snedecor ('57) had the following values: palmitoleic acid $b = -0.0298$; myristic acid $b = -0.0730$; oleic acid $b = +0.212$; lauric acid $b = -0.262$. The corresponding data for interscapular and mesenteric fats were similar to those shown here.

tive percentages" of the short-chain saturated fatty acids, lauric and myristic, were decreasing (fig. 7). The ratios of the sums of unsaturated to those of saturated fatty acids are shown in table 1. These ranged from 0.74 in the mesenteric fat of males fed coconut oil to 4.17 in the same tissue of males fed safflower oil for 9 weeks. This is in contrast with the nearly constant ratio observed in plasma lipids. Adipose

TABLE 1

*Ratio of the sum of unsaturated to the sum of saturated fatty acids*

| Diet[1] | Male rats | | | Female rats | | |
|---|---|---|---|---|---|---|
| | Fats | | | Fats | | |
| | Subcutaneous | Mesenteric | Interscapular | Subcutaneous | Mesenteric | Interscapular |
| CN-12 (17)[2] | 0.89 | 0.76 | 1.24 | 1.21 | 0.99 | 1.19 |
| CN-3, Saff-9 (16) | 3.79 | 4.17 | 2.54 | 3.22 | 3.39 | 2.22 |
| CN-3, Saff-3, CN-6 (16) | 1.09 | 0.74 | 1.27 | 1.37 | 0.98 | 1.16 |

[1] CN-12: Groups of animals fed coconut oil for 12 weeks. CN-3, Saff-9: Groups of animals fed coconut oil for three weeks followed by safflower oil for 9 weeks. CN-3, Saff-3, CN-6: Groups of animals fed coconut oil for three weeks followed by safflower oil for three weeks and returned to coconut oil for 6 weeks.

[2] Numbers in parentheses indicate number of animals in the group.

tissue appears to maintain its physical characteristics by other mechanisms.

The data in the present experiment indicate that the rat has the capacity for rather prompt adipose tissue storage of linoleic acid. It is probable that after a certain critical level of essential fatty acids is reached preferential storage may cease. Conversely, rate of depletion may decrease after another, lower critical level is reached. More probably, two regulatory mechanisms function at first, and one is displaced as the depletion progresses.

From previous observations, it was to be expected that there might be sex differences in the metabolic activity of adipose tissue. McKerns ('61), for instance, noted greater fatty acid synthesis in fat pads from female rats than in those from male rats. Tove and Smith ('59) reported a faster depletion of linoleate from female than from male adipose tissue, and larger regression coefficients for some of the other fatty acids in the female tissues than for those in the corresponding male tissues. Our data show shorter half-times of replacement of linoleate for adipose tissues from females than for those from males. Generally, the data indicate a faster equilibration of fatty acids in adipose tissue of females than in that of males in response to changes in the diet. The discontinuity of the curves for the concentration of fatty acids of coconut oil-fed females (fig. 4) is largely unexplained. As mentioned previously, it cannot be decided whether the animals fed safflower oil showed a similar break. Due to insufficient numbers of experimental points, the exact location of this break is unknown. The change occurred in females that had been fed the diet between 7 and 11 weeks, that is those between 10 and 14 weeks old. A possible explanation of the phenomenon might be a change of hormonal balance associated with cessation of rapid growth.

## SUMMARY

Changes in the fatty acid composition of three selected adipose tissues (subcutaneous, mesenteric and interscapular fat) of rats in response to changes of the dietary fat were determined. Safflower oil, containing about 75% of linoleic acid, and coconut oil, furnishing some 68% of lauric and myristic and less than 3% of linoleic acids, were fed as 10% of adequate semisynthetic diets. Three groups of rats were compared: those fed coconut oil from weaning age, those fed safflower oil after an initial three-week period of coconut oil, and those changed back to coconut oil after a period of safflower oil feeding. Groups of animals were killed, and the tissues were analyzed at intervals of one, three, 6 and 9 weeks after change of diet.

Changes in the dietary fat produced changes in the composition of the adipose tissue within one week or less. Most changes observed were in the direction that might have been predicted from the composition of the dietary fat. Some variations from this pattern were evidently due to variations in age, sex and growth rate of the experimental animals.

Generally, the changes were qualitatively similar in all three tissues observed. The interscapular fat had higher levels of nitrogen and lower levels of fatty acids than did the other two tissues, and its fat content varied less.

Equilibration of the composition of the adipose tissue with that of the dietary fat occurred earlier in female rats than in males. Of the three tissue fats, the interscapular reached an equilibrium level earliest, followed by mesenteric and finally by subcutaneous fat. The half-time of replacement for linoleic acid ranged from 11 to 53 days, depending on the sex of the animals and the tissue in question. Lauric and myristic acids had shorter half-times of replacement and were accumulated in lower proportions in relation to their intake.

The most striking difference between male and female rats was a decrease in the total fatty acid content, which occurred only in females at 10 to 14 weeks of age. The decrease was accompanied by an increase in water content.

Since the observed ratio of unsaturated to saturated fatty acids was not constant, it is possible that the animal tends instead to maintain adipose tissue of constant physical properties.

The data support the hypothesis that the rat can store dietary linoleic acid in its adipose tissue during a period of high intake for slow release when the dietary supply is low.

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# Changes in Tissue Lipids in Response to Diet

## II. FATTY ACID COMPOSITION OF LIVER AND PLASMA LIPIDS IN RELATION TO FED AND STORED FAT[1]

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The concept that adipose tissue constituents serve as relatively inert stores of nutrients, that those of the liver are the ready supplies for immediate use, and that blood serves only as a carrier, has been greatly modified as a result of the demonstration of rapid turnover of some of those constituents (Jeanrenaud, '61). Too little is known, however, of the effect of diet on the equilibriums between stored and circulating lipids. This is evident from recent sweeping conclusions concerning lipid metabolism drawn from serum lipid data without regard to accompanying changes in tissue lipids. It seemed desirable, therefore, to study the changes in composition of the different lipid fractions of liver and plasma in relation to changes in fatty acid content of the adipose tissue of animals at different intervals after changes in the composition of dietary fat.

The adipose tissue data are reported in the preceding paper (Ostwald et al., '62). When young rats were shifted from a linoleate-poor to a linoleate-rich diet, and later back to the linoleate-poor diet, there was some evidence of preferential storage of linoleic acid in mesenteric and subcutaneous fats. Lauric and myristic acids (from dietary coconut oil) had shorter half-times of replacement than did linoleic, and were stored in smaller proportions in relation to intake. Arachidonic acid was noted only in trace amounts in adipose tissues.

### METHODS

Details of the experimental plan are given in the preceding paper (Ostwald et al., '62). All diets contained 10% of fat and 1% of cholesterol. Coconut oil (CN) was fed to all animals for the first three weeks after weaning, and the first groups were killed one week after part of them had been transferred to the safflower oil diet (Saff), i.e., the 4-week groups were fed coconut oil for three weeks then safflower oil (CN-Saff 3-1, table 1) for one week, as contrasted with those fed coconut oil (CN-4) for 4 weeks.

All rats were killed 15 to 18 hours after removal of food cups from the cages. Liver and plasma lipid extracts were prepared, fractionated on silicic acid columns, and analyzed by methods described in previous papers (Lis et al., '61; Lis and Okey, '61; and Okey et al., '61).[2]

### RESULTS

Changes in body and liver weights and in liver and plasma lipids are summarized in table 1. Comparisons should be made between animals of the same ages. Cholesterol ester levels of both liver and plasma differed with sex. Males fed the CN diet had plasma levels of cholesterol ester which averaged approximately 10 mg per 100 ml higher than those of the males fed the Saff diet. This was not true of females. Indeed, a large proportion of the females that continued to receive the Saff diet for 9 weeks had very high (mean 129 ± 26 mg per 100 ml) plasma cholesterol ester values. Both males and females of

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[2] When the size of the fraction permitted, determinations of cholesterol, of total fatty acid, and of lipid phosphorus were made in addition to the gas liquid chromatography of the methylated fatty acids. Data were used to compute percentages of the individual fatty acids to "absolute" values, i.e., milligrams per gram of tissue. An internal standard (a saturated $C^{17}$ fatty acid) was used for the same purpose for some of the later analyses (Tinoco et al., unpublished data). See footnote on chromatographic measurements in previous paper (Ostwald et al., '62).

TABLE 1

*Composition of liver and plasma*

| Diets, weeks fed | No. rats | Body weight | Livers | | | | | | Plasma | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Weight | Moisture | Total lipid | Cholesterol | | Phospholipid | Cholesterol | |
| | | | | | | As ester | Free | | As ester | Free |
| | | *gm* | *gm* | % | | *% moist weight* | | | *mg/100 ml* | |
| **Males** | | | | | | | | | | |
| **CN** | | | | | | | | | | |
| 4 | 5 | 198 | 7.6 ± 0.5 | 63.5 | 11.9 ± 1.3 | 1.70 ± 0.37 | 0.27 ± 0.01 | 3.8 ± 0.1 | 53 ± 4 | 12 ± 1 |
| 6 | 5 | 275 | 9.8 ± 0.4 | 63.3 | 13.0 ± 1.2 | 1.94 ± 0.26 | 0.27 ± 0.02 | 3.8 ± 0.1 | 40 ± 4 | 10 ± 2 |
| 9 | 8 | 354 | 11.9 ± 0.4 | 64.2 | 9.3 ± 0.8 | 1.27 ± 0.17 | 0.27 ± 0.01 | 4.3 ± 0.1 | 45 ± 4 | 12 ± 1 |
| 12 | 8 | 408 | 12.6 ± 0.5 | 64.1 | 9.2 ± 1.1 | 1.38 ± 0.26 | 0.28 ± 0.01 | 3.9 ± 0.1 | 46 ± 3 | 15 ± 1 |
| **CN-Saff** | | | | | | | | | | |
| 3-1 | 8 | 215 | 7.8 ± 0.3 | 62.2 | 13.1 ± 1.6 | 2.28 ± 0.24 | 0.30 ± 0.01 | 3.7 ± 0.1 | 36 ± 3 | 8 ± 1 |
| 3-3 | 9 | 266 | 9.7 ± 0.5 | 58.3 | 16.9 ± 1.3 | 2.96 ± 0.30 | 0.31 ± 0.01 | 3.9 ± 0.1 | 29 ± 2 | 7 ± 1 |
| 3-9 | 10 | 411 | 13.7 ± 0.5 | 56.4 | 19.1 ± 1.8 | 4.29 ± 0.57 | 0.32 ± 0.01 | 3.5 ± 0.1 | 37 ± 3 | 11 ± 1 |
| **CN-Saff-CN** | | | | | | | | | | |
| 3-3-1 | 7 | 313 | 12.6 ± 0.4 | 58.2 | 16.0 ± 1.2 | 2.34 ± 0.20 | 0.27 ± 0.01 | 3.7 ± 0.1 | 38 ± 3 | 11 ± 1 |
| 3-3-3 | 8 | 355 | 15.5 ± 1.4 | 53.9 | 21.1 ± 2.9 | 3.50 ± 0.41 | 0.31 ± 0.01 | 3.5 ± 0.2 | 32 ± 3 | 8 ± 1 |
| 3-3-6 | 7 | 414 | 12.3 ± 0.8 | 61.1 | 13.8 ± 0.6 | 2.20 ± 0.31 | 0.27 ± 0.01 | 3.9 ± 0.2 | 38 ± 5 | 10 ± 1 |
| **Females** | | | | | | | | | | |
| **CN** | | | | | | | | | | |
| 4 | 5 | 164 | 5.8 ± 0.4 | 68.7 | 7.1 ± 0.6 | 0.95 ± 0.27 | 0.29 ± 0.02 | 4.0 ± 0.2 | 43 ± 4 | 10 ± 1 |
| 6 | 5 | 205 | 6.5 ± 0.9 | 67.1 | 8.1 ± 0.8 | 1.17 ± 0.26 | 0.32 ± 0.03 | 4.2 ± 0.1 | 55 ± 5 | 9 ± 1 |
| 9 | 8 | 256 | 7.4 ± 0.2 | 66.8 | 7.6 ± 0.5 | 0.97 ± 0.20 | 0.30 ± 0.01 | 4.4 ± 0.2 | 64 ± 9 | 12 ± 1 |
| 12 | 8 | 264 | 7.1 ± 0.4 | 67.2 | 7.0 ± 0.3 | 0.85 ± 0.18 | 0.29 ± 0.01 | 3.9 ± 0.1 | 64 ± 17 | 12 ± 2 |
| **CN-Saff** | | | | | | | | | | |
| 3-1 | 10 | 162 | 5.7 ± 0.1 | 64.6 | 11.0 ± 0.5 | 1.80 ± 0.13 | 0.30 ± 0.01 | 3.8 ± 0.1 | 35 ± 3 | 7 ± 1 |
| 3-3 | 10 | 210 | 6.7 ± 0.2 | 61.9 | 12.7 ± 0.9 | 2.86 ± 0.28 | 0.33 ± 0.01 | 3.8 ± 0.2 | 60 ± 7 | 8 ± 1 |
| 3-9 | 5 | 286 | 8.2 ± 0.2 | 62.8 | 11.3 ± 0.8 | 3.16 ± 0.38 | 0.33 ± 0.01 | 3.9 ± 0.1 | 129 ± 26 | 13 ± 3 |
| **CN-Saff-CN** | | | | | | | | | | |
| 3-3-1 | 8 | 227 | 7.0 ± 0.2 | 62.8 | 9.2 ± 0.7 | 1.55 ± 0.22 | 0.31 ± 0.01 | 4.2 ± 0.1 | 64 ± 9 | 11 ± 1 |
| 3-3-3 | 8 | 246 | 7.2 ± 0.3 | 66.0 | 8.5 ± 0.4 | 1.39 ± 0.20 | 0.30 ± 0.01 | 3.9 ± 0.1 | 72 ± 15 | 13 ± 1 |
| 3-3-6 | 7 | 256 | 7.4 ± 0.1 | 65.8 | 9.3 ± 0.6 | 1.56 ± 0.27 | 0.30 ± 0.01 | 3.9 ± 0.1 | 52 ± 7 | 11 ± 1 |

the CN-Saff groups had higher liver cholesterol ester values than those for the groups continuing to receive coconut oil. Females, however, tended to have lower liver cholesterol ester values than males of the same age and diet groups. Sex differences in percentage of liver esters were statistically significant for the following groups only: CN 6 weeks and CN-Saff 3-3 weeks ($P < 0.02$); CN 12 weeks and CN-Saff 3–9 weeks ($P < 0.05$). The livers of females were not as large as those of males; hence the total amounts of cholesterol ester were much smaller.

In general, variations in total cholesterol values for both liver and plasma were accounted for by variations in the esterified fraction. There was, however, a tendency toward slightly higher values for "free" cholesterol in the plasma of the CN-fed than in the plasma of the Saff-fed rats.

Glyceride fatty acids of liver, in males only, showed a transient increase following return to the CN diet after safflower oil feeding. Otherwise, liver glyceride fatty acid values for the CN-Saff rats tended to be higher than for the CN and CN-Saff-CN groups. Samples were too small to permit determination of total glyceride fatty acids in most of the plasma extracts.

Phospholipid phosphorus values for liver extracts varied within rather narrow ranges (table 1). For reasons given later, the total phospholipid values for plasma are not reported.

*Fatty acid moieties of the plasma and liver lipids.* Esterified cholesterol accounted for about four-fifths of the total plasma cholesterol in the rats that had values within normal ranges, and for a much higher percentage in the CN-Saff 3–9 week females with the very high plasma cholesterol values. With cholesterol in the diet, at least nine-tenths of the liver cholesterol was esterified. Changes in the ester fatty acids in relation to concentration of cholesterol in liver and plasma are, therefore, interesting in relation to possible function in transport and storage of cholesterol.

*Arachidonic acid* (fig. 1) made up a substantial percentage of the ester fatty acid in plasma, a smaller one in liver in all rats. In neither was the percentage as high as that observed in rats fed no cholesterol (Okey et al., '61). Females tended to have higher plasma arachidonic acid values than males when fed the coconut oil diet, and responded to the return to coconut oil after safflower feeding with a larger and longer sustained increase in plasma arachidonic acid. Percentages of plasma ester arachidonic acid were maintained and amounts increased in the animals that responded to continued feeding of safflower oil with high plasma cholesterol values. Percentages of ester arachidonic acid in the livers of both sexes were so small as to make for considerable analytical error. Liver storage of arachidonic acid was usually increased in males at the time that females responded to the change from safflower oil to coconut oil by an increase in proportion and amount of plasma ester arachidonic acid.

*Linoleic acid* (fig. 1) of plasma cholesterol ester, expressed as weight percentage of the ester fatty acid, was two to three times as high one week after change to safflower oil as it was in the controls continuing to be fed coconut oil. Percentages and amounts of linoleic acid in plasma cholesterol ester continued to increase in females continuing to be fed safflower oil. In males the increase (expressed as mg per 100 ml) was less marked because of the lower cholesterol ester values for the animals fed safflower oil. The decrease in ester linoleic acid following return to coconut oil was less abrupt in males than in females. Linoleic acid of liver cholesterol ester reflected changes in diet even more quickly than did that of plasma ester (fig. 2). *Percentages* of ester linoleic acid in livers of rats returned to the coconut oil diet after safflower oil feeding did not quite reach those of control levels in 6 weeks. *Amounts* more nearly approached control level because of the decrease in total amount of cholesterol ester following return to the CN diet. In males only, there were some indications that linoleic acid was being selectively retained in the liver during the first week after withdrawal of the safflower oil diet.

Percentages of *ester monoenoic acid* (figs. 1, 2) tended to increase in plasma and liver as the percentages of more highly

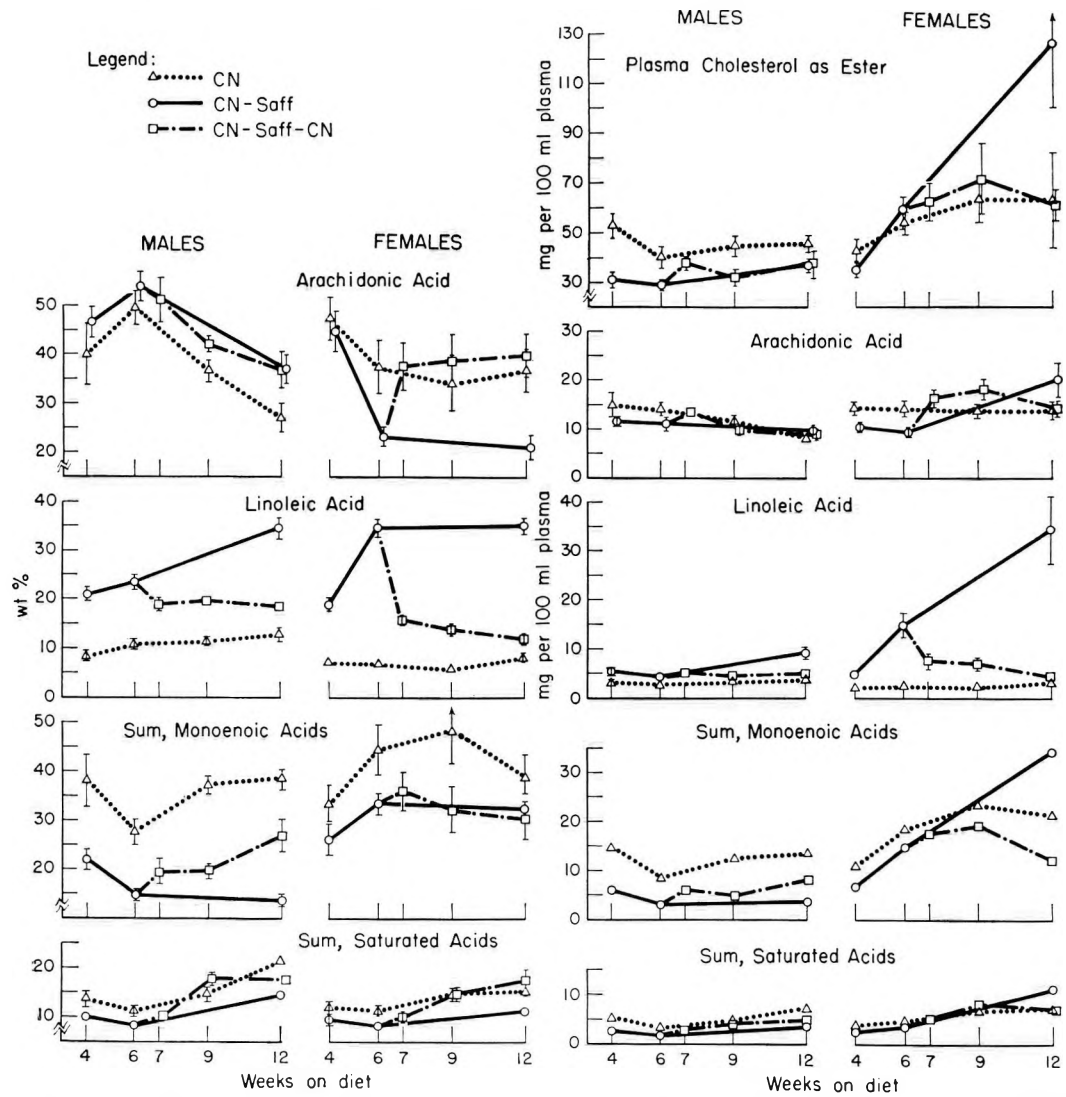


Fig. 1 Fatty acids of plasma cholesterol esters. "Weight percentages" represent the corrected chromatographic data (footnote 3) for the specified fatty acids expressed as percentages of the total fatty acid in the cholesterol ester fraction separated by the silicic acid column. The "mg per 100 ml" shown in the right hand columns were computed by applying these percentages to the total cholesterol ester fatty acid figures obtained by chemical analysis. All animals were fed the coconut oil diet for the first three weeks after weaning. First autopsies took place one week thereafter. The CN-Saff data represent the means for rats fed the coconut oil diet for three weeks, and the safflower oil diet thereafter; the CN-Saff-CN data represent means for rats fed the coconut oil diet for three weeks, then the safflower oil diet for three weeks, and the coconut oil diet thereafter. Standard errors of means are shown by vertical lines when they exceed the size of the diet symbols.

unsaturated acids decreased. Amounts of monoenoic acid were high in the plasma of female rats with high cholesterol values. In the livers of males transferred from safflower to coconut oil there was a transient, but very large, increase in the amount of ester monoenoic acid, which reflected an increase in liver weight rather than an increase in percentage of oleic and palmitoleic acids.

*Saturated acids of cholesterol ester* showed the same general trends of varia-

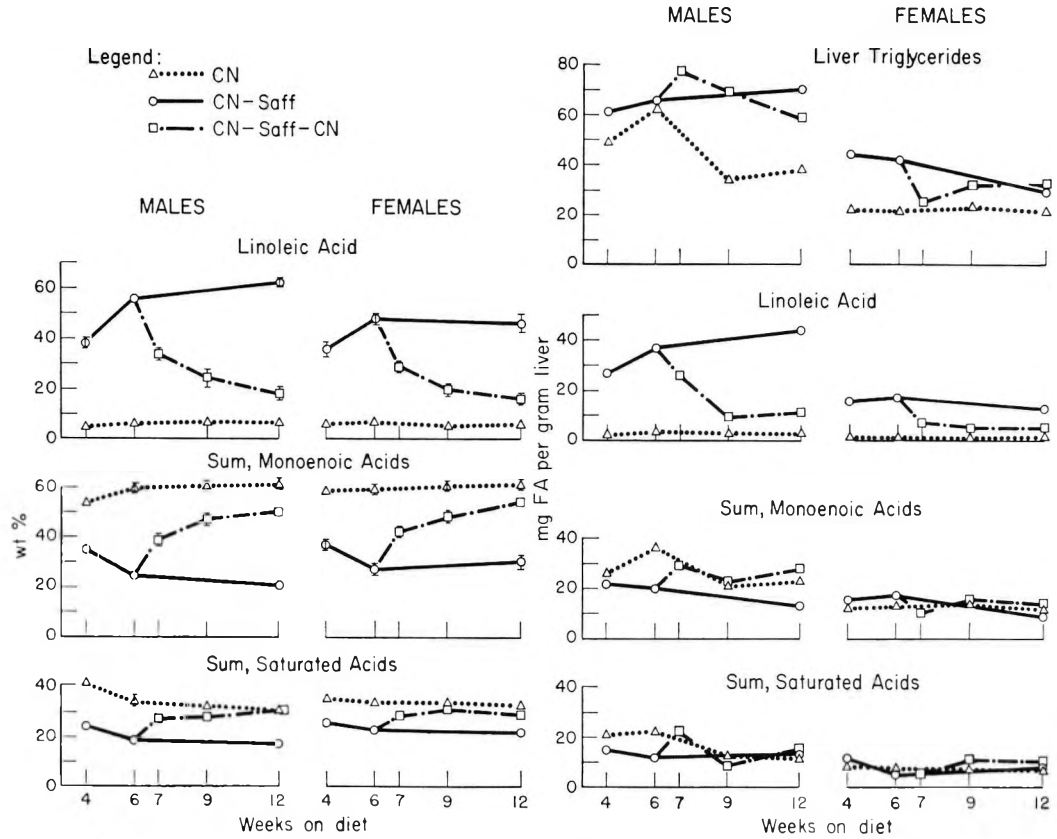


Fig. 2 Fatty acids of liver cholesterol esters. Data are arranged as in figure 1.

tion with diet as did the monoenoic. Differences were due primarily to changes in palmitic and stearic acid. There was never more than 1% of myristic or lauric acid in plasma ester fatty acid, or about 5% in the liver ester fatty acid.

*Triglyceride fatty acids* (figs. 3, 4). Concentration of total triglyceride fatty acids was higher in the livers of male rats than in those of female rats of all groups. Because of the small size of the plasma samples obtainable from the 4- and 6-week groups, the entire samples were used for gas liquid chromatography, and accurate data for total plasma triglyceride fatty acids were not obtained. Variations in the proportions of the different triglyceride fatty acids were rapid during the early period following a change in the composition of the dietary fat, and became increasingly slower thereafter. Proportions of *arachidonic* acid in liver and plasma triglycerides were higher than the trace amounts observed in adipose tissue. In males, the highest values were observed in animals fed safflower oil (7 to 15% in plasma triglycerides and 2 to 4% in liver triglyceride). In females the highest values were observed three weeks after return to the coconut oil diet, following the feeding of safflower oil (12% for plasma and 8% for liver). Although determination of small proportions of the acid by gas liquid chromatography is not accurate enough to justify statistical analysis, the data at least suggest differences in function between adipose tissue and liver triglycerides.

Liver triglyceride *linoleic acid* variations with diet closely resembled those observed in linoleic acid of cholesterol ester. Proportions of lauric and myristic acids reached 5% of the plasma triglyceride fatty acid and 9% of the liver triglyceride fatty acid of the rats fed coconut oil. These percentages were, however, never more than a third as high as in the adipose

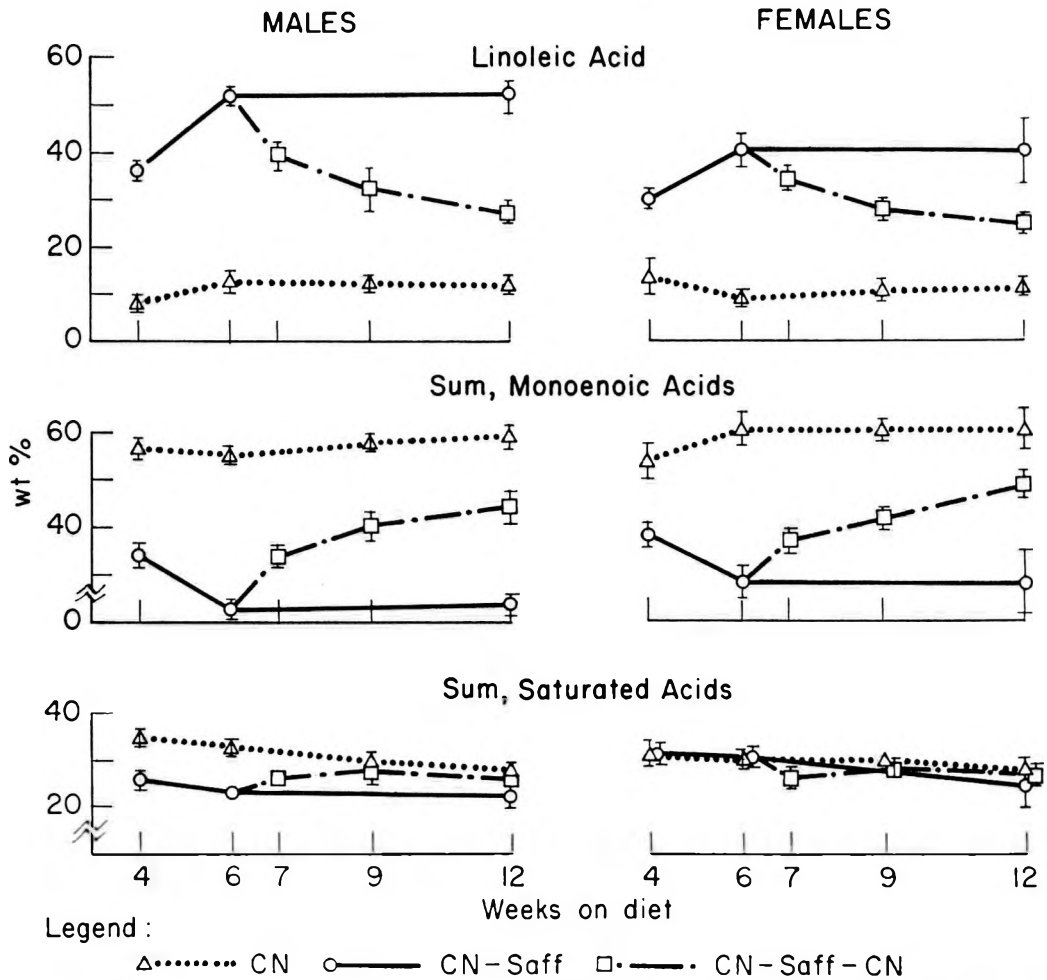


Fig. 3 Fatty acids of plasma triglycerides. Samples were too small to permit chemical analyses of the total triglyceride fatty acids; therefore only the chromatographic data are given.

tissues of the same rats. The effect of feeding coconut oil was still evident three weeks after the change to the safflower oil diet. The transient increases in the total amounts of liver triglyceride fatty acid (fig. 4) shown when male rats were transferred back to coconut oil after safflower oil were largely accounted for as monoenoic and saturated acid.

*Fatty acids of fraction III* (mono- and diglycerides, free fatty acids). This fraction was very small, proportionately, both in plasma and in liver. Absolute amounts were not determined in plasma. In liver, the fatty acids of this fraction constituted about 1 to 2 mg per gm wet weight of the liver. Distribution of the fatty acids following changes in dietary fat showed much the same pattern as in the triglyceride fraction. Saturated acids, including myristic and stearic, were present in somewhat greater proportion than in the triglyceride fraction, and oleic and linoleic acids were in smaller proportions. Arachidonic acid also was present in slightly higher percentages than in the triglycerides.

*Phospholipid fatty acids* (table 2). Changes in the liver and plasma phospholipids differed markedly from those of the other lipid fractions. This might possibly have been expected from the differences in the proportions of certain fatty acids in all phospholipids. Although the

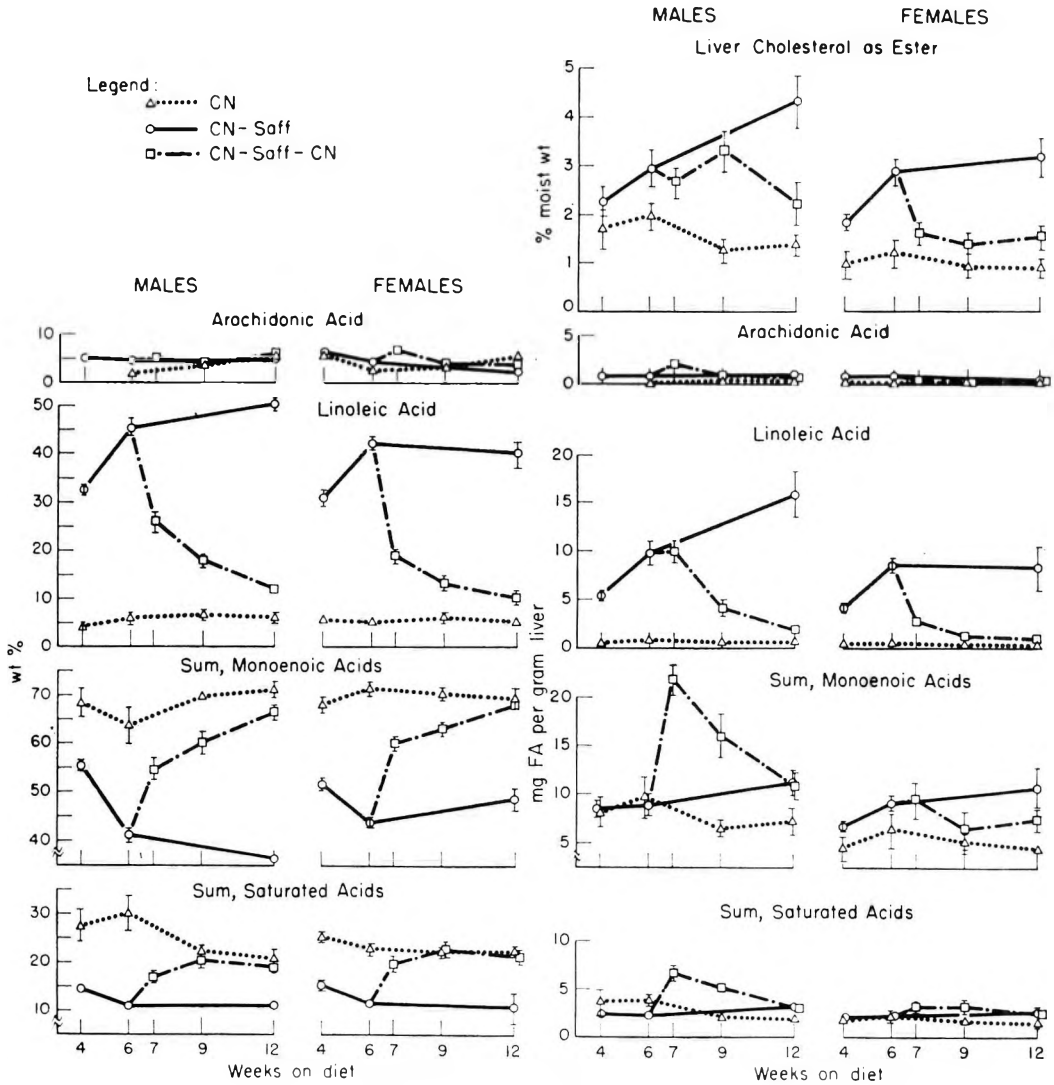


Fig. 4 Fatty acids of liver triglycerides. Data are arranged as in figure 1.

alcohol-ether-soluble phosphorus of liver was 95% soluble in petroleum ether, solubility of the plasma extracts varied greatly. The insoluble fraction was largest in the plasma extracts of rats fed safflower oil. Fatty acids of this insoluble fraction were largely saturated and monoenoic. Evidence indicated that the proportion of petroleum ether-insoluble phospholipid might vary with small changes in the opportunity for oxidation of polyunsaturated fatty acids which might occur inadvertently during extraction and transfer of the lipid extracts, thus making these data of doubtful significance. Plasma phospholipid fatty acids are therefore reported for the petroleum ether-soluble fraction only. Data for liver, in contrast, represent nearly all the phospholipid originally extracted.

Phospholipid of liver was present in the largest amounts in the animals killed 9 weeks after weaning, namely, when values for triglyceride and cholesterol ester tended to decrease (Okey and Lyman, '56). Proportions of arachidonic acid were about 10% higher in the liver phospho-

TABLE 2

*Fraction IV: phospholipid fatty acids (as weight per cent of fraction)*

| Diet | Coconut oil | | | | Coconut — safflower | | | Coconut — safflower — coconut | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Weeks | 4 | 6 | 9 | 12 | 3-1 | 3-3 | 3-9 | 3-3-1 | 3-3-3 | 3-3-6 |
| Livers | | | | | | | | | | |
| Males | | | | | | | | | | |
| 12:0[1] | | 0.1 | 0.2 | 0.3 | tr | | tr | | tr | |
| 14:0 | 0.8 | 0.8 | 1.3 | 1.0 | 0.7 | 0.4 | 1.4 | 0.7 | 0.8 | 1.0 |
| 16:0 | 16.3 | 16.2 | 15.4 | 15.7 | 16.3 | 14.8 | 15.4 | 16.6 | 16.9 ± 1.2 | 15.4 |
| 16:1 | 2.8 | 2.6 | 3.2 | 2.9 | 1.5 | 1.5 | 1.3 | 2.1 | 2.7 | 2.2 |
| 18:0 | 23.9 ± 1.2[2] | 22.0 ± 2.0 | 20.6 | 20.0 | 22.0 | 21.3 | 19.1 ± 2.0 | 21.0 | 21.0 | 19.0 |
| 18:1 | 12.5 | 11.7 ± 1.2 | 13.7 | 14.7 | 8.2 | 6.9 | 7.5 | 8.5 | 10.9 | 12.2 |
| 18:2 | 9.1 | 8.9 ± 1.1 | 11.4 | 11.8 | 15.6 | 17.7 | 18.6 | 15.3 | 13.4 | 13.4 |
| 20:4 | 34.6 | 38.0 ± 2.3 | 34.4 | 33.8 | 35.8 | 37.2 | 37.9 ± 1.3 | 35.7 | 34.5 ± 1.4 | 36.8 |
| Females | | | | | | | | | | |
| 12:0 | | 0.1 | 0.1 | 0.2 | 0.2 | | 0.1 | tr | 0.1 | 0.1 |
| 14:0 | 1.3 | 0.6 | 0.7 | 0.5 | 0.6 | 0.3 | 0.8 | 0.3 | 0.6 | 0.5 |
| 16:0 | 14.8 ± 1.1 | 12.1 | 12.6 | 12.1 | 14.2 | 12.5 | 12.8 | 12.5 | 11.8 | 12.9 |
| 16:1 | 2.1 | 1.8 | 2.1 | 1.8 | 1.1 | 0.9 | 1.1 | 1.1 | 1.7 | 1.9 |
| 18:0 | 28.1 | 26.6 | 27.7 | 26.1 | 27.4 ± 1.1 | 27.1 | 26.1 ± 1.2 | 27.8 | 26.1 ± 1.1 | 24.2 |
| 18:1 | 11.6 | 11.5 | 11.5 | 10.9 | 6.9 | 6.0 | 7.3 | 7.6 | 8.7 | 9.8 |
| 18:2 | 7.2 | 8.4 | 7.5 | 7.4 | 13.8 | 14.7 | 17.3 | 12.3 | 10.0 | 10.5 |
| 20:4 | 34.9 ± 2.2 | 39.1 | 38.0 | 41.1 | 36.1 | 38.9 | 35.2 ± 1.2 | 38.6 | 41.1 | 40.2 |
| Plasma (petroleum ether-soluble) | | | | | | | | | | |
| Males | | | | | | | | | | |
| 12:0 | | | 0.6 | 0.6 | | | | | | 0.4 |
| 14:0 | 1.1 | 1.1 | 0.9 | 2.2 | 1.6 | 0.3 | 0.6 | 0.8 | tr | 1.5 |
| 16:0 | 20.5 | 21.3 | 20.1 | 21.8 | 24.8 ± 1.2 | 24.8 | 22.5 | 23.8 | 23.2 ± 3.7 | 21.5 |
| 16:1 | 2.8 | 4.6 ± 1.4 | 4.5 | 4.6 | 2.3 | 2.8 | 2.1 | 2.0 | 2.6 | 3.6 |
| 18:0 | 20.2 ± 1.7 | 19.5 ± 1.7 | 15.2 ± 1.4 | 13.3 | 16.3 ± 1.2 | 17.3 | 13.9 | 18.3 | 16.5 ± 2.0 | 12.9 |
| 18:1 | 19.9 ± 1.9 | 16.6 ± 3.7 | 19.7 ± 1.5 | 23.5 | 10.7 ± 1.1 | 9.2 ± 1.7 | 9.2 | 8.9 | 12.0 ± 1.6 | 18.5 |
| 18:2 | 13.6 | 10.0 ± 2.2 | 13.0 | 13.3 | 18.8 | 19.8 ± 1.2 | 26.0 ± 1.7 | 15.5 | 16.7 ± 2.7 | 20.4 |
| 20:4 | 22.7 | 28.0 ± 5.7 | 25.7 ± 1.6 | 21.1 ± 1.4 | 26.8 ± 2.0 | 25.4 ± 1.4 | 26.6 ± 1.5 | 30.9 ± 2.3 | 28.8 ± 2.6 | 21.3 ± 1.3 |
| Females | | | | | | | | | | |
| 12:0 | tr | | | | | | | | 0.3 | |
| 14:0 | 1.2 | 0.5 | 0.9 | 0.9 | 0.7 | 0.7 | 0.6 | 0.8 | 0.6 | 0.7 |
| 16:0 | 17.0 | 16.8 | 14.8 | 15.9 | 18.9 ± 1.3 | 19.9 ± 1.5 | 14.5 | 15.5 | 14.7 | 16.5 |
| 16:1 | 3.5 | 1.5 | 3.1 | 2.9 | 2.7 | 1.4 | 2.9 ± 2.0 | 1.8 | 2.6 | 2.1 |
| 18:0 | 23.8 ± 1.5 | 27.8 | 23.5 ± 1.4 | 22.9 | 21.7 ± 1.1 | 27.2 ± 1.7 | 22.9 ± 1.4 | 25.5 ± 1.3 | 23.5 ± 1.9 | 22.5 ± 1.3 |
| 18:1 | 14.4 ± 1.5 | 12.5 | 18.4 ± 1.4 | 18.3 ± 1.6 | 11.3 | 6.3 ± 1.3 | 8.7 ± 1.1 | 9.3 | 11.4 | 11.3 ± 1.1 |
| 18:2 | 10.3 | 6.3 | 8.9 | 8.4 | 16.6 ± 1.3 | 14.2 | 15.1 ± 1.7 | 11.8 | 12.4 | 11.7 |
| 20:4 | 30.2 ± 3.3 | 34.3 ± 1.4 | 30.9 ± 1.6 | 31.3 ± 2.0 | 29.5 ± 1.6 | 30.7 ± 2.7 | 35.5 ± 2.0 | 35.3 | 34.8 ± 2.1 | 35.7 ± 1.6 |

[1] The first figure represents the number of carbon atoms and the second, the number of double bonds.
[2] Standard errors of the means are included only when they exceeded 1%. They were calculated for all except the C12 acids.

lipids than in the plasma phospholipids of males; differences were smaller for females, other than the 12-week CN controls. (Ranges: 21 to 31% for plasma, and 34 to 38% for livers of males; 30 to 36 % for plasma and 35 to 41% for livers of females.)

Thirty-five to 50% of the phospholipid fatty acid was saturated, both in plasma and in liver. The older animals showed a sex difference in the chain length of the saturated acids; stearic was present in the larger proportion in the phospholipids of females, and palmitic in males. For liver phospholipids, some of the differences in stearic were great enough to be significant: CN diet, 9 weeks ($P < 0.01$); CN-Saff-CN 3–3–1 weeks and CN-Saff-CN 3–3–6 weeks ($P < 0.02$). Differences in proportions of plasma phospholipid fatty acids were smaller, but variations were in the same direction. It is possible that these differences indicate different proportions of individual phospholipids. Marcus et al. ('61) recently reported higher percentages of stearic and arachidonic acids in the phosphatidyl serine and ethanolamine fractions of human platelets, together with higher palmitic and oleic values for the lecithin and sphingomyelin fractions. Getz et al. ('61) showed similar observations for rat livers.

Phospholipid linoleic acid percentages were increased and those of oleic acid decreased by feeding of safflower oil, but both changes were smaller than those for the other lipid fractions, in liver as well as in plasma. Apart from these changes in proportions of linoleic and oleic acids with diet, it seems quite probable that the other changes noted for phospholipid fatty acids may be attributed to the effects of age, sex, and rate of growth of the experimental animals.

## DISCUSSION

The outstanding difference between plasma and adipose tissue lipids was that, whereas the latter consisted chiefly of triglyceride and free fatty acid, a high proportion of the plasma lipid was made up of cholesterol ester and phospholipid. Although the high proportions of cholesterol ester in the livers were to be attributed to cholesterol feeding (Okey et al., '61), the extent to which the feeding of linoleate increased liver cholesterol ester levels was worthy of note. Liver phospholipid was maintained within a range of 3.5 to 4.4% regardless of diet, but there was some indication that the higher percentages of liver phospholipid were to be expected at the times when liver cholesterol ester percentages were decreasing. This was true whether the drop in cholesterol ester was to be attributed to age of the animal or to change in diet.

The proportions of unsaturated fatty acid in the phospholipid and cholesterol ester of liver and plasma were high, and seemed to be altered somewhat less readily by changes in the composition of the dietary fat than was the case with adipose tissue triglycerides. Moreover, there was less tendency for the "unusual" short-chain fatty acids, such as lauric and myristic, to accumulate in plasma and liver lipids than in adipose tissue, even when the amounts of these acids supplied by the diet were as great as for the coconut oil-fed rats. This observation may be taken to indicate that liver lipid represents a selected store of constituents, ready for circulation in plasma. That the liver may also serve to remove excess cholesterol from the bloodstream is indicated by the large increases in liver cholesterol ester incidental to cholesterol feeding. This effect was found to be augmented by feeding linoleate and was concomitant with increases in the linoleic acid content of the liver lipids as well as with the accumulation of linoleate in adipose tissue.

The sex differences in accumulation of cholesterol and other lipids in liver might be linked with the greater capacity for liver enlargement manifested by the cholesterol-fed male. However, the possibility that the high plasma cholesterol values of the female Saff-fed rats was due to a direct effect of ovarian hormones was suggested by a previous experiment in which estradiol dosage had resulted in increases in the circulating cholesterol of male castrate rats (Okey and Lyman, '56).

Sex differences in the distribution of fatty acids in liver and plasma lipids were observed in the present experiment. Mean

percentages of stearic acid were greater in the plasma cholesterol ester of the female rats, whereas palmitic acid percentages were greater in the ester of males. Phospholipid fatty acids likewise varied with sex of the rats as noted earlier.

A sex difference in the facility with which arachidonic acid was synthesized from linoleic acid was also suggested by the data. Males maintained stationary or only slightly increased proportions of plasma ester arachidonic acid with prolonged feeding of safflower oil. At the same time, their plasma cholesterol levels were not elevated. But females responded to prolonged feeding of safflower oil with decreased proportions of plasma ester arachidonic acid, although with such high levels of plasma cholesterol ester that the actual amount of ester arachidonic acid was increased (fig. 2). The immediate response of the female rats transferred back from the safflower to the coconut oil diet was a very sharp rise both in proportion and amount of plasma ester arachidonic acid. This coincided in time with sexual maturity, with a decrease in liver cholesterol and triglyceride, and with a decrease in the proportion of linoleic acid in the adipose tissue stores which was considerably steeper in females than in males.

The data as a whole suggest that, when the supply of dietary linoleic acid is limited, the female rat has a greater facility for maintenance of plasma ester arachidonic acid supply than the male. But, under the double stress imposed by a diet high in cholesterol and rich in a fat that facilitated cholesterol absorption, dietary linoleate failed to prevent an increase in plasma cholesterol in females as it did in males.

Numerous tests were tried for correlation between times of change in the composition of the dietary fat and changes in the fatty acid constituents of plasma, liver, and adipose tissue. None of them gave entirely satisfactory results, but the following deserve mention.

Tove's postulation (Tove and Smith, '59), that when linoleic acid intake is cut off its content in tissue decreases rapidly until a critical level is reached, after which it is conserved, was tested. It was found to be more nearly in agreement with our data for adipose tissue than with those for liver and plasma lipids. One reason, apparently, was the sharp rise in plasma arachidonic acid of females during the first week following the change from the Saff to the CN diet. If, instead of subtracting the percentages of linoleic acid from the total cholesterol ester fatty acids, we subtracted percentages of arachidonic acid and then carried through Tove's plotting of plasma cholesterol ester linoleic acid against the total minus arachidonic, the distribution of the resulting data indicated some positive correlation between percentage of linoleic acid and of the total minus arachidonic acid for the males of the CN-Saff-CN 3–3–6 and the CN-Saff 3–9 week groups. Data for females of the same groups suggested negative correlations between changes in arachidonic acid and relative percentages of linoleic acid. Scatter of the data for the remaining groups made calculation of regression slopes of doubtful significance. Means for each of the groups were, however, treated individually and slopes of regression lines were calculated as noted above. Values were $+0.75$ for males and $-0.97$ for females. Although it is doubtful whether such a test constitutes valid evidence of differences in the kinetics of fatty acid metabolism, results suggest that rats have a characteristic sex difference in the use of linoleic and arachidonic acids for the esterification of plasma cholesterol.

Differences occurred in the time pattern of change of composition and amounts of the lipids other than cholesterol ester. The large but transient increase in liver triglycerides of males, after their removal from the safflower oil diet, took place when the total liver triglyceride concentration in the livers of females was decreasing. The concomitant decrease in the linoleic acid of liver triglyceride was steeper in males than in females, as was the decrease in adipose tissue linoleate. Even in the triglyceride fraction, however, the changes in fatty acid composition following changes in dietary fat tended to be slower in liver than in adipose tissue.

If unesterified fatty acids of liver and plasma can be taken to represent those in transit or in the process of being supplied to tissue, the lower proportions of oleic and linoleic acids, and the higher percentages of saturated and arachidonic acids in fraction III, as compared with the triglyceride fractions of the same tissue samples, may be of metabolic importance. The small size of the fraction containing the free fatty acid unfortunately makes accurate analysis difficult. The differences described were observed consistently, and are in agreement with the evidence that the rat tissues use arachidonic acid in preference to linoleic.

The maintenance of a relatively high proportion of arachidonic acid in the phospholipid fractions, as well as the fact that this proportion was higher in liver than in plasma, suggests that these compounds represent important sources of essential fatty acids, and that phospholipids may even be the functional compounds of these acids.

That the low percentages of lauric and myristic acids in the liver and plasma lipids may reflect a specific effect of short-chain fatty acids is possible. Rats fed coconut oil had comparatively low liver cholesterol concentrations and rather high adipose tissue reserves of lauric and myristic acids. Fluck and Pritham ('61) recently reported inhibition of cholesterol synthesis by liver slices when saturated fatty acids of chain lengths under 16 carbons were added to the reacting medium. But rats fed cholesterol, such as those in the present experiment, might be expected to derive a large percentage of their liver and plasma cholesterol from their diets.

Changes in the plasma and tissue lipids in response to changes in the composition of the dietary fat apparently follow different patterns in different species of animals. A recent report by Dhopeshwarkar and Mead ('61) gives tissue data for guinea pigs of one age group. Wissler et al.[3] made a preliminary report of an extensive study with *Cebus* monkeys, and Dam and Engle ('58) reported isomerization data for liver and adipose tissue fatty acids of female rats given fat-free rations with supplements of varying amounts of peanut oil. Both guinea pigs and monkeys incorporated lauric and myristic acids into cholesterol ester to a greater extent than did our rats. Neither species had the high percentages of cholesterol ester arachidonic acid characteristic of the rat, regardless of diet. The latter may be important, metabolically, because of the postulation of Swell et al. ('60) to the effect that the arachidonic acid content of cholesterol ester may be taken as an index of the resistance of a given animal to the development of atherosclerosis. The data in the present paper suggest that, in the rat at least, the liver has a selective function in the maintenance of a supply of essential fatty acid. Although the male rat evidently uses linoleic acid readily in the esterification of liver and plasma cholesterol, the female tends to retain higher levels of ester arachidonic acid when the supply of dietary linoleic acid is limited. The female also is more likely to have high plasma cholesterol values when excess linoleic acid is fed with cholesterol.

## SUMMARY

Liver and plasma data were reported for young rats at intervals following change from a diet containing 10% of a largely saturated fat (coconut oil) to one containing the same amount of a linoleate-rich oil (safflower oil) and return to the saturated fat. Diets were semisynthetic, adequate to support normal growth, and identical except for the kind of fat. All contained 1% of cholesterol. The lipid extracts from liver and plasma were fractionated on silicic acid columns, and the methylated fatty acids of each fraction were analyzed by gas liquid chromatography. Fatty acid composition of mesenteric, subcutaneous and interscapular fat of the same animals are reported in the preceding paper.

In contrast to adipose tissues, which contained largely triglycerides, the liver and plasma lipids of all groups contained relatively large proportions of cholesterol esters and phospholipids, and appreciable amounts of the fraction containing mono-

[3] Wissler, R. W., R. H. Hughes, L. E. Frazier and R. A. Rasmussen 1961 Serum lipids and fatty aortic intimal lesions in *Cebus* monkeys. Federation Proc., 20: 94 (abstract).

and diglycerides and free fatty acids as well as free cholesterol.

The lipids of liver and plasma contained little lauric or myristic acid, regardless of diet, whereas the adipose tissue of the animals fed coconut oil sometimes contained as much as 30% of lauric and myristic acids. But there was always considerable arachidonic acid in liver and plasma lipids and little or none in adipose tissue.

Although the linoleic acid content of liver and plasma lipids was increased by feeding safflower oil and decreased by feeding coconut oil, there seemed to be some tendency toward retention of linoleic acid in liver lipids, following change of the rats back to the coconut oil diet. If phospholipid fatty acids were included, however, the variations of the liver lipid fatty acids with diet stayed within narrower ranges than those of the adipose tissue.

Characteristic sex differences were observed both in the concentration of the different liver and plasma lipids, and in their fatty acid patterns. Plasma cholesterol levels for males fed coconut oil averaged about 10 mg per 100 ml higher than those for males fed safflower oil. Females did not show this difference. For the females fed safflower oil for 9 weeks, the plasma cholesterol values of most doubled during the period of safflower oil feeding. Females fed coconut oil had higher cholesterol ester arachidonic acid values than males. Also, when females were changed back from the safflower oil diet to coconut oil, they responded with greater increases in percentages of plasma ester arachidonate than the males. But the males fed safflower oil showed more liver enlargement and more liver storage of both cholesterol ester and linoleic acid. In neither sex did the proportion of linoleic acid in the glyceride fraction of liver and plasma return to its original level within 6 weeks after return to the coconut oil diet following three weeks of safflower feeding.

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# Effects of Chronic Food Restriction in Swine[1,2]

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Actuarial studies have uniformly shown that obesity is associated with shortened life expectancy and increased incidence of cardiovascular and renal disease, particularly when comparisons are made between overweight and underweight populations (Gubner, '57). Controlled underfeeding of nutritionally adequate diets to rats has been shown to increase life span and decrease the incidence of several chronic diseases (McCay et al., '43; Berg and Simms, '60, '61). But some investigators (Rodbard et al., '51; Goldner et al., '54) have reported an enhancement of atherosclerosis when cholesterol feeding and undernutrition were combined. In a careful study of the role of undernutrition upon cholesterol-induced atherosclerosis in rabbits, McMillan et al. ('54) observed that food restriction favored hypercholesterolemia but did not change the incidence of aortic atherosclerosis.

The experiment reported here was performed to determine the effects of underfeeding a human diet on common parameters of lipid metabolism and vascular pathology of swine. This species was selected because obesity develops early in life; vascular lesions occur spontaneously (Gottlieb and Lalich, '54); and blood lipoprotein components are said to closely resemble those of man (Havel et al., '55) and to vary depending upon relative body leanness (Lewis and Page, '56).

## MATERIALS AND METHODS

Ten "miniature," vasectomized, male swine were purchased from the Hormel Institute at 12 weeks of age. Upon receipt, the animals were fed a normal pig-growing ration for a two-week standardization period, during which initial blood samples were obtained. Two groups were then designated on the basis of body weight, serum lipid characteristics, and hematologic findings.

One group was permitted to eat ad libitum during the next 44 weeks of study. The second group was restricted in food intake to limit body weight gain to 50% of that of paired ad libitum-fed pigs. The basal diet for both groups was a blended military combat ration. This C ration differs from a typical American diet in that it is lower in dairy products and eggs and that all foods are processed. Salt content is rather high, 2.56% of dry solids (see table 1). The protein quality of the ration is approximately equal to casein. Methionine, at 0.37% of dry solids (plus 0.11% of cystine), constitutes the only limiting amino acid. To this basal ration were added sufficient calcium phosphate and vitamin D (10 IU per 100 gm) to permit adequate bone growth in young animals. The diet of the restricted group was fortified with the full complement of B vitamins in excess of requirement and with 2% of corn oil, in order to double the linoleic acid content of the diet. To maintain equivalent caloric distribution, 2% of hydrogenated vegetable fat[6] was added to the diet fed ad libitum. Thus, the diets provided 236 Cal. per 100 gm, 40% of which

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[6] Primex B and C, Procter and Gamble Company, Cincinnati.

TABLE 1

*Composition of ration, individual, combat diet as fed*

| | |
|---|---|
| | *gm/100 gm* |
| Moisture | 52.30 |
| Protein | 8.37 |
| Crude fat | 10.64[1] |
| Ash | 1.99 |
| Carbohydrate (by difference) | 26.70 |
| Sodium chloride | 1.22 |
| Calcium | 0.38[2] |
| Phosphorus | 0.28[2] |
| Potassium | 0.36 |
| Magnesium | 0.039 |
| | *mg/100 gm* |
| Iron | 2.2 |
| Zinc | 0.6 |

[1] Of this fat, 8.64 gm was derived from the basal diet of C ration; the remainder was added fat as noted in the text.

[2] With added calcium phosphate. The unsupplemented diet contained 0.058% of calcium and 0.12% of phosphorus.

were derived from fat, 14% from protein, and 46% from carbohydrate.

For the first 24 weeks, the swine were housed in dog metabolism cages in a controlled environment (26° ± 2°C). At this time the dimensions of the group fed ad libitum approximately equaled those of the cages. The animals were moved to an unheated basement, individually penned, and bedded on 2 to 3 inches of wood shavings over concrete. Temperature fluctuated between 10° and 20°C, and strict control of rate of gain became difficult.

The animals were weighed weekly and thickness of back fat was measured intermittently by a lean meter (Andrews and Whaley, '54).[7] Fasting blood samples (30 ml) were taken from the anterior vena cava at 2- or 4-week intervals throughout the test. Serum lipid components and electrophoretic patterns were determined on all samples. Total serum cholesterol was measured by the classical Liebermann-Burchard reaction, as modified by Sperry and Webb ('50). Serum lipid phosphorus was determined, with *p*-semidine hydrochloride as the color reagent (Dryer et al., '57). For the determination of lipoprotein lipid, electrophoresis was performed with a hanging strip paper electrophoretic apparatus. After a 16-hour migration period with pH 8.6 veronal buffer, of ionic strength 0.05, the paper was dried and stained with oil red O. Density measurments were made and lipid content was estimated by comparison to a standard lipid run simultaneously and treated in an identical manner as serum. Serum protein electrophoresis was performed in the same way, with bromphenol blue as the dye.

Other determinations designed to evaluate nutritional status and functional integrity of important organ systems were made from time to time. These included hematocrit, hemoglobin, and blood-formed elements (conventional clinical methods); serum total protein (Cohn and Wolfson, '48) and nonprotein nitrogen (Koch and McMeekin, '24); activities of serum glutamic-oxalacetic and glutamic-pyruvic transaminases[8] and of alkaline and acid phosphatases;[9] serum bilirubin (Malloy and Evelyn, '37); protein-bound iodine (Thompson et al., '56); blood sugar (Nelson, '44) and ketones (Greenberg and Lester, '44).

After terminal blood samples were drawn, the swine were anaesthetized with sodium pentobarbital and exsanguinated. At necropsy, tissues were removed for histopathologic examination by Drs. Paul B. Szanto and Leo Williams of the Hektoen Institute for Medical Research. Quick-frozen samples of liver and adrenal tissues were reserved for measurement of hepatic glucose-6-phosphatase activity (Swanson, '55) and activity of adenosine triphosphatase (DuBois and Potter, '43) and 5′-nucleotidase (Cochran et al., '51).

## RESULTS

As indicated in figure 1, very little gain was achieved in the restricted group from the thirtieth week to the end of the test but the goal of half-gain was reasonably well met. The back-fat layer of the group fed ad libitum was 1.3 in. at 6 months and rose to a terminal average of 1.9 in. The final range was 1.10 to 2.82 in. The re-

[7] Andrews, F. N., and R. M. Whaley 1954 A method for the measurement of subcutaneous fat and muscular tissues in the live animal. Presented at the Annual Meeting of the American Society of Animal Production, Chicago.

[8] Sigma Chemical Company 1958 A simplified method for the clinical determination of serum glutamic-oxalacetic transaminase and serum glutamic-pyruvic transaminase. Tech. bull. no. 505.

[9] Sigma Chemical Company 1958a Determination of serum acid and alkaline phosphatase and "prostatic" acid phosphatase using Sigma 104 phosphatase substrate. Tech. bull. no. 104.

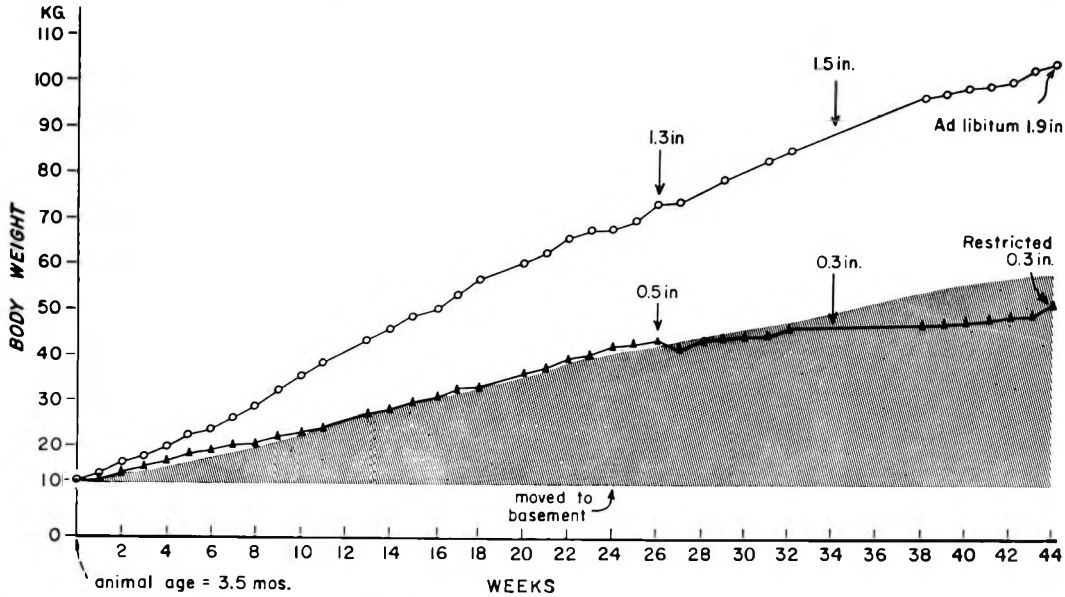


Fig. 1 Growth of swine fed C ration ad libitum or restricted to allow 50% gain (shaded area) numbers indicate back-fat thickness.

stricted group varied from 0.25 to 0.42 in. in back-fat, averaging 0.3 in. (see table 2).

Variations in body type were noticeable among the group fed ad libitum. One animal (no. 14) was of placid disposition and tended to gain weight more efficiently than the others. A second pig (no. 16), although weighing very nearly the same amount (120 and 116 kg, respectively), had only half the thickness of back-fat. Pig no. 12 was the leanest of the group fed ad libitum and was an apprehensive type that reacted to routine blood sampling by a marked drop in caloric efficiency for a few days.

The caloric intake of the two groups is shown in figure 2. Food consumption of the group fed ad libitum increased steadily from 2,400 to 8,000 Cal. daily during the first 20 weeks of study and remained essentially constant thereafter. The restricted group was fed one-fourth to one-

TABLE 2

*Body weight, fat thickness, and serum cholesterol*

| Pig no. | Litter no. | Body weight | | | Back-fat thickness | | Serum total cholesterol 44-week average |
|---|---|---|---|---|---|---|---|
| | | Initial | 6 months | 10 months | 6 months | 10 months | |
| | | *kg* | *kg* | *kg* | *inches* | *inches* | *mg/100 ml* |
| | | | Ad libitum group | | | | |
| 11 | 519 | 9.2 | 72.6 | 106.2 | 1.79 | 2.28 | 88 |
| 12 | 523 | 10.9 | 62.2 | 82.9 | 0.75 | 1.10 | 105 |
| 14 | 523 | 11.8 | 76.5 | 120.2 | 1.72 | 2.82 | 90 |
| 15 | 516 | 13.3 | 72.8 | 98.5 | 1.05 | 1.80 | 123 |
| 16 | 514 | 16.4 | 83.6 | 116.4 | 1.38 | 1.45 | 103 |
| | | | Restricted group | | | | |
| 21 | 515 | 9.3 | 40.6 | 56.3 | 0.58 | 0.25 | 133 |
| 22 | 519 | 11.4 | 37.6 | 46.7 | 0.35 | 0.25 | 113 |
| 24 | 522 | 12.8 | 44.6 | 52.2 | 0.55 | 0.42 | 97 |
| 25 | 516 | 13.5 | 45.6 | 53.4 | 0.55 | 0.38 | 115 |
| 26 | 517 | 14.3 | 49.4 | 53.4 | 0.55 | 0.30 | 121 |

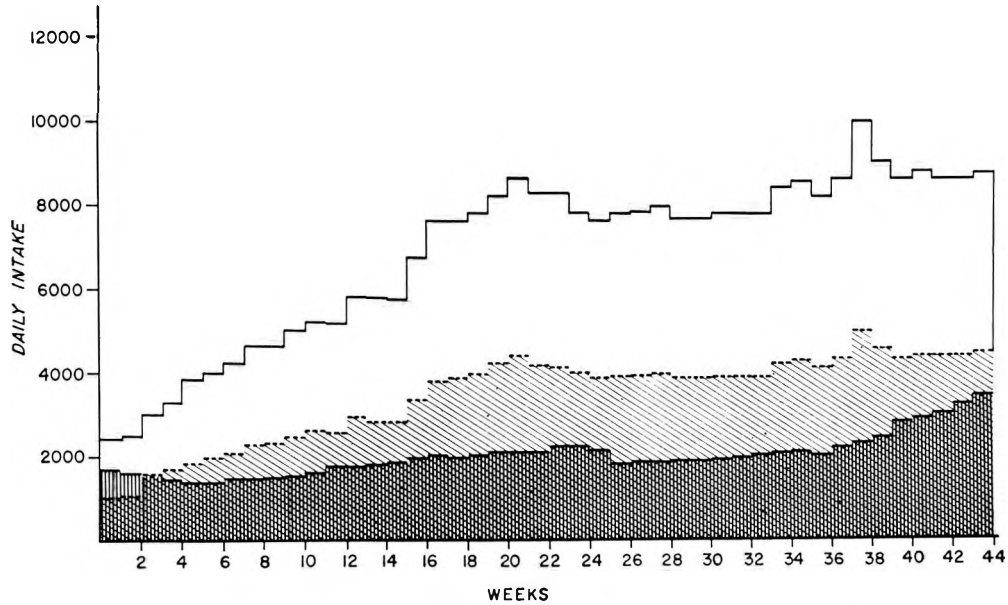


Fig. 2 Mean daily caloric intake of swine fed C ration ad libitum or restricted to allow 50% weight gain. Upper solid line indicates ad libitum intake; shaded area 50% caloric intake; lower solid line, intake of restricted group.

third as much; their caloric intake was a little less than half that of the ad libitum animals, on a body weight basis. During the first 36 weeks, the percentage weight gain of the restricted group was about 65% of that of the ad libitum group and was achieved with 45% of their caloric intake.

The change in serum total cholesterol during the experimental period is illustrated in figure 3. An immediate elevation from the pre-experiment level was observed in both groups of animals, the restricted animals showing a greater increase. During the early phases of the experimental period, the level remained elevated. At the eighth week, serum cholesterol content of the ad libitum animals had returned to initial values and remained at this level for the remainder of the experiment. But serum cholesterol in the restricted animals remained slightly elevated throughout most of the experimental period, returning to initial values by the end of the thirtieth week. From the thirtieth week until the end of the experiment, there was no indication of any difference between the two groups. No correlation between serum cholesterol and body weight or fat thickness within groups was found (see table 2).

A similar pattern was seen when the serum phospholipid was examined (see fig. 3). An immediate marked increase was obtained in all animals, with a peak reached by the fourth week. In the ad libitum animals, phospholipid levels returned to initial value 8 weeks after the start of the experiment and remained below that level from the twelfth week onward. With the restricted animals, a more gradual decrease from the peak value was observed. By the twenty-fourth week, phospholipid content had returned to the initial values and remained in this range for the rest of the experiment. The restricted animals showed consistently higher serum phospholipid content when compared with their ad libitum-fed pair mates.

Cholesterol/phospholipid (C/P) ratios were calculated as another possible indication of lipid metabolic derangement. As noted in figure 3, an initial decrease in both groups was observed with values returning to pre-experimental levels by the twelfth week. The ratios of ad libitum animals showed an additional increase be-

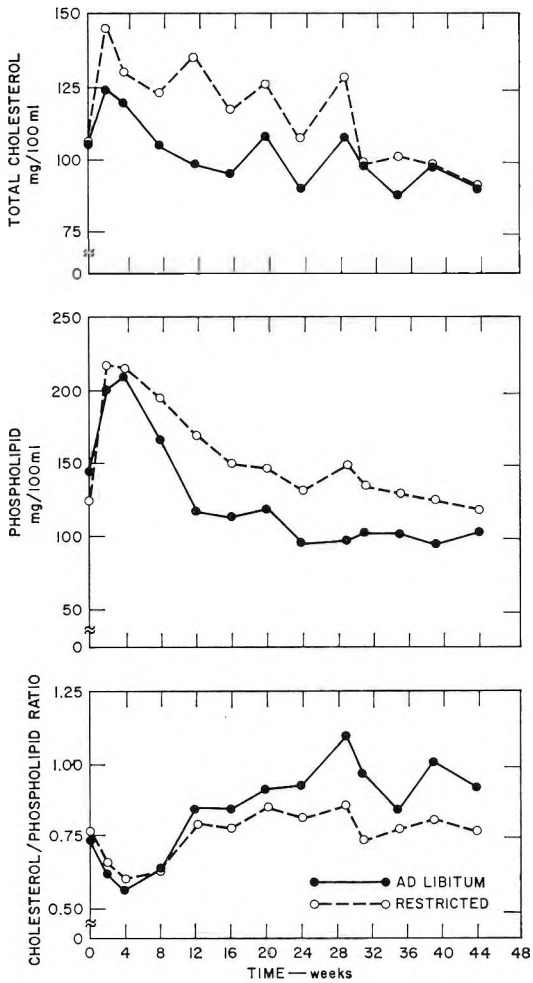


Fig. 3 Serum total cholesterol and phospholipid content and the ratios of these in swine fed ad libitum or restricted to allow 50% weight gain.

yond the twelfth week and remained higher than the restricted animals for the remainder of the experimental period.

In table 3 are shown the results of electrophoretic separations of lipoprotein components. Three distinct peaks were obtained by electrophoresis under the conditions employed. A nonmigrating staining area was usually distinct from the α-lipoprotein peak. These nonmigrating lipids were termed "origin" as listed in the table. Total lipoprotein lipid showed, as with cholesterol and phospholipid, an increase in both groups with a peak reached on the fourth week. Thereafter, a general decrease was noted with a return to initial values near termination of the experiment. On both the thirty-ninth and forty-fourth week of experimentation, the lipoprotein lipid content of the sera of the restricted animals was below the initial values. The β-lipoprotein levels remained relatively constant, with the restricted group showing a greater decrease after the twenty-fourth week. The most marked differences were in the α-(high density) lipoproteins. Concerning this component, the restricted animals showed a higher content throughout most of the experimental period than the animals fed ad libitum. This also accounts for the higher α/β lipoprotein ratios in the restricted animals. The nonmigrating lipids appeared to be higher in the ad libitum-fed animals.

Serum protein electrophoresis and analysis of albumin and globulin components showed no differences between experimental groups (table 4). Both serum total protein and nonprotein nitrogen levels

TABLE 3
*Serum lipoproteins*

| Time | Ad libitum | | | | Alpha/beta ratio | Restricted | | | | Alpha/beta ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| | Lipoprotein | | | | | Lipoprotein | | | | |
| | Alpha | Beta | Origin | Total | | Alpha | Beta | Origin | Total | |
| *weeks* | *mg/100 ml* | *mg/100 ml* | *mg/100 ml* | *mg/100 ml* | | *mg/100 ml* | *mg/100 ml* | *mg/100 ml* | *mg/100 ml* | |
| 0 | 99 | 191 | 46 | 336 | 0.52 | 118 | 209 | 56 | 383 | 0.56 |
| 2 | 159 | 187 | 100 | 446 | 0.85 | 158 | 168 | 93 | 419 | 0.94 |
| 4 | 272 | 271 | 162 | 705 | 1.00 | 409 | 252 | 83 | 744 | 1.62 |
| 8 | 236 | 216 | 95 | 547 | 1.09 | 251 | 136 | 59 | 446 | 1.85 |
| 16 | 249 | 216 | 71 | 536 | 1.15 | 343 | 255 | 71 | 669 | 1.35 |
| 24 | 142 | 169 | 85 | 396 | 0.84 | 236 | 143 | 62 | 441 | 1.65 |
| 29 | 150 | 159 | 71 | 380 | 0.94 | 284 | 155 | 39 | 478 | 1.83 |
| 35 | 131 | 128 | 68 | 327 | 1.02 | 244 | 112 | 57 | 413 | 2.18 |
| 39 | 134 | 150 | 35 | 319 | 0.89 | 160 | 66 | 15 | 231 | 2.42 |
| 44 | 134 | 137 | 75 | 346 | 0.98 | 169 | 83 | 45 | 297 | 2.04 |

TABLE 4

*Enzyme activities and tissue components of metabolic interest*

| | Ad libitum | Restricted |
|---|---|---|
| Serum | | |
| Glutamic-oxalacetic transaminase activity[1] | 44 ± 13[2] | 34 ± 4 |
| Glutamic-pyruvic transaminase activity[1] | 23 ± 4 | 24 ± 3 |
| Alkaline phosphatase activity[3] | 2.28 ± 0.70 | 1.97 ± 0.22 |
| Acid phosphatase activity[3] | 1.32 ± 0.18 | 0.85 ± 0.16[4] |
| Protein-bound iodine, μg/100 ml | 2.3 ± 0.3 | 2.3 ± 0.3 |
| Protein, gm/100 ml | 7.4 ± 0.6 | 7.3 ± 0.4 |
| Albumin, % | 49.5 ± 1.5 | 51.5 ± 1.9 |
| Alpha globulin, % | 20.0 ± 2.9 | 18.2 ± 2.2 |
| Beta globulin, % | 16.0 ± 1.4 | 17.1 ± 1.0 |
| Gamma globulin, % | 14.5 ± 3.6 | 13.2 ± 1.5 |
| Nonprotein nitrogen, mg/100 ml | 33.0 ± 2.0 | 31.2 ± 4.7 |
| Bilirubin, μg/100 ml | 49 ± 23 | 62 ± 13 |
| Whole blood | | |
| Sugar, mg/100 ml | 58.1 ± 8.7 | 68.0 ± 6.5 |
| Ketones, mg/100 ml | 0.96 ± 0.07 | 1.19 ± 0.15[4] |
| Liver | | |
| Adenosine triphosphatase activity[5] | 11.3 ± 5.3 | 5.5 ± 2.6 |
| 5′-Nucleotidase activity[5] | 2.0 ± 0.6 | 3.5 ± 0.7[4] |
| Glucose-6-phosphatase activity[5] | 6.3 ± 1.5 | 4.8 ± 1.2 |
| Adrenal | | |
| Cholesterol, mg/gm | 5.79 ± 1.06 | 5.99 ± 2.24 |
| Adenosine triphosphatase activity[5] | 3.4 ± 1.1 | 3.1 ± 0.8 |
| 5′-Nucleotidase activity[5] | 12.3 ± 4.2 | 15.8 ± 8.3 |

[1] Sigma units per milliliter.
[2] Mean and standard deviation.
[3] Sigma units per liter.
[4] Differs significantly from ad libitum group, $P < 0.05$.
[5] Micrograms of phosphorus released per milligram of tissue.

were also uniform, suggesting normal protein synthesis and renal clearance mechanisms.

Few of the other components listed in table 4 differentiated the two groups. Serum transaminase and alkaline phosphatase activities were comparable and acid phosphatase activity only slightly reduced in the restricted group. Equivalent protein-bound iodine levels indicate that caloric insufficiency did not alter thyroid function. Normal serum bilirubin levels also attested to the adequacy of liver function. Fasting blood ketone content was slightly higher in the restricted group.

Hematologic measurements, shown in table 5, did not reveal any major differences between the two groups. Total leukocyte counts were high initially and showed a steady decline during the period of study. This transition was noticeable after 4 weeks in the group fed ad libitum but only after 24 weeks in the restricted group. Differential counts revealed the same proportions of each of the cell forms in both groups.

Although some differences in mean tissue enzyme activities existed, the range was large (table 4). Only one enzyme of the high-energy phosphate system was significantly affected, the 5′-nucleotidase activity of liver, which was increased in the restricted animals. Each of the measures given in tables 3–5 was evaluated for correlation with body fatness within groups, and none was related significantly.

Measurements made at necropsy revealed that skeletal growth was not significantly retarded by caloric restriction (table 6). Whereas the difference in body weight between groups was 50%, the difference in femur length was only 2%. Kidney and spleen weight were reduced to the same extent as body mass, and thyroid weight depression was proportionate to the calculated decrease in body surface area. Heart and liver weights were 25% lower in the restricted group than in those

TABLE 5

*Formed elements of the blood*

| | Initial | 4 weeks | 12 weeks | 24 weeks | 44 weeks |
|---|---|---|---|---|---|
| | | Hemoglobin, gm/100 ml | | | |
| Ad libitum | | 16.0 | 17.7 | 19.9 | 20.2 |
| Restricted | | 15.8 | 17.9 | 19.8 | 17.9 |
| | | Hematocrit, % | | | |
| Ad libitum | | 50.8 | 47.8 | 53.3 | 54.2 |
| Restricted | | 49.0 | 46.3 | 50.6 | 49.9 |
| | | Red cells, million per $mm^3$ | | | |
| Ad libitum | 8.29 | 7.76 | 8.71 | 9.26 | 8.84 |
| Restricted | 8.22 | 7.54 | 8.90 | 9.12 | 7.70 |
| | | White cells, per $mm^3$ | | | |
| Ad libitum | 21718 | 16710 | 16585 | 15512 | 13944 |
| Restricted | 21945 | 20030 | 21558 | 16400 | 15570 |

TABLE 6

*Organ weights of swine*

| | Ad libitum | | Restricted | | Δ |
|---|---|---|---|---|---|
| | Mean | Range | Mean | Range | |
| | | | | | % |
| Body weight, kg | 104.8 | 82.9 – 120.2 | 52.4 | 46.7 – 56.3 | 50 |
| Surface area, $m^2$ [1] | 1.84 | 1.59– 2.01 | 1.19 | 1.11– 1.21 | 36 |
| Femur length, cm | 19.9 | 18.5 – 21.5 | 19.5 | 18.4 – 20.5 | 2 |
| Heart, gm | 246 | 209 – 271 | 190 | 170 – 222 | 23 |
| Liver, gm | 1540 | 1355 –1740 | 1159 | 1090 –1241 | 25 |
| Kidneys, gm | 327 | 291 – 363 | 188 | 164 – 225 | 42 |
| Spleen, gm | 137 | 102 – 158 | 76 | 49 – 85 | 46 |
| Brain, gm | 103.3 | 93.6 – 107.5 | 98.7 | 92.0 – 105.1 | 4 |
| Testes, gm | 264 | 198 – 290 | 241 | 206 – 267 | 9 |
| Adrenals, gm | 3.35 | 2.88– 3.84 | 2.98 | 2.42– 3.63 | 11 |
| Pituitary, gm | 0.32 | 0.22– 0.41 | 0.26 | 0.20– 0.29 | 16 |
| Thyroid, gm | 12.88 | 9.08– 17.03 | 8.11 | 5.64– 11.57 | 37 |

[1] Calculated from the formula: Surface area, $m^2 = 0.097 \times BW, kg^{0.633}$ (Brody et al., '28).

fed ad libitum. Brain, testes, pituitary, and adrenal glands did not differ significantly on a total wet weight basis. Obviously, if organ weights are adjusted to an equivalent body weight basis, the ratios will be found to be much greater in the restricted group.

Only one observation of major clinical significance was made during the course of the experiment. Soon after receipt of pig no. 26, assigned to the restricted group, it was found to have a small wart-like growth on the right shoulder. After 5 months, this lesion had grown to 7 × 5 × 2 cm and became quite irritated. The growth was removed under ether anesthesia and identified as a benign melanoma. Careful examination of the herd revealed a progressive increase in size and number of similar melanotic lesions in this animal, in no. 25 and, later to a limited extent, in no. 16.

Grossly, pathologic changes were observed in the ascending portion of the arch of the aorta in most of the animals. The lesions were small (1 × 1 to 2 × 4 mm) white or yellowish areas of nodular thickening, with slight or no induration, of the intimal surface. The number of lesions grossly observable varied from zero to 20, with most showing one to three lesions (see table 7). Four animals (one in the ad libitum group and three restricted) had minimal longitudinal wrinkling of the intimal surface of the ascending aorta, and 7 animals (three fed ad libitum, 4 restricted) had a pinkish-yellow discoloration spread diffusely over the intima. Two

pigs fed ad libitum had small nodules present in the abdominal aorta while all others were located about the orifices of the brachiocephalic and bicarotid arteries. None of the lesions significantly narrowed the lumen of the vessel involved. Histologically, these nodular lesions resembled those described by Gottlieb and Lalich ('54), consisting of proliferative changes with hyalinization, fibrosis and calcification. No stainable lipid was seen.

A moderate but progressive development of arteriosclerosis was recorded and the frequency of calcified lesions was greater in the group fed ad libitum than among restricted animals. Focal loss or depletion of the elastic fibrils of the media of the aorta was also noted. These changes were more marked in the ad libitum group, but independent of any arteriosclerotic lesions which might occur in the same animal.

Neither gross nor microscopic examination revealed pathologic changes in coronary, cerebral, renal or retinal vessels.

In addition to the vascular alterations, a number of general pathologic processes were observed, most of which were judged to be unrelated to the dietary management. They are listed in table 7. The lesions in the genital organs and contiguous structures were concluded to be sequelae of the bilateral vasectomies performed by the breeder. Three of the cases of lymphadenopathy were melanin deposits associated with the development of multi-

TABLE 7

*Pathologic observations*

| | Ad libitum | | | | | Restricted | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 11 | 12 | 14 | 15 | 16 | 21 | 22 | 24 | 25 | 26 |
| Gross, general | | | | | | | | | | |
| Melanotic tumors | | | | | ? | | | | × | × |
| Cutaneous abscess | | | | | | | × | | | |
| Lymphadenopathy | × | | × | | × | × | × | | × | × |
| Subserosal petechia, lung | | | | × | | | | | | |
| Submucosal petechia, gallbladder | | × | | | | | | | | |
| Adhesions, inguinal ring | | | | | | × | | | | |
| Hypertrophy, seminal vesicle | | × | | | | | | | | |
| Granulomatous lesions, epididymitis | | × | × | × | × | | × | × | × | × |
| Atrophy, olfactory lobe of brain | | | | | | | × | | | |
| Fibrosis, kidney | × | | | | | | | | | |
| Bursitis, carpus | × | | | | | | | | | |
| Microscopic, general | | | | | | | | | | |
| Melanomata | | | | | × | | | | × | × |
| Melanin deposit lymph node | | | | | × | | | | × | × |
| Chronic lymphadenitis | × | | × | | | × | × | | | |
| Interstitial fibrosis of kidney | × | | | | | | | | | |
| Dilatation of tubules of epididymis | × | × | × | × | × | × | × | × | × | × |
| Gross, vascular | | | | | | | | | | |
| Pinkish yellow streaking of intima of aorta | | | × | × | × | × | × | × | × | |
| Wrinkling of intimal surface of aorta | | | | × | | | | × | × | × |
| No. of intimal nodules | 1 | 1 | 0 | 20 | 2 | 0 | 1 | 1 | 2 | 0 |
| Microscopic, vascular | | | | | | | | | | |
| Maximal stage of development of arteriosclerotic lesion[1] | 4 | 4 | 3 | 4 | 0 | 0 | 4 | 1 | 4 | 1 |

[1] The pathologic changes were graded as follows:
Stage 1 Proliferative changes in the intima, all of mild to moderate degree.
Stage 2 Proliferative changes with hyalinization.
Stage 3 Proliferative changes with hyalinization and fibrosis.
Stage 4 Calcification in addition to the above changes.

ple primary melanomas, and the remainder were chronic lymphadenitis. The neoplasms will be discussed in detail elsewhere.[10] Renal fibrosis and bursitis of the carpus were observed in one member of the ad libitum group. Histologic examination of all other major organs, including both exocrine and endocrine glands, produced negative findings.

## DISCUSSION

Our data agree with observations cited earlier, in that controlled underfeeding was accompanied by slightly elevated serum cholesterol levels. The lack of positive correspondence within groups between relative body fatness and cholesterol levels is at variance with the observation of Lewis and Page ('56) in this same strain of swine and with some human studies (Walker, '53; Keys et al., '55; Olson, '59). Other workers have, however, failed to detect a relationship between body fatness and serum cholesterol levels in man (Keys, '55; Hawthorne et al., '56).

The pathologic findings in the vasculature support the concept of retarded aging in the restricted group. The delayed fall from high juvenile leukocyte counts to typically lower adult levels also shows this trend. In only one respect do our data conflict with observations of comparably restricted rats: spontaneous tumorous processes occurred earlier, more frequently, and more severely in the underfed group. The tumors appearing spontaneously in the present experiment were melanomas, a type not found in the species used by other investigators.

## SUMMARY

Male swine were fed a mixed human diet ad libitum for 44 weeks. Paired animals were given the diet in amounts that allowed only 50% of the weight gain of the fully fed group. The back-fat thickness of the ad libitum group averaged 1.9 in. terminally and that of the restricted animals was 0.3 in. The restricted group showed slightly elevated levels of serum cholesterol, phospholipid and ketones and of hepatic 5′-nucleotidase activity. Vascular pathology was indicative of delayed aging in the underfed group but tumorigenesis was enhanced. There was no positive correlation within groups between relative fatness and any other measured characteristic.

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# A Reinvestigation of Methylglyoxal Accumulation in Thiamine Deficiency[1]

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One of the most recurrent reports in the older literature is that of the accumulation of methylglyoxal in thiamine deficiency. The latest experimental evidence was reported by Salem ('54, '55). The accumulation of methylglyoxal has been attributed to a decrease in glyoxalase activity, but the evidence is conflicting. Most authors agree that there is some decrease in enzyme activity, but this is, at least in some tissues, due to a decrease in glutathione (Drummond, '61). Such a decrease in glutathione is not uniform in all tissues (Hsu and Chow, '61).

The biochemical lesion in thiamine deficiency is less clear now than it was thought to be after Peter's classical work on pyruvic oxidase (Kinnersley and Peters, '30). The work of Jones and d'Angeli ('60) raises serious questions whether a functional decrease in pyruvic acid oxidase is present in the intact deficient animal. A derangement in a specific tissue is not excluded, but the overall symptoms of thiamine deficiency cannot be attributed to a decrease in thiamine pyrophosphate. Furthermore, erythrocyte transketolase is nonfunctional at an early stage in the deficiency (Brin et al., '58), long before gross symptoms appear. This suggests that the animal can survive for prolonged periods without transketolase.

Recently it was observed that α-glycerolphosphate dehydrogenase is very sensitive to thiamine deficiency (van Eys, '61a). Lactic dehydrogenase was somewhat less sensitive to a deficiency provoked by dietary means or by pyrithiamine, but very sensitive to oxythiamine-induced deficiency. These observations suggest the possibility of deranged glycolysis. A derangement of any type in this area of metabolism might result in the accumulation of methylglyoxal in thiamine deficiency. Although the results are not conclusive, they do indicate that it may accumulate in thiamine deficiency and that methylglyoxal could be further metabolized to pyruvate through D-lactic acid.

There is at present only one enzymatic synthesis of methylglyoxal known in the animal organism: the degradation of threonine through aminoacetone to methylglyoxal (Elliot, '60), although this is not the major pathway of threonine degradation.

## EXPERIMENTAL

*Growth and maintenance of animals.* Wistar rats[3] were fed a commercial thiamine-deficient diet and were treated with oxythiamine and pyrithiamine as described previously (van Eys, '61a). Some tissues were obtained from the rats described in the previous report; others were taken from a new series of animals. In studies on some 70 rats it was confirmed that oxythiamine does not provoke neurological lesions in rats. Pyrithiamine at all dose levels induces neurological signs in all animals treated for a sufficient time. All pyrithiamine-treated rats were maintained until neurological symptoms appeared. Dietary-deficient rats or oxythiamine-treated rats were maintained until the weight was at least 5 gm below the starting weight. Urine collections were made daily and the urine was frozen until the analysis could be made.

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[2] Investigator, Howard Hughes Medical Institute.

[3] The rats were obtained from the Harlem Small Animal Company, Cumberland, Indiana.

*Enzyme preparations.* Glyoxalase I and II were prepared as described by Racker ('55). Triose-phosphate isomerase was prepared by the method of Meyerhof and Beck ('44). A D-lactic acid specific lactic dehydrogenase was prepared from *Leuconostoc mesenteroides*.[4]

The organism was grown at 30°C in a medium consisting of: $KH_2PO_4$, 2 gm; Difco Bactopeptone, 5 gm; dextrose, 10 gm; Difco yeast extract, 2 gm; and the supernatant, obtained by centrifugation of tomato juice, 100 ml per liter of culture medium. The inclusion of tomato juice increased the yield of organisms by as much as 100%.

After a 24-hour growth period the cells were collected by centrifuging, and washed with saline. Usually a two-liter batch was worked up at a time. The cells were suspended in a buffer 0.01 M pyrophosphate, containing 500 mg per liter of versene at pH 6.7 and disrupted in a Mickle disintegrator for one-half hour, the debris and glass beads separated by centrifuging and the supernatant brought to 75% saturation with a saturated ammonium sulfate solution. This and subsequent precipitates were collected after centrifugation for 10 minutes at 10,000 rpm. All centrifugations and manipulations were carried out at 4°C. The precipitate was subfractionated and the fraction precipitating between 35 and 75% saturation was retained, redissolved in water and an equal volume of a 1% protamine sulfate solution added. The resultant precipitate was discarded, the supernatant again subfractionated with ammonium sulfate solution and the fraction between 55 and 65% saturation retained. This fraction was poured over a 20 × 1 cm column of Sephadex G-50 to desalt and remove protamine. The enzyme so obtained was stable to freezing and thawing for several weeks. The results of a typical purification are shown in table 1.

The enzyme so obtained showed a preference for the DPN analogue: acetylpyridine-*DPN. When assayed in a medium containing 0.1 M pyrophosphate buffer, pH 9.3, and $3.3 \times 10^{-3}$ M D-lactic acid, the analogues of DPN had the following relative activity (in per cent of DPN): DPN:100, acetylpyridine-*DPN: 135; pyridinealdehyde-*DPN:40; TPN: inactive. The Michaelis constants were as follows: for acetylpyridine-*DPN at $3.3 \times 10^{-3}$ M lactic acid: $5.6 \times 10^{-4}$. For lactic acid at an acetylpyridine-*DPN concentration of $4.4 \times 10^{-4}$:$2.4 \times 10^{-2}$.

The preparation showed no DPNH oxidase activity and was inactive toward L-lactic acid, oxalic acid, glycolic acid, ethanol, glycerol, glycerol phosphate, glutamic acid, glucuronic acid, glucose, 1,2-propanediol, acetaldehyde, mercaptoethanol, 3-phosphoglyceraldehyde, sorbitol, and α-hydroxybutyric acid. It showed a small activity with glyoxylic acid, apparently due to the same enzyme. With DPN, but not with acetylpyridine-*DPN, glucose 6-phosphate was slowly oxidized due to a contaminating protein.

*Determinations.* The methods used were as follows: total lactic acid by the method of Barker and Summerson ('41); pyruvate as the 2,4-dinitrophenylhydra-

[4] The strain used was an organism classified as *Betacoccus arabinosaceus* in the Orla Jensen collection. It was kindly supplied by Dr. A. H. Harrison, Jr., from the Department of Biology at Vanderbilt University.

TABLE 1

*Purification of* D-*lactic dehydrogenase from L. mesenteroides*[1]

| Fraction, % saturated $(NH_4)_2SO_4$ | Volume | Protein | | Units | | Specific activity |
|---|---|---|---|---|---|---|
| | | mg/ml | Total mg | /ml | Total | |
| | *ml* | | | | | |
| Crude extract | 19.0 | 3.2 | 60.0 | $6.1 \times 10^4$ | $1.35 \times 10^6$ | 22.5 |
| 0–75 | 10.0 | 4.5 | 44.6 | $1.8 \times 10^5$ | $1.8 \times 10^6$ | 41.3 |
| 35–75 | 10.0 | 1.6 | 15.6 | $1.3 \times 10^5$ | $1.3 \times 10^6$ | 82.7 |
| 55–65 | 5.0 | 0.62 | 3.1 | $1.1 \times 10^5$ | $0.5 \times 10^6$ | 171.0 |

[1] One unit is that amount of enzyme which caused a change in optical density of 0.001/minute when assayed in the following system: DPNH: 0.4 μmoles; phosphate buffer, pH 7.6: 50 μmoles; pyruvate: 10 μmoles; and enzyme, in a final volume of 3.0 ml. Specific activity is defined as units per milligram of protein.

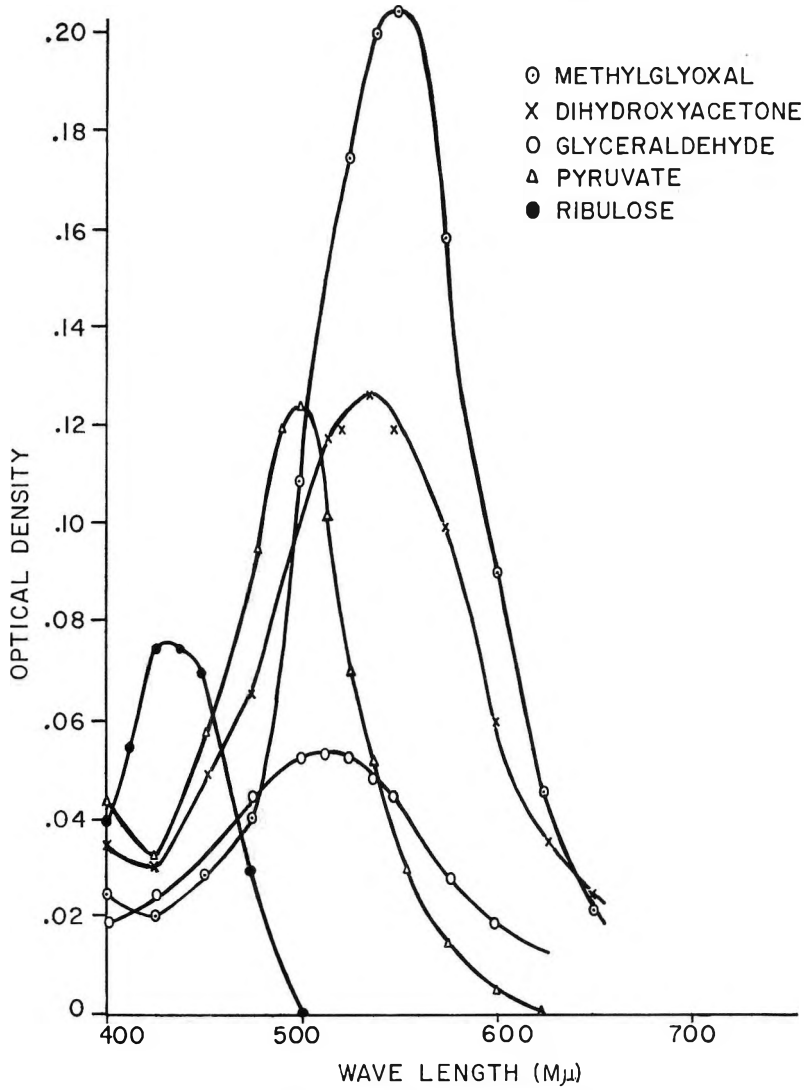


Fig. 1 The color response of various carbohydrate derivatives in the Dische test. Methylglyoxal was tested at 10 μg/ml, all other compounds at 400 μg/ml.

zone (Friedemann, '57); D-α-hydroxy acids microbiologically using the D-lactic acid-requiring mutant of *Lactobacillus casei* 280–16 (Camien and Dunn, '53; Judge and van Eys, '62); protein in tissues by the biuret test (Weichselbaum, '46); and methylglyoxal by the method of Dische and Robbins ('34). Although the latter method is not absolutely specific, the sensitivity for methylglyoxal is such that a reliable estimate can be obtained (fig. 1). Furthermore, a barium-zinc filtrate eliminates interference of phosphorylated compounds.

D-Lactic acid was estimated enzymatically by allowing the enzyme to act on an unknown sample in the presence of acetylpyridine-*DPN. This estimation was made on urine freed of pyruvate as follows: one milliliter of urine was treated with Lloyd's reagent and centrifuged. To the supernatant was added 0.02 ml of 30% hydrogen peroxide and the urine was allowed to stand for one hour. Then 50 μg

of crystalline catalase were added and again the urine was allowed to stand one hour. Recovery experiments indicated that all pyruvate was destroyed and that no lactate was lost during these manipulations.

The treated urine was then diluted with 0.1 M pyrophosphate buffer, pH 9.3 to a volume of 2.8 ml. Two-tenths of a milliliter of a solution containing 10 mg per ml of acetylpyridine-*DPN and enzyme were added, and the reaction was allowed to proceed to completion as judged by the optical density at 365 mμ. From the molar extinction coefficient of $9.1 \times 10^3$ (Siegel et al., '59), the lactic acid concentration could be calculated.

## RESULTS AND DISCUSSION

*Attempted identification of methylglyoxal in urine.* Methylglyoxal is both chemically unstable and volatile. Ordinary collection of urine did not allow recovery of methylglyoxal added initially unless the urine was quick-frozen with dry ice during collection. Even then, however, the identification of methylglyoxal posed serious problems. Deionization of the urine over ion exchange columns apparently destroyed methylglyoxal. Concentration of urine, even at room temperature, volatilized the compound. Untreated urine had such a large amount of interfering materials for both the chemical assay or the assay with glyoxalase I and II that a direct determination was excluded. These results indicated that a quantitative estimation of methylglyoxal is very difficult. Nevertheless, methyglyoxal was not noted in the quantities indicated by Salem ('54), in agreement with the recent observations by Drummond ('61).

*"Enzymatic" formation of methylglyoxal.* Because of the failure of the direct demonstration of methylglyoxal in urine (or blood) a different approach was taken. For efficient pyruvate formation, if this formation is not coupled to reduction to lactic acid, some means of removal of glycolytically reduced DPN must operate. In brain the α-glycerophosphate cycle, postulated by Estabrook and Sacktor ('58) and by Zebe et al. ('59), probably is the means by which such DPNH is removed. A lowering of both lactic dehydrogenase and glycerolphosphate dehydrogenase would result in a blocking of glycolysis and an accumulation of triose-phosphates, which could result in production of methylglyoxal. In liver other means, such as an enzyme analogous to cytochrome c reductase (van Eys, '61b), may exist to remove glycolytically generated DPNH. Thus the glycerophosphate cycle is relatively less important.

TABLE 2

*Methylglyoxal formation in thiamine-deficient brains*[1]

| Group | No. animals | Methylglyoxal[2] |
|---|---|---|
| | | *mμmoles/mg brain* |
| Controls | (7) | 16.3 ± 6.78 |
| Oxythiamine | (8) | 36.0 ± 7.67[3] |
| Pyrithiamine | (6) | 36.3 ± 4.68[3] |

[1] Each Warburg flask contained 20 μmoles of fructose-diphosphate as substrate and 50 mg of brain homogenate. The flasks were fortified with 15 units of hexokinase, 30 μmoles of 2-deoxyglucose, 1.5 μmoles of DPN, 3.0 μmoles of ATP, 50 μmoles of phosphate buffer, pH 7.5, 20 μmoles of $MgCl_2$, 120 μmoles of nicotinamide and 0.03 μmoles of cytochrome c. KOH was added to the centerwell. The gas phase was air, incubation time 40 minutes. Methylglyoxal was assayed by the method of Dische and Robbins ('34) on a barium-zinc filtrate.

[2] All figures are given with their standard deviation.

[3] Significantly higher than control group ($P = 0.01$).

Therefore, to find methylglyoxal formation, tissues were incubated in a Warburg respirometer with a complete medium for glycolysis patterned after one published previously (van Eys and Warnock, '59). The details are given in the legend of table 2. When so examined, the homogenates of brains of thiamine-deficient rats form more methylglyoxal than normal tissues do. Representative data are shown in table 2. In liver, however, methylglyoxal accumulation was only evident after blocking the glycolysis with fluoride ion (table 3). Under those conditions a glycerophosphate cycle must operate to further oxidize trioses.

The question whether the formation of methylglyoxal is an artifact or an enzymatic function is open. Chemically dihydroxyacetone-phosphate is more stable than is glyceraldehyde 3-phosphate. The enzymatic equilibrium, as achieved by triose-phosphate isomerase between the two trioses, is far in favor of dihydroxyacetone-phosphate (Meyerhof and Beck, '44). Thus the methylglyoxal accumula-

TABLE 3

*Methylglyoxal accumulation in liver homogenates*[1]

| Group | No. animals | Methylglyoxal[2] | |
|---|---|---|---|
| | | − F | + F |
| | | *μmoles/mg protein/20 minutes* | |
| Control | 8 | 6.33 ± 1.34 | 5.37 ± 0.94 |
| Restricted[3] | 9 | 5.29 ± 1.74 | 7.87 ± 1.64 |
| Deficient | 9 | 6.72 ± 0.76 | 11.78 ± 2.24[4] |

[1] Rat liver homogenates, prepared in isotonic KCl, were incubated with 12 μmoles of fructose-diphosphate for 20 minutes. When fluoride was present, a level of 0.01 M was used. The samples were deproteinized with barium/zinc and analyzed for methylglyoxal by the method of Dische and Robbins ('34).

[2] All figures are given with their standard deviation.

[3] Pair-fed with the deficeint animals.

[4] Statistically significantly higher than controls at $P < 0.05$.

tion should be low if it was nonenzymatic (compare with table 4). But unknown factors in a homogenate may play a non-enzymatic catalytic role.

*Formation of* D-*lactic acid.* Assays for glyoxalase indicated that the enzyme was somewhat, but not drastically, decreased in tissues from deficient rats, in agreement with the observations of Drummond ('61). Thus, although in homogenates glyoxalase would be inactive, since no glutathione was added, in the whole animal one might expect that D-lactic acid would be formed when tissues, which are blocked in glycolysis, accumulate methylglyoxal. Therefore an effort to detect D-lactic acid was made. Initially only the lactic acid-requiring mutant of *L. casei* was used. Considerable quantities of "D-lactic acid" were excreted by rats. However, other D-α-hydroxy acids may substitute as a growth factor for this organism (Camien and Dunn, '53), and it became evident that more than lactic acid was being measured.[5] That D-lactic acid was one component of the urine was indicated by the fact that lactic acids isolated through countercurrent distribution (Camien et al., '59) had growth promoting activity (compare with Judge and van Eys, '62). D-Alanine also has a feeble growth promoting activity for the *L. casei* mutant (Camien and Dunn, '53). Pyrithiamine-treated rats have a generalized aminoaciduria, as indicated by the analysis of their urine. (An automatic amino acid analyzer was used.) However, the increase in alanine, or even total amino acids, could not account for more than a very small fraction of the growth stimulation.

To get a more specific measure of D-lactic acid an enzymatic assay was used. This method is not quantitative since recoveries from urine of known amounts of D-lactic acid were poor, largely because of inhibition of the enzyme by the urine. Thus the *rate* of acetylpyridine-*DPN reduction by urine was only one-third that of an equivalent amount of D-lactic acid in pure solution. An approximation was gained, however, which was probably an underestimate. The results indicated that both deficient and normal animals excreted D-lactic acid and that the deficient animals did not excrete a great deal more. But since the total lactic acid excretion dropped markedly, the percentage of D-

TABLE 4

*Spontaneous formation of methylglyoxal from triosephosphates*[1]

| Additions | Methylglyoxal formed |
|---|---|
| | *mμmoles* |
| DL-Glyceraldehyde-3-P | 290 |
| DL-Glyceraldehyde-3-P + triosephosphate isomerase | 165 |
| Dihydroxyacetone-P | 0 |
| Dihydroxyacetone-P + triosephosphate isomerase | 47 |

[1] Five micromoles of DL-glyceraldehyde 3-phosphate or dihydroxyacetone phosphate were incubated at 37°C for 30 minutes in 3 ml 0.05 M glycylglycine, pH 6.5. When added, 1,200 units of triosephosphate isomerase were used. After incubation the samples were analyzed for methylglyoxal by the method of Dische and Robbins ('34).

[5] The rats that received pyrithiamine treatment showed a sudden increase in microbiological D-lactic acid excretion not paralleled by the enzymatic assay; this increase was not shared by the oxythiamine-treated rats. This precipitous increase coincided with the sudden onset of neurological symptoms about 24 hours before death. The conclusion is inescapable that some compound (or compounds) is excreted by pyrithiamine-treated rats, other than D-lactic acid. The nature of this growth-promoting factor will be investigated.

TABLE 5

*D-Lactic acid excretion by pyrithiamine-treated rats*[1]

| Group | Period | Average urine volume | D-Lactic acid excretion | | Total lactic acid | D-Lactic acid/total lactic acid ratio | |
|---|---|---|---|---|---|---|---|
| | | | Enzymatic | Micro-biological | | Enzymatic | Micro-biological |
| | | *ml/24 hours* | *μmoles/ 24 hours* | *μmoles/ 24 hours* | *μmoles/ 24 hours* | | |
| Controls | 1 | 5.0 ± 0.42 | 2.24 ± 0.465 | 3.95 ± 1.093 | 28.68 ± 7.98 | 0.09 ± 0.017 | 0.06 ± 0.008 |
| Pyrithiamine | 1 | 4.9 ± 0.29 | 2.12 ± 0.345 | 4.91 ± 0.694 | 36.26 ± 6.20 | 0.14 ± 0.013 | 0.16 ± 0.036 |
| Controls | 2 | 4.7 ± 0.33 | 2.83 ± 0.558 | 15.03 ± 2.55 | 54.38 ± 15.74 | 0.08 ± 0.030 | 0.27 ± 0.964 |
| Pyrithiamine | 2 | 4.7 ± 0.34 | 2.05 ± 0.146 | 8.65 ± 0.638 | 11.64 ± 2.27[2] | 0.19 ± 0.039[3] | 1.02 ± 0.309[4] |

[1] A group of 5 controls and a group of 10 rats, treated with pyrithiamine at a level of 10 μg/ml of drinking water as described previously (van Eys, '61), were housed in individual metabolism cages. Urine was collected daily and pooled to give sufficient material for analysis. After 6 days the pyrithiamine-treated rats started rapid weight loss and at the eleventh day all treated animals showed neurological symptoms. They were killed on the twelfth day. The periods refer to the first and second period of 6 days, which were averaged to give an estimate of the significance of the data obtained. All figures are given with their standard deviation.
[2] Significantly lower than controls at a level of $P < 0.05$.
[3] Significantly higher than controls at a level of $P < 0.02$.
[4] Significantly higher than controls at a level of $P < 0.05$.

TABLE 6

*D-Lactic acid oxidation by thiamine-deficient rats*[1]

| Group | No. animals | Liver | | | Kidney | | |
|---|---|---|---|---|---|---|---|
| | | Protein | | $O_2$ uptake | Protein | | $O_2$ uptake |
| | | Whole homogenate | "Particles" | | Whole homogenate | "Particles" | |
| | | *mg/mg tissue* | *mg/mg tissue* | *mμmoles/hour/ mg liver* | *mg/mg tissue* | *mg/mg tissue* | *mμmoles/hour/ mg kidney* |
| Controls | 4 | 0.50 ± 0.179 | 0.32 ± 0.110 | 1.7 ± 0.90 | 0.38 ± 0.059 | 0.18 ± 0.044 | 3.2 ± 3.60 |
| Oxythiamine | 4 | 0.46 ± 0.074 | 0.23 ± 0.159 | 1.2 ± 0.73 | 0.35 ± 0.139 | 0.24 ± 0.067 | 4.3 ± 4.73 |
| Pyrithiamine | 4 | 0.55 ± 0.230 | 0.28 ± 0.059 | 1.7 ± 1.53 | 0.49 ± 0.183 | 0.29[2] ± 0.117 | 2.3 ± 1.63 |

[1] A 20% homogenate was prepared in isotonic KCl. The particulate matter was collected by centrifugation and resuspended in the original volume of KCl. Each Warburg flask contained, in a final volume of 3.0 ml, the following: 30 μmoles of D-lactate, 50 μmoles of phosphate buffer, pH 7.6; 0.3 μmoles of methylene blue. KOH was in the centerwell, air was the gas phase. The rates were corrected for endogenous $O_2$ uptake. All figures are given with their standard deviation.
[2] Significantly different from control at $P < 0.02$.

lactic acid out of the total increased toward the end of the deficiency period, especially in animals treated with pyrithiamine (table 5).

*Pyruvate levels in thiamine deficiency.* If D-lactic acid resulted chemically or enzymatically from methylglyoxal, accumulation of methylglyoxal would result in accumulation of D-lactic acid unless this compound could be further metabolized.

Recent reports (Tubbs and Greville, '59) re-emphasized the occurrence of a nonpyridine nucleotide dependent enzyme that oxidizes D-lactic acid to pyruvic acid (Kaplan et al., '51; Mahler et al., '53). Thiamine-deficient animals do not appear to be deficient in this enzyme (table 6), although the data show wide scatter.

It has long been known that in thiamine deficiency the pyruvate/lactate ratio increases (Stolz and Bessey, '42). Pyruvate excretion apparently increases and lactate excretion decreases. But the increase in pyruvate may be only a paired-feeding artifact. Pair-fed animals lose weight, but not as rapidly as their deficient mates. Considerable quantities of body material are metabolized during weight loss. This involves considerable amount of cellular material. When, as a rough measure of total metabolism, the grams of food consumed are corrected for a change in body weight, the pyruvate excretion remains about the same (fig. 2). Thus the animal can handle the bulk of the pyruvate produced and a small percentage spills over in the urine. Thiamine-deficient rats produce more pyruvate due to tissue catabolism and hence excrete more than their pair-fed mates.

However, the ratio pyruvate/lactate increases toward the end of the deficiency, even when compared with pair-fed controls (table 7). Starvation tends to lower the L-lactic acid excretion, making the difference less marked, but still significant when the animals are pair-fed. The results obtained in a group of pyrithiamine-treated rats are shown in figure 3.

Under our conditions of study the reported accumulation of methylglyoxal is apparently not of the order of magnitude that has been claimed. The accumulation in brain homogenates is, however, significantly elevated in thiamine deficiency. Under the latter circumstances methyl-

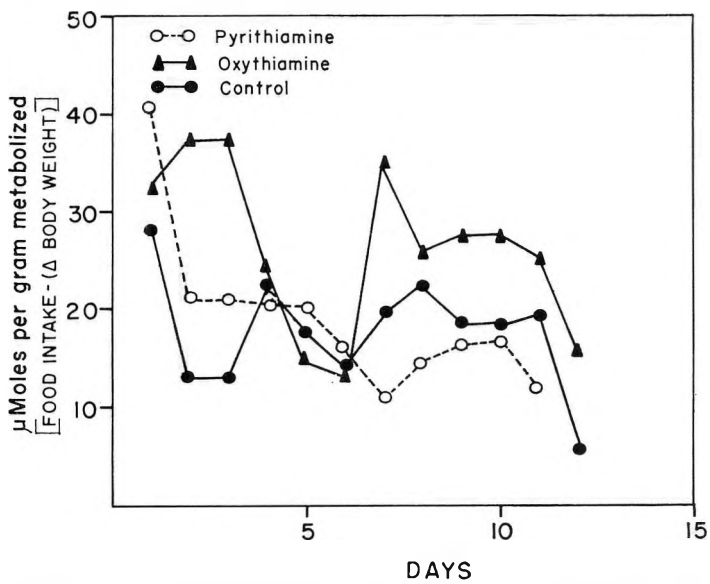


Fig. 2 Average daily pyruvate excretion by thiamine-deficient rats. The control animals were not pair-fed. The dose of antimetabolites administered was 50 µg/ml of drinking water for oxythiamine and 5 µg/ml of drinking water for pyrithiamine. The rats were weighed daily and their change in body weight subtracted from their food intake to gain an approximate measure of their amount of metabolic load. The figures are the average of 5 controls and 10 rats in each deficient group.

TABLE 7

*Lactate and pyruvate excretion in thiamine-deficient rats*[1]

| Weeks fed diet | Lactate | | Pyruvate | | Pyruvate/lactate ratio | |
|---|---|---|---|---|---|---|
| | Control | Deficient | Control | Deficient | Control | Deficient |
| | *μmoles/24 hours* | | *μmoles/24 hours* | | | |
| 3 | 55.5 ± 14.8 | 99.3 ± 6.19[2] | 2.71 ± 0.35 | 20.23 ± 4.47[2] | 0.05 | 0.20 |
| 4 | 33.0 ± 9.91 | 163.5 ± 60.10[2] | 2.74 ± 2.17 | 5.28 ± 1.13[3] | 0.08 | 0.03 |
| 5 | 28.3 ± 5.23 | 89.2 ± 39.5[2] | 2.11 ± 0.93 | 6.02 ± 0.93 | 0.08 | 0.07 |
| 6 | 19.5 ± 7.62 | 47.9 ± 19.84[3] | 3.10 ± 0.90 | 14.31 ± 3.71[2] | 0.16 | 0.30 |

[1] Controls were pair-fed with the deficient animals. Six rats were used per group. The urines were pooled per rat for each week. All figures are given with their standard deviation.
[2] Deficient group significantly higher than control group ($P < 0.05$).
[3] No statistically significant difference.

glyoxal is derived from fructose-diphosphate, and therefore probably from triosephosphates. The evidence cited favors the view that this is an enzymatic conversion. Even if this transformation is nonenzymatic, it is possible that such a transformation occurs *in vivo*. The excretion of traces of D-lactic acid argues in favor of such an *in vivo* formation of methylglyoxal.

The decrease in L-lactic acid in thiamine deficiency is probably partially the result of inactivity of the rats. But if this were the only explanation, a parallel decrease in pyruvate should occur (Stolz and Bessey, '42). The data (fig. 2) show that such a parallel decrease does not occur.

Tissue pyruvate/lactate ratio is an indication of the DPN/DPNH ratio in the tissues (Bücher and Klingenberg, '58).[6] Urinary levels of pyruvic and lactic acid also would tend to reflect changes in such

[6] This is not a thermodynamic equilibrium since most nucleotide is bound to dehydrogenase; thus, a DPNH/DPN ratio of one corresponds to a lactate/pyruvate ratio of approximately 20.

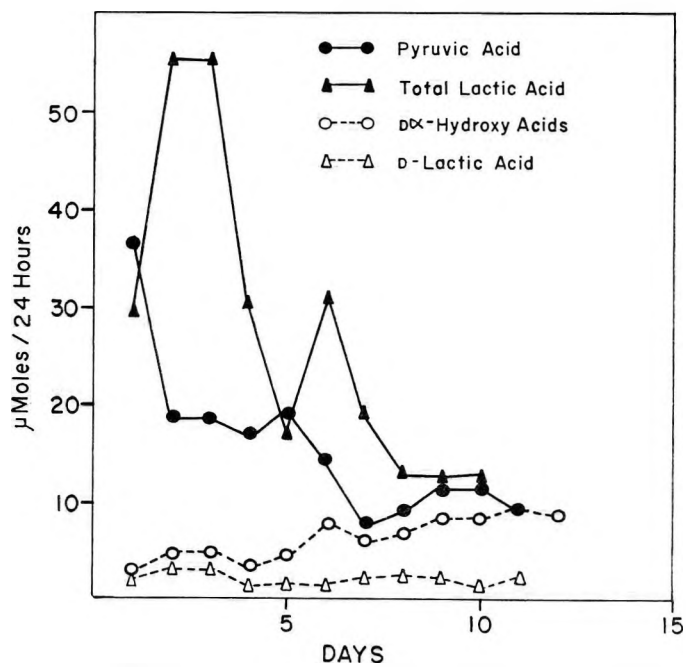


Fig. 3 The average daily excretion of total lactate, pyruvate and D-lactic acid measured both microbiologically and enzymatically on 10 rats receiving 5 μg of pyrithiamine/ml of drinking water.

a ratio in the animal. If all pyruvate resulted from glycolysis, then the increase in this ratio could result only from a rapid removal of DPNH other than by L-lactate formation. However, the glycerophosphate cycle is blocked, and the overall catabolism of the animals would be expected to generate reduced nucleotides rather than oxidized DPN and TPN. Thus a source of pyruvate other than glycolysis must operate. This could be the result of extensive amino acid degradation,[7] but is quite likely that at least part is derived from D-lactic acid. If that is the case, the known enzymes would effect the transformation methylglyoxal → D-lactic acid → pyruvate. This sequence is not in contradiction to the low oxidized pyridine nucleotide levels since the latter enzyme is nonpyridine nucleotide-dependent.

Whether the methylglyoxal and D-lactic acid are derived *in vivo* from threonine or from carbohydrate (as was the case in homogenates) cannot be settled. Experiments are in progress to elucidate this point.

A bypass through methylglyoxal would result in the loss of generation of *glycolytic* ATP.[8] This could be a serious metabolic defect. In fact, in thiamine deficiency the shunt is blocked at the transketolase stage, pyruvic acid oxidation may become impaired, glycolysis is indirectly blocked; thus no avenue of carbohydrate metabolism is open.

A corollary is that the high pyruvate/lactate ratio would not allow gluconeogenesis through reversal of the glycolytic pathway because of the very low DPNH and TPNH levels. Thus the low glycogen content of thiamine-deficient tissues is again a natural consequence of the syndrome.

Of the three pathways mentioned the Krebs cycle is probably the least impaired. This is especially indicated by the fact that a thiamine-deficient animal may survive for prolonged periods of time when fed a pure lipid diet.

## SUMMARY

Thiamine-deficient animals did not excrete large quantities of methylglyoxal, but their tissues accumulated the intermediate when incubated with fructose-diphosphate.

D-Lactic acid was excreted by both normal and thiamine-deficient rats, but the ratio of D/L-lactic acid was significantly greater in deficient animals. The pyruvate/lactate ratio increased in deficient animals, especially the pyruvate/L-lactate ratio.

Since glyoxalase and D-lactate oxidase were present in normal and deficient rats in significant amounts, it was proposed that most formed methylglyoxal was further metabolized to pyruvate and that this pathway is at least partially responsible for increased pyruvate/lactate ratios.

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[7] Analysis of average animal protein shows that the majority of the constituent amino acids are made up of a very select few: aspartic acid, glutamic acid, serine, glycine and alanine. Of these, 4 readily give rise to pyruvate.

[8] There is considerable evidence that glycolytic metabolites are not in ready equilibrium with oxidative metabolites. Thus glycolytic DPNH is not mitochondrial DPNH, and some evidence has been put forth to show that glycolytic ATP is not equivalent to mitochondrial ATP. For a review see van Eys ('61b).

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# Calcium, Phosphorus and Citrate Interactions in Oxalate Urolithiasis Produced with a Low-Phosphorus Diet in Rats[1,2]

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Despite the large number of studies on urolithiasis, the etiology of calculi in man and domestic animals has not been elucidated. Since albino rats rarely produce calculi under normal conditions, some method of inducing calculogenesis is necessary before urolithiasis can be studied. Schneider and Steenbock ('40) and Morris and Steenbock ('51), using a low-phosphorus diet containing egg whites, produced calcium citrate calculi in weanling rats. Sager and Spargo ('55) produced citrate stones in mature rats, using a low-phosphorus diet having a high calcium:phosphorus ratio. Sager and Spargo felt that the low-phosphorus level allowed excessive calcium absorption from the gut resulting in a high level of nonprotein bound calcium in the blood (calcium citrate complex). The amount of calcium citrate complex in the blood was felt to be further increased by hypoproteinemia. They concluded that the resulting large excretion of calcium and citrate in the urine caused the formation of calcium citrate calculi. Van Reen et al. ('59a, '59b), however, using a diet containing 0.18% of phosphorus instead of the 0.03% levels in the diets mentioned above, published data indicating that a constant calcium:phosphorus ratio that results in calculi formation with a low-phosphorus level will not necessarily produce calculi with a higher phosphorus level.

In all of these studies, calcium citrate stones were formed. Since calcium citrate stones and extremely low dietary phosphorus levels are rare in both man and livestock, the existing means of inducing urolithiasis do not appear to depict urolithiasis as it commonly occurs in man and livestock. This paper describes a moderately low phosphorus diet which will produce a high incidence of calcium oxalate stones in weanling rats. The effect of the dietary phosphorus level, calcium:phosphorus ratio, vitamin D, age and mineral metabolism on calculogenesis is also described.

## EXPERIMENTAL

One-hundred-and-twenty weanling, male Wistar rats were divided into 5 groups which received either the basal diet A, shown in table 1,[3] or a modification of diet A as to calcium, phosphorus and vitamin D levels as shown in table 2. In groups D and E the extra phosphorus was added as $Na_2HPO_4$. The ratio between calcium carbonate and calcium lactate indicated in table 1 was maintained in all the diets. Twenty-eight mature rats averaging 20 weeks of age and 380 gm in weight were distributed equally into groups receiving diet A (calculogenic) or diet D (noncalculogenic) to determine whether age had an effect on susceptibility to urolithiasis. All animals were kept in an air-conditioned room and received feed and tap water ad libitum. Water consumption was measured and the mineral content was included in all metabolism data. Preliminary experiments with 100 weanling rats showed no differences in incidence of calculi between tap and distilled water.

To follow any histological and metabolic changes that might occur during the development of urolithiasis, one to three rats were sacrificed from each group at

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[3] Vitamins A and D were supplied by the Nopco Chemical Company, Harrison, New Jersey.

TABLE 1

*Basal diet A*

| | *gm/kg of diet* |
|---|---|
| Casein | 180 |
| Glucose[1] | 527 |
| Corn starch[2] | 200 |
| Cotton seed oil[3] | 50 |
| Salt mix[4] | 40 |
| Vitamin mix[5] | 3 |

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Argo Cornstarch, Corn Products Company, Argo, Illinois.
[3] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.
[4] Salt mix (gm): NaCl, 105; KCl, 290; $CaCO_3$, 210; $MgSO_4$, 90; Ca lactate, 236; ferric citrate, 16.15; $K_2Al_2(SO_4)_4 \cdot 12H_2O$, 0.09; $CuSO_4 \cdot 5H_2O$, 0.39; NaF, 0.57; KI, 0.05.
[5] To supply the given number of mg per kg of diet: thiamine·HCl, 5; riboflavin, 10; pyridoxine, 5; niacin, 100; p-aminobenzoic acid, 200; inositol, 1,000; folic acid, 0.2; biotin, 0.06; ascorbic acid, 400; Ca pantothenate, 20; choline chloride, 500; menadione 1, α-tocopherol, 50; vitamin A, 20,000 USP units; vitamin D, 5,000 USP units.

TABLE 2

*Experimental outline showing treatment variables*

| Group | Dietary composition | | | | No. rats | |
|---|---|---|---|---|---|---|
| | P[1] | Ca[1] | Ca/P | Vitamin D[2] | Weanling[3] | Mature[4] |
| | % | % | | | | |
| A | 0.16 | 0.56 | 3.52 | 5 | 35 | 14 |
| B | 0.16 | 0.26 | 1.66 | 5 | 21 | — |
| C | 0.16 | 0.55 | 3.48 | 10 | 10 | — |
| D | 0.33 | 0.55 | 1.64 | 5 | 22 | 14 |
| E | 0.35 | 1.36 | 3.92 | 5 | 22 | — |

[1] By chemical analysis.
[2] Thousands of USP units/kg of diet.
[3] Weanling (about 28 days old).
[4] Mature (about 20 weeks old).

10 weeks and at two-week intervals to 40 weeks. The right femur was saved for bone ash determinations. Blood and urine samples were taken for mineral analysis and the kidneys and bladders were examined with a 20 × microscope for calculi. One-half of each kidney was fixed in alcoholic formalin and one-half saved for mineral analysis. Prior to sacrifice, 7-day mineral metabolism trials were conducted on at least 10 rats from each group during the 9- to 40-week interval. The exact number are shown in the tabular data. Since the only changes in mineral metabolism were those expected with the normal growth process and these changes were parallel in all groups, the data are presented as averages for each group treatment.

Serum inorganic phosphorus, and total urine, kidney and fecal phosphorus were determined by appropriate adaptations of Fiske and Subbarow ('25). Serum and urine magnesium were determined according to Kunkel et al. ('47). Oxalic acid, citric acid and urea were determined according to Powers and Levatin ('44), Taylor ('53) and Conway ('57), respectively. The petroleum ether used in the citric acid determination was purified according to Weil-Malherbe and Bone ('49). Serum calcium was determined by the method of Ferro and Ham ('57). Urinary calcium was determined with a Coleman model 21 flame photometer after adjusting samples and standards to the same sodium level. Calcium and magnesium in the kidney and feces digests were determined according to Michaels et al. ('49) except that the calcium oxalate precipitate was dissolved in sulfuric acid and titrated with potassium permanganate. The pH of the urine was determined on fresh samples using a Beckman model G pH meter with a one-drop electrode.

The bladder calculi were analyzed by the infrared method of Weissman et al. ('59). Confirmatory chemical analyses for calcium, magnesium, phosphorus, oxalate and citrate were made using the methods of Ferro and Ham ('57), Kunkel et al. ('47), Hirata and Appleman ('59), Powers and Levatin ('44) and Taylor ('53), respectively. An x-ray diffraction pattern was made and the *d* values checked with those reported by Prien and Frondel ('47).

The fixed kidneys were imbedded in paraffin and stained with the von Kossa silver nitrate stain for calcium and the periodic acid-Schiff (PAS) stain for mucopolysaccharides. We found, as Moffat did ('58), that an aqueous periodic acid solution removed the silver deposits. Although Moffat remedied this by gold toning, we found that changing to an alcoholic periodic acid solution as suggested by Baker and Sison ('54) also corrected this difficulty. A ferric mannitol stain (Lillie, '54) for acid mucopolysaccharides and the ferrous iron replacement stain for calcium as given by Casselman ('59) were each used in a few instances to gain some insight into the nature of the PAS-positive

material and the calcium deposits. Data were analyzed statistically by the use of analysis of variance (Snedecor, '61).

## RESULTS

*Incidence and composition of calculi.* Renal urolithiasis was manifested in three ways: production of stones, formation of small white flakes detectable with the dissecting microscope, and calcification in the renal tubules and in the renal tissue. Only weanling group A had a significant amount of actual stone formation (table 3). Urolithiasis was evident at the end of 9 weeks, the time of earliest observation; hence the initial occurrence is not known. The severity of urolithiasis increased with time. Bladder stones were not noted until 14 weeks. The calculi were brownish yellow, hard, crystalline aggregates very similar to the calcium oxalate crystals from urine sediment pictured by Hawk et al. ('54). The renal stones were readily visible in the pelvis with the dissecting microscope, but were not large enough to isolate and analyze chemically. The stones did not appear to interfere with renal function as indicated by urea clearance tests. Three of the 16 rats in group A with renal stones also had larger bladder stones, which infrared analysis showed to be predominantly calcium oxalate. The curves for calcium oxalate, calcium citrate[4] and one of these calculi known from chemical and x-ray analysis to contain 90% of calcium oxalate dihydrate and 10% of calcium citrate are shown in figure 1. The 10% citrate calculus was the only calculus that showed the small peak at 7 μ, indicating that the other two contained even less citrate. The magnesium and phosphorus content was negligible.

TABLE 3

*Effect of treatment on incidence of renal calculi in weanling rats*

| Group | No. rats | Norm[1] | Hist[2] | Fl[3] | St[4] |
|---|---|---|---|---|---|
| A | 35 | 7 | 2 | 10 | 16 |
| B | 21 | 13 | 7 | 0 | 1 |
| C | 10 | 5 | 0 | 5 | 0 |
| D | 22 | 19 | 3 | 0 | 0 |
| E | 22 | 13 | 6 | 2 | 1 |

[1] Norm indicates normal animals with no gross or histological symptoms of urolithiasis.
[2] Hist indicates rats showing only histological evidence of nephrocalcinosis.
[3] Fl indicates rats having white flakes but no stones in the kidneys.
[4] St indicates rats showing actual stone formation detectable with a 20× dissecting microscope.

None of the rats that were mature at the start of the experiment developed calculi; hence the data in the tables include only those for the weanling rats.

The stones from the only rat in group B that developed gross signs of urolithiasis proved to be uric acid. Since the condition was accompanied by respiratory

[4] Calcium citrate was supplied by Chas. Pfizer and Company, Brooklyn, New York.

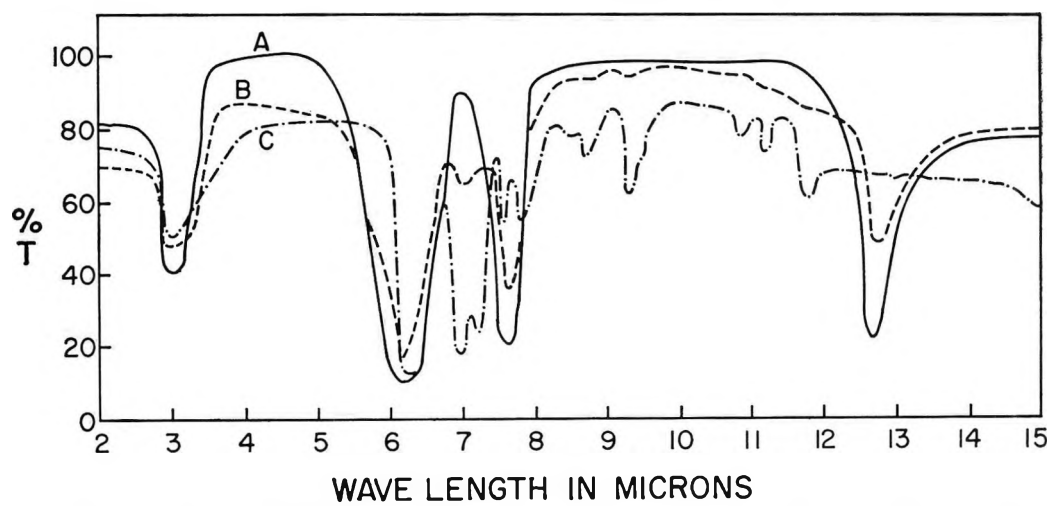


Fig. 1 Infrared curves: A, calcium oxalate; B, calculus containing 90% of calcium oxalate dihydrate and 10% of calcium citrate; C, calcium citrate.

TABLE 4

*Effect of dietary treatment on calcium metabolism*

| Group | No. rats | Serum Ca | Ca intake[1] | Urinary Ca | Fecal Ca | Apparent Ca absorption | Ca retention |
|---|---|---|---|---|---|---|---|
| | | *mg/100 ml* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* |
| A | 22 | 9.32 | 92.0 | 13.2 | 56.0 | 36.0 | 22.8 |
| B | 15 | 8.45** | 45.3 | 1.78 | 24.0 | 21.3** | 19.5 |
| C | 10 | 9.56 | 78.3 | 14.9 | 35.0 | 43.3 | 28.4 |
| D | 16 | 8.94 | 99.2 | 0.936 | 75.8 | 23.4* | 22.5 |
| E | 17 | 9.10 | 225 | 5.94 | 182 | 42.8 | 37.0** |

[1] Includes calcium in both feed and water.
* Significant difference ($P < 0.05$) from group A.
** Highly significant difference ($P < 0.01$) from group A.

trouble resulting in death, it was concluded that factors other than dietary treatment were involved. The single rat in group E that was indicated in table 3 as having stones had a single small stone.

This is the first report of the production of oxalate stones by a low-phosphorus diet. The previously mentioned explanation by Sager and Spargo ('55) may adequately explain the formation of calcium citrate stones with extremely phosphorus-deficient diets. Van Reen et al. ('59a), however, produced calcium citrate stones with a diet containing a higher level of phosphorus which was comparable to the calcium and phosphorus levels of basal diet A. Serum calcium was normal in all rats (table 4), indicating that large increases in the calcium citrate complex in the blood did not occur with these diets. That Van Reen et al. ('59b) reported a urinary calcium excretion of about 6 mg per 100 gm of body weight per day, which was similar to the excretion obtained in group C (table 5), indicates that their serum levels were probably similar to ours. But they reported a citrate excretion of about 10 mg per 100 gm of body weight per day, which was twice as great as was observed in group C (table 6). Van Reen et al. ('59b) did not give any data on urinary pH, but since all of the calcium and most of the magnesium was supplied as the carbonate, it seems quite likely that their diet was more alkaline than ours. This probably caused both the increase in urinary citrate excretion and an alkaline urinary pH, which together may explain the formation of calcium citrate stones in their experiments.

Another manifestation of urolithiasis was the occurrence of small white flakes in the pelvis of the kidneys. These white flakes were distinguished from the stones because they did not have the color, crystalline appearance or hardness of the stones. Presumably, these flakes represented an early stage of stone formation since they were seen in the greatest amounts in weanling group A and sometimes occurred along with the stones. Only trace-to-moderate amounts of flakes were noted in 5 of the 10 rats in group

TABLE 5

*Urinary calcium concentration and excretion*

| Group | Concentration | Daily excretion |
|---|---|---|
| | *mg/100 ml* | *mg/100 gm body weight* |
| A (34)[1] | 108 | 4.23 |
| B (20) | 18.3** | 0.558** |
| C (10) | 242** | 6.84* |
| D (22) | 8.88** | 0.237** |
| E (22) | 56.0** | 2.29** |

[1] Numbers in parentheses indicate number of animals included in the averages.
* Significant difference ($P < 0.05$) from group A.
** Highly significant difference ($P < 0.01$) from group A.

TABLE 6

*Urinary citric acid*

| Group | No. rats | Concentration | Daily excretion |
|---|---|---|---|
| | | *mg/100 ml* | *mg/100 gm body weight* |
| A | 34 | 46.6 | 1.92 |
| B | 19 | 14.5* | 0.45** |
| C | 10 | 151** | 4.38* |
| D | 21 | 34.3 | 0.89 |
| E | 21 | 102** | 3.83* |

* Significant difference ($P < 0.05$) from group A.
** Highly significant difference ($P < 0.01$) from group A.

C, and slight amounts in two of the 22 rats in group E. Samples were not sufficient for composition analysis.

Urolithiasis also was manifested by calcium deposition in the lumen of renal tubules or in the renal tissue, or both. In weanling group A these deposits sometimes occurred in the form of plaques beneath the epithelium of the minor calyces or just below the surface of the papilla. In one instance the plaque had broken through the surface of the epithelium. We found no evidence, however, that these plaques were serving as the point of development of free stones as suggested by Randall and Melvin ('37) and Randall ('40). Occasionally, these plaques were associated with PAS-positive material. Since the plaques stained with both the silver and ferrous iron substitution techniques, it was concluded that the plaques contained some form of calcium other than or in addition to the oxalate.

Renal papillary calcification was seen frequently only in the weanling rats of group A. Sixteen of these rats had either plaques or tubular calcification in the papillae. Only one rat in group B, two in group C and one in group D had slight calcification in the papillae. Several large deposits were observed in a renal papilla of a rat which had been fed diet E for 36 weeks. This was not the same rat that was reported to have a single renal stone.

Only small deposits, usually in one or two collecting tubules in the medulla, were noted in the other weanling rats of groups B, C, D and E. Such deposits were also seen in mature rats. Sometimes the calcification appeared to be in the basement membrane of the tubule.

PAS-positive casts, sometimes partially calcified, were observed in the renal tubules of all groups. There was a tendency for them to be larger and more numerous in the young rats of group A, although even here casts were not observed in every kidney. Of course, our studies were limited to a few longitudinal sections of the kidney. Our observations that some casts were partially calcified and that some plaques were associated with PAS-positive material indicate that this PAS-positive material may be involved in calculogenesis. Whether PAS-positive material was contained in the flakes or stones is not known. Results of the ferric mannitol stain showed that the casts possibly contained acid mucopolysaccharides.

*Urine volume and pH.* Data on urinary pH (table 7), water consumption, volume and specific gravity of urine indicated that neither low water intake, low urine volume, concentrated urine nor alkaline urine was the cause of urolithiasis in group A. In fact the average water consumption and urine volume were higher and the average specific gravity of urine was lower than in any other group.

*Mineral metabolism.* Magnesium metabolism was studied because magnesium is frequently an important stone component and because Watchorn ('32), Hammarsten as quoted by Jeghers and Murphy ('45) and Vermeulen et al. ('51) have some evidence that magnesium can influence stone formation. But, other than a slight decrease in absorption and urinary excretion of magnesium in groups D and E with added phosphorus, no differences in magnesium metabolism were noted. Considering this and that magnesium was not a significant stone component, we felt assured that under the conditions of our experiments magnesium was not playing a major role in calculogenesis.

It has been postulated that the increased calcium absorption and excretion in the urine resulting from a high calcium: phosphorus ratio with low-phosphorus diets is the cause of urolithiasis. In our studies group C showed a higher concentration and excretion of calcium in the urine than group A and yet did not produce stones (tables 4 and 5).

TABLE 7

*Effect of dietary treatment on daily gains and urinary pH*

| Group | Gain[1] | pH |
|---|---|---|
| | *gm/day* | |
| A | 1.99 (35)[2] | 6.58 (35) |
| B | 3.23 (21)** | 6.15 (20)** |
| C | 1.58 (10) | 6.23 (10) |
| D | 4.85 (18)** | 6.79 (22) |
| E | 3.52 (22)** | 7.44 (22)** |

[1] For first 5 weeks of the experiment.
[2] Numbers in parentheses indicate number of animals included in the averages.
** Highly significant difference ($P < 0.1$) from group A.

Since oxalate stones were unexpected, a few urinary oxalate determinations were made but showed no differences, the average excretion (mg per day) being 1.12 (10 rats), 1.03 (5 rats), 1.00 (3 rats), 1.21 (6 rats) and 1.18 (4 rats) for groups A, B, C, D and E, respectively. Therefore, it was apparent that the increased incidence and severity of urolithiasis in group A was not due to a difference in urinary oxalate excretion.

There is a great deal of controversy over the importance of urinary citric acid in preventing stone formation by forming soluble complexes with calcium. As shown in table 6, doubling the vitamin D content of the diet (group C) significantly increased the citric acid concentration and excretion. But the increase in citric acid excretion was accompanied by a greater increase in calcium excretion. Considering that the most calcium that can be complexed is less than 20% of the weight of citric acid, the increase in citric acid excretion in group C was not sufficient to complex the additional amount of urinary calcium. In fact, the amount of calcium in excess of that which could combine with citrate was higher in group C than in group A. Therefore, the lack of stone formation in group C cannot be explained by the increase in urinary citric acid excretion.

Since calculogenesis in group A cannot be explained by changes in the calcium, citrate or oxalate composition of the urine, some of the more subtle changes indicated by the data were examined. First of all, group B, with a lower calcium level (also reduced Ca:P ratio) than group A, had significantly increased growth ($P < 0.01$) (table 7), but no significant changes in calcium (table 4) or phosphorus retention (table 8). Group C, with added vitamin D, had the poorest growth of all the groups, yet had the same phosphorus retention as group D, which had the best growth, and slightly greater calcium retention than group D. Schneider and Steenbock ('39) and Cramer and Steenbock ('56) have postulated that the decrease in growth caused by adding vitamin D to low-phosphorus diets is due to a differential shunting of phosphorus from the metabolic pool to bone instead of to soft tissues. In our experiment, however, three bone ash assays from each group at 18 weeks showed that group C had the lowest average bone ash (56%). This was significantly lower ($P < 0.01$) than group D (60%) although not significantly lower than weanling group A (58%). Bone ash assays at 10 weeks showed the same differences but a lower percentage of ash. In addition, the phosphorus content of the kidneys of the various groups was extremely constant, varying from 0.28 to 0.29%. Group E had a lower growth rate than group D although phosphorus retention was only slightly lower than that of group D and calcium retention was increased. These differences in growth indicate that the low growth rate in group A was due at least partially to other factors than a gross dietary phosphorus deficiency. This conclusion was further supported by the fact that the serum inorganic phosphorus levels (table 8), while tending to be low in group A, were not outside the normal range.

## DISCUSSION

The basal low-phosphorus calculogenic diet used in this experiment has several advantages over the extremely low-phos-

Table 8

*Phosphorus metabolism*

| Group | No. rats | Serum inorganic P | P intake | Urine P | Feces P | Apparent P absorption | P retention |
|---|---|---|---|---|---|---|---|
| | | *mg/100 ml* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* |
| A | 22 | 7.50 | 24.3 | 0.229 | 15.2 | 9.05 | 8.82 |
| B | 15 | 8.66* | 24.4 | 1.96 | 12.7 | 11.7 | 9.70 |
| C | 10 | 7.31 | 21.0 | 0.174 | 7.46 | 13.5* | 13.3* |
| D | 16 | 7.90 | 57.2 | 10.6 | 33.0 | 24.2** | 13.6* |
| E | 17 | 7.64 | 56.0 | 0.640 | 43.0 | 13.0* | 12.4 |

* Significant difference ($P < 0.05$) from group A.
** Highly significant difference ($P < 0.01$) from group A.

phorus diets that include egg white rather than casein as the protein source. (1) The phosphorus level is high enough to permit reasonably good growth and to prevent any gross phosphorus deficiency symptoms; (2) it produces a mild form of calculi, which avoids interference from anuria or uremia in the physiological measurements; and (3) it produces calcium oxalate stones, which are clinically much more prevalent than citrate stones. The urolithiasis produced by our low-phosphorus diet differs, however, from that seen in humans in that it is not readily produced in mature rats; whereas in human urolithiasis is now mainly a disease of middle age. An exception to this occurs in underdeveloped countries where malnutrition results in urolithiasis at rather high rates in infants.

Vitamin A deficiencies and infection can be eliminated as causes of urolithiasis under the conditions of the experiment. All of the diets were made with the same vitamin mix, which contained adequate levels. The animals were kept in adjacent cages so that infection certainly would not have been limited to one group.

Apparently from the lack of stone formation in groups C and E, factors in addition to increased calcium absorption are involved in the production of urolithiasis with low-phosphorus diets. Also, under the conditions of this experiment both the low phosphorus level and the high calcium:phosphorus ratio are required for stone formation.

In recent years considerable work has been carried out to elucidate the possible role of an organic matrix material in urolithiasis (Boyce and Sulkin, '56; Sulkin and Boyce, '56). Considering our histological observations of PAS-positive casts and the lack of any changes in mineral metabolism which can adequately explain the occurrence of severe urolithiasis only in group A, it seems possible that the low phosphorus level and high calcium:phosphorus ratio may be a prerequisite for the formation of an organic matrix material in our experiments.

Our data are not complicated by changes in protein level of the diet and, since they include the fecal as well as urinary excretion of calcium and phosphorus, may give further insight into the results of Van Reen et al. ('59a,b). For example, these workers produced calculi with a 15% casein diet, containing 0.19% of phosphorus and 0.43% of calcium, but not with a 30% casein diet containing 0.37% of phosphorus and 0.86% of calcium. Since the latter diet had the same calcium:phosphorus ratio as the first and twice the mineral content, they concluded that the protein level may have been a factor in calculogenesis. We obtained similar results in groups A (0.16% of P, 0.56% of Ca) and E (0.35% of P and 1.36% of Ca) without changing the protein level. Therefore, apparently an adequate phosphorus level was more important in preventing urolithiasis than the increased protein level. Our data and that of Van Reen et al. ('59a) indicate that, at least in growing rats, the calcium:phosphorus ratio may be very critical in urolithiasis production as well as growth. None of the ratios used were very high and relatively small changes made the difference between producing and not producing urolithiasis.

## SUMMARY

A low-phosphorus (0.16%), normal calcium (0.56%) diet containing casein which produced a high incidence (80%) of mild urolithiasis in weanling male rats was described. This is the first experimental low-phosphorus diet that has been reported to produce calcium oxalate rather than calcium citrate stones. Calculi were produced in young rats by 9 weeks. Mature rats, receiving the diets for a 40-week period, did not develop stones. Both the low phosphorus level and the high calcium:phosphorus ratio were essential for stone formation in weanling rats.

Mineral metabolism and urine composition data indicated that urolithiasis was not a direct result of increased urinary calcium or oxalate excretion. This, combined with the frequent occurrence of large PAS-positive casts, led to the postulation that the low phosphorus level and high calcium:phosphorus ratio in the basal diet caused an increase in the production of an organic matrix material, which, combined with the high urine calcium level, resulted in severe urolithiasis. Doubling

the vitamin D level of the basal diet reduced the incidence and severity of urolithiasis. This effect was not the direct result of increased urine citrate excretion.

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# The Subcommissural Organ of the Vitamin $B_{12}$-Deficient Rat[1]

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Richardson and Hogan ('46) observed hydrocephalus in a significant number of the offspring from inadequately nourished female rats. Subsequent investigations in a number of laboratories have confirmed these observations (Giroud and Boisselot, '51; Nelson, '57). It was determined that the diet of Richardson and Hogan was deficient in both folic acid and vitamin $B_{12}$ (O'Dell et al., '48). Later work (O'Dell et al., '51) showed that a deficiency of vitamin $B_{12}$ alone in the maternal diet resulted in a much higher incidence of hydrocephalus in infant rats than the diet deficient in both folic acid and vitamin $B_{12}$.

Histological studies (Overholser et al., '54; Newberne and O'Dell, '58) have been made of brains from offspring littered by female rats maintained with diets such as those described above. Overholser et al. ('54) concluded that the hydrocephalus in rats littered by folic acid-deficient or vitamin $B_{12}$-deficient dams was a result of occlusion of the cerebral aqueduct which occurred between the sixteenth and eighteenth day of gestation. These investigators attributed the occlusion of the cerebral aqueduct to absence of a specialized group of "columnar ependymal cells" in the roof of the aqueduct and posterior part of the third ventricle. It was postulated that these cells secrete fluid into the ventricular system during embryonic development before the choroid becomes fully developed. The secretion at this time was considered to be of sufficient quantity to prevent collapse and closure of the aqueduct which is the narrowest part of the ventricular system. Newberne and O'Dell ('58) observed missing and detached ependymal cells, fragmentation of choroid epithelium, and lipid in choroid and ependymal cells.

Newberne and O'Dell ('59) extended the studies and observed that hydrocephalus in vitamin $B_{12}$-deficient embryonic rats was associated with alterations in mitosis of ependymal cells. The available evidence indicated that an abnormal histological pattern developed sometime between the fourteenth and sixteenth day of gestation in the deficient brain. It had been reported previously (Newberne and O'Dell, '58) that the "tall columnar cells" in the roof of the aqueduct and third ventricle were irregularly present in the hydrocephalus of vitamin $B_{12}$ deficiency. These cells, which are in fact those composing the subcommissural organ, were present in some of the brains with severe hydrocephalus. This observation indicated that these cells served some purpose during embryogenesis not necessarily related to elaboration of fluid sufficient simply by force of volume to maintain patency of the aqueduct.

The subcommissural organ (fig. 1) is comprised of a specialized area of ependyma located on the anteroinferior surface of the posterior commissure of the midbrain where the third ventricle continues into the cerebral aqueduct (Wislocki and Leduc, '52). The cells of this area are tall columnar epithelial cells and appear to be secretory in nature. The organ elaborates a substance that reacts positively with chrome alum-hematoxylin, aldehyde fuchsin, and periodic acid-Schiff reagents (Wislocki and Leduc, '54). The substance elaborated clings to the lumenal surface of the cells and accumulates within the lumen of the aqueduct. In all but a few vertebrates, including man, the secretion condenses to give rise to the proteinaceous fiber known as Reissner's fiber. This fiber begins as minute fibrils which unite within the aqueduct to form the structure known

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[1] This investigation was supported in part by grant A-3947 N.I.H., U.S.P.H.S.

as Reissner's fiber; this structure extends from the optic tectum through the lumen of the fourth ventricle and central spinal canal to terminate in the filum terminal at the posterior extremity of the spinal canal.

Selective staining of the secretion of the subcommissural organ and of the substance of Reissner's fiber has been reported by a number of investigators on many species (Stutinsky, '50, frog; Wislocki and Leduc, '52, rat; Bargmann and Schiebler, '52, dog, cat; Wingstrand, '53, chick; Wislocki and Roth, '58, man; Gilbert, '60, man). Wislocki and Leduc ('54) demonstrated by histochemical methods that, in the rat, the subcommissural organ is the site of pronounced enzymatic activity. The cells of the organ elaborate a secretion that extends from the nucleus to the lumenal surface and is remarkably similar in histochemical properties to the material stored in the posterior pituitary. Gilbert ('60) produced evidence that the subcommissural organ, in the rat, is related to water metabolism and postulated that the organ may release a hormone-like substance that acts at the renal level to influence water metabolism. By use of histochemical methods Gilbert ('60) demonstrated the secretory nature of the organ in 9 adult human brains.

In view of the numerous reports in recent literature relating to the subcommissural organ in many species it seemed worthwhile to attempt to elucidate the relationship, if any, between the organ and vitamin $B_{12}$- deficiency hydrocephalus. Because it has been observed that the kidney of the vitamin $B_{12}$-deficient newborn rat is markedly immature and exhibits positive tubular damage, the kidney was included in this study. Most vitamin $B_{12}$-deficient newborn exhibit widespread subcutaneous edema, a point of probable significance. The purpose of this report is to describe and illustrate certain histochemical and morphological differences that exisit in the brain and kidney of the vitamin $B_{12}$-deficient newborn rat as compared with its vitamin $B_{12}$-supplemented control.

## MATERIALS AND METHODS

Two types of basal diets were used in these experiments. The soybean meal diet (O'Dell et al., '51) reported previously was used in one group of animals. It consisted of the following ingredients: (in per cent) soybean oil meal, 70; glucose, 22; lard, 4; salts (Richardson and Hogan, '46), 4 and a vitamin supplement.[2] Another group of animals was maintained with the following diet: (in per cent) alpha protein (purified soybean protein), 30; sucrose, 55.7; soybean oil, 10; salts (Richardson and Hogan, '46), 4;[3] DL-methionine, 0.3.[4] CD strain rats were used. The animals were maintained in raised screen-bottom cages in an air-conditioned room with food and water supplied ad libitum from weaning. The females were fed the respective vitamin $B_{12}$-deficient diets at weaning in order to deplete body stores of the vitamin. At maturity the females were mated with normal males, and newborn were observed for gross abnormalities and early mortality. Control animals were fed the basal diets supplemented with 30 μg of vitamin $B_{12}$ per kg of diet. Criteria used to determine state of deficiency were a high incidence of hydrocephalus, subcutaneous edema, cyanosis, mortality of 90% or more during the first week, and histological evaluation based on pronounced immaturity of organs and tissues in deficient offspring.

Morphological and histochemical studies were made on brains and kidneys of 21 vitamin $B_{12}$-deficient hydrocephalic animals, 16 littermates of these, and 12 vitamin $B_{12}$-supplemented controls. Brains used for study were fixed by injecting the fixative through the left ventricle of the heart according to a modification of the method of Davies ('59). Following decapitation the cranial cap was carefully removed and the entire brain placed in fixative. Kidneys were removed and placed directly into the respective fixatives. Fixatives included Bouin's, Orth's, Susa, and 10% buffered formalin. Routine Technicon procedures were used for paraffin embedding. Medial sagittal sections of the

[2] A vitamin supplement supplied in mg/100 gm of diet: thiamine·HCl, 1.6; riboflavin, 1.6; pyridoxine·HCl, 1.6; Ca pantothenate, 4.0; choline chloride, 100; biotin, 0.02; folacin, 0.5; α-tocopherol, 3.0; 2-methyl-1,4-naphthoquinone, 1.0; vitamin A, 2,000 IU and vitamin D, 280 IU. The author wishes to thank Merck & Company for supplying the water-soluble vitamins, and Distillation Products Industries for vitamin A and α-tocopherol.

[3] Cobalt was omitted from salts mix in this diet.

[4] The same vitamin mix was used as in the above diet except that choline chloride was increased to 300.

brain and cross sections of the kidney were cut at 6 μ; stains that were used included hematoxylin and eosin, Gomori's chrome alum-hematoxylin, Gomori's aldehyde fuchsin, alcian blue-PAS, Masson's trichrome and protein bound sulfhydryl and disulfide by the method of Barnett and Seligman ('52).

Past observations had shown that there was a variation in degree of severity of ventricular dilatation of the hydrocephalic animals. Furthermore, most litters that contained animals exhibiting hydrocephalus also contained littermates with no evidence of gross dilatation. Certain animals revealed an occluded or stenotic cerebral aqueduct and others showed grossly dilated ventricles and patent aqueducts. Thus it appeared that hydrocephalus was not a result of an occluded aqueduct per se. For these reasons the offspring selected for this study were animals with patent aqueducts. These were divided into groups to include (1) those showing gross hydrocephalus, (2) nonhydrocephalic littermates of these, and (3) vitamin $B_{12}$-supplemented controls.

## RESULTS

Performance of the dam and the percentage of abnormalities observed in offspring differed in the two diets used. The results of a 12-month test period are shown in table 1. These indicate reduced breeding or conception in the group maintained on alpha protein or both. The animals maintained with this diet were somewhat obese and averaged 40 to 50 gm more in weight at 8 months of age than the animals consuming the soybean meal diet. The increased sugar content plus a slight increase in fat may have made this diet more palatable. Thus, obesity may have accounted in part for the decreased reproduction. There was a higher incidence of hydrocephalus in the offspring littered to dams maintained with alpha protein. Since the alpha protein diet appeared to be adequate in all respects except for vitamin $B_{12}$ and cobalt, the increase in hydrocephalus was attributed to a more severe borderline deficiency of vitamin $B_{12}$ as a result of omitting cobalt from this diet.

*Brain.* The subcommissural organ (fig. 1) from a newborn control rat shows the location of the organ beneath the posterior commissure and pineal body where the third ventricle narrows to become the cerebral aqueduct. Control brains showed a stainable substance within the cells of the organ extending from the nucleus to the lumenal surface. The cells of the subcommissural organ stained vividly and selectively with chrome alum-hematoxylin, periodic acid-Schiff (PAS) and, to a lesser degree, with Gomori's aldehyde fuchsin. Cell nuclei, cytoplasm and droplet material stained faintly for protein bound sulfhydryl and moderately heavy for protein bound disulfide. There was a variation in affinity for the different stains (table 2), depending upon the type fixative used.

The classification of staining intensity shown in table 2 is based on sections from which glycogen had been removed with several changes of saliva. PAS-positive and, to a lesser degree, aldehyde fuchsin-positive material that appeared to be secreted by the cells of the subcommissural organ clung to the lumenal surfaces of the cells and accumulated within the lumen as

TABLE 1

*Reproductive performance of females for 12-month period*

| Type diet | No. females | No. litters | Litters/female (av.) | No. offspring | Av. no. in litter | Hydrocephalus |
|---|---|---|---|---|---|---|
| | | | | | | % |
| Soybean oil meal | 45 | 150 | 3.33 | 810 | 5.4 | 14.8 |
| Soybean oil meal + vitamin $B_{12}$ | 9 | 54 | 6.00 | 378 | 7.0 | 0.0 |
| Alpha protein | 32 | 75 | 2.34 | 382 | 5.1 | 19.6 |
| Alpha protein + vitamin $B_{12}$ | 9 | 38 | 4.22 | 277 | 7.3 | 0.0 |

TABLE 2

*Tinctorial characteristics of the subcommissural organ*

| Fixative | Stain and intensity[1] | | | |
|---|---|---|---|---|
| | Aldehyde fuchsin | PAS | Protein-bound sulfhydryl | Protein-bound disulfide |
| Bouin's | 2 | 4 | 2 | 3 |
| Formalin, 10% | 0 | 2 | 1 | 2 |
| Orth's | 1 | 2 | 0 | 0 |
| Susa | 1 | 4 | 0 | 1 |

[1] Graded 1 to 4 with a trace as grade 1 and strongest reaction as grade 4.

droplets (fig. 2). The subcommissural organ was intact in the hydrocephalic animals selected to demonstrate changes observed in this study, athough in some of the brains examined the organ was smaller than normal. The degree of ventricular dilatation and brain damage seemed to vary little among hydrocephalics. There was a marked difference, however, in staining characteristics of the organ in hydrocephalics, their nonhydrocephalic littermates and the supplemented controls. The cells of the subcommissural organ from a hydrocephalic animal showing severe ventricular dilatation contained little stainable material (fig. 3) and there was no evidence of droplets clinging to the lumenal surface or free within the lumen. In addition, the cytoplasm of the cells was vacuolated and length of the cells diminished with many nuclei placed near the lumenal border. Nonhydrocephalic littermates revealed moderate amounts of stainable material within the cells and clinging to their lumenal border, or free within the lumen (fig. 4).

A characteristic observation in control brains and, to a lesser extent, in the nondistended brains of littermaters of hydrocephalics was the presence of strongly PAS-positive, faintly aldehyde fuchsin-positive material at the poles of the nuclei of cells of the subcommissural organ (fig. 5). This material was not observed in brains of hydrocephalic animals (fig. 6). Caudal to the subcommissural organ in the lumen of the aqueduct of control brains there were moderate to heavy accumulations of strongly PAS-positive, faintly aldehyde fuchsin-positive material (fig. 7). The droplets coalesced to form heavy concentrations and there were thick bands of stainable material at the lumenal surface of the cells. Little, if any, such material was seen in the aqueducts of hydrocephalic brains (fig. 8). Littermates to hydrocephalics showed moderate amounts within the lumen of the aqueduct.

Scattered about in the gray substance bordering the third ventricle and the aqueduct were variable numbers of glial-type cells. They were numerous in control brains, fewer in number in littermates to hydrocephalics, but were sparse or absent in hydrocephalics. These cells contained granules that stained selectively with PAS and aldehyde fuchsin; the latter stain, however, was less vivid (figs. 9–11). These cells probably correspond to those of the normal adult rat brain described by Wislocki and Leduc ('54) who showed that this cell type accumulated silver intravitally and also contained PAS-positive material and droplets of golden brown pigment.

*Kidney.* The immature and damaged kidney of the vitamin $B_{12}$-deficient newborn rat has been described (Jones et al., '55; Newberne and O'Dell, '59). Sections included in this report were treated with several changes of saliva to remove glycogen and were then stained by the alcian blue-PAS technique.

The most prominent observation in the kidney of the vitamin $B_{12}$-deficient newborn rat is gross immaturity. Many tubules and glomeruli in the cortical zone fail to differentiate into functional units (figs. 12 and 13). Glomeruli are poorly differentiated and have the appearance of a contracted, highly cellular structure compared with the control animal (fig. 14). Both proximal and distal tubules are markedly dilated. The poorly differentiated glomeruli and the damaged tubules of the hydrocephalic newborn rat observed in this

study are demonstrated in figure 15. In figure 16 is shown the near normal pattern of a nonhydrocephalic littermate to the animal shown in figure 15. Even more striking were the alterations in the papillary area (figs. 17 and 18). The papillary tubules were dilated and many were cystic (fig. 18); casts were not observed and occlusion of the renal system was not apparent to account for the dilatations. The severe tubular dilatation in the papillary zone of the hydrocephalic animal is compared with that of the control animal in figures 19 and 20. Note in figure 20 the accumulation of PAS-positive globules accumulated within the epithelium of many of the tubules. Subsequent studies showed these structures to be histochemically identical to the structures observed in the same location of the potassium-deficient rat. Littermates to hydrocephalic animals showed kidneys that were less damaged than those of the latter.

## DISCUSSION

In our studies, the soybean oil meal diet supported animals that appeared to be at a lower plane of nutrition than animals maintained with alpha protein-type diets. This occurred despite approximate equality in proximate nutrients. The difference in protein could hardly account for this. Animals fed the alpha protein-type diet probably became somewhat obese from increased intake of a more palatable diet. Obesity in rats is known to reduce reproduction and this factor probably accounted for the poor reproduction with the alpha protein diet. Increased incidence of hydrocephalus with the latter diet was attributed to lack of cobalt and thus reduced synthesis of vitamin $B_{12}$ in females consuming this type diet. Alpha protein is nearly devoid of cobalt and this constituent was omitted from salts used in the alpha protein diet. The alpha protein diet, however, contained an increased fat level. Increasing fat in the diet is usually considered to increase the vitamin $B_{12}$ requirement.

The vitamin $B_{12}$-deficient newborn rat exhibits widespread subcutaneous edema irrespective of whether hydrocephalus is present. The presence of edema plus a significant incidence of hydrocephalus indicates a severe defect in the regulation of body fluid volume. The newborn rat brain, as shown in our control animals, exhibits considerable quantities of stainable material in the cells of the subcommissural organ and in the lumen of the cerebral aqueduct. Littermates to hydrocephalic animals showed moderate amounts of stainable material in the same locations as observed in control animals. We were unable, however, to demonstrate significant quantities of stainable material in the subcommissural organ and aqueduct of the hydrocephalic animals. These observations seem to indicate that the secretions of this organ may be related in some manner to the hydrocephalus present. Furthermore, the material observed at the poles of the nuclei in control brains was absent in hydrocephalic brains. Staining characteristics of this material were the same as those of the cytoplasm of the organ and of droplets within the lumen of the aqueduct. Histochemical studies indicate that the material elaborated is protein in nature and the work of Gilbert ('60) suggests a hormone-like effect when extracted and administered to normal rats. If the material is elaborated by the subcommissural organ as available evidence indicates, the elaboration or storage is heavier in the area of the nucleus.

There has been no obvious explanation for the occurrence of litters containing hydrocephalic animals along with nonhydrocephalic littermates. Presence of PAS-positive and aldehyde fuchsin-positive material in the cells of the subcommissural organ and in the lumen of the aqueduct of such animals may offer some explanation for absence of hydrocephalus. If the material elaborated by the organ is related to fluid balance, the nonhydrocephalic offspring may secrete sufficient amounts to prevent severe fluid imbalance and gross distention of the ventricles. These animals exhibit subcutaneous edema; thus the fluid volume control is not entirely adequate.

The kidney of the vitamin $B_{12}$-deficient hydrocephalic animal is radically altered. If there is a relation between the subcommissural organ and fluid balance it seems logical that the kidney would be involved. A kidney such as that observed in the vitamin $B_{12}$-deficient offspring would be more

likely to compound the defect in fluid dynamics already present. The histochemical nature of the globular material observed in the papillary tubules is identical to the globules observed in the same location in the potassium-deficient rat kidney. Recently, Newman et al. ('58) showed that lesions of the periaqueductal gray matter involving the posterior commissure markedly alter aldosterone secretion. Aldosterone-stimulating activity was highest in the "pineal complex" that contained the pineal gland and subcommissural organ (Farrell, '59). Fluid retention by the postulated subcommissural volume receptor could be mediated through adrenal aldosterone stimulation. In man, diseases of the brain stem are sometimes associated with disturbances of fluid and electrolyte balance. Studies on the electrolyte balance of the vitamin $B_{12}$-deficient offspring might help elucidate the state of fluid balance. Such studies have been initiated in our laboratories.

Glial cells containing stainable material in periventricular and periaqueductal gray substance of control brains were not observed in hydrocephalic brains and were reduced in number in littermates of hydrocephalics. Wislocki and Leduc ('54) observed that these cells stored silver that was administered to the animal over long periods of time. It was also revealed that they store, in addition, material with staining characteristics identical to the substance of the subcommissural organ. If the material observed in the cells of animals in this study is derived from secretions of the subcommissural organ, one may postulate that in the absence of organ function none is available to be taken up by the glial cells. The observed absence in hydrocephalic brains and reduced numbers in littermates may indicate simply a lack of material supplied by the organ. The staining characteristics of the substance in brains of our animals varied slightly from those described by Wislocki and Leduc ('54) in that the material observed in our animals stained faintly with aldehyde fuchsin, whereas the substance was vividly stained by this agent in Wislocki and Leduc's animals. These investigators were working with normal adult rats and our material was embryonic. Otherwise staining characteristics of our material were typical in every way of that seen in other mammals but more closely paralleled the intensity of reactions described by Wislocki and Roth ('58) in material obtained from a normal human fetus.

The morphological and histochemical alterations observed in the brains of normal, hydrocephalic and littermates seem to relate this organ and the substance elaborated by it to the presence of disturbed fluid balance in the vitamin $B_{12}$-deficient animal. The kidney may also be involved but any inference here would be highly speculative. In view of postulations made by others (Gilbert, '60; Farrell, '59) that the secretions of the subcommissural organ do stimulate water intake and influence fluid balance, it is difficult to harmonize the condition observed in the vitamin $B_{12}$-deficient rat and the postulations of Gilbert. The vitamin $B_{12}$-deficient rat has an excess of fluid and reduction or absence of subcommissural secretions. The interrelationship of the subcommissural organ and organs of internal secretion may be of some significance, as suggested by Farrell ('59). The retained water and obvious defect in fluid volume control of the vitamin $B_{12}$-deficient animal suggest a block of some important mechanism for fluid balance. The mechanism *in utero* may differ remarkably from that in postnatal life. It is significant that there is a correlation between the hydrocephalus and edema observed and the state of activity of the subcommissural organ as well as kidney damage. These observations warrant further investigation.

## SUMMARY

Depleting the female rat of vitamin $B_{12}$ results in a significant incidence of hydrocephalus, subcutaneous edema and defects in organs including the kidney. A purified diet low in cobalt and vitamin $B_{12}$ resulted in a higher incidence of hydrocephalus than a practical type diet also low in cobalt and vitamin $B_{12}$. Reproduction was poor in females consuming the purified diet. This was attributed to increased food intake and moderate obesity in females consuming the purified diet.

The subcommissural organ and certain periventricular and periaqueductal cells of the control newborn rat brain ex-

hibited considerable quantities of stainable material positive to periodic acid-Schiff, chrome alum-hematoxylin, Gomori's aldehyde fuchsin, sulfhydryl and disulfide stains. Only insignificant amounts of the material were observed in brains of hydrocephalic animals but moderate quantities were present in littermates to hydrocephalics. Kidney damage seemed to vary directly with the degree of severity of hydrocephalus and edema. The possible relationship of the observed alterations in the vitamin $B_{12}$-deficient newborn to fluid dynamics was discussed.

ACKNOWLEDGMENTS

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## PLATE 1

### EXPLANATION OF FIGURES

1 Subcommissural organ, control newborn rat. Third ventricle is to left of photograph; the cerebral aqueduct continues into the fourth ventricle to right. The group of tall columnar cells dorsal to the opening of the aqueduct make up the subcommissural organ. It is situated on the anteroinferior surface of the posterior commissure of the midbrain — the light staining tissue just above the organ. The dark staining irregularly oval structure above the organ is the pineal body. Note material within the lumen of the aqueduct and adhering to the lumenal border of the subcommissural organ. This is the proteinaceous material elaborated by the organ observed in control brains. Alcian blue-PAS stain. × 100.

2 Lumenal surface of subcommissural organ of control newborn rat. Note material clinging to margin of cells and accumulated within lumen of aqueduct. This material is PAS-positive, aldehyde fuchsin-positive and stains with moderate intensity for protein bound disulfide (cystine). Alcian blue-PAS stain. × 1,100.


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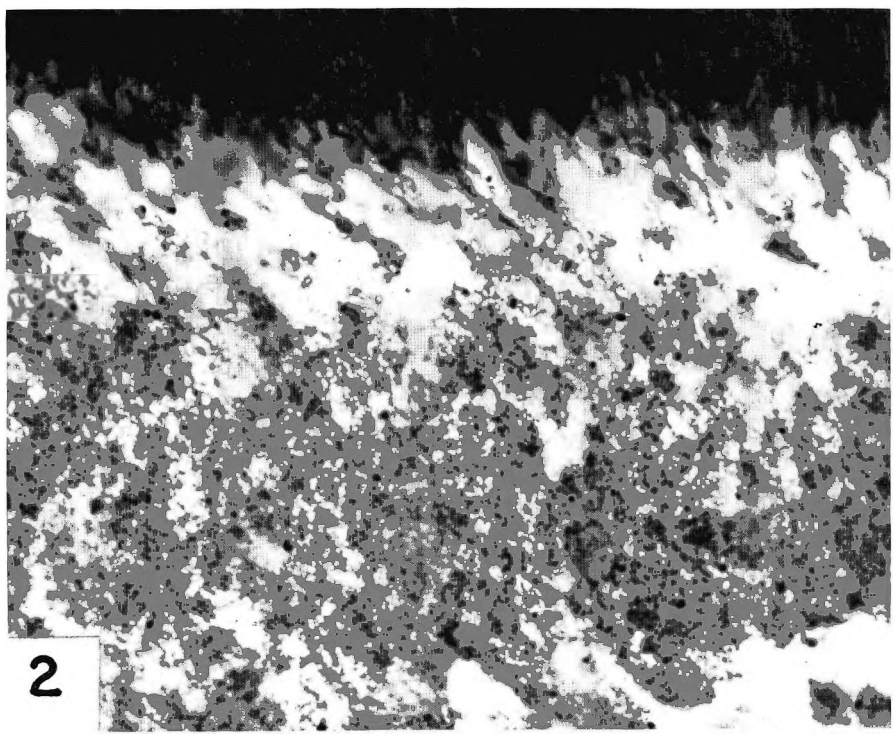
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## PLATE 2

EXPLANATION OF FIGURES

3 Subcommissural organ from vitamin $B_{12}$-deficient newborn rat that showed the typical gross ventricular distention of hydrocephalus. Only small amounts of stainable material are present at lumenal surface of cells and none within lumen of the aqueduct. Note also vacuolation of cytoplasm of cells of the subcommissural organ. Alcian blue-PAS stain. × 440.

4 Lumenal surface of subcommissural organ from vitamin $B_{12}$-deficient nonhydrocephalic littermate of animal of figure 3. Considerable quantities of material cling to lumenal border of cells of the organ. Moderate amounts were also present within the lumen of the aqueduct. Vacuolation of cytoplasm is not observed here. Alcian blue-PAS stain. × 1,100.

5 Subcommissural organ from newborn control animal. Photograph shows dense zone of nuclei dorsal to elongated cytoplasmic extensions of the cells. Note dark staining material at poles of nuclei (arrows). This material has the same histochemical characteristics as the stainable material located in cytoplasm and aqueductal lumen. Alcian blue-PAS. × 1,100.

6 Subcommissural organ from newborn vitamin $B_{12}$-deficient hydrocephalic animal. This area is comparable to that shown in figure 5. Note absence of stainable material at poles of nuclei. Alcian blue-PAS stain. × 1,100.

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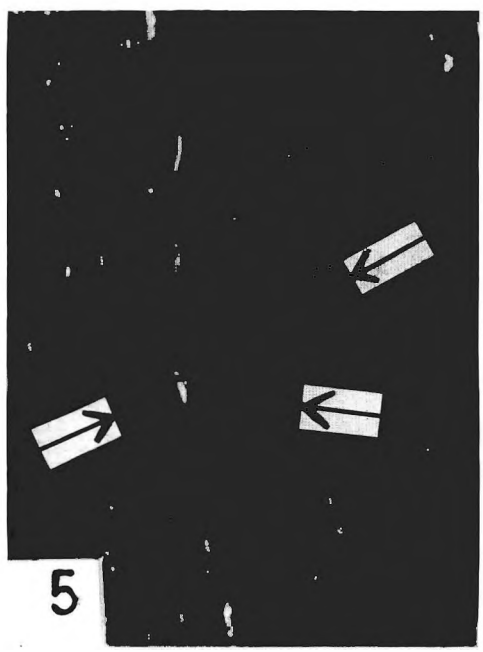

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## PLATE 3

### EXPLANATION OF FIGURES

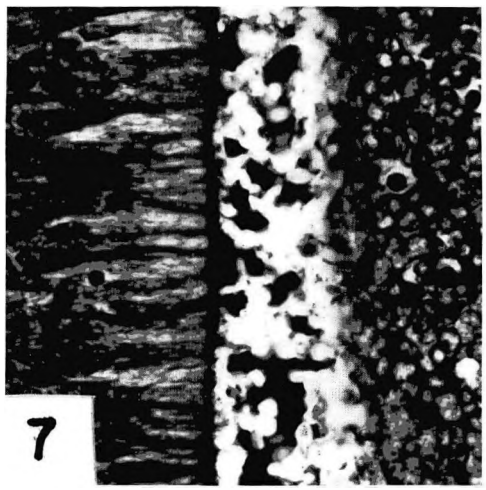
7 Aqueduct of control brain. Note accumulation of material within lumen of aqueduct with strong affinity for PAS stain. Alcian blue-PAS stain. × 440.

8 Aqueduct of vitamin $B_{12}$-deficient hydrocephalic brain. Small amount of stainable material is observed within aqueduct lumen. Compare with figure 7. Alcian blue-PAS stain. × 440.

9 Periaqueductal area of control newborn rat. Dark staining cells (arrows) shown in the subependymal gray matter beneath the aqueduct contain material strongly positive for chrome alum hematoxylin and for the PAS stain; weakly positive for aldehyde fuchsin. Note material in aqueduct at upper left of photo. Alcian blue-PAS stain. × 440.

10 Periaqueductal area of control newborn rat. Higher magnification of figure 9. Six cells in center of photo contain stainable material. Alcian blue-PAS stain. × 1,100.

11 Periaqueductal gray matter in newborn vitamin $B_{12}$-deficient hydrocephalic rat. None of the cells contain stainable material. The cells in center (arrows) are probably the cell type that accumulates material; however, no stainable material is present in any of the cells. Alcian blue-PAS stain. × 1,100.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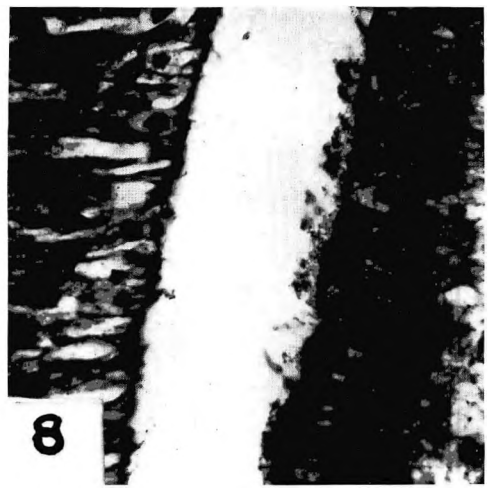
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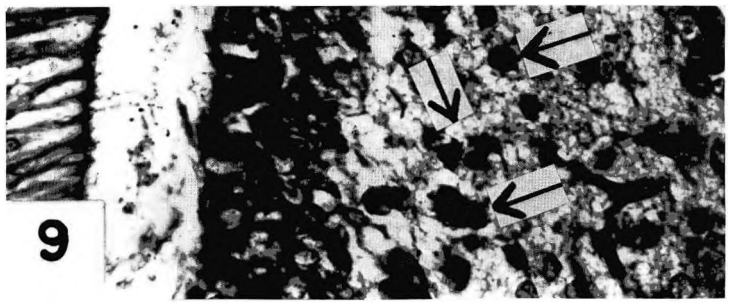
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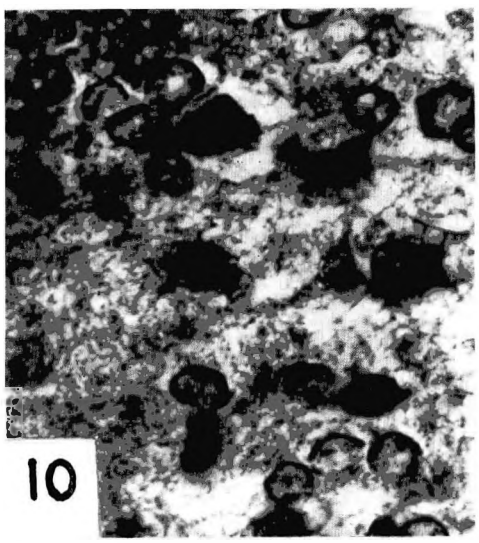
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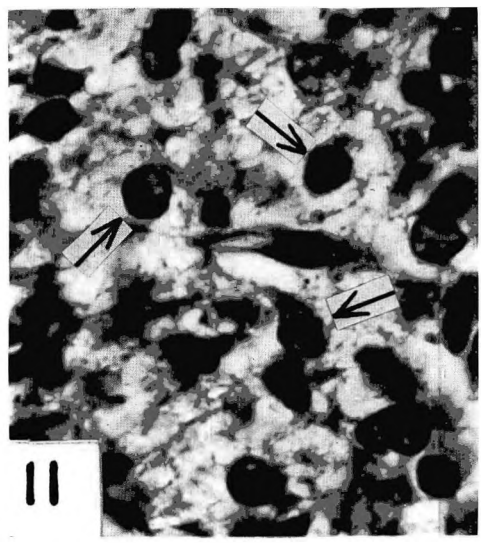
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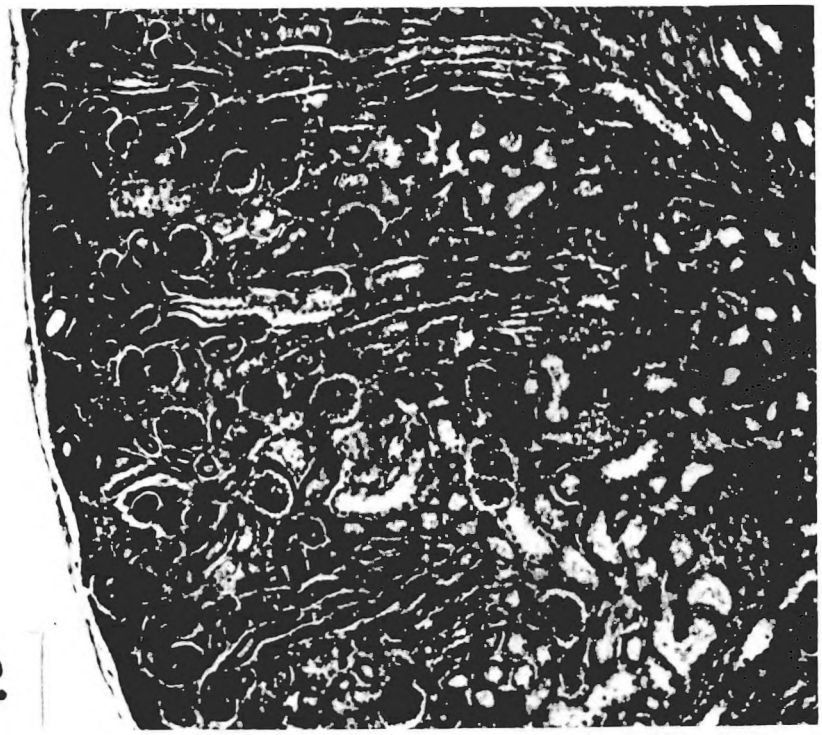
## PLATE 4

EXPLANATION OF FIGURES

12 Kidney, newborn control rat. Differentiation into tubules and glomeruli is normal for this stage of development. Hematoxylin and eosin stain. × 100.

13 Kidney, newborn vitamin $B_{12}$-deficient hydrocephalic rat. Glomeruli are contracted and show high degree of cellular density as a result of less advanced differentiation. Proximal and distal tubules are markedly dilated and contain cellular debris. Hematoxylin and eosin stain. × 100.



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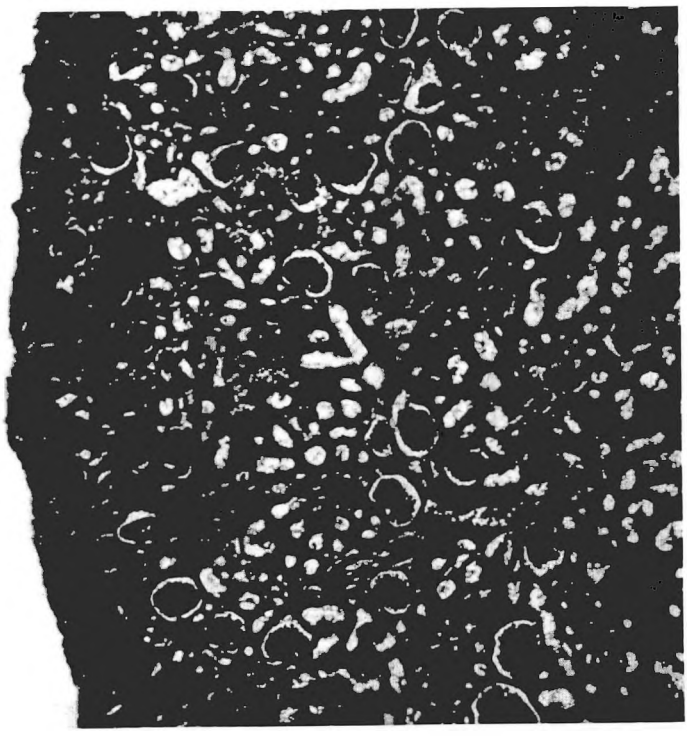

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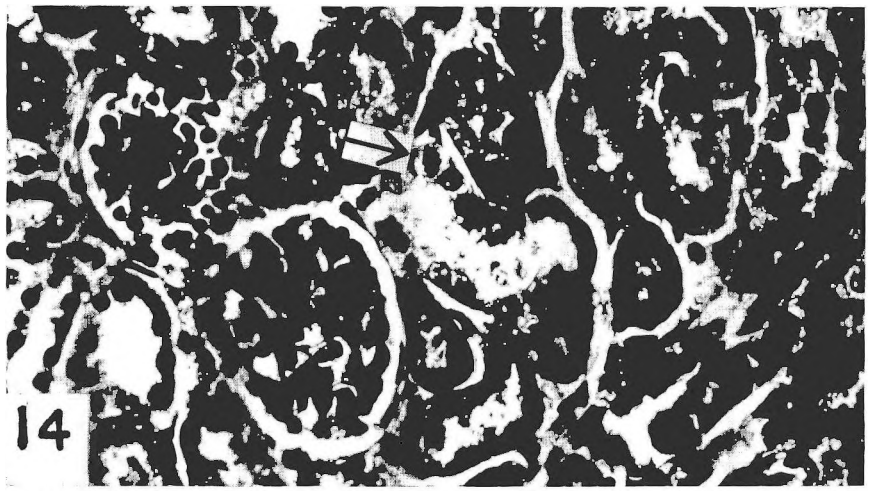
## PLATE 5

EXPLANATION OF FIGURES

14 Kidney, newborn control rat. Glomeruli and tubules are normal for this stage of development. Note mitotic figure in cell of distal tubule in center of photograph. Hematoxylin and eosin stain. × 440.

15 Kidney from vitamin $B_{12}$-deficient hydrocephalic rat. Glomeruli have contracted, highly cellular appearance; proximal and distal tubules are markedly dilated and contain debris within the lumen. Epithelial cells have pale nuclei and the cytoplasm contains granular, PAS-positive material. Alcian blue-PAS stain. × 440.

16 Kidney from vitamin $B_{12}$-deficient nonhydrocephalic littermate of animal with kidney shown in figure 15. Mild damage to tubular epithelium is present but appearance is near normal. Compare with figure 14. Alcian blue-PAS stain. × 440.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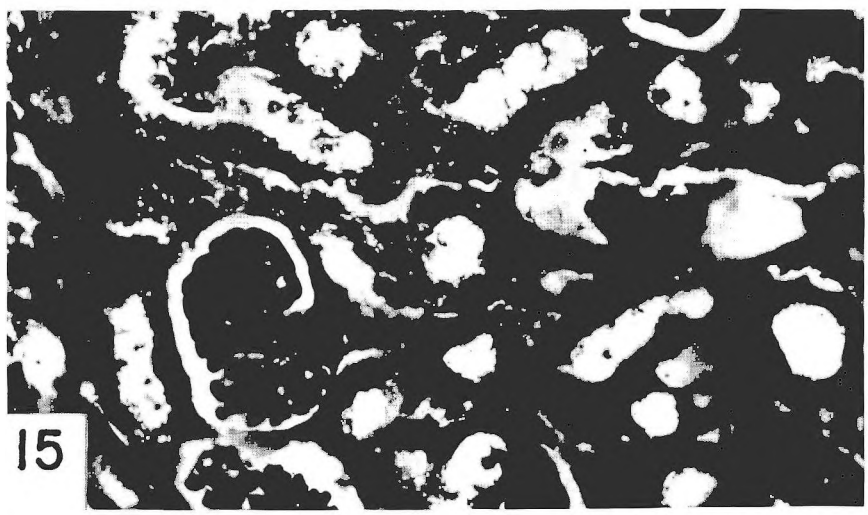
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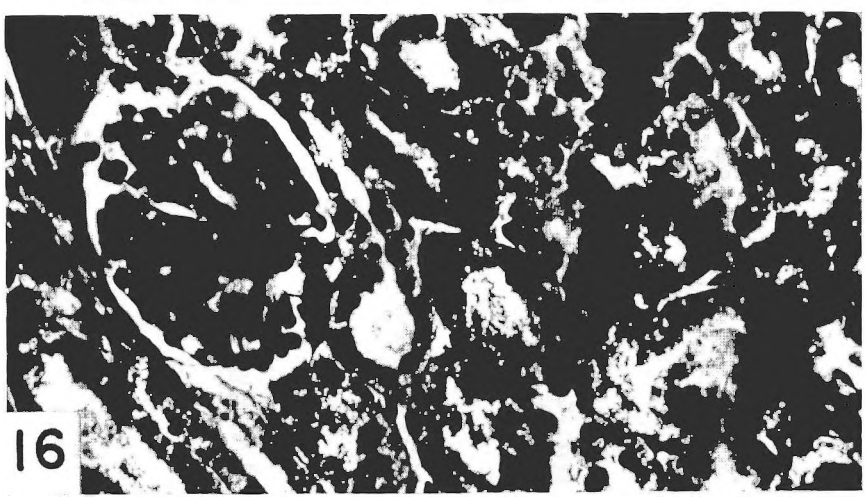
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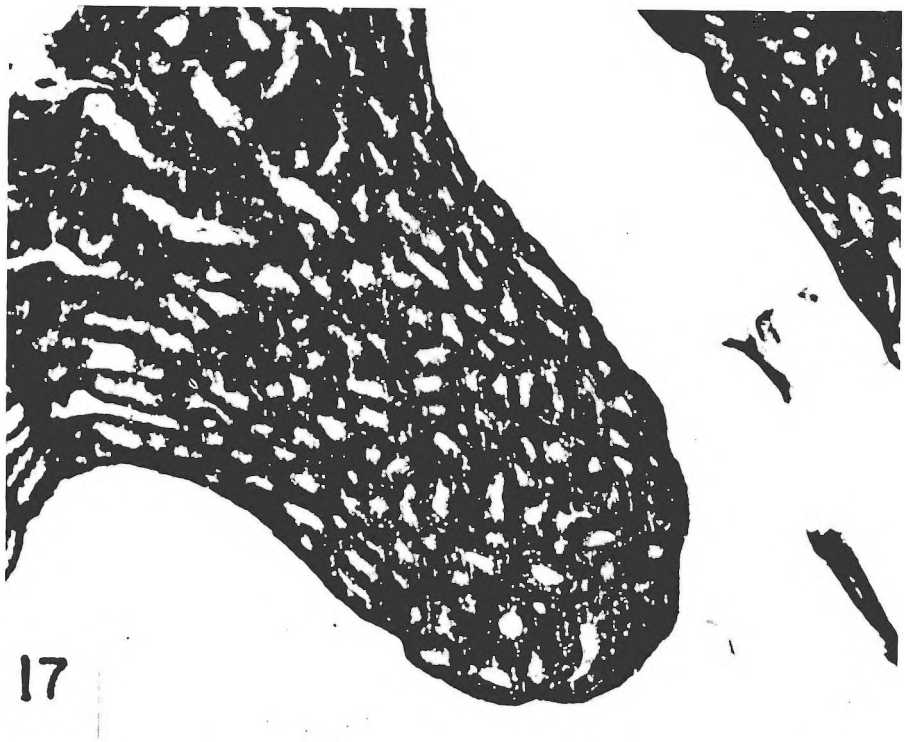
## PLATE 6

EXPLANATION OF FIGURES

17 Papillary zone of kidney from newborn control rat. The open space is the pelvis that continues into the ureter. Hematoxylin and eosin stain. × 100.

18 Papillary zone of the kidney from vitamin $B_{12}$-deficient hydrocephalic newborn rat. Note severe dilatation of many of the collecting tubules. Hematoxylin and eosin stain. × 100.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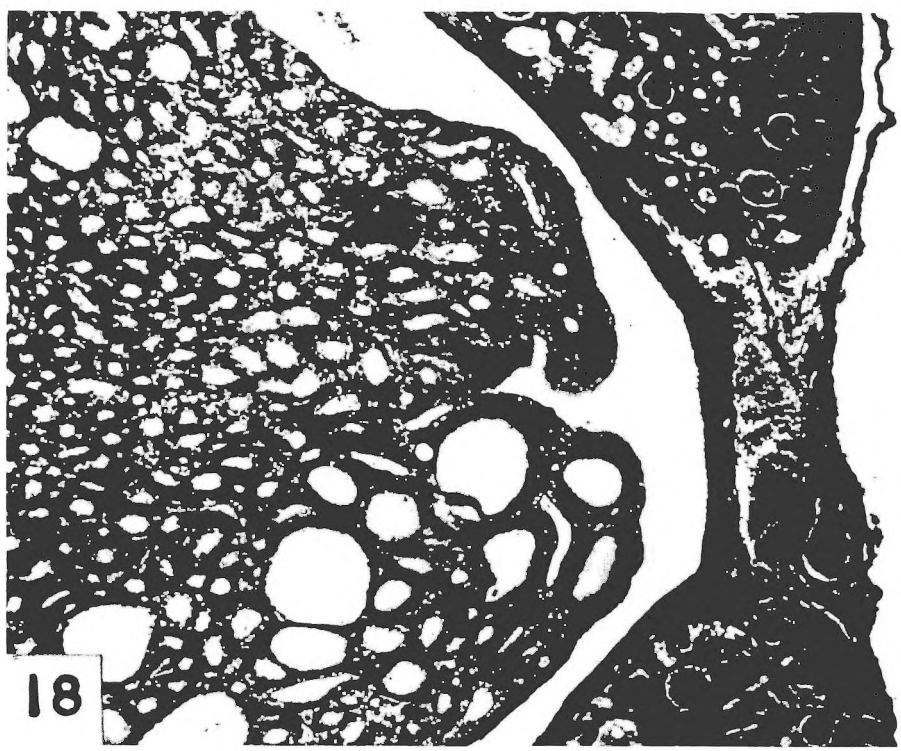
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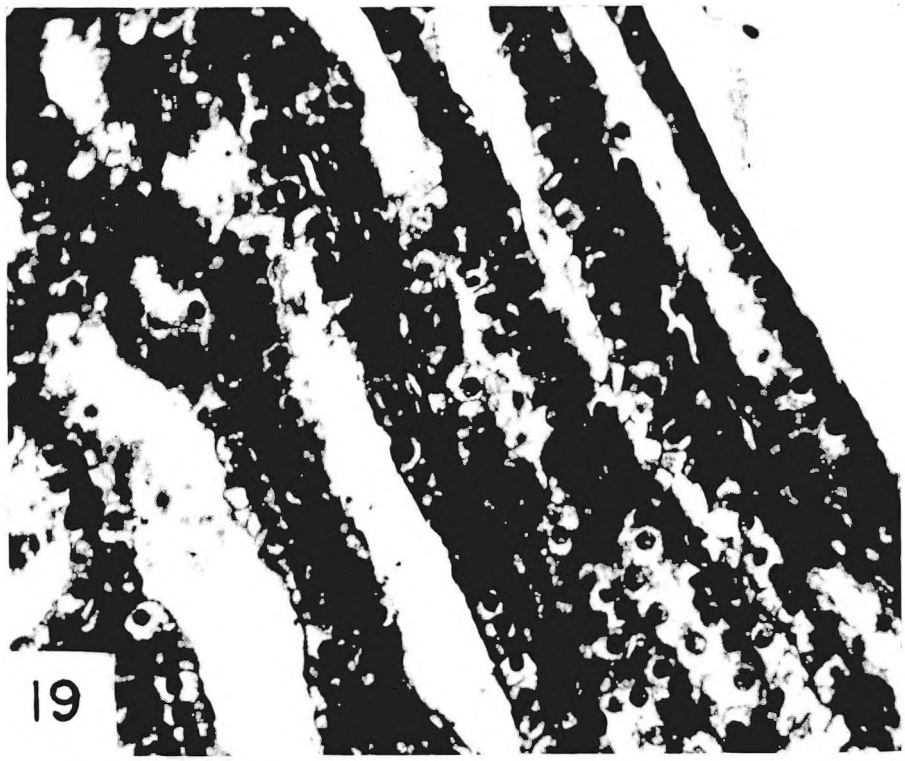
## PLATE 7

EXPLANATION OF FIGURES

19 Papillary collecting tubules from kidney of newborn control rat. Epithelial cells are billowy with normal cytoplasmic density. Alcian blue-PAS stain. × 440.

20 Papillary collecting tubules from kidney of vitamin $B_{12}$-deficient hydrocephalic newborn rat. Tubules are dilated, epithelium is flattened in some of them; in others the cytoplasm contains PAS-positive globular material. Alcian blue-PAS stain on sections treated with several changes of saliva to remove glycogen. × 440.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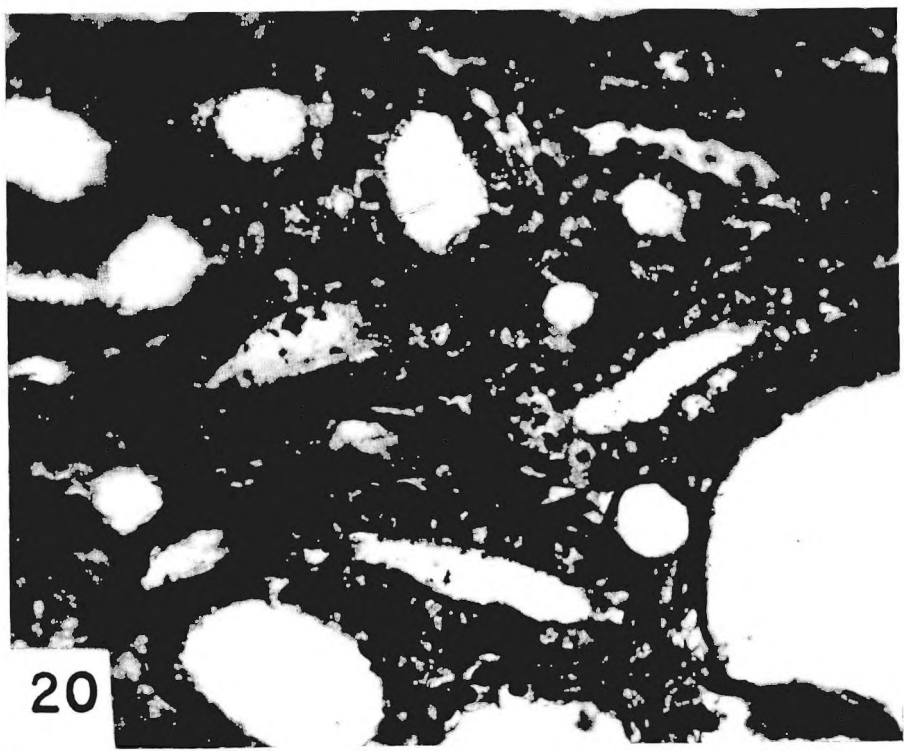
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# Adaptation of an *In Vitro* System to the Study of Starch Fermentation by Rumen Bacteria[1,2,3,4]

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The mechanism of starch fermentation by rumen microorganisms has been studied by several techniques. Baker ('42), Baker et al. ('50) and van der Wath ('48) used microscopic techniques in observing the microbial degradation of starch granules in rumen contents. Many *in vivo* studies have dealt with the production of volatile fatty acids and lactic acid in the rumen of animals receiving diets with varied concentrate:roughage ratios. Balch and Rowland ('57) reported a decreasing molar ratio of acetate to propionate coinciding with a decreasing ratio of fibrous to starchy carbohydrates in the ration. They observed this phenomenon especially when flaked maize replaced the fibrous portion of the ration. Similar observations were made by Phillipson ('52) and Shaw et al. ('60). Reid et al. ('57) observed a relationship between high dietary intakes of wheat, wheat starch or crushed maize and high proportions of propionate in rumen fluid. A decreased acetate:propionate ratio has implications for the energy efficiency of the ruminant because of the evidence for the high specific dynamic action of acetate (McClymont, '52; Armstrong et al., '57).

The transient occurrence of large quantities of lactate in the rumen following ingestion of diets containing starch has indicated that lactate may be an intermediate in the pathway of starch fermentation (Phillipson, '52; Waldo and Schultz, '56; Balch and Rowland, '57; Reid et al., '57). The decline of lactate levels in the rumen has been correlated with increased levels of propionate but not with increased levels of acetate or butyrate (Waldo and Schultz, '56).

These and many other *in vivo* investigations have suggested certain problems of rumen microorganism metabolism that might best be investigated by controlled *in vitro* techniques. Doetsch et al. ('53) and Robinson et al. ('55) used the washed suspension technique which allows study of a "controlled, nonproliferating population which exhibits little activity in the absence of added substrate." This group of investigators has studied the formation of volatile fatty acids from glucose, maltose and cellobiose. Maltose was fermented most vigorously and the resultant volatile fatty acid mixture exhibited a lower acetate:propionate ratio than that from the other substrates. Lactate accumulation was found to be related to the pH, the level of substrate and the centrifugal fraction of bacteria used as inoculum. Sijpesteijn and Elsden ('52), using a simillar technique, reported the conversion of succinate to propionate.

Jayasuriya and Hungate ('59) studied the conversion of lactate to volatile fatty acids by incubating rumen contents from hay-fed and grain-fed steers in short-term experiments. Lactate was converted to acetate more rapidly by cultures from grain-fed than from hay-fed steers.

Stewart and Schultz ('58) used rumen fluid from roughage-fed steers as inocula for studies on the fermentation of hay-concentrate-urea mixtures *in vitro*. Corn meal additions were observed to increase

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[2] These investigations were supported in part by a U.S.P.H.S. Research Grant, E-3418, from the National Institutes of Health, Public Health Service.
[3] Data taken from portions of a dissertation presented by the Senior author to the Graduate School, The Ohio State University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
[4] A preliminary report of this work was presented at the annual meeting of the American Society of Animal Production, 1960.
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propionate production. Hershberger et al. ('56), studied the degradation of cellulose, starch and metabolic intermediates by proliferating mixed cultures centrifuged from the rumen fluid of roughage-fed animals. When starch was fermented, volatile fatty acid mixtures were produced with lower acetate:propionate ratios than when cellulose was fermented. Added lactate increased the formation of acetate and propionate equally.

An all-glass *in vitro* fermentation system has been used successfully in this laboratory in studies on cellulose digestion by proliferating rumen microorganisms (Bentley et al., '55; Johnson et al., '58; Dehority et al., '60). The present paper reports the adaptation of this system to the study of the mechanism of starch fermentation and includes studies on the preparation of inocula, evaluation of the requirements for optimal microbial activity, observations on the proliferating organisms, and comparisons of the *in vitro* activities of mixed cultures from the rumen of 4 sheep fed identical diets.

## EXPERIMENTAL

*In vitro fermentation technique.* Fermentations were carried out at a volume of 100 ml in 180-ml glass bottles placed in a constant temperature water bath at 39°C. Anaerobiosis was achieved by bubbling a continuous stream of $CO_2$ through the media. The basal media contained urea, sodium carbonate and buffered mineral mixture (Bentley et al., '55). Powdered cornstarch[6] was used as the substrate, the level of which will be given. Biotin, 20 μg, and *p*-aminobenzoic acid (PABA), 50 μg, were added routinely except as indicated. Adjustments of pH were made periodically with 20% (w/v) aqueous sodium carbonate or 50% (v/v) aqueous ortho-phosphoric acid.

*Inoculum source.* Four fistulated wethers were maintained with a diet of 9 parts of ground shelled corn, 3 parts of chopped mixed hay and 1 part of soybean oil meal fed at the rate of approximately 2 pounds per day. It was necessary to feed approximately 0.2 pounds of long hay per day to prevent loss of appetite. Trace mineralized salt and water were offered ad libitum.

*Inoculum preparation.* Rumen fluid was withdrawn through the fistula by use of a polyethylene tube attached to a suction flask. A minimum of suction was used and the polyethylene tube was moved horizontally and vertically within the rumen during sampling. Fluid was taken in this manner two hours after the morning feeding. Sampling at this time provided mixed cultures of rumen microorganisms which were most consistent from day to day and which proliferated *in vitro* without apparent gross changes in microscopically observed morphological types.

The rumen fluid was immediately diluted with equal parts of 0.01 M phosphate buffer (pH 7.0), warmed to 39°C and saturated with $CO_2$. Dilution was required to insure adequate centrifugal sedimentation in subsequent steps. Centrifugation at $250 \times g$ and $3{,}000 \times g$ was carried out in lusteroid tubes using an International centrifuge, size 2, and a Servall type "SP" angle head centrifuge, respectively.

*Measurement of starch fermentation.* Aliquots of the media were added to 2 N NaOH to dissolve the starch, and, after one hour, neutralized with 2 N HCl (Carroll and Cheung, '60). After dilution, the carbohydrate concentration was estimated colorimetrically with the anthrone reagent and using starch as a standard. The recovery of added starch with this procedure was $103.2 \pm 5.7\%$.[7] The difference between the concentrations of anthrone reactive material in inoculated flasks at the time of inoculation and after a given period of incubation was taken as an index of fermentation. Figures obtained in this manner were considered as estimates of starch fermentation rather than starch hydrolysis since anthrone is sensitive to the products of starch hydrolysis.

*Organic acid production.* Total volatile fatty acids (TVFA) were determined by steam distillation using a micro-Kjeldahl distillation apparatus. Samples were deproteinized with an equal volume of 0.1 N HCl prior to distillation from acid solution.

[6] A. E. Staley and Company, Decatur, Illinois.
[7] Mean and standard deviation.

Individual volatile fatty acids were separated by vapor-phase chromatography using an apparatus constructed in this laboratory. Sixteen- foot columns of 6 mm (I.D.) glass tubing were filled with glass beads 150 to 250 μ in diameter[8] coated with stearic and orthophosphoric acids in the ratio 50 to 1 by weight. A glass heating jacket with refluxing xylene as the heat source was used to maintain a temperature of 138°C. Helium gas was used to elute the volatile fatty acids. The eluate passed through a heated cupric oxide oxidation chamber, a phosphorus pentoxide dessicant, and a thermal conductivity cell. Elution patterns were recorded by a Leeds and Northrup Speedomax Recorder. Quantitative determination of the individual acids was accomplished by weighing the chart paper circumscribed by the elution peaks and comparing to a standard curve obtained with known mixtures of authentic acids. Twenty-five-milliliter samples were deproteinized with 25 ml of 95% ethanol, made alkaline to phenol red by adding 2 N NaOH, and evaporated to dryness. The residue was redissolved in 2 ml of 5% orthophosphoric acid and the weight of this solution was determined. An aliquot of known weight was injected into the column. Evaluation of this technique was done by separating the volatile fatty acids of 8 samples by both vapor-phase chromatography and by silica-gel partition chromatography (Bulen et al., '52) using *n*-butanol: benzene mixtures as eluants.[9] Vapor phase chromatography gave 102% recovery of acetate and 110% recovery of both propionate and butyrate relative to silica-gel partition chromatography.

Lactic acid was determined colorimetrically by the method of Barker and Summerson ('41).

When whole rumen fluid was to be analyzed the material was strained through two layers of cheesecloth and centrifuged at 250 × *g*. The resultant supernatant, free of food particles, was analyzed.

*Bacterial growth.* The amount of nitrogen insoluble in 10% (w/v) trichloroacetic acid (TCA-N) was taken as an estimate of bacterial protein nitrogen in the fermentation media (Cline et al., '58). The difference between concentrations of TCA-N in flasks at inoculation time and after incubation was taken as an estimate of bacterial growth.

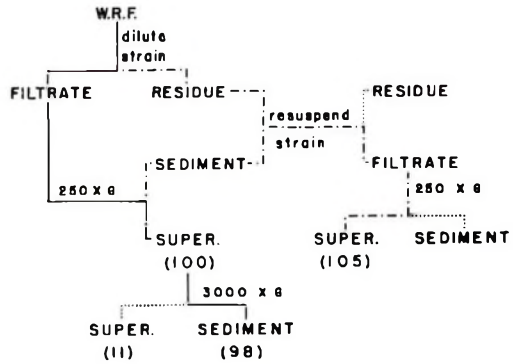


Fig. 1 Flow chart of steps taken in separating whole rumen fluid (WRF) into various fractions for *in vitro* inocula, with relative starch fermenting activities shown in parentheses.

*Microscopic examinations.* All bacterial preparations and fermentation media were examined microscopically for bacterial morphology using the Gram-stain (Hucker modification). Only cursory observations were made. Photomicrographs of these Gram-stain preparations were taken using a Kodak Wratten B. filter, no. 58.

## RESULTS

*Inoculum preparation.* The various steps used in developing a routine inoculum are shown in figure 1. The diluted whole rumen fluid (WRF) was strained through cheesecloth and the resultant filtrate was centrifuged at 250 × *g* for 5 minutes. The cheesecloth residue and 250 × *g* sediment, found to consist of food particles, protozoa, and bacteria in association with the food particles, were combined and resuspended in 0.01 M phosphate buffer (pH 7.0). From this suspension, a second 250 × *g* supernatant was prepared as shown in figure 1 (refer to the broken line). The average relative starch fermenting activities of these supernatants used as inocula representing 20 ml of WRF are shown in parentheses in figure 1. The mean ratio between TVFA production and starch fermentation for

[8] Minnesota Mining and Manufacturing Company, St. Paul, Minnesota.

[9] Linke, F. G. 1952 Chromatographic separation of volatile fatty acids produced in the artificial rumen. M. S. Thesis, The Ohio State University, Columbus, Ohio.

both inocula was 8.37 ± 0.85[10] mEq per gm of starch fermented. Microscopic examination of the proliferating organisms revealed no apparent differences between inocula. The 250 × *g* supernatant prepared by the flow of material along the solid line of figure 1 was used in further experiments.

The 250 × *g* supernatant was fractionated by centrifuging at 3,000 × *g* for 20 minutes (fig. 1). The 3,000 × *g* sediment was resuspended in 0.01 M phosphate buffer (pH 7.0). The average relative starch fermenting activities of the 250 × *g* supernatant and 3,000 × *g* fractions are shown in parentheses in figure 1. That the mixed culture sedimented at 3,000 × *g* and proliferating therefrom was representative of the mixed culture in whole rumen fluid is indicated by the photomicrographs in figure 2. Therefore, in all future work reported herein the inoculum used was the 3,000 × *g* sediment resuspended in buffer and representing 20 ml of WRF.

*Optimal pH for starch fermentation.* The inoculum preparation studies reported were carried out at pH 6.9, the pH indicated in preliminary experiments to support satisfactory starch fermentation. To define the pH optimum more precisely the following experiment was conducted. By adjusting the relative proportions of the phosphate salts in the mineral mixture of Bentley et al. ('55), three mineral mixtures were prepared, buffered at pH 7.1, 6.8 and 6.5, respectively. The contents of duplicate flasks, with the appropriate mineral mixture, were adjusted to pH 7.1, 6.9, 6.8, 6.7 and 6.5 immediately after inoculation and every hour thereafter. The inoculum was prepared from a composite of equal parts of WRF from three sheep fed identical diets. Autoclaved supernatant was omitted since preliminary experiments had indicated no strict requirement for this nutrient source. The substrate level was 1.50 gm per 100 ml. The experiment was conducted three times, with incubation times of 9, 12 and 24 hours, respectively. Residual starch was estimated before and after incubation. Analysis of variance indicated that the variation in starch fermentation associated with pH was highly significant ($P < 0.01$). Furthermore, the regression of starch fermented on pH was adequately described by a quadratic equation. This equation accounted for 97.8% of the variation associated with pH ($P < 0.01$) and indicated that the maximal starch fermentation would occur when the flask contents were readjusted hourly to pH 6.8.

*Media evaluation.* To determine whether autoclaved supernatant or biotin and PABA were essential components of the media, the following experiment was conducted. Four media were prepared with the following additions to the urea and mineral mixture: none, 20 μg of biotin and 50 μg of PABA, 20 ml of autoclaved supernatant, and a combination of autoclaved supernatant, biotin and PABA. A composite of rumen fluid from three sheep was used to prepare two inocula. The first (regular) inoculum was prepared as previously described. The second (washed) inoculum was prepared from a portion of the first by recentrifugation at 3,000 × *g* for 20 minutes and resuspending the sediment in fresh 0.01 M phosphate buffer (pH 7.0). The washing process reduced the TCA-N concentration to 88.9% of the regular inoculum. Each inoculum was used with each of the 4 media. Three replications were conducted and the substrate level was 1.50 gm per 100 ml. After 12 hours' incubation, analyses were made for residual starch, TVFA and TCA-N. The milliequivalents of TVFA produced per gram of starch fermented and the milligrams of TCA-N synthesized per gram of starch fermented were computed. The means of starch fermentation for the different treatments covered the range 0.88 to 1.02 gm with an overall range of 0.21 to 1.46 gm.

Analysis of variance revealed no significant differences between nutrient or inoculum means for the 5 variables measured or computed. There was, however, an indication of depressed *in vitro* activity with no nutrient additions and the washed inoculum. Either biotin and PABA or autoclaved supernatant appeared to alleviate the depression. Biotin and PABA were chosen as routine additions to maintain a more closely controlled *in vitro* media than would be achieved with autoclaved supernatant.

[10] Mean and standard deviation.


Fig. 2 Photomicrographs of bacteria from the rumen of sheep fed 3 corn: 1 hay. A, Whole rumen fluid with food particle; B, 3,000 × *g* Sediment used as inoculum; C, 3,000 × *g* Sediment after 9 hours incubation. Gram-stain. × 1,296.

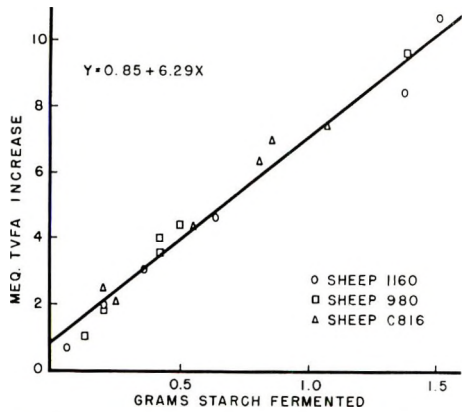


Fig. 3 Regression of the increase in total volatile fatty acids (TVFA) on starch fermented and mean values obtained in simultaneous incubations of inocula from three sheep in 6 trials.

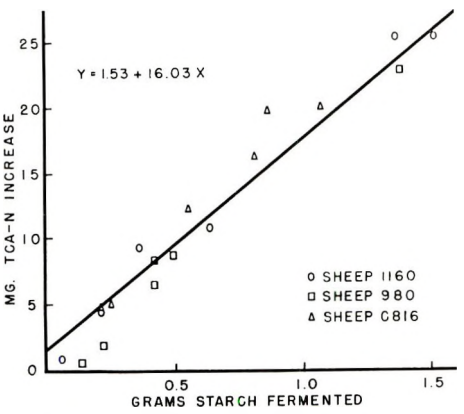


Fig. 4 Regression of the increase in trichloroacetic acid insoluble nitrogen (TCA-N) on starch fermented and mean values obtained in simultaneous incubations of inocula from three sheep in 6 trials.

*Comparison of inocula from different sheep.* Considerable day-to-day variation in the amount of starch fermented in a given time period was observed in the previous experiments. In addition, there seemed to be some slight differences between sheep in the relative numbers of the predominant types of rumen bacteria. Therefore, it seemed desirable to compare the mixed culture from sheep receiving identical diets using the *in vitro* criteria of TVFA produced and TCA-N synthesized per unit of starch metabolized. Inocula were prepared simultaneously from each of three sheep and incubated for 9 hours in 4 trials and for 12 hours in two additional trials. The substrate level was 1.50 gm per 100 ml. The data obtained were subjected to analysis of variance.

The regressions of TVFA and TCA-N on starch are shown in figures 3 and 4. The linear regressions accounted for 97.4% of the variation in TVFA means ($P < 0.01$) and 95.4% of the variation in TCA-N means ($P < 0.01$). These data indicated that the relationships between the fermentation of starch and the production of TVFA or TCA-N for the inocula from the three sheep were similar. This suggested that, although slight differences in microscopic appearance of the mixed cultures were observed, these 18 mixed cultures may have had the same gross metabolic activities.

These experiments again demonstrated considerable variation in the amount of starch fermented in a given time period. To determine whether this variation was the result of different maximal rates of fermentation or of differences in lag phase, namely, the time required to reach the maximal rate of fermentation, additional simultaneous experiments were conducted using inocula from 4 sheep. The contents of duplicate flasks were analyzed at inoculation time, at the first sign of a pH depression, 6 hours after the pH depression, and after 24 hours' total incuba-

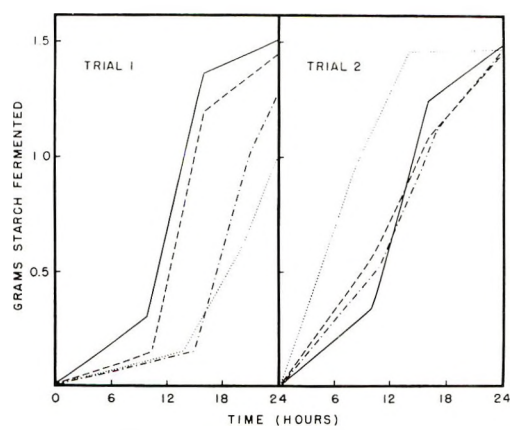


Fig. 5 Time curves, obtained from simultaneous incubation of inocula from 4 sheep. Duplicate flasks analyzed at each time indicated by a change in slope. Trial 1 conducted on October 24, 1960 and trial 2 on December 8, 1960. (—— sheep no. 1160; – – – – sheep no. 980; –·–·–· sheep no. C840; ..... sheep no. C816).

TABLE 1

*Molar percentages of the volatile fatty acids and levels of lactic acid observed in vivo[1] and in vitro[2]*

| Sample | No. of samples | Molar percentage of volatile fatty acids | | | Lactic acid |
|---|---|---|---|---|---|
| | | Acetic | Propionic | Butyric | |
| | | % | % | % | *mg/100 ml* |
| *In vivo* | | | | | |
| Pre-feed | 15 | 57.4 ± 10.2[3] | 22.8 ± 9.2 | 19.8 ± 4.7 | 0.47 ± 0.19[4] |
| 2 hours post-feed | 11 | 54.7 ± 9.0 | 27.0 ± 7.1 | 18.3 ± 5.4 | 0.57 ± 0.19 |
| 7 hours post-feed | 15 | 56.6 ± 9.7 | 24.9 ± 8.1 | 18.5 ± 6.1 | 0.58 ± 0.21 |
| *In vitro* | | | | | |
| 0.10 gm/hour[5] | 10 | 44.6 ± 13.0 | 35.2 ± 8.5 | 20.2 ± 10.1 | 1.5 ± 0.84[6] |
| 0.17 gm/hour[5] | 6 | 56.0 ± 6.4 | 33.4 ± 2.3 | 10.6 ± 3.6 | 50.1 ± 13.2[6] |

[1] Sampled on 4 days over 10-weeks.
[2] Inocula from 4 sheep, two trials, duplicate flasks each sheep.
[3] Mean and standard deviation.
[4] One sample, analyzing 6.78 mg/100 ml, omitted from calculation of mean and standard deviation.
[5] Mean rate of starch fermentation for 6 hours prior to sampling.
[6] Increase over inoculation time analysis.

tion time. The resultant starch fermentation curves from two such trials are shown in figure 5. Apparently differences in starch fermentation in a given period of time are the result of differences both in lag phase and in the maximal rate of fermentation. No consistent differences between the inocula from the 4 sheep were noted. Five additional trials at one- to two-week intervals confirmed these observations.

In the two trials shown in figure 5, samples were taken from the flasks 6 hours after the pH depression for volatile fatty acid separation and lactate estimation. Samples of rumen fluid taken before and 2 and 7 hours after the morning feeding were similarly analyzed. These data are presented in table 1. Two types of *in vitro* fermentations occurred. Apparently the accumulation of lactate was associated with the higher maximal rates of starch fermentation. These higher rates of fermentation and lactate accumulation occurred with sheep 1,160 in trials 1 and 2 and with sheep 980 in trial 1 (fig. 5). With sheep C816 in trial 2 an apparently short lag phase and rapid maximal rate was observed. However, no lactate was noted when sampled. It is considered possible that lactate degradation may have occurred prior to sampling since starch fermentation was essentially complete at that time.

## DISCUSSION

Differential centrifugation was used in these studies to prepare a bacterial sediment that was microscopically representative of the mixed culture found in the whole rumen fluid of sheep fed a 3 parts corn to 1 part hay ration. The mixed culture thus obtained proved to have active amylolytic properties. Among the many morphological types present in the population it is possible that some are not amylolytic. No attempt was made to isolate the organisms to differentiate them metabolically.

Using the criteria of starch fermentation, TVFA production and TCA-N synthesis, added biotin and PABA or autoclaved rumen fluid supernatant had no apparent effect on the fermentation. Washing the cells one time by recentrifugation to further reduce rumen fluid carryover failed to depress microbial activity even though the concentration of TCA-N was thereby reduced. With the apparently large number of species present in the mixed cultures it seems plausible to suspect that a symbiotic relationship may exist with respect to any required growth factors. Dehority et al. ('60) studying cellulose digestion with a similar *in vitro* technique, demonstrated an absolute requirement for biotin as well as valeric acid; but the cellulolytic culture proliferating in those studies was found to consist

of one predominant morphological type. Under those conditions symbiosis might not be expected to provide the required growth factors.

Simultaneous comparisons of mixed cultures from three sheep fed identical diets revealed no differences between sheep or trials in the relationships between TVFA production or TCA-N synthesis and the amount of starch fermented. Rosenberger and Elsden ('60) and Bauchop and Elsden ('60) demonstrated the constancy of the relationship between the weight of substrate metabolized and the weight of cellular material synthesized for a given organism in the steady state. This relationship differs between species when the energy securing or retaining metabolic pathways differ. It would seem that a similar relationship would apply to metabolic end-products. The data obtained in these studies with 18 mixed cultures from three sheep fed identical diets suggests that the end-product- and cellular material-substrate relationships are parameters that might be applied in defining the gross activities of a mixed culture. Further work with mixed cultures from ruminants fed varying diets is necessary to investigate this hypothesis.

The apparent relationship between the accumulation of lactate and the maximal rates of starch fermentation suggests that the rate of lactate degradation may have been exceeded by the rate of lactate production when the fermentation proceeded at a rapid rate. The differences in fermentation rates could not be related to the microscopic appearances of the mixed cultures either at inoculation time or after incubation. Analyses of the organic acids in the rumen fluid would not serve to predict the *in vitro* differences observed.

However, a cyclic variation in pH occurred during the periods of maximal fermentation rates since a constant pH was not maintained with this *in vitro* system. Furthermore, with the fermentations having the higher maximal rates a greater depression of pH between adjustments was noticed. Karush et al. ('56), Reid et al. ('57) and Rosenberger and Elsden ('60) have presented data indicating the modification of bacterial metabolism by varied pH levels. It may be that in the case of a rapid fermentation the higher early production of volatile fatty acids was effective in lowering the pH to a level inhibitory to lactate degradation. The effect of pH depression on the metabolism of the mixed cultures used in these studies is being investigated.

## SUMMARY

An *in vitro* system was described for the study of starch fermentation by microorganisms from the rumen of sheep fed a high grain diet. A centrifugal procedure was used to obtain the predominant rumen bacteria as a sediment which appeared to proliferate *in vitro* without gross changes in morphological types. Under the conditions of these experiments the maximal starch fermentation occurred when flask contents were readjusted to pH 6.8. The addition of biotin and *p*-aminobenzoic acid or autoclaved rumen fluid supernatant was not necessary for maximal starch fermentation.

Eighteen mixed cultures from the rumen of three sheep fed identical diets were compared and found to be similar in the relationships between the production of total volatile fatty acid or the synthesis of trichloroacetic acid-insoluble nitrogen and the extent of starch fermentation. Differences in the amount of starch fermented in a given time were apparently the result of differences in the time required to reach the maximal rate of fermentation and to differences in the maximal rates of fermentation. These differences were not consistent with respect to sheep or to trials.

There appeared to be two types of fermentations *in vitro* related to the maximal rates of fermentation attained. Accumulation of lactate was associated with the higher rates of fermentation. Possible explanations for this phenomenon are suggested including the effect of a cyclic pH variation on the metabolic pathways of fermentation.

## ACKNOWLEDGMENT

We wish to thank Dr. C. R. Weaver and his staff for the statistical analyses; Clark Robey and Glenn Berkey for the photomicrography; and Harold Scott for developing the vapor-phase chromatography pro-

cedure and for assistance with the application of the technique.

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# Protein Value and the Amino Acid Deficiencies of Various Algae for Growth of Rats and Chicks

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Numerous reports have appeared in recent decades concerning the use of algae and plankton as a source of food; these reports have been reviewed in a recent monograph by Schwimmer and Schwimmer ('55). Although considerable interest has been expressed about the role of algae as a food and much has been accomplished in developing methods for the mass culture of algae (Gummert et al., '53; Tamiya, '59), there have been few studies on its nutritional adequacy.

Several reports concerning the amino acid composition of various species of algae have demonstrated a low level of sulfur amino acids although the level of most other "essential" amino acids appeared adequate (Eny, '49; Fowden, '52a, b; Schieler et al., '53; Smith and Young, '55). As a result of the sulfur amino acid deficiency, algae protein would be expected to stimulate growth to a lesser degree than proteins of high biological value; such results have been reported for the rat by several workers (Hayami and Shino, '58; Geoghegan, '53; Fisher and Burlew, '53). In contrast, Fink and Herold ('55, '56, '57, '58) have concluded that for growth of rats the protein of the alga *Scenedesmus obliquus* was equal or slightly superior to proteins of skim milk, egg white and various plants.

In a more recent study, Prosky et al. ('61)[1] demonstrated that the addition of algae to a 10% casein diet for rats stimulated growth; also, that rats fed algae as the sole source of protein at a level of 10 or 30% of the diet grew less well than animals receiving the same level of protein from casein; however, equal growth was observed in animals receiving 20% of dietary protein from either algae or casein.

Combs ('52) reported that the substitution of 10% of chlorella for soybean meal in the diet of chicks resulted in a marked improvement in growth and feed efficiency; however, this growth stimulation was apparently due to the carotene content of the algae and not its protein.

The present report presents data on the protein value of various algae for growth in the chick and rat, and also presents evidence concerning the amino acid inadequacies of these materials.

## EXPERIMENTAL

*Chick studies.* Male Hy-line chicks were maintained in cages with raised wire floors and fed a practical diet for one week, at which time 10 chicks were assigned per group on the basis of weight. The chicks were distributed among the experimental groups in such a manner that the initial average weights for each group were within plus or minus 1 gm. The experimental diets were fed for three weeks. The animals were weighed and food consumption was determined weekly.

The composition of the basal diet, expressed as a percentage, was as follows: starch, 16.20; salt mixture,[2] 5.31; vitamin mixture,[3] 0.40; choline·Cl, 0.20; corn oil, 5.00; fiber, 3.00; glucose to 100. Algae and amino acids were added to the basal diet at the expense of glucose.

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[1] Prosky, L., J. F. Karinen and H. E. Sauberlich 1961 The nutritional adequacy of algae in weanling rats. U. S. Army Medical Research and Nutrition Laboratory, unpublished report.

[2] Chick salt mixture, supplied/kg of diet when fed at the rate of 5.31% of the diet: (in grams) $CaCO_3$, 3.0; $Ca_3(PO_4)_2$, 28.0; $K_2HPO_4$, 9.0; $MgSO_4$, 1.25; Fe gluconate, 2.24; NaCl, 8.80; and (in milligrams) $ZnSO_4 \cdot 7H_2O$, 60; KI, 40; $CuSO_4$, 20; $H_3BO_3$, 9; $CoSO_4 \cdot 7H_2O$, 1; $MnSO_4$, 650.

[3] Chick vitamin mix, supplied in mg/kg of diet when fed at the rate of 0.40% of the diet: thiamine·HCl, 25.0; riboflavin, 16.0; Ca pantothenate, 20.0; pyridoxine, 6.0; biotin, 0.6; folic acid, 4.0; *p*-aminobenzoic acid, 2.0; menadione, 5.0; and vitamin $B_{12}$, 20 $\mu$g; inositol, 100; ascorbic acid, 250; niacin, 150; vitamin A, 10,000 IU; vitamin $D_3$, 1,000 ICU; $\alpha$-tocopheryl acetate, 100 mg.

*Rat studies.* Weanling male, albino rats of the Holtzman strain weighing between 40 to 50 gm were used. The animals were housed in individual cages with raised wire floors. A stock diet was fed for three or 4 days, following which 10 rats were distributed per group on the basis of weight in such a manner as to have almost identical initial weights for all groups. The animals were maintained with the experimental diets for approximately three weeks. The exact length of the experimental periods is indicated in the tables of results.

The composition of the basal diet used in the rat studies, expressed as a percentage of the diet, was as follows: starch, 13.00; salt mixture (USP 14), 4.00; vitamin mixture,[4] 0.40; choline·Cl, 0.30; fiber, 3.00; corn oil, 5.00; glucose to 100. Algae and amino acids were added at the levels indicated in the results section at the expense of glucose.

Three algae were tested: (1) a mixture of *Scenedesmus obliquus* and *Chlorella ellipsoida;*[5] (2) *Chlorella pyrenoidosa;*[6] and (3) *Spongiococcum excentricum.*[7] The "crude" protein (N × 6.25) content of these algae was determined by Kjeldahl analysis, total lipids by acid hydrolysis of the algae followed by ether extraction of the hydrolysate. The total lipid extract was evaporated to dryness redissolved in petroleum ether and applied to a magnesia : supercell (1:1) column and the lipids free of chlorophyll and other chromogens were eluted with petroleum ether : acetone (9:1). An additional sample of each algae was used for ash determination by conventional analysis. The results of these analyses are presented in table 1.

## RESULTS

The protein value of the algae used was ascertained by means of growth and protein efficiency ratios (gm gain per gm of protein consumed) in both chicks and rats; these results are presented in tables 2 and 3. These data indicate that all of

[4] Rat vitamin mix, supplied in mg/kg of diet when fed at the rate of 0.4% of the diet: thiamine·HCl, 22.0; riboflavin, 22.0; Ca pantothenate, 66.0; pyridoxine, 22.0; biotin, 0.44; folic acid, 2.0; p-aminobenzoic acid, 110; menadione, 0.05; and vitamin $B_{12}$, 30 μg; inositol, 110; ascorbic acid, 1.0 gm; niacin, 100; vitamin A, 10,000 IU; vitamin D, 2,000 IU; α-tocopheryl acetate, 50 mg.

[5] Obtained from Japan Chlorella Institute, Tokyo, Japan.

[6] Generously supplied by Dr. R. H. Lowry, Boeing Airplane Company, Aero Space Division, Seattle 24, Washington.

[7] Purchased from Grain Processing Corporation, Muscatine, Iowa. This material was especially prepared for this laboratory.

TABLE 1

*Protein, total lipid, chlorophyll-free lipid and ash content of various algae*

| Algae | Crude protein (N × 6.25) | Total lipid | Chlorophyll-free[1] lipid | Ash |
|---|---|---|---|---|
| | % | % | % | % |
| *Scenedesmus obliquus* + *Chlorella ellipsoida* | 55.56 | 4.49 | 0.72 | 3.62 |
| *Chlorella pyrenoidosa* | 59.96 | 11.93 | 7.60 | 4.91 |
| *Spongiococcum excentricum* | 31.04 | 11.32 | 7.18 | 5.29 |

[1] Total lipid extract freed of chlorophyll and other chromogens.

TABLE 2

*Influence of various algae protein on growth and protein efficiency of the growing chick*

| Protein source[1] | 3-Week gain | Protein efficiency ratio |
|---|---|---|
| | *gm* | *gm gain/gm protein consumed* |
| Soybean + DL-methionine[2] | 237 ± 26[3] | 3.04 |
| *Scenedesmus obliquus* + *Chlorella ellipsoida* | 87 ± 22 | 1.55 |
| *Chlorella pyrenoidosa* | 9 ± 13 | 0.31 |
| *Spongiococcum excentricum* | 13 ± 8 | 0.43 |

[1] All proteins supplied at a level of 18% of the diet (% N × 6.25).
[2] 0.54% added DL-methionine.
[3] Mean ± standard deviation.

TABLE 3

*Influence of various algae proteins on gain and protein efficiency ratio in growing rats*

| Protein source | Dietary protein level (N × 6.25) | 3-Week gain | Protein efficiency ratio |
|---|---|---|---|
| | % | *gm* | *gm gain/gm protein consumed* |
| Casein | 14.88 | 112 ± 10[1] | 2.50 ± 0.09 |
| *Scenedesmus obliquus* + *Chlorella ellipsoida* | 15.31 | 60 ± 7 | 1.38 ± 0.14 |
| *Chlorella pyrenoidosa* | 15.31 | 38 ± 12 | 0.94 ± 0.19 |
| *Spongiococcum excentricum* | 14.81 | 9 ± 6 | 0.34 ± 0.24 |
| *Spongiococcum excentricum* + DL-methionine | 15.12 | 23 ± 4[2] | 1.22 ± 0.18 |

[1] Mean ± standard deviation.
[2] The animals receiving the *S. excentricum* diet were maintained with the same diet to which had been added 0.5% of DL-methionine for 11 days (21st through 32nd day).

the algae tested were far inferior to either the casein or soybean protein controls used for the rat and chick studies respectively. The mixture of *S. obliquus* and *C. ellipsoida* was superior to either of the other two algae studied (tables 2 and 3). The data in table 3 also demonstrate that the diet containing the algae *S. excentricum* was deficient in methionine for the growing rat. At the termination of the three-week experimental period, the *S. excentricum* diet was supplemented with 0.5% of DL-methionine and fed to the same animals for an additional 11 days. The animals gained 23 gm in 11 days and had a protein efficiency ratio of 1.22 when fed the methionine-supplemented diet as compared with a gain of only 9 gm in the previous 21-day period and a protein efficiency ratio of only 0.34 with the unsupplemented diet (table 3).

TABLE 4

*Influence of amino acid supplementation of algae protein (Scenedesmus obliquus + Chlorella ellipsoida) on growth and feed efficiency in the growing chick*[1]

| Amino acid mixture[2] | 3-Week gain | Feed efficiency |
|---|---|---|
| | *gm* | *gm gain/gm feed consumed* |
| None | 83 ± 11[3] | 0.32 |
| Complete | 124 ± 21 | 0.47 |
| Minus threonine | 117 ± 15 | 0.45 |
| Minus glycine | 96 ± 9 | 0.38 |
| Minus arginine | 122 ± 19 | 0.47 |
| Minus tryptophan | 124 ± 14 | 0.47 |
| Minus methionine | 78 ± 9 | 0.36 |
| Minus lysine | 114 ± 14 | 0.42 |
| Minus histidine | 132 ± 15 | 0.47 |

[1] All diets supplied 23.45% crude protein (% N × 6.25) from algae.
[2] Amino acids were supplemented at the following levels as a percentage of the diet: DL-methionine, 0.80; DL-tryptophan, 0.20; L-arginine·HCl, 0.73; glycine, 0.75; DL-threonine, 0.60; L-histidine·HCl·$H_2O$, 0.20; L-lysine·HCl, (95% purity), 0.59.
[3] Mean ± standard deviation.

The influence of amino acid supplementation of the mixed algae (*S. obliquus* and *C. ellipsoida*) diet on growth of the chick was tested. Reports of amino acid analyses of various algae (Eny, '49; Fowden, '52a, b; Schieler et al., '53; Smith and Young, '55) indicated that methionine was the most limiting amino acid and that deficiencies of arginine, glycine and threonine might also exist for the chick. Based on these data and those of Prosky et al. ('61),[8] indicating that methionine, lysine, tryptophan and histidine might be deficient in algae for the growing rat, chicks were fed diets supplemented with methionine, tryptophan, arginine, glycine, threonine, histidine and lysine (table 4). These data indicate that supplementation of the algae diet with all 7 amino acids resulted in a significant increase in weight gain and feed efficiency. The omission of a single amino acid at a time from the mixture was without significant effect with the exception of glycine and methionine. When glycine or methionine was omitted from the mixture of amino acids, growth was depressed. The omission of histidine from the mixture of amino acids resulted in a slight improvement in weight gain although this increase was not statistically significant.

Based on the above results, a second study was initiated with chicks, using the same mixed algae as the protein source.

[8] See footnote 1.

The results of this study are presented in table 5. Supplementation of the algae diet with either methionine or glycine alone, the two amino acids observed to be limiting, did not stimulate growth, although the addition of methionine appeared to improve feed efficiency. When both methionine and glycine were added together, a highly significant growth response and improvement in feed efficiency were noted. The addition of threonine to the methionine-glycine supplemented diet was without effect. Lysine, however, significantly depressed growth when added to a diet supplemented with methionine, glycine and threonine; the addition of histidine to this diet depressed growth further. Supplementation of the diet with histidine alone also resulted in a depression in weight gain below that of the group fed the unsupplemented algae. The addition of leucine alone or in combination with the 5 other amino acids tested had no effect.

A similar study on the effect of amino acid supplementation was initiated with

TABLE 5

*Effect of amino acid supplementation of algae protein (Scenedesmus obliquus + Chlorella ellipsoida) on growth and feed efficiency of the growing chick*

| Amino acid supplement[1] | | | | | | 3-Week gain | Feed efficiency |
|---|---|---|---|---|---|---|---|
| DL-Methionine 0.80% | Glycine 1.00% | DL-Threonine 1.20% | L-lysine·HCl (95% purity) 1.10% | L-Histidine·HCl·$H_2O$ 0.30% | L-Leucine 0.70% | | |
| | | | | | | *gm* | *gm gain/gm feed consumed* |
| − | − | − | − | − | − | 87 ± 22[2] | 0.28 |
| + | − | − | − | − | − | 93 ± 19 | 0.34 |
| − | + | − | − | − | − | 89 ± 25 | 0.28 |
| + | + | − | − | − | − | 127 ± 28 | 0.39 |
| + | + | + | − | − | − | 124 ± 28 | 0.40 |
| + | + | + | + | − | − | 102 ± 30 | 0.34 |
| + | + | + | + | + | − | 89 ± 41 | 0.34 |
| + | + | + | + | + | + | 81 ± 41 | 0.32 |
| − | − | − | − | + | − | 71 ± 22 | 0.26 |
| − | − | − | − | − | + | 86 ± 25 | 0.30 |

[1] All diets supplied 18.00% protein (% N × 6.25) from algae.
[2] Mean ± standard deviation.

TABLE 6

*Influence of amino acid supplementation of algae protein (Chlorella pyrenoidosa) on body weight gain and feed efficiency of albino rats*

| Amino acid supplement[1] | | | | | 19-Day gain | Feed efficiency |
|---|---|---|---|---|---|---|
| DL-Methionine 0.40% | DL-Tryptophan 0.40% | L-Lysine·HCl (95% purity) 1.33% | DL-Threonine 1.00% | L-Histidine·HCl·$H_2O$ 0.53% | | |
| | | | | | *gm* | *gm gain/gm feed consumed* |
| − | − | − | − | − | 51 ± 8[2] | 0.23 ± 0.02[2] |
| + | + | + | + | + | 70 ± 18 | 0.33 ± 0.04 |
| + | + | + | + | − | 52 ± 10 | 0.26 ± 0.04 |
| + | + | + | − | + | 76 ± 14 | 0.28 ± 0.03 |
| + | + | − | + | + | 70 ± 14 | 0.29 ± 0.03 |
| + | − | + | + | + | 71 ± 16 | 0.32 ± 0.03 |
| − | + | + | + | + | 50 ± 8 | 0.22 ± 0.02 |
| + | − | − | − | − | 65 ± 11 | 0.23 ± 0.03 |

[1] All diets supplied 18.00% crude protein (% N × 6.25) from algae.
[2] Mean ± standard deviation.

weanling rats using the algae, *C. pyrenoidosa,* as the protein source; the results of this study are presented in table 6. A significant improvement in weight gain and feed efficiency was observed as a result of supplementing the algae diet with methionine, tryptophan, lysine, threonine and histidine. The omission of single amino acids from the mixture resulted in a growth depression only in the case of histidine and methionine, indicating a deficiency of these two amino acids for the growing rat. Supplementation of the algae diet with methionine alone resulted in a significant growth response in comparison with animals receiving the unsupplemented control diet, although feed efficiency was not improved.

## DISCUSSION

The studies reported demonstrate that all of the algae fed are deficient in methionine, an observation that would be anticipated from various reports concerning the amino acid composition of algae (Eny, '49; Fowden, '52a, b; Schieler et al., '53; Smith and Young, '55). Glycine also was found to be deficient for the growing chick in the mixed algae used (*S. obliquus* and *C. ellipsoida*). Based on reports of analyses previously mentioned this also would be expected, although this observation has not previously been made since most studies have used the rat, which does not require glycine.

The growth-depressing effect of histidine observed in chick studies is indicative of an amino acid imbalance (Harper and Kumta, '59). Data thus far obtained, however, are inadequate to determine the mechanism of the imbalance.

In the rat growth studies with *C. pyrenoidosa,* the omission of histidine from the mixture of amino acids used to supplement the diet resulted in a growth depression. Again, this observation is in accord with amino acid analyses showing histidine to be low in this species of algae (Schieler et al., '53) and is in agreement with the data of Prosky et al. ('61).[9]

The results obtained with rats fed diets containing the algae, *S. excentricum,* indicate methionine to be one of the most limiting amino acids; however, further study is required to corroborate this observation and to delineate other amino acid deficiencies that may exist. The high levels of algae fed to chicks in these studies did not result in the impacted beak condition reported by Combs ('52). Diarrhea was observed in chicks fed the algae diets, but was not apparent in the rat studies.

Also worthy of further study is the poor growth observed in both rats and chicks with the algae diets supplemented with amino acids. There may be amino acids which are limiting other than those tested or the problem may be one of availability. Since the protein of algae is enclosed in a cellulose membrane, digestibility might be expected to present a problem. It has been reported that rats fed dried chlorella grew poorly, but that treatment of the algae with methanol improved growth and digestibility and that when further supplemented with methionine, the methanol-treated algae supported optimal growth (Tamura et al., '58a, b). Further similar studies would, therefore, appear to be of considerable value.

## SUMMARY

Chicks and weanling rats were fed diets containing the algae *Scenedesmus obliquus* and *Chlorella ellipsoida,* or *Chlorella pyrenoidosa,* or *Spongiococcum excentricum* as the sole source of protein.

All of the algae tested were inferior to the control diets in which soybean oil meal, supplemented with methionine or casein, served as the protein source for chicks and rats, respectively. The animals receiving the control diets showed superior weight gains and protein efficiency ratios. Of the algae tested, the mixture of *S. obliquus* and *C. ellipsoida* was superior to either of the other algae fed singly.

Data were presented showing all of the algae tested to be deficient in methionine for the growing rat and chick. The mixture of algae was also deficient in glycine for the chick and the algae *Chlorella pyrenoidosa* was found to be deficient in histidine for the growing rat.

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[9] See footnote 1.

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# Tissue Cholesterol Ester and Triglyceride Fatty Acid Composition of Rabbits Fed Cholesterol Diets High and Low in Linoleic Acid[1]

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Several reports (Kritchevsky et al., '54, '56) have indicated that rabbits fed high-cholesterol diets supplemented with fats rich in linoleic acid develop less atherosclerosis than animals receiving similar diets containing fats poor in that acid. The action of the polyunsaturated fatty acids in lowering the blood cholesterol level in man and retarding the development of atherosclerosis in rabbits may be related to the role of those acids in the transport and metabolism of cholesterol. Increased formation of cholesterol esters containing a high proportion of saturated and monounsaturated fatty acids may also be a factor involved in the genesis of atherosclerosis (Sinclair, '56). Several reports (Swell et al., '60a, b) have shown that the atherosclerotic plaque cholesterol esters of human aorta contain a higher percentage of oleic and a lower percentage of linoleic acids than that fraction of the serum. The cholesterol esters of the aortas of cockerels and rabbits with atherosclerosis have also been shown to contain more oleic and less linoleic acids than the serum fraction (Blomstrand and Christensen, '61; Swell et al., '61a). These observations suggest that cholesterol oleate may be preferentially deposited in the aorta from the serum and that further studies on the cholesterol ester transport system are needed. Several important questions regarding the role of linoleic acid in atherosclerosis remain to be investigated. This includes how the level of that acid in the diet influences tissue lipid fatty acid composition and in particular, the aorta cholesterol esters. The present report is a continuation of our earlier studies (Swell et al., '61a) on the changes in the tissue lipid fatty acids accompanying the production of atherosclerosis.

## EXPERIMENTAL

*Animals and diets.* New Zealand white male rabbits weighing 2.5 to 3 kg were used. Nineteen animals were divided into three groups of 6, 7 and 6 each. Each of the groups received, in the morning, 50 gm of powdered commercial pellet chow[2] with one of the following: group A, no additions; group B, 1 gm of cholesterol and 8% of corn oil; and group C, 1 gm of cholesterol and 8% of olive oil. The animals consumed their meal in the morning, and in the afternoon were given an additional 50 gm of the diets without cholesterol. The diets were prepared as previously described (Swell et al., '61a). After the animals had received the diets for 9 weeks, they were fasted overnight and then killed under pentobarbital sodium anesthesia by exsanguination and the aorta and liver removed. The aorta was carefully cleared of adherent fat and graded for atherosclerosis on a scale from zero to 4 +.

*Methods.* The tissues were weighed and extracted according to procedures described earlier (Swell et al., '60a). Free and total cholesterol of serum lipid extracts were determined by the method of Sperry and Webb ('50). The cholesterol esters and triglycerides were separated on silicic acid columns and the fatty acid composition of the isolated lipid fractions determined by gas-liquid chromatography

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[2] Purina Pellet Chow, Ralston Purina Company, St. Louis.

(Swell et al., '60a, '61b). Statistical analyses were carried out by the *t* test and values of $P < 0.01$ were considered to be significant.

## RESULTS

*Fatty acid composition of diets (table 1).* The diet (pellet chow) fed to group A contained a high percentage of linoleic acid (48.6%), but a total fat content of only 2.4%. The average daily intake of fat per rabbit fed the control diet was 2.4 gm and of that 1.2 gm was linoleic acid. The fat in the diet fed to group B also contained a high proportion of linoleic acid (55.3%), but the total fat intake of that group was considerably higher (10.4 gm per day or 5.6 gm of linoleic acid) than in group A. The fat in the diet fed to group C contained the lowest percentage of linoleic acid (18.4%). The average fat intake per day per rabbit was the same as for group B (10.4 gm), but the intake of linoleic acid was only 1.9 gm.

*Tissue lipid levels (table 2).* At the time of sacrifice both groups (B and C) had greatly elevated serum, liver and aorta cholesterol levels when compared with the group receiving the diet (A) with no addi-

TABLE 1

*Fatty acid composition of diets*

| Fatty acid | | Diets | | |
|---|---|---|---|---|
| No. carbons | No. double bonds | A (6)[1] No additions | B (7) High cholesterol Corn oil | C (6) High cholesterol Olive oil |
| | | *% total fatty acids* | | |
| 6 to 14 | | 0.9 | 0.4 | 0.4 |
| 16 | 0 | 18.3 | 13.7 | 15.3 |
| 16 | 1 | 0.9 | 0.2 | 0.8 |
| 18 | 0 | 2.5 | 1.9 | 2.1 |
| 18 | 1 | 23.4 | 26.7 | 61.3 |
| 18 | 2 | 48.6 | 55.3 | 18.4 |
| 18 | 3 | 5.4 | 1.8 | 1.7 |
| Dietary fat content, % or fat intake, gm/day[2] | | 2.4 | 10.4 | 10.4 |
| Linoleic acid intake, gm/day | | 1.2 | 5.6 | 1.9 |

[1] Values in parentheses represent the number of rabbits per group.
[2] Food consumption per animal was 100 gm/day.

TABLE 2

*Effect of diets on cholesterol levels in tissues*

| Group | Cholesterol | | | Ester/total cholesterol |
|---|---|---|---|---|
| | Free | Ester | Total | |
| | | | | % |
| | Serum,[1] mg/100 ml | | | |
| A | 17 ± 4[3] | 45 ± 7 | 62 ± 12 | 72.0 ± 2.4 |
| B | 462 ± 101 | 1360 ± 350 | 1822 ± 448 | 74.6 ± 3.6 |
| C | 555 ± 151 | 1380 ± 405 | 1935 ± 540 | 71.3 ± 2.0 |
| | Aorta,[2] mg/gm fresh tissue | | | |
| A | 1.0 ± 0.2 | 0.1 ± 0.1 | 1.1 ± 0.3 | 9.1 ± 2.5 |
| B | 3.3 ± 1.4 | 5.7 ± 2.0 | 9.0 ± 3.2 | 63.3 ± 3.0 |
| C | 3.4 ± 1.8 | 4.9 ± 2.5 | 8.3 ± 3.5 | 59.0 ± 4.1 |
| | Liver, mg/gm fresh tissue | | | |
| A | 1.7 ± 0.3 | 0.6 ± 0.2 | 2.3 ± 0.5 | 26.1 ± 3.0 |
| B | 6.4 ± 1.0 | 46.4 ± 6.1 | 52.8 ± 7.0 | 87.9 ± 2.0 |
| C | 7.0 ± 1.6 | 46.7 ± 7.2 | 53.7 ± 9.0 | 87.0 ± 1.8 |

[1] Represents the serum cholesterol values at death.
[2] The degree of atherosclerosis for group B was 2.1 ± 0.7 and for group C, 3.0 ± 1.0.
[3] Standard deviation.

tions. The percentage of total cholesterol as ester increased markedly in both aorta and liver. There were no significant differences between the levels of cholesterol in the aorta, serum and liver of groups B and C. The degree of atherosclerosis for group B was less (2.1) than for group C (3.0), but this difference was found to be not significant at the 1 or 5% levels.

*Tissue cholesterol ester fatty acids (table 3). Serum:* The corn oil (B) and olive oil (C) diets had significant effects on the serum cholesterol ester fatty acid composition. The major changes in the cholesterol ester fatty acids of the groups fed those diets were in the proportions of oleic, linoleic and palmitic acids. Group B (corn oil diet) had a significantly greater percentage of oleic acid and a significantly lower percentage of palmitic acid than the control group (A). A slight increase occurred in the percentage of linoleic acid, but this was found not be significant. The most marked change in that serum lipid fraction occurred in the group fed the olive oil diet (group C). Oleic acid became the major acid of the serum cholesterol ester fatty acids (58.1%). Also significant proportionate decreases occurred in the percentages of palmitic and linoleic acids in the serum cholesterol ester fraction of group C when compared with that serum fraction of the other two groups (A and B).

*Aorta*: The aortas of the group fed the control diet (A) did not contain sufficient cholesterol esters for fatty acid analysis. The major fatty acid of the cholesterol esters deposited in the aorta of the animals receiving diets B and C was oleic acid; the group receiving the corn oil diet (B) had significantly less oleic acid than the group fed the olive oil diet (C). The level of linoleic acid was quite low in both groups (group B, 22.3%; group C, 10.3%). Comparison of the aorta and serum cholesterol ester fatty acid composition indicated significant differences in fatty acid composition. The cholesterol esters in the aorta of groups B and C had significantly less linoleic acid and more saturated fatty acids than that fraction of the serum. That the cholesterol esters of the aorta were influenced by the fatty

TABLE 3

*Effect of diets on tissue cholesterol ester fatty acid composition*

| Fatty acid[1] | | Serum | | | Aorta[2] | | Liver | | |
|---|---|---|---|---|---|---|---|---|---|
| No. carbons | No. double bonds | A | B | C | B | C | A | B | C |
| | | % *total fatty acids* | | | % *total fatty acids* | | % *total fatty acids* | | |
| 6 to 15 | | 2.1 ± 0.5[3] | 1.7 ± 0.4 | 1.7 ± 0.3 | 2.9 ± 1.0 | 2.8 ± 1.2 | 2.4 ± 0.7 | 1.7 ± 0.7 | 1.5 ± 0.4 |
| 16 | 0 | 21.7 ± 1.8 | 12.6 ± 1.1 | 13.0 ± 2.1 | 19.5 ± 3.9 | 15.6 ± 2.0 | 28.0 ± 2.1 | 15.6 ± 3.3 | 11.0 ± 1.6 |
| 16 | 1 | 3.9 ± 0.5 | 1.5 ± 0.5 | 2.2 ± 0.4 | 3.2 ± 0.7 | 4.0 ± 0.7 | 2.6 ± 0.5 | 2.3 ± 0.9 | 2.3 ± 0.6 |
| 16 | 2 | 0.6 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.1 | 1.9 ± 0.4 | 2.1 ± 0.5 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 |
| 17 | 0 | 1.5 ± 0.4 | 0.6 ± 0.3 | 0.5 ± 0.2 | 0.3 ± 0.2 | 0.2 ± 0.1 | 0.7 ± 0.2 | 0.3 ± 0.1 | 0.3 ± 0.1 |
| 18 | 0 | 3.9 ± 1.1 | 3.8 ± 1.0 | 3.9 ± 1.5 | 6.4 ± 1.4 | 5.0 ± 0.7 | 16.0 ± 4.3 | 4.2 ± 1.2 | 4.3 ± 1.1 |
| 18 | 1 | 27.2 ± 5.0 | 36.2 ± 4.9 | 58.1 ± 5.6 | 39.9 ± 5.4 | 57.1 ± 4.3 | 30.0 ± 4.6 | 44.8 ± 3.4 | 68.5 ± 5.9 |
| 18 | 2 | 36.4 ± 4.2 | 40.4 ± 3.6 | 18.0 ± 3.1 | 22.3 ± 4.2 | 10.3 ± 1.7 | 17.5 ± 3.6 | 28.5 ± 2.5 | 10.6 ± 4.4 |
| 18 | 3 | 0.6 ± 0.2 | 1.5 ± 0.2 | 1.2 ± 0.2 | 1.7 ± 0.3 | 1.5 ± 0.6 | 1.1 ± 0.3 | 1.2 ± 0.5 | 0.5 ± 0.2 |
| 20 | 0 | 0.3 ± 0.2 | 0.2 ± 0.1 | 0.6 ± 0.2 | 1.0 ± 0.5 | 0.6 ± 0.3 | 0.7 ± 0.2 | 0.5 ± 0.2 | 0.4 ± 0.1 |
| 20 | 4 | 1.8 ± 0.4 | 0.9 ± 0.5 | 0.6 ± 0.2 | 0.9 ± 0.3 | 0.8 ± 0.2 | 0.8 ± 0.4 | 0.7 ± 0.2 | 0.4 ± 0.2 |

[1] Represents the major fatty acids determined; small amounts of others were also detected.
[2] Insufficient cholesterol ester was present in normal rabbit aorta for analysis.
[3] Standard deviation.

TABLE 4

*Effect of diets on tissue triglyceride fatty acid composition*[1]

| Fatty acid | | Serum | | | Aorta | | | Liver | | |
|---|---|---|---|---|---|---|---|---|---|---|
| No. carbons | No. double bonds | A | B | C | A | B | C | A | B | C |
| | | *% total fatty acids* | | | | | | | | |
| 6 to 15 | | 2.5 ± 1.0[2] | 2.6 ± 1.1 | 3.0 ± 0.7 | 2.9 ± 0.7 | 3.3 ± 0.6 | 3.8 ± 0.8 | 2.4 ± 0.8 | 3.4 ± 0.9 | 3.6 ± 0.7 |
| 16 | 0 | 37.5 ± 3.6 | 21.9 ± 3.4 | 22.0 ± 5.5 | 36.4 ± 5.0 | 30.0 ± 5.3 | 26.8 ± 3.4 | 37.0 ± 3.5 | 28.2 ± 3.0 | 26.0 ± 2.7 |
| 16 | 1 | 2.4 ± 0.8 | 1.7 ± 0.6 | 3.0 ± 1.5 | 2.7 ± 0.8 | 2.0 ± 0.4 | 2.6 ± 1.4 | 2.1 ± 0.4 | 1.5 ± 0.4 | 2.3 ± 0.6 |
| 16 | 2 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 |
| 17 | 0 | 0.4 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.4 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.4 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.1 |
| 18 | 0 | 5.5 ± 1.4 | 6.9 ± 1.5 | 7.0 ± 2.1 | 5.6 ± 1.0 | 6.5 ± 0.5 | 6.4 ± 2.5 | 4.3 ± 0.6 | 5.2 ± 1.1 | 5.5 ± 2.2 |
| 18 | 1 | 24.6 ± 2.0 | 26.5 ± 1.5 | 48.0 ± 6.8 | 29.0 ± 2.4 | 26.0 ± 1.7 | 40.8 ± 3.4 | 21.1 ± 3.0 | 26.6 ± 2.3 | 47.0 ± 2.7 |
| 18 | 2 | 25.0 ± 2.4 | 38.5 ± 6.5 | 15.1 ± 2.4 | 20.7 ± 3.0 | 29.4 ± 4.5 | 17.3 ± 4.2 | 30.7 ± 3.2 | 33.4 ± 2.9 | 14.1 ± 2.2 |
| 18 | 3 | 1.1 ± 0.3 | 0.8 ± 0.3 | 0.9 ± 0.3 | 1.5 ± 0.3 | 1.7 ± 0.3 | 1.2 ± 0.6 | 1.2 ± 0.5 | 0.6 ± 0.1 | 0.5 ± 0.1 |
| 20 | 0 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.5 ± 0.2 | 0.3 ± 0.2 | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 |
| 20 | 4 | 0.5 ± 0.2 | 0.5 ± 0.1 | 0.4 ± 0.2 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 |

[1] See footnotes to table 3.
[2] Standard deviation.

acid composition of the diet is of particular interest.

*Liver:* The liver cholesterol esters of the group receiving the control diet (A) contained a higher percentage of saturated fatty acids (palmitic and stearic) and a lower percentage of linoleic acid than that fraction of the serum. The percentage of oleic acid in both the serum and liver cholesterol esters was approximately the same. The diets (B and C) produced pronounced changes in the fatty acid composition of the liver cholesterol esters. As with the other tissues, the most pronounced effect was seen in the group fed the olive oil diet (C). The liver cholesterol esters of that group contained 68.5% of oleic acid and a very low level of linoleic acid (10.6%). The corn oil diet (B) produced significant increases in the oleic and linoleic acid levels and a lowering in the percentage of saturated fatty acids.

*Triglyceride fatty acid composition (table 4). Serum:* The corn oil diet (B) produced a significant decrease in the percentage of palmitic acid and an increase in linoleic acid in the serum triglycerides. The olive oil diet (C) produced a decrease in palmitic and linoleic acids and an increase in oleic acid.

*Aorta:* The aorta triglycerides of the group fed the corn oil diet (B) showed a decrease in palmitic acid and an increase in linoleic acid when compared to the control group (A). The group fed the olive oil diet (C) showed the most marked changes in triglyceride fatty acid composition. There was a decrease in the proportion of palmitic and linoleic acids and an increase in oleic acid. Comparison of the serum and aorta triglycerides did not indicate any major differences.

*Liver:* The olive oil diet (C) produced the most marked change in liver triglyceride fatty acid composition. Less palmitic and linoleic acids and more oleic acid were observed than in the other two groups (A and B).

## DISCUSSION

This study extends the previous report (Swell et al., '61a) on the cholesterol ester fatty acid pattern in sera and aortae of atherosclerotic rabbits. It was also shown that the fatty acid composition of the de-

posited aorta cholesterol esters is not constant, but can be altered by changing the fatty acid composition of the dietary fat. The aorta cholesterol esters of rabbits fed the high cholesterol-corn oil diet contained a higher percentage of linoleic acid than that fraction in the aorta of animals receiving a similar diet with olive oil. Even in the group receiving the corn oil diet, however, the major fatty acid of the aorta cholesterol esters was oleic acid. Oleic acid has also been shown to be the major fatty acid of the cholesterol ester fraction in the human plaque lipids and thickened intima (Swell et al., '60a, b). The fatty acid composition of the serum cholesterol esters was likewise dependent upon the type of dietary fat; a large increase in the level of serum cholesterol oleate occurred when the rabbits were fed the olive oil diet. The results of the present study suggest that in the relationship between the serum cholesterol level and the development of atherosclerotic plaques in the rabbit there are both quantitative and qualitative factors, namely, the absolute level of cholesterol esters in the serum and fatty acid composition of those esters. Thus, below a certain serum cholesterol level atherosclerosis does not develop; also, there is a relationship between the level of the serum cholesterol and the time required for the development of the disease. As for the qualitative aspect, it has been shown (Field et al., '60; Biggs and Kritchevsky, '51) that the major part of the plaque cholesterol in man and rabbit is derived from the serum, and yet, the fatty acid composition is quite different between plaque and serum cholesterol esters (Swell et al., '60a, b, '61a; Blomstrand and Christensen, '61). Thus, the data of the present and previous studies suggest that certain cholesterol esters, and in particular cholesterol oleate, may be preferentially deposited in the aorta and that the deposition is dependent upon the level of those esters in the serum cholesterol ester fraction.

No significant differences were noted in the degree of atherosclerosis between the groups receiving the high-cholesterol diets supplemented with corn or olive oils. These differences might have been significant if a diet with less cholesterol had been fed over a shorter period of time.

The major fatty acid of the liver cholesterol esters of the animals receiving the high cholesterol-oil supplemented diets (B and C) was oleic acid. Similar observations have been made (Evans et al., '59) in rabbits fed cholesterol supplemented with tallow or corn oil. This also suggests that there are marked differences in the handling of individual cholesterol esters in the tissues.

The diets (B and C) produced some changes in the tissue triglyceride fatty acid composition, but these were not as striking as with the cholesterol ester fraction. The major changes occurred in the group receiving the olive oil diet. In the serum similar changes in fatty acid composition were observed as with the cholesterol ester fraction. In general, the serum and aorta triglyceride fractions of the several groups were similar in fatty acid composition. The data suggest that the tissue triglycerides may be derived from common fatty acid pools.

## SUMMARY

The observations indicate that the nature of the dietary fat incorporated into a high-cholesterol diet influences the serum and tissue cholesterol ester and triglyceride fatty acid composition. The aorta cholesterol esters are not of constant composition, but are dependent upon the fatty acid composition of the dietary fat fed. The data also suggest that derangements of cholesterol oleate metabolism may be an important factor involved in the deposition of cholesterol esters in liver and aorta of rabbits fed high-cholesterol diets.

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# Dietary Protein and Utilization of Vitamin A

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Numerous studies in the past have suggested a relationship exists between vitamin A utilization and nitrogen metabolism. A decreased rate of growth (Sampson et al., '32; Sampson and Korenchevsky, '32; Braman et al., '35; Patterson et al., '42; Mayer and Krehl, '48a, b), lowered food and protein efficiency (Sampson and Korenchevsky, '32; Mayer and Krehl, '48a, b; Brown and Morgan, '48) coupled with increased nitrogen excretion (Sampson et al., '32; Émerique, '37; Brown and Morgan, '48) and decreased protein synthesis (Leutskaya, '57) were observed in animals suffering from avitaminosis A. Moreover, liberal amounts of vitamin A are known to lessen the ill effects of a severe protein deficiency (Moore et al., '52). The reverse relationship, namely, the effect of dietary protein on vitamin A metabolism, has been studied also, but the results are not conclusive (Randoin and Queuille, '34; Basu and De, '41; Baumann et al., '42; Dye et al., '45; Deuel et al., '46; McCoord et al., '47; Mayer and Krehl, '48b; Johnson and Baumann, '48; Leutskii and Leutskaya, '55; Peretianu and Lupu, '57). The understanding of this relationship is of particular interest because of the high incidence of xerophthalmia and keratomalacia, attributable to vitamin A deficiency in children suffering from kwashiorkor and other protein malnutrition states (Esh et al., '60; Gopalan et al., '60).[3]

Several investigators (Basu and De, '41; Baumann et al., '42; Leutskii and Leutskaya, '55; Peretianu and Lupu, '57) claimed that protein is necessary for the utilization and storage of vitamin A. The more recent work, however, conducted in our laboratory (Rechcigl et al., '59a, b) as well as elsewhere[4] (Olsen et al., '59; Esh et al., '60) suggests that dietary protein is not absolutely essential for the storage of vitamin A. Our studies indicate that rats are able to store ample amounts of vitamin A despite an amino acid imbalance or in the complete absence of dietary protein.

The object of the present investigation was to study the effect of the amount and quality of dietary protein on the utilization of vitamin A.

## EXPERIMENTAL

Male weanling albino rats of the Holzman strain[5] were housed individually and given food and water ad libitum. Two days prior to the start of the experiments a large dose of vitamin A acetate in oil solution was given orally to all rats in order to increase their vitamin A reserves. Several representative animals were killed as a control group at the beginning of the experiments to determine the initial storage of vitamin A in the liver and kidneys (Ames et al., '54). The remaining rats were divided into appropriate experimental groups usually consisting of 4 or 5 rats and totaling the same weight at the onset of the treatments. The body weight changes and food consumption were recorded daily during the three-week experimental period.

At the termination of the experiments the animals were killed, the livers and kidneys removed, weighed and analyzed for vitamin A content. The liver fat was determined by weighing the residue from

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[3] These publications list numerous references on the subject.

[4] Arnrich, L., and D. J. Pederson 1956 Dietary protein and carotene utilization. Federation Proc., 15: 212 (abstract).

[5] Purchased from Holtzman, Madison, Wisconsin.

an ether extract (evaporated to dryness under nitrogen) of the livers, dehydrated with $Na_2SO_4$. Nitrogen analyses were carried out on the fat-extracted liver residues by the Kjeldahl method.

The composition of the basal protein-free diet shown in table 1, is similar to that of the diets used previously in this laboratory (Rechcigl et al., '58; Rechcigl and Williams, '61). Different experimental diets were prepared by inclusion of the protein source, compensating for the different levels by adjusting the percentage of dextrin.

The experiments were carried out in two phases with respect to the utilization of vitamin A. The first part deals with the effect of quantity of protein; the second part with effect of quality of protein.

TABLE 1

*Composition of the basal diet*

| | *gm/100 gm* |
|---|---|
| MAJOR COMPONENTS | |
| Dextrin | 58.0 |
| Glucose[1] | 15.0 |
| Hydrogenated vegetable oil[2] | 14.0 |
| Salt mixture[3] | 4.0 |
| Cellulose[4] | 2.0 |
| Fat soluble vitamins (in corn oil) | 2.0 |
| B vitamins (in starch) | 5.0 |
| | *mg* |
| VITAMIN COMPONENTS | |
| B Vitamins in 5.0 gm of starch | |
| Thiamine | 0.25 |
| Riboflavin | 0.25 |
| Pyridoxine | 0.25 |
| Ca pantothenate | 2.00 |
| Nicotinic acid | 1.00 |
| p-Aminobenzoic acid | 5.00 |
| Inositol | 10.00 |
| Choline chloride | 100.00 |
| | *mg* |
| Fat-soluble vitamins in 2.0 gm of corn oil | |
| Vitamin D (calciferol) | 0.0045 |
| α-Tocopherol | 11.00 |

[1] Cerelose, Corn Products Company, New York.
[2] Crisco, Procter and Gamble Company, Cincinnati.
[3] Jones and Foster ('42).
[4] Solka-Floc, Brown Company, Berlin, New Hampshire.

## RESULTS

*Influence of level of protein.* The results of these experiments are summarized in table 2. Rats receiving no protein in their diet lost weight and suffered from anorexia These animals, however, utilized less vitamin A than any other group, as evident from the high liver content of vitamin A. With an increase in the dietary protein there was a progressive increase in the body weight gain accompanied by higher food consumption and efficiency of food utilization. The total vitamin A content of the liver, in general, was lowered as the protein level in the diet was increased. But the content of vitamin A in the kidney became greater with an increase in the dietary protein.

The differences in the vitamin A content of the organs were increased by expressing them per 100 gm of body weight. This is understandable because of the large differences in the body weights of

TABLE 2

*Effect of level of protein in the diet on depletion of liver and kidney vitamin A*

| | Control | Protein-free | 6% Casein | 12% Casein | 18% Casein |
|---|---|---|---|---|---|
| Av.weight gain,[1] gm | | −14 ± 0.6[2] | 19 ± 3.1 | 71 ± 7.0 | 97 ± 7.3 |
| Food intake, gm | | 81 | 148 | 223 | 223 |
| Food efficiency[3] | | −0.17 | 0.13 | 0.32 | 0.43 |
| Liver weight, gm | 2.8 ± 0.23 | 1.6 ± 0.03 | 2.7 ± 0.13 | 5.3 ± 0.53 | 6.3 ± 0.15 |
| Liver fat, % | 2.2 | 3.3 | 3.4 | 3.4 | 3.3 |
| Liver vitamin A total μg | 377 ± 19.6 | 317 ± 5.8 | 303 ± 3.3 | 268 ± 15.1 | 241 ± 6.4 |
| μg/100 gm body weight | | 682 | 379 | 203 | 152 |
| μg/gm organ | 135 | 198 | 112 | 50 | 38 |
| Kidney weight, gm | 0.74 ± 0.07 | 0.46 ± 0.03 | 0.63 ± 0.02 | 1.00 ± 0.04 | 1.28 ± 0.05 |
| Kidney vitamin A total μg | 6.3 ± 0.29 | 5.6 ± 0.64 | 3.7 ± 0.18 | 7.3 ± 0.69 | 12.2 ± 0.53 |
| μg/100 gm body weight | | 12.0 | 4.6 | 5.5 | 7.2 |
| μg/gm organ | 8.5 | 12.2 | 5.9 | 7.3 | 9.5 |

[1] The average initial weight of the rats was 60 gm.
[2] Standard error of the mean.
[3] Grams gain per gram of food consumed.

various experimental groups and the apparent relationship between the vitamin A content and growth. Increase in the protein level also resulted in larger size and weight of the organs which is reflected in even more striking diminution of vitamin A reserves when the values are expressed per unit of weight. The fat content of the livers did not show significant changes between the various experimental groups.

The total vitamin A depletion or utilization, calculated by subtracting the final storage of vitamin A in the liver and the kidneys of the experimental animals from the initial storage of the controls, was lowest in rats ingesting a protein-free diet and became progressively greater as the protein level in the diet was increased. Apparently a linear relationship exists between weight gain and vitamin A utilization as the plotting of these two parameters resulted in a straight line (fig. 1), over the range studied.

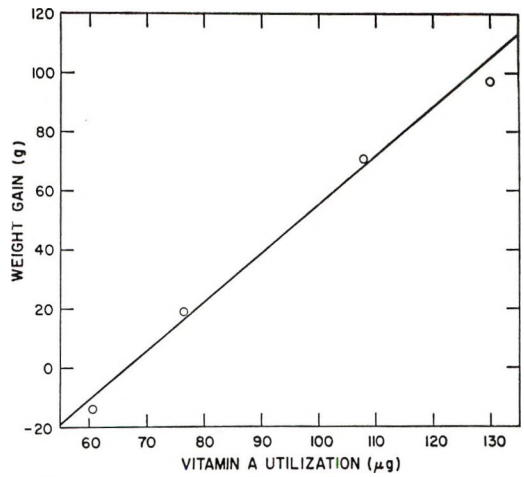


Fig. 1 Linear correlation between vitamin A utilization and the growth rate of rats fed different levels of casein.

*Influence of quality of protein.* The experimental results of this study are shown in table 3. Of the three proteins tested, feeding of casein resulted in the best growth of rats. The animals ingesting gluten as a sole source of protein grew very slowly, whereas those maintained with the zein diet gradually lost weight. The poor growth response observed in rats fed gluten was corrected by supplementation of the diet with 0.8% of L-lysine and 0.3% of DL-threonine. The addition of a complex amino acid mixture to zein increased only slightly the body weight gains of the animals. The pattern of food consumption followed very closely the changes in growth.

Feeding of gluten or zein resulted in a greater fat content of the liver than the casein diet. Although with zein this effect

TABLE 3

*Effect of quality of protein in the diet on depletion of liver and kidney vitamin A*

| | Control | 18% Casein | 18% Gluten | 18% Gluten + AA[1] | 18% Zein | 18% Zein + AA[2] |
|---|---|---|---|---|---|---|
| Av. weight gain,[3] gm | | 123 ± 9.4[4] | 25 ± 4.5 | 109 ± 9.7 | −6 ± 1.3 | 17 ± 3.0 |
| Food intake, gm | | 269 | 163 | 258 | 94 | 127 |
| Food efficiency[4] | | 0.46 | 0.15 | 0.42 | −0.06 | 0.13 |
| Liver weight, gm | 2.1 ± 0.05 | 7.9 ± 0.75 | 3.6 ± 0.42 | 8.5 ± 0.45 | 1.9 ± 0.04 | 2.8 ± 0.23 |
| Liver fat, % | 3.6 | 2.8 | 4.4 | 4.9 | 5.5 | 2.4 |
| Liver nitrogen, % | | 2.34 | 2.44 | 2.37 | 2.72 | 2.88 |
| Liver vitamin A total μg | 265 ± 5 | 149 ± 11 | 179 ± 1 | 168 ± 5 | 179 ± 13 | 162 ± 7 |
| μg/100 gm body weight | | 86 | 236 | 105 | 398 | 238 |
| μg/gm organ | 126 | 19 | 50 | 20 | 94 | 58 |
| Kidney weight, gm | 0.57 ± 0.02 | 1.45 ± 0.08 | 0.76 ± 0.07 | 1.37 ± 0.04 | 0.50 ± 0.02 | 0.78 ± 0.05 |
| Kidney vitamin A total μg | 4.8 ± 0.5 | 8.3 ± 1.8 | 3.0 ± 0.25 | 8.3 ± 1.3 | 1.8 ± 0.1 | 2.2 ± 0.2 |
| μg/100 gm body weight | | 4.8 | 3.9 | 5.2 | 4.0 | 3.2 |
| μg/gm organ | 8.4 | 5.7 | 3.9 | 6.1 | 3.6 | 2.8 |

[1] AA included 0.8% L-lysine and 0.3% DL-threonine.

[2] AA included 1.0% L-lysine, 1.15% DL-valine, 0.15% DL-histidine, 0.3% DL-tryptophan, 0.8% DL-threonine, 0.2% L-arginine and 0.3% DL-isoleucine.

[3] The average initial weight of rats was 51 gm.

[4] See appropriate footnotes in table 2.

was reversed by amino acid supplementation, no change was observed on supplementing the gluten diet. The increased concentration of liver nitrogen observed on zein may be a reflection of dehydration, commonly seen in starvation.

The rats ingesting a casein diet had the lowest amount of vitamin A in the liver at the end of the three-week test. Those fed gluten and zein showed identical values, which were quite high in comparison with those from the casein group. Amino acid supplementation of gluten and zein resulted in slight decrease of hepatic vitamin A values.

The vitamin A content of the kidneys, however, was highest in the casein group. The relatively low vitamin A content of the kidneys of rats fed the gluten ration was increased by amino acid supplementation, and essentially no change in kidney vitamin A was observed by the supplementation of the zein diet.

The observed differences in the vitamin A content of organs were increased by expressing the values per unit of organ weight or by correcting them for the differences in body weight.

## DISCUSSION

The above experiments demonstrated that depletion of liver vitamin A stores is affected both by the amount and the quality of dietary protein. These data are in agreement with previous studies from this laboratory (Rechcigl et al., '59a, b) showing that the oral administration of equal amounts of vitamin A to growing rats resulted in greater hepatic vitamin A storage in animals fed a protein-free diet than in animals maintained with an 18% casein diet. Similarly, rats whose growth was retarded by an amino acid imbalance stored more vitamin A than the control animals. Arnrich and Pederson[6] also reported in a preliminary communication that following vitamin A administration the concentration of vitamin A per gram of liver was almost double in animals receiving diets low in protein. In the more recent work of Olsen et al. ('59), with chicks, an inverse relationship was noted between the protein level and vitamin A storage.

These observations are also in accord with less recent observations showing that in vitamin A-deficient animals, increased levels of dietary protein either had no effect upon time of development of xerophthalmia (Randoin and Queuille, '34) or actually increased severity of the symptoms of vitamin A deficiency (Brown and Morgan, '48). In the recent work of McLaren ('59) protein depletion delayed the development of xerophthalmia in rats fed diets deficient in vitamin A. In the studies of Dye et al. ('45) the level of dietary protein had no effect on the utilization of vitamin A as judged by weight gain and incidence of foci of keratinized epithelium.

A direct relationship was observed between growth and vitamin A utilization in the present experiments, particularly in the study on the effect of protein quantity (fig. 1). A similar relationship was noted earlier by Johnson and Baumann ('48), using diets fed in limited amounts or deficient in thiamine or tryptophan. The observed relationship is primarily due to changes of the vitamin A content of the liver where most of the vitamin is stored. The kidney vitamin A, however, is apparently inversely related to the rate of growth. This is apparent from this as well as the work of Moore ('57).

In table 4 vitamin A utilization for growth with various diets was estimated by calculating the amount of vitamin A depleted from the liver and the kidneys per gram of gain. In addition, a second formula was used which allows calculation of vitamin A needed by the growing animal for growth and maintenance. Using this procedure it may be concluded that the quantity of dietary protein does not significantly alter the efficiency of vitamin A utilization.

Vitamin A requirement for maintenance can also be estimated in an alternative way from the plot of growth and vitamin A utilization, shown in figure 1, as the amount utilized at zero gain. By this procedure the maintenance requirement was found to approximate 66 μg for the three-week experimental period or 3 μg per day. The efficiency of vitamin A utilization can

[6] See footnote 4.

TABLE 4

*Efficiency of vitamin A utilization with experimental diets*

| Series no. | Diet | Total vitamin A depletion[1] | Efficiency of utilization | |
|---|---|---|---|---|
| | | | Growth[2] | Growth — maintenance[3] |
| | | μg | μg | μg |
| I | Protein-free | 60.7 | −4.3 | 1.1 |
| | 6% Casein | 76.6 | 4.0 | 1.1 |
| | 12% Casein | 108.0 | 1.5 | 1.1 |
| | 18% Casein | 130.1 | 1.3 | 1.2 |
| II | 18% Casein | 113 | 0.9 | 1.0 |
| | 18% Gluten | 88 | 3.5 | 1.4 |
| | 18% Gluten + AA[4] | 93 | 0.9 | 0.9 |
| | 18% Zein | 89 | −15.0 | 1.9 |
| | 18% Zein + AA[4] | 106 | 6.2 | 1.8 |

[1] Initial reserves minus final reserves of vitamin A.

[2] $\frac{\text{Total vitamin A depletion}}{\text{Body gain}}$.

[3] $\frac{\text{Total vitamin A depletion}}{\text{Initial body weight} + \left(\frac{\text{Body gain}}{2}\right)}$.

[4] See appropriate footnotes in table 3.

then be calculated by subtracting the former value from the amount of vitamin A depleted from the liver and kidneys, and by dividing the difference by the animals' weight gain. In this way it was calculated that 0.6, 0.6 and 0.7 μg of vitamin A were necessary to produce a 1-gm gain for 6, 12 and 18% casein diets, respectively. This is in agreement with the above results showing that under the present conditions the quantity of protein had no apparent effect on the efficiency with which stored vitamin A was utilized. The vitamin A utilization values calculated from the results obtained in the experiments in which proteins of different quality were fed suggest, however, that the efficiency of vitamin A utilization is affected by protein quality.

## SUMMARY

The effect of protein quantity and quality on vitamin A utilization was studied by administering a large dose of vitamin A to rats at the start of the test and measuring the vitamin A remaining in the liver and kidneys after the test diets were fed for three weeks. Highest vitamin A content was observed in the livers of rats fed a protein-free diet. With an increase in the dietary protein there was a progressive decrease in the amount of vitamin A in the liver and an increase in the amount of vitamin A in the kidneys. A linear relationship was observed between weight gain and vitamin A utilization.

The utilization of vitamin A was also affected by protein quality. In rats ingesting an 18% casein diet, less vitamin A was observed in the liver and more in the kidneys than in slower-growing animals fed 18% of either gluten or zein. Amino acid supplementation of gluten and zein resulted in an improved growth rate, increased depletion of hepatic vitamin A and greater total utilization of the vitamin. The efficiency of vitamin A utilization, calculated after correction for the maintenance requirement, was decreased by inferior protein quality, but was not affected by the level of dietary protein.

## ADDENDUM

After the completion of this manuscript, papers by Jagannathan and Patwardhan ('60a, b) bearing on this problem appeared. Although the experiments are not strictly comparable, their results are essentially in agreement with those of the present study. Stoewsand and Scott ('61) have shown that in chicks a high-protein diet decreases blood and liver vitamin A levels and favors the onset of deficiency symptoms in comparison with low-protein diets.

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# Methylation of Nicotinamide in Vitamin $B_{12}$- and Vitamin $B_6$-Deficient Rats

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The role of vitamin $B_{12}$ on methylation is not yet established. On one hand, vitamin $B_{12}$-deficient chicks can utilize equal molecular amounts of homocysteine and betaine in place of methionine, and monomethylaminoethanol and betaine in place of dietary choline for growth and for the prevention of perosis[2] for which choline per se is required. In baby pigs vitamin $B_{12}$ deficiency did not influence the level of choline (Firth et al., '54) in liver nor its urinary excretion. These data were interpreted to indicate that transmethylation from betaine to methionine or from methionine to choline is not affected by vitamin $B_{12}$.

On the other hand, liver homogenates from vitamin $B_{12}$-deficient rats exhibit a reduced ability to form methionine from homocysteine and cholines (Oginsky, '50). Dietrich et al. ('52) and Fatterpaker et al. ('55) demonstrated that the excretion of N-methylnicotinamide is decreased in vitamin $B_{12}$ deficiency, and reported that creatine formation is increased under the influence of vitamin $B_{12}$.

Because of the discrepancy in interpretation of the results cited above, it was deemed worthwhile to determine as far as possible, whether methylation by vitamin $B_{12}$-deficient animals is impaired. And if so, whether the impairment is due to the deficiency of vitamin $B_{12}$ rather than to nonspecific factors such as reduction of food intake or weight differences between groups. Furthermore, will the deficiency of vitamin $B_6$, which reduces vitamin $B_{12}$ absorption, result in a sufficient reduction of the total liver vitamin $B_{12}$ content so as to effect the methylation capacity of vitamin $B_6$-deficient animals? The results of this study are reported here.

## EXPERIMENTAL

### 1. *Preparation of animals*

*Vitamin $B_{12}$-deficient animals.* Male and female progeny of rats of the McCollum strain fed a soybean diet (Ling and Chow, '52) low in vitamin $B_{12}$ content during pregnancy and lactation, were placed in individual cages with large screen bottoms, and offered the same soybean diet. Unless otherwise stated, the treated littermates were fed the same basal diet supplemented with vitamin $B_{12}$ (100 μg per kg of diet). These diets were offered to the two respective groups of animals for 6 to 8 months, to provide an ample opportunity for the development of the deficiency of vitamin $B_{12}$ in the group deprived of this vitamin.

*Vitamin $B_6$-deficient animals.* Male weanling rats of the McCollum strain, as well as of the Wistar strain, were offered, ad libitum the vitamin $B_6$-deficient diet consisting of casein, sucrose, minerals and all known vitamins except vitamin $B_6$ (Tulpule and Williams, '55). The animals were housed in screen-bottom cages. For comparison, the littermates were supplied daily (1) by the injection of 50 μg of pyridoxine hydrochloride or (2) by the supplementation of the basal diet with pyridoxine hydrochloride (100 μg per kg). The animals were placed under these dietary regimens for several months, and the usual symptoms of vitamin $B_6$ deficiency such as acrodynia and loss of weight and appetite developed in the group deprived of this vitamin. Even in the last group most of the animals survived.

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TABLE 1

*Excretion of N-methylnicotinamide (MNA) in the urine of vitamin $B_{12}$-deficient and treated rats and their vitamin $B_{12}$ status*[1]

| Experiment | A[2] | | B[3] | |
|---|---|---|---|---|
| | Males | | Females | |
| | Vitamin $B_{12}$-deficient | Vitamin $B_{12}$-treated | Vitamin $B_{12}$-deficient | Vitamin $B_{12}$-treated |
| Body weight, gm | 201 ± 3 | 319 ± 14 | 241 ± 4 | 235 ± 8 |
| Test 1[4] | 3.07 ± 0.12 | 3.64 ± 0.16 | 2.34 ± 0.21 | 3.06 ± 0.11 |
| Test 2[5] | 5.71 ± 0.25 | 7.60 ± 0.12 | 4.67 ± 0.24 | 6.37 ± 0.26 |
| Liver, gm, % body weight | 5.16 ± 0.59 | 3.25 ± 0.17 | 3.13 ± 0.10 | 3.06 ± 0.14 |
| Liver vitamin $B_{12}$, mμg/gm liver | 27.1 ± 6.9 | 124.8 ± 8.0 | 56.9 ± 4.8 | 328.7 ± 19.3 |
| Total liver vitamin $B_{12}$, mμg % body weight | 127.4 ± 23.5 | 401.7 ± 23.4 | 176.9 ± 14.1 | 1009.7 ± 79.2 |
| Serum vitamin $B_{12}$, μμg/ml | 116.7 ± 15.2 | 1358.0 ± 91.0 | 65.3 ± 7.0 | 954.1 ± 37.5 |

[1] All results are expressed as their averages ± standard error of the mean.
[2] Five rats were used for experiment A in each group.
[3] Ten rats were used for experiment B in each group.
[4] Test 1 = (5.0 mg nicotinamide/100 gm of body weight) MNA excreted (mg/100 gm of body weight/24-hour urine).
[5] Test 2 = (20 mg nicotinamide/100 gm of body weight) MNA excreted (mg/100 gm of body weight/24-hour urine).

### 2. *Methods of analysis*

*A. Serum glutamic oxalacetic acid transaminase activity.* The serum glutamic oxalacetic acid transaminase activity (SGOT) was measured by the procedure of Steinberg et al. ('56) and was taken as an index of vitamin $B_6$ deficiency.

*B. Vitamin $B_{12}$ determinations.* Vitamin $B_{12}$ concentrations in sera or plasma were determined by the modified procedures of Gaffney et al. ('57), the details of which have been published elsewhere. Livers from experimental animals were homogenized in Potter-Elvehjem glass homogenizers with 10 times their weight of distilled water, treated with potassium cyanide and assayed microbiologically for vitamin $B_{12}$ activity.

*C. Determination of the methylation capacity.* For the determination of the methylation, the urinary excretion of N-methylnicotinamide (MNA) after oral administration of 5, 10 and/or 20 mg of nicotinamide (NA)[3] per 100-gm rat was measured in the 24-hour urine after conversion to trigonelline according to Sarett's procedure ('43). Four days before the first assay, 5 mg of NA were given to each rat by stomach tube to assure full saturation.

## RESULTS

### 1. *Effect of vitamin $B_{12}$ deprivation of the urinary excretion of MNA*

The following experiments were conducted to study the effect of vitamin $B_{12}$ deficiency on the urinary excretion of MNA:

*A. Male rats.* In experiment A, 5 vitamin $B_{12}$-deficient and 5 vitamin $B_{12}$-treated male rats were given orally 5 mg of NA per 100 gm of body weight. Urine collection was made 24 hours later for the determination of MNA. The results (table 1) indicate a greater amount of MNA in the urine of the vitamin $B_{12}$-treated group than that of the vitamin $B_{12}$-deficient group. One week after the first test, these animals were given a second dose of this load compound, which was increased to 20 mg per 100 gm of body weight. The results (table 1) show a smaller amount of MNA in the urine of the vitamin $B_{12}$-deficient group. The vitamin $B_{12}$ status of the two groups of animals

[3] Dissolved in water by stomach tube.

may be assessed by the serum and liver vitamin $B_{12}$ contents.

*B. Female rats.* In experiment B, the MNA excretion test was carried out with female rats (10 in the vitamin $B_{12}$-deficient and 10 in the treated groups). After 12 months of deprivation of dietary vitamin $B_{12}$, the animals were distributed into two groups, one of which continued to receive the vitamin $B_{12}$-deficient diet and the other one received the vitamin $B_{12}$-supplemented diet (100 μg per kg). Two test dosages of 5 and 20 mg of NA per 100 gm of body weight were then given at the time intervals identical to those used for the male rats. Our results (table 1) demonstrate that vitamin $B_{12}$-deficient female rats excreted less MNA than the treated ones. The two groups of animals, unlike those in experiment A, had essentially the same body and liver weights but marked difference in vitamin $B_{12}$ status.

### 2. *The duration of vitamin $B_{12}$ deprivation and the excretion of MNA*

To study the effect of the duration of dietary deprivation of vitamin $B_{12}$ on MNA excretion, NA was given to two groups of rats. The vitamin $B_{12}$-deficient rats (group 1) and the vitamin $B_{12}$-treated rats (group 2) were fed the respective diets for 4 months following weaning. The first and second tests were carried out three and 12 months afterwards. The results of both tests (table 2) demonstrated that vitamin $B_{12}$ deprivation resulted in a reduction of excretion of MNA after three months of feeding. The severity did not increase materially with prolonged deprivation.

### 3. *The urinary excretion of MNA by pair-fed rats*

Twelve vitamin $B_{12}$-deficient male progeny born by mothers fed the soybean diet during the period of pregnancy were divided randomly into two groups. One group continued to be fed the soybean diet and the second group was given the soybean diet supplemented with vitamin $B_{12}$. The food intake of the latter group was pair-fed to that of the first group. At the end of 6 months of feeding, 20 mg of NA was given as a test dose to all the animals. The results tabulated in table 3 demonstrated that there was no significant difference in the body weight, but a marked difference existed in the vitamin $B_{12}$ serum levels. The data show further that the MNA excretion is significantly higher in the vitamin $B_{12}$-treated group than the deprived group. The probability of this difference being due to chance is less than 5%.

### 4. *Effect of pyridoxine deficiency on the urinary excretion of MNA*

All male animals were fed the vitamin $B_6$-deficient diet for 11 weeks. They were then randomly divided into two groups of 6 rats each. The control group (group T) received intraperitoneally 50 μg of pyridoxine hydrochloride, while the deficient group (group D) received a saline solution. Both groups of animals received by intraperitoneal injection 2.5 μg of vitamin $B_{12}$ three times weekly. After 10 weeks of these regimens, the animals were given a test dose of 10 mg NA per 100 gm of body weight by mouth. At that time, the average body weights of the treated group were

TABLE 2

*Duration of dietary deprivation of vitamin $B_{12}$ and N-methylnicotinamide (MNA) excretion by female rats*

| Group[1] | Vitamin $B_{12}$ diets | Duration | | | |
|---|---|---|---|---|---|
| | | Three months | | Twelve months | |
| | | Body weight | MNA | Body weight | MNA |
| | | *gm* | *mg/100 gm body weight* | *gm* | *mg/100 gm body weight/ 24-hour urine* |
| 1 | Deficient | 134 ± 4[2] | 4.20 ± 0.18 | 235 ± 7 | 4.44 ± 0.22 |
| 2 | Supplemented | 128 ± 6 | 5.17 ± 0.21 | 248 ± 6 | 6.37 ± 0.17 |

[1] Six rats were used in each group.

[2] All results are expressed as their averages ± standard error of mean.

TABLE 3

*Effect of vitamin $B_{12}$-deficiency on urinary excretion of N-methylnicotinamide (MNA) by pair-fed male rats*

| Group | Body weight | MNA excreted | Vitamin $B_{12}$ serum levels |
|---|---|---|---|
| | *gm* | *mg/100 gm body weight/ 24-hour urine* | *μμg/ml* |
| Vitamin $B_{12}$-deficient | 208 ± 10[1] | 5.07 ± 0.18 | 128 ± 6 |
| Vitamin $B_{12}$-treated | 214 ± 6 | 6.16 ± 0.14 | > 1,000 ± 68 |

[1] All results are expressed as their averages ± standard error of the mean.

TABLE 4

*Excretion of N-methylnicotinamide (MNA) in pyridoxine-deficient rats receiving vitamin $B_{12}$ by injection before and after cross experiment*

| | Group T (+ vitamin $B_6$) | | Group D (− vitamin $B_6$) | |
|---|---|---|---|---|
| | Body weight | MNA | Body weight | MNA |
| | *gm* | *mg/100 gm body weight/ 24-hour urine* | *gm* | *mg/100 gm body weight/ 24-hour urine* |
| Before cross experiment | 248.2 ± 17.5[1] | 3.77 ± 0.28 | 144.5 ± 12.6 | 4.31 ± 0.29 |
| | Group T (− vitamin $B_6$) | | Group D (+ vitamin $B_6$) | |
| After cross experiment | 270.0 ± 24.2 | 3.85 ± 0.27 | 272.3 ± 20.2 | 4.29 ± 0.15 |

[1] All results are expressed as their averages ± standard error of the mean.

almost twice that of the deficient group (table 4). The average transaminase activity in sera of these two groups of rats was 86.9 ± 0.8 and 23.7 ± 0.7 units per ml, respectively. However, there was a slight but insignificant increase in the amount of MNA excreted in the urine of the deficient animals.

A week after the test, the vitamin $B_6$ treatments were reversed; thus, those in group T now received intraperitoneal injections of a normal saline solution; whereas, those in group D received vitamin $B_6$ by injection. The treatments were continued for 10 additional weeks, at which time the body weights of both groups were essentially the same, that is, body weights of animals in group D approached those of the animals in group T. A test dose of NA was again given. There was no significant difference (table 4) in the urinary excretion of MNA between their two different groups or between the same groups.

### 5. *The effect of prolonged vitamin $B_6$ deficiency on MNA excretion*

Dietary deprivation of vitamin $B_6$ results in a reduction of vitamin $B_{12}$ absorption (Hsu and Chow, '57). If this regimen were to continue for a long period of time, vitamin $B_{12}$ reserve may be expected to be partially depleted (Ranke et al., '60) and some biochemical aberrations of vitamin $B_{12}$ deficiency may appear. It seemed worthwhile, therefore to determine whether prolonged dietary deprivation of vitamin $B_6$ would result in a decrease of the urinary excretion of MNA. In this experiment, three groups of weanling female rats from our colony were given a vitamin $B_6$ deficient diet: to group A, 100 μg vitamin $B_{12}$ was added to 1 kg of diet, to group B a total of 100 mμg of vitamin $B_{12}$ was injected in equal amounts 5 times a week subcutaneously, and group C received both vitamin $B_6$ (5 mg) and 100 μg of vitamin $B_{12}$ per kg of diet. Twenty milligrams of NA per 100 gm of body weight were given to each rat three and 12 months after the dietary regimens were begun.

The data (table 5) show that after three months of feeding there was no difference in the excretion of MNA among the three groups. After 12 months of vitamin $B_6$ deprivation, the following changes were noticed. The vitamin $B_{12}$ serum level of group A was reduced from an average of

TABLE 5

*Effect of prolonged dietary vitamin $B_6$ deprivation on methylation*

| Group[1] | Body weight | Vitamin $B_{12}$ in serum | MNA[3] in urine |
|---|---|---|---|
| | *gm* | *μμg/ml* | *mg/100 gm body weight/ 24-hour urine* |
| | | Test 1[2] | |
| A[4] | 110 ± 4 | 388 ± 76[5] | 5.48 ± 0.32 |
| B[6] | 138 ± 6 | 2,000 ± 180 | 5.88 ± 0.20 |
| C[7] | 186 ± 6 | 960 ± 97 | 6.28 ± 0.16 |
| | | Test 2[8] | |
| A[4] | 178 ± 12 | 68 ± 10.5 | 4.72 ± 0.26 |
| B[6] | 192 ± 20 | 2,000 ± 97 | 5.99 ± 0.31 |
| C[7] | 246 ± 6 | 1,000 ± 110 | 5.92 ± 0.22 |

[1] Ten rats were used in each group.
[2] Test 1 was carried out after 3 months of vitamin $B_6$ deprivation.
[3] N-Methylnicotinamide.
[4] Group A indicates vitamin $B_6$-deficient rats given vitamin $B_{12}$ orally.
[5] All results are expressed as their averages ± standard error of the mean.
[6] Group B indicates vitamin $B_6$-deficient rats given vitamin $B_{12}$ by injection.
[7] Group C indicates vitamin $B_6$-treated rats given vitamin $B_{12}$ orally.
[8] Test 2 was carried out after 12 months of vitamin $B_6$ deprivation.

388 μμg per ml to 68 μμg per ml despite the presence of an adequate amount of vitamin $B_{12}$ in the diet. This reduction was accompanied by a diminution of MNA to a value significantly lower than those of groups B and C.

## DISCUSSION

Early growth experiments with chicks and rats showed that vitamin $B_{12}$ had a methionine-sparing action, especially if homocysteine was supplied (Arnstein, '55; Jukes and Stokstad, '51). At first, this was interpreted as an effect of this vitamin on transmethylation, that is, the transfer of the labile methyl group from choline or betaine to homocysteine to give methionine. More recently, Larrabee et al. ('61) reported that "vitamin $B_{12}$ enzyme" is involved in the formation of methionine from homocystine. Similarly, vitamin $B_{12}$ could replace at least part of the choline requirement of the chick, the rat and the baby pig. Tracer studies showed that vitamin $B_{12}$ had no effect upon transmethylation but that it is involved in the direct synthesis of the labile methyl group from more highly oxidized precursors, such as formate, the α-carbon of glycine or the β-carbon of serine; this subject has been well reviewed by Arnstein ('55).

However, there are difficulties with the interpretation of results in experiments with intact animals. For example, appetite is seriously impaired by vitamin $B_{12}$ deficiency, and the results noted may often be due merely to a lower food intake compared with that of control animals and to the differences in body weight. These uncertainties can be resolved, in part, by restricting food intakes to the level acceptable to the deficient animals ("paired feeding" technique) or by using female rats where vitamin $B_{12}$ treatment does not result in an increase of body weights. The results of the present study aimed at ruling out nonspecific factors show a statistically significant difference in the urinary excretion of MNA between the vitamin $B_{12}$-deficient and treated animals of both sexes, following a test dose of NA. This is also true of pair-fed animals in which instance, as well as in the females, there was no appreciable difference in the body weights or food intake. Therefore, the observed difference is unlikely to be due to these two factors. Furthermore, long-term starvation which usually accompanies vitamin depletion did not alter the urinary excretion of MNA, contrary to the effect of a 48-hour fasting which can result in slightly higher MNA excretion.[4] With these controlled studies we feel justified to conclude that the observed difference is specifically due to vitamin $B_{12}$ deficiency. This belief is supported by the observation that in the cross design

[4] Unpublished data.

study, vitamin $B_6$ deficiency in rats did not effect methylation. Since the nicotinamide dosages were given according to the body weight and since the methylation takes place mainly in the liver, the total amount of liver vitamin $B_{12}$ should be of importance for the evaluation of methylating capacity. This was found to be true regardless of the method of depletion of vitamin $B_{12}$ reserve. Thus, when the vitamin $B_{12}$ reserve is diminished by dietary deprivation or by the impairment of absorption because of vitamin $B_6$ deficiency, the methylation process by the latter group of animals was reduced. These observations support our concept that deficiency of vitamin $B_6$ can bring about the reduction of vitamin $B_{12}$ reserve to such a level as to effect an important metabolic role of vitamin $B_{12}$, — in this case methylation, as measured by the amount of methylnicotinamide found in the urine of rats dosed with NA.

## SUMMARY

The effect of vitamin $B_{12}$ deficiency on methylation, as indicated by the urinary excretion of methylnicotinamide following the administration of nicotinamide, was studied. It was found in male and female rats that dietary deprivation of this vitamin brought about a decrease in methylation. This effect is not due to nonphysiologic causes since pair-fed animals from both deficient and treated groups, as well as female rats in these two groups, exhibited a significant difference in excretion. Furthermore, vitamin $B_6$ deficiency does not bring about such a difference. However, if vitamin $B_6$ deficiency were prolonged, vitamin $B_{12}$ serum level of these vitamin $B_6$-deprived animals was reduced. Only then did a decrease in methylation occur. From these studies, it was concluded that vitamin $B_{12}$ deficiency by dietary deprivation or by poor absorption of this vitamin decreases the urinary excretion of MNA.

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# The Level of Isocitric Acid Dehydrogenase in Tissues of Vitamin E-Deficient Rabbits[1]

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Using the *in vitro* reduction of triphenyltetrazolium chloride (TPTZ) as an index of dehydrogenase activity, Rosenkrantz and Laferte ('60) demonstrated a 30% decline in the total dehydrogenase activity in the skeletal musculature of vitamin E-deficient rabbits. Upon introducing specific substrates these workers were able to show that the levels of isocitric acid dehydrogenase decreased some 54% in animals with nutritional muscular dystrophy.

Since it was not known at which point in the respiratory chain TPTZ accepted electrons (namely, coenzyme II, flavoprotein, or cytochrome oxidase), it was not certain which component(s) was being estimated (Nachlas et al., '60). It thus appeared to be of interest to investigate by more direct means the titers of isocitric acid dehydrogenase in control and vitamin E-deficient rabbits. In the present study a purified enzyme preparation was used for determining enzyme activity by spectrophotometric analysis of its reduced coenzyme.

## METHODS

Littermate, New Zealand white rabbits were reared with a vitamin E-deficient diet as previously reported (Rosenkrantz, '55). The control animals received triweekly oral supplements of α-tocopheryl acetate. The vitamin E-deficient rabbits developed nutritional muscular dystrophy within 4 to 5 weeks after initiation of the purified diet. The relative state of the disease was followed by determining the urinary creatine titers according to the diacetyl method of Ennor and Stocken ('47).

At urinary creatine levels of 40 to 80 mg per 24 hours, the rabbits were killed by a blow in the medullary region. Ochoa's procedure ('55) for isolating isocitric acid dehydrogenase from pig heart was adopted for concentrating the same enzyme from skeletal muscle. The musculature of both hind legs of each rabbit was excised in the cold. Equal wet weights (287 to 300 gm) of muscle from three control and three deficient animals were respectively pooled to provide sufficient tissue for the purification of the enzyme. At each major fractionation step, dry-weight and total nitrogen determination were made The enzymatic activity was determine by following the reduction of triphosphopyridine nucleotide (TPN) on equivalent amounts of nitrogen from the purified isocitric acid dehydrogenase fractions. A Cary model 11 automatic recording spectrophotometer was used.

A secondary study was also made to determine the levels of isocitric acid dehydrogenase by manometric techniques (Umbreit et al., '48). Each flask contained approximately 250 mg wet weight of muscle fiber strips from the rectus femoris in 3 ml of Krebs-Ringer-phosphate buffer at pH7.4 (Rosenkrantz, '55). The liberation of carbon dioxide was followed in the presence and absence of 0.1 ml of 0.25 M isocitric acid for 20 minutes at 37.5°C.

The opportunity was available to determine the blood level of isocitric acid dehydrogenase by the spectrophotometric procedure of Taylor and Friedman ('60). Blood samples were collected by direct cardiac puncture and serum obtained with avoidance of red cell rupture. Deprotein-

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[2] This investigation was carried out in partial fulfillment of the requirements for the degree of Master of Arts.

TABLE 1

*Dry-weight and nitrogen analysis on muscle fractions from control and vitamin E-deficient rabbits*[1]

| Fraction | Animal condition | Total volume | Dry weight | Nitrogen | Nitrogen/ dry weight | Total nitrogen | Total protein |
|---|---|---|---|---|---|---|---|
| | | *ml* | *mg/ml* | *mg/ml* | *mg/100 mg* | *mg* | *gm* |
| Buffer extract | Control | 530 | 9.7 | 1.34 | 13.80 | 710 | 4.44 |
| | Deficient | 530 | 7.9 | 1.07 | 13.50 | 565 | 3.53 |
| $(NH_4)_2SO_4$ ppt | Control | 40 | 69.9 | 12.90 | 18.50 | 516 | 3.23 |
| | Deficient | 40 | 50.2 | 8.10 | 16.20 | 324 | 2.06 |
| Ethanol ppt | Control | 10 | 4.8 | 0.37 | 7.7 | 3.7 | 0.023 |
| | Deficient | 10 | 2.4 | 0.21 | 8.7 | 2.1 | 0.015 |

[1] 287 gm of skeletal muscle tissue were pooled from three rabbits in each group. The creatinuria of each vitamin E-deficient animal fell between 60 to 85 mg/24 hours.

ized serum aliquots were also used for the estimation of citric acid by the method of Ettinger et al. ('52).

## EXPERIMENTAL AND RESULTS

The dry-weight and total nitrogen data obtained during a representative fractionation sequence from one group each of control and vitamin E-deficient rabbits are shown in table 1. Of the 287 gm of initial skeletal muscle fractionated, 5.1 gm of the dry-weight material in the control group (column 3 × 4) and 4.2 gm of dry-weight material in the deficient group were recovered in the buffer extract. The protein content of these same final fractions was 4.44 and 3.53 gm, respectively.

Further fractionation with ammonium sulfate reduced the dry-weight material from 5.1 to 2.8 gm for the control and from 4.2 to 2.0 gm for the dystrophic. In the final ethanol precipitate fraction the total dry-weight values were reduced to 0.048 gm for the control and 0.024 gm for the deficient. The corresponding protein concentrations present in the same fractions were found to be 0.023 gm and 0.015 gm, respectively.

The enzyme activities were measured only on the reconstituted ethanol fractions. The activity was defined as the optical density attained at 340 mμ in 5 minutes by 0.1 ml of the purified isocitric acid dehydrogenase preparation. The total activity was obtained by multiplying the optical density achieved by 0.1 ml of aliquot by the actual volume of the fraction tested. In turn the value for the total activity was divided by the total nitrogen content in order to express the values as

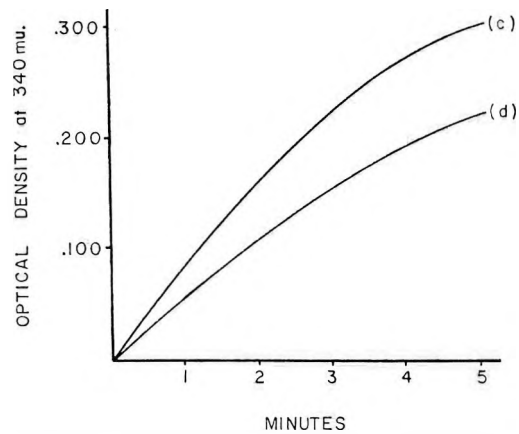


Fig. 1 Reduction of triphosphopyridine nucleotide (TPN) by equivalent amounts of purified isocitric acid dehydrogenase from control (c) and vitamin E-deficient (d) rabbits.

activity per milligram of nitrogen. The activity of purified isocitric acid dehydrogenase from a control and a deficient group is shown in figure 1 which is an automatic recording of the reaction. The reaction mixture contained 0.3 ml of 0.25 M glycylglycine buffer, pH7.4, 0.1 ml of 0.018 M manganese dichloride, 0.1 ml of 0.00135 M TPN, 0.1 ml of the enzyme preparation, and 2.3 ml of distilled water. The reaction was begun by adding 0.1 ml of 0.006 M DL-isocitric acid to the sample cell. The control cell received 0.1 ml of distilled water.

A comparison of the enzymatic activity from the skeletal musculature of three different colonies of control and vitamin E-deficient rabbits yielded essentially the same results, which are summarized in table 2. The activities were calculated on

TABLE 2

*Comparison of the activity of highly purified isocitric acid dehydrogenase from skeletal muscle of control and vitamin E-deficient rabbits*[1]

| Animal condition | Before fractionation | | After fractionation | | | After fractionation | | Change |
|---|---|---|---|---|---|---|---|---|
| | | | | | | OD attained at 340 mμ by 0.1 ml aliquot in 5 minutes | | |
| | Total wet weight | Total dry weight | Total wet weight | Total nitrogen | Total protein | Total[2] activity | Activity/ mg[2] nitrogen | |
| | *gm* | *gm* | *mg* | *mg* | *mg* | | | % |
| 1 Control | 300 | 45 | 58.0 | 5.63 | 32.5 | 115.0 | 20.4 | −27 |
| Deficient | 300 | 51 | 49.6 | 4.20 | 26.2 | 63.0 | 15.0 | |
| 2 Control | 287 | 46 | 44.1 | 5.38 | 33.7 | 42.4 | 7.9 | −25 |
| Deficient | 287 | 43 | 47.5 | 6.67 | 41.7 | 39.6 | 5.9 | |
| 3 Control | 300 | 42 | 48.0 | 3.70 | 23.2 | 29.0 | 7.9 | −30 |
| Deficient | 300 | 45 | 44.0 | 3.85 | 24.0 | 21.0 | 5.5 | |

[1] The creatinuria of each vitamin E-deficient animal fell between 60 to 85 mg/24 hours.
[2] The total activity was obtained by multiplying the optical density achieved by 0.1 ml aliquot by the actual volume of the fraction tested. In turn the value for the total activity was divided by the total nitrogen content in order to express the values as activity per milligram of nitrogen.

the basis of equivalent milligrams of nitrogen present in the final purified fraction. The results consistently indicated that there was a decrease in the specific activity of isocitric acid dehydrogenase based on nitrogen content in the skeletal musculature of vitamin E-deficient rabbits. The percentage decrease in the enzyme activity in the three different colonies tested was 27, 25 and 30, respectively.

In a preliminary exploration, manometric techniques were used to estimate skeletal muscle isocitric acid dehydrogenase by the liberation of carbon dioxide from exogenous isocitric acid. One vitamin E-deficient group liberated 104 μ liters of $CO_2$ per hour per 100 mg of dry weight of tissue, whereas the value for the corresponding control group was 149. A second group of vitamin E-deficient rabbits liberated 146 μ liters of $CO_2$ per hour per 100 mg and the corresponding controls averaged 183. Overall, the figures disclosed a decrease of approximately 25% in the production of $CO_2$ from added isocitrate by isocitric acid dehydrogenase in the vitamin E-deficient animals.

To investigate the possible leakage of muscle enzyme into the plasma during development of vitamin E-deficiency, a comparative study was carried out on the isocitric acid dehydrogenase levels in serum samples of both groups. The serum levels of the enzyme were followed by measuring the production of α-ketoglutaric acid formed from exogenous isocitric acid (Taylor and Friedman, '60). The results are outlined in table 3, and the comparison of control and deficient animals was made on the basis of α-ketoglutarate present in total serum. The usual 3.1% of body weight for the rabbit was used to calculate total serum. The alteration in the serum isocitric acid dehydrogenase levels appeared to parallel the severity of the dystrophy. Rabbits with early vitamin E-deficiency (colony A) showed little change in serum enzyme, the mildly dystrophic (colony B) had a 35% decrease, and the moderately dystrophic (colonies C and D) showed a further decrement to 51 and 49%.

But in severe and terminal dystrophic cases the reverse condition was noted. The severely dystrophic group (colony E) showed 52% increase in serum isocitric acid dehydrogenase levels, whereas the titers in the terminal dystrophic group (colony F) were elevated by 108%.

Since a difference was noted in the serum isocitric acid dehydrogenase titers of control and vitamin E-deficient animals, it was of interest to see whether the content of Krebs cycle intermediates before isocitric acid were altered. The estimation of serum citric acid was selected for this study. A comparison of the citric acid levels in the serum from control and vitamin E-deficient rabbits at various stages of dystrophy is also presented in table 3.

TABLE

*Levels of isocitric acid dehydrogenase and citric acid in serum from control and vitamin E-deficient rabbits*

| Colony | Condition | No. of rabbits | Urinary creatine | Exogenous α-ketoglutarate | α-Keto-glutarate in total serum[1] | Change in total serum deficient/ control | Endogenous citrate | Citrate in total serum[1] | Change in total serum deficient/ control |
|---|---|---|---|---|---|---|---|---|---|
| | | | *mg/24 hours* | *mg/ml* | *mg* | *%* | *μg/ml* | *mg* | *%* |
| A Control | normal | 2 | 5.7 | 2.19 ± 0.31[2] | 73.2 | | 95.2 ± 1.2[2,3] | 3.19 | |
| Deficient | no dystrophy | 2 | 15.3 | 2.05 ± 0.35 | 75.4 | +3 | 104.5 ± 7.6 | 3.45 | +8 |
| B Control | normal | 2 | 5.4 | 1.87 ± 0.11 | 78.8 | | 35.6 ± 7.0 | 1.50 | |
| Deficient | mild dystrophy | 3 | 66.0 | 1.38 ± 0.19 | 51.7 | −35 | 51.9 ± 7.6 | 1.73 | +13 |
| C Control | normal | 2 | 11.2 | 1.50 ± 0.11 | 42.6 | | | | |
| Deficient | moderate dystrophy | 2 | 87.0 | 0.56 ± 0.20 | 20.9 | −51 | | | |
| D Control | normal | 3 | 6.0 | 2.72 ± 0.33 | 116.0 | | 41.9 ± 5.9 | 1.69 | |
| Deficient | moderate dystrophy | 3 | 84.0 | 1.38 ± 0.22 | 65.7 | −49 | 47.3 ± 7.4 | 2.32 | +37 |
| E Control | normal | 4 | 9.1 | 1.07 ± 0.02 | 48.4 | | 55.5 ± 2.9[3] | 2.51 | |
| Deficient | severe dystrophy | 4 | 112.0 | 1.97 ± 0.35 | 73.9 | +52 | 29.8 ± 1.3 | 1.12 | −55 |
| F Control | normal | 3 | 6.8 | 1.46 ± 0.21 | 54.9 | | 37.8 ± 7.3 | 1.45 | |
| Deficient | terminal dystrophy | 2 | 124.0 | 2.91 ± 0.45 | 114.5 | +108 | 30.1 ± 10.5 | 1.13 | −22 |

[1] Calculated on the basis that 3.1% of body weight is equal to the total serum. Duplicate determinations on each sample of blood from each animal were within 10% of each other.

[2] Mean ± standard deviation.

[3] These values were obtained on the first animals studied using trichloroacetic acid as a protein precipitant. The other values were derived from serum which was deproteinized with tungstic acid.

Animals that were still in a reversible condition of vitamin E-deficiency (mild and moderate dystrophy) showed an increase of 8 to 37% in the serum citric acid level. However, severely dystrophic rabbits showed a 22 to 55% decrease in the concentration of serum citrate. The somewhat higher control values for colonies A and E seemed to be due to the use of trichloroacetic acid as the protein precipitant. All other values were obtained on serum deproteinized with tungstic acid.

## DISCUSSION

Unlike the use of tetrazolium salts in estimating the dehydrogenase activity in tissues, the more direct approach of enzyme isolation permitted a more definitive observation. The specificity of the present method used confirmed the decline of muscle isocitric acid dehyrogenase levels obtained with the TPTZ procedure. This was also borne out by the manometric studies. A greater decrease was seen in the TPTZ method, but this may in part reflect the measurement of other reducing systems in addition to that of isocitric acid dehydrogenase.

The results observed with the serum isocitric acid dehydrogenase may be interpreted in two ways: (1) a decreased synthesis of enzyme in the liver would be reflected by the blood level, or (2) an increased degradation of the serum isocitric acid dehydrogenase could occur. Either of the mechanisms could yield a decline in the serum enzyme titers during moderate severity of nutritional muscular dystrophy. Where terminal signs of the disease appear, it would be reasonable to interpret elevated serum enzyme levels as being in part due to leakage of the enzyme from the muscle into the serum.

The concentration of serum citrate appeared to be correlated with the content of isocitric acid dehydrogenase in the blood. It is not clear whether this suggests conversion of citrate to isocitrate in serum or the existence of a more complicated picture.

Although the reduced levels of serum isocitric acid dehydrogenase do not correlate with enzyme leakage from the muscle in the earlier stages of the disease, the decreased activity of this enzyme in the muscle itself may be of primary importance. A partial block at the isocitric acid dehydrogenase level in the Krebs cycle must be circumvented since oxygen consumption proceeds normally (Rosenkrantz, '55). Perhaps amino acids from the degenerating muscle protein entering via α-ketoglutaric acid and oxalacetic acid maintain the Krebs cycle. A partial block at the isocitric acid dehydrogenase level would also shift the equilibrium of the Krebs cycle from isocitric acid back toward acetate; this result may account for the increased cholesterol synthesis observed in muscle (Alfin-Slater, '60).

## SUMMARY

1. Using a purified enzyme preparation it was found that the isocitric acid dehydrogenase titers in the skeletal musculature of vitamin E-deficient rabbits decreased approximately 25 to 30% from the level of the corresponding control animals.

2. The levels of serum isocitric acid dehydrogenase in rabbits with moderate dystrophy declined, whereas those with terminal symptoms showed an increase.

3. It was also observed that serum citrate levels declined with an increase in the isocitric acid dehydrogenase titers.

4. The possibility that a partial block at the isocitric acid dehydrogenase level may account for increased cholesterol levels and be consistent with normal oxygen uptake was discussed.

## ACKNOWLEDGMENT

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# The Limiting Amino Acid Sequence in Raw and Roasted Peanut Protein

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A number of reports have appeared in the literature on the nutritive value of the peanut (Mitchell et al., '49; Buss and Goddard, '48; Reugamer et al., '50; Rutgers University Bureau of Biological Research, '46–50); but none of these concern measurements of the nutritive value of the whole peanut after it has been roasted — the form in which most peanuts are consumed. As roasted peanuts are an important constituent of the diet, an evaluation of the nutritive value of roasted peanuts was needed.

In the experiments reported here, the nutritive value of several peanut pastes was determined. The effect of roasting under conditions that simulate practical roasting with respect to time and temperature was determined. The limiting amino acid sequence in roasted peanut paste was shown to be lysine, threonine, and methionine. In unroasted peanut paste lysine, methionine, and threonine were equally limiting.

## EXPERIMENTAL

*Peanut pastes.* All peanut pastes used were prepared from U. S. no. 1 Spanish peanuts. The peanuts were roasted in a commercial, radiant heat roaster to the end point temperature given below. After roasting, the nuts were reduced to paste by grinding. During grinding, 2% of hydrogenated peanut oil was added to reduce oil separation.

The raw peanut paste was prepared from nuts roasted to an end point temperature of 95°C which required 14 minutes. This treatment resulted in nuts that were essentially unroasted but blanched sufficiently that the testa could be removed. A roasted peanut paste was prepared from nuts heated to an end point of 170°C which required 36 minutes. The 170°C end point temperature was selected because it approximates that sometimes used in the practical roasting of peanuts.

*Feeding experiments.* All experiments were carried out using 15 male, weanling Sprague-Dawley rats per group and an experimental period of 28 days. The composition of the diets is shown in table 1. The amino acid supplements were mixed with a portion of the sucrose to assure uniform distribution in the diet. All diets were made isonitrogenous through the addition of glycine to the amino acid supplement.

The animals were housed in individual cages and given feed and water ad libitum. Body weights were recorded once each week and feed consumption three times each week. Body weight gain and feed efficiency were used as criteria of animal response for the various diets. The feed efficiency and body weight gain data were analyzed for statistical significance using the analysis of variance. Minimal significant differences between means were determined by the method of Tukey ('52).

The lysine, threonine, and methionine content of the peanut protein was determined as follows. A sample (about 500 mg) of fat-free peanut meal was hydrolyzed at reflux with 20 ml of redistilled, constant boiling HCl for 18 hours. The hydrolysates were filtered and made to a known volume. A sample was withdrawn, adjusted to pH 6.8, and diluted to an assayable amino acid concentration.

Lysine was measured using *Leuconostoc mesenteroides* and the medium of Kuiken et al. ('43). *Streptococcus faecalis* and the above medium were used to determine methionine. Threonine was determined

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TABLE 1

*Composition of diets*

| | Control diet | Experimental diet |
|---|---|---|
| | % | % |
| Casein | to 15% protein (N × 6.25) | |
| Peanut paste | | to 15% protein (N × 6.25) |
| Peanut oil | to 28% fat | to 28% fat |
| Fat-soluble vitamin mix[1] | 1.0 | 1.0 |
| Water-soluble vitamin mix[2] | 5.0 | 5.0 |
| Cellulose[3] | 5.0 | 5.0 |
| Salt mix USP 14 | 4.8 | 3.7 |
| Amino acid supplement[4] | 5.0 | 5.0 |
| Sucrose | to 100% | to 100% |

[1] Composition in grams: vitamin A, 6.25; vitamin D (400,000 IU/gm), 1.56; α-tocopherol, 5.0; peanut oil, 487.25.

[2] Menadione, 60; thiamine·HCl, 80; riboflavin, 100; niacin, 400; Ca pantothenate, 400; folic acid (1% trituration with sucrose), 500; pyridoxine·HCl, 80 mg. Vitamin $B_{12}$ (0.1% trituration with mannitol), 3; choline, 60; inositol, 40; ascorbic acid, 2; *p*-aminobenzoic acid, 2 gm. Biotin (100 μg/ml), 60 ml. Sucrose to 1,000 gm.

[3] Cellu Flour, Chicago Dietetic Supply Company, Chicago.

[4] Amino acid content as described in the following tables made up as a mixture with sucrose.

with S. *faecalis* and the medium of Henderson and Snell ('48) as modified by Sirny et al. ('54). In all assays an incubation period of 72 hours at 37°C was used. Acid production, determined by titrating with 0.05 N potassium hydroxide, served as the measure of bacterial growth.

## RESULTS

Data showing the effect roasting has on the nutritive value of peanut protein is presented in table 2. The feed and protein efficiency data point up the inferiority of both peanut preparations compared with casein as a protein source for the rat. Although less obvious, the differences in feed and protein efficiency obtained between the raw and roasted peanuts were shown to be significant by statistical analysis.

TABLE 2

*Effect of roasting on the nutritive value of peanuts*

| Protein source | Average feed efficiency[1] | Average protein efficiency[2] | Average weight gain[3] |
|---|---|---|---|
| | | | *gm* |
| Casein | 36.8 | 2.44 | 150.8 |
| Raw peanut[4] | 27.1 | 1.81 | 95.6 |
| Roasted peanut[4] | 22.5 | 1.50 | 70.5 |

[1] Feed efficiency = $\frac{\text{body weight gain}}{\text{feed intake}} \times 100$.

$F = 36.18$; $P = 0.05$; d (least significant difference) = 4.5.

[2] Protein efficiency = $\frac{\text{body weight gain}}{\text{protein intake}}$.

$F = 34.16$; $P = 0.05$; d (least significant difference) = 0.29.

[3] $F = 31.86$; $P = 0.05$; d (least significant difference) = 26.7.

[4] Roasting condition stated in text.

The low nutritive value of the raw peanut protein was assumed, on the basis of reports in the literature, to be due primarily to a deficiency of methionine. As shown in table 3, however, the addition of methionine in the absence of lysine (diet 3) or threonine (diet 5) did not improve the feed efficiency or body weight gain significantly. The addition of lysine and threonine, individually or in combination (diets 4, 11, 13), showed no improvement in feed efficiency. The combined addition of lysine, threonine, and methionine (diets 6, 7, 8) produced a significant increase in feed efficiency and body weight gain. The addition of either tryptophan (diet 7) or histidine (diet 6) or tryptophan and histidine (diet 8) to diets containing lysine, threonine, and methionine failed to produce a further improvement in feed efficiency. However, body weight gain was somewhat better with diet 8 than with diets 6 and 7. The addition of the three most limiting amino acids to the raw peanut protein supported better growth than the diet that contained 15% of casein. The omission of threonine (diet 5) produced a significant decrease in feed efficiency and body weight gain relative to the unsupplemented peanut protein diet (diet 2). Because it appeared that an amino acid imbalance may have been produced, a second experiment (experiment 2, table 3) was carried out to determine what

TABLE 3
*Determination of the limiting amino acids in raw peanut protein*

| Experiment | Diet[1] | Supplemental amino acids (percentage of diet) | | | | | | Average feed efficiency[4] | Average weight gain[5] |
|---|---|---|---|---|---|---|---|---|---|
| | | L-Lysine | DL-Methionine | DL-Threonine | DL-Tryptophan | L-Histidine[2] | Glycine[3] | | |
| | | | | | | | | | *gm* |
| 1 | 1 | — | — | — | — | — | 1.53 | 45.0 | 120.0 |
| | 2 | — | — | — | — | — | 1.53 | 29.5 | 76.0 |
| | 3 | — | 0.61 | 0.38 | 0.09 | 0.13 | 0.72 | 29.5 | 85.0 |
| | 4 | 0.71 | — | 0.38 | 0.09 | 0.13 | 0.30 | 30.5 | 80.0 |
| | 5 | 0.71 | 0.61 | — | 0.09 | 0.13 | 0.24 | 24.5 | 60.0 |
| | 6 | 0.71 | 0.61 | 0.38 | — | 0.13 | 0.07 | 40.5 | 140.0 |
| | 7 | 0.71 | 0.61 | 0.38 | 0.09 | — | 0.19 | 43.5 | 147.0 |
| | 8 | 0.71 | 0.61 | 0.38 | 0.09 | 0.13 | — | 43.0 | 157.0 |
| 2 | 9 | — | — | — | — | — | 1.53 | 46.2 | 140.3 |
| | 10 | — | — | — | — | — | 1.53 | 30.5 | 76.5 |
| | 11 | 0.71 | — | — | — | — | 0.80 | 31.7 | 78.3 |
| | 12 | — | 0.61 | — | — | — | 1.22 | 30.8 | 83.1 |
| | 13 | — | — | 0.38 | — | — | 1.29 | 32.7 | 99.6 |
| | 14 | 0.71 | 0.61 | — | — | — | 0.50 | 29.6 | 70.9 |
| | 15 | 0.71 | 0.61 | — | 0.09 | — | 0.43 | 29.6 | 70.3 |
| | 16 | 0.71 | 0.61 | — | 0.09 | 0.13 | 0.24 | 29.7 | 68.7 |

[1] Diets 1 and 9 contained 15% of casein. All other diets contained 15% of raw peanut protein.
[2] Added as the amino acid hydrochloride.
[3] Amount added to keep diets isonitrogenous.
[4] For experiment 1 — $F = 104.38$; $P = 0.05$; d (least significant difference) = 3.4.
For experiment 2 — $F = 35.72$; $P = 0.05$; d (least significant difference) = 4.0.
[5] For experiment 1 — $F = 99.43$; $P = 0.05$; d (least significant difference) = 15.7.
For experiment 2 — $F = 32.39$; $P = 0.05$; d (least significant difference) = 18.0.

amino acid(s), if any, was responsible for the imbalance. The animal performance with the peanut protein (diets 2, 10) and the casein control diets (diets 1, 9) in the two experiments shows that these experiments are comparable. The feed efficiencies obtained for diets 11 through 16, which contain the important combinations of the 4 amino acids (excluding threonine), are not significantly different. In fact, diet 16, which was made up from the same components as diet 5, did not produce a significant decrease in either feed efficiency or body weight gain. In a third experiment, the details of which are not reported, the omission of threonine from the otherwise complete amino acid mixture did not depress feed efficiency. Thus, it seems likely that an amino acid imbalance does not exist and that lysine, threonine, and methionine are equally limiting in raw peanut protein.

An initial experiment (not reported) using a peanut paste prepared from roasted peanuts as the dietary protein source indicated, contrary to the published reports (Grau, '46; Cama et al., '55), that lysine, and not methionine, was the most limiting amino acid under our experimental conditions. Furthermoer, it suggested that methionine was not the second most limiting acid in roasted peanut protein. Consequently, the limiting amino acid sequence for roasted peanut protein was determined, as shown in table 4 (experiment 3). The addition of lysine (diet 3) improves the peanut control diet as measured by the feed efficiency. This improvement was not sufficient to be reflected in a significant increase in body weight gain. Data concerning diet 4 show that methionine is not even the second most limiting acid, but rather diets 5 and 6 demonstrate threonine and methionine to be the second and third limiting acids, respectively. The further addition of tryptophan (diet 7) or of tryptophan and histidine (diet 8) produced some increase in feed efficiency and weight gain, but neither was considered statistically significant.

In experiment 3, reported in table 4, the amino acids were added as mixtures of 4 amino acids except for diet 3 which contained only lysine. Since the results of this experiment do not agree with those reported in the earlier literature, it was important to ascertain whether a se-

TABLE 4

*Determination of the limiting amino acids in roasted peanut protein*

| Experiment | Diet[1] | Supplemental amino acids (percentage of diet) | | | | | | Average feed efficiency[4] | Average weight gain[5] |
|---|---|---|---|---|---|---|---|---|---|
| | | L-Lysine[2] | DL-Methionine | DL-Threonine[3] | DL-Tryptophan | L-Histidine[2] | Glycine | | |
| | | | | | | | | | *gm* |
| 3 | 1 | — | — | — | — | — | 1.53 | 43.9 | 122.1 |
| | 2 | — | — | — | — | — | 1.53 | 22.9 | 45.7 |
| | 3 | 0.71 | — | — | — | — | 0.80 | 27.4 | 48.4 |
| | 4 | 0.71 | 0.61 | — | 0.09 | 0.13 | 0.24 | 27.7 | 55.1 |
| | 5 | 0.71 | — | 0.38 | 0.09 | 0.13 | 0.30 | 33.9 | 89.6 |
| | 6 | 0.71 | 0.61 | 0.38 | — | 0.13 | 0.07 | 43.8 | 138.2 |
| | 7 | 0.71 | 0.61 | 0.38 | 0.09 | — | 0.19 | 47.3 | 151.4 |
| | 8 | 0.71 | 0.61 | 0.38 | 0.09 | 0.13 | — | 47.5 | 152.9 |
| 4 | 9 | — | — | — | — | — | 1.53 | 37.1 | 99.4 |
| | 10 | — | — | — | — | — | 1.53 | 27.8 | 60.8 |
| | 11 | 0.71 | — | — | — | — | 0.80 | 32.5 | 71.9 |
| | 12 | 0.71 | — | 0.38 | — | — | 0.56 | 32.0 | 91.5 |
| | 13 | 0.71 | 0.61 | 0.38 | — | — | 0.26 | 46.0 | 148.2 |
| | 14 | 0.71 | 0.61 | 0.38 | 0.09 | — | 0.19 | 48.4 | 159.5 |
| | 15 | 0.71 | 0.61 | 0.38 | 0.09 | 0.13 | — | 50.0 | 160.0 |

[1] Diets 1 and 9 contained 15% of casein. All other diets contained 15% of roasted peanut protein.
[2] Added as the amino acid hydrochloride.
[3] Equivalent to 0.19% of available threonine since the D- form is not utilized.
[4] For experiment 3 — $F = 142.9$; $P = 0.05$; d (least significant difference) = 3.7.
For experiment 4 — $F = 98.5$; $P = 0.05$; d (least significant difference) = 3.7.
[5] For experiment 3 — $F = 154.6$; $P = 0.05$; d (least significant difference) = 16.6.
For experiment 4 — $F = 81.2$; $P = 0.05$; d (least significant difference) = 19.9.

quential addition of the limiting amino acids would yield similar results (experiment 4, table 4). The results generally confirm those of experiment 3. There are, however, two differences which should be pointed out. First, the addition of threonine to the lysine supplemented protein (diet 12) did not produce a significant increase in feed efficiency over that produced by supplementation with lysine alone (diet 11). The inconsistency with respect to threonine probably represents a difference in the peanut stock or a difference in the amount of change in the protein during roasting since the same peanut stock was not used in the two experiments. This is further borne out by the feed efficiency of the peanut control diets — 22.9 in experiment 3 and 27.8 in experiment 4. The latter feed efficiency is very close to that obtained with the raw peanut preparations and is thus indicative of a lesser amount of alteration during roasting.

Second, the addition of tryptophan and histidine caused a significant increase in feed efficiency. This increase in feed efficiency is of questionable importance since the difference in feed efficiency between diets 6 and 8 in experiment 3 is 3.7 ($d = 3.7$) and in experiment 4 the difference obtained between similar diets (diet 13 and diet 15) was 4.0 ($d = 3.7$). It is important, however, that supplementation of either raw or roasted peanut protein with lysine, threonine, and methionine improved the peanut protein to such a degree that it was better than the control diet which contained 15% of casein.

The amount of each of the three most limiting amino acids in normal roasted peanut protein necessary to produce an optimal feed efficiency is shown in figure 1. Four supplemental levels were chosen so that two levels would be suboptimal and two levels would be more than adequate. The point of intercept between the two straight lines should equal the minimal requirement of the amino acid under test when adequate quantities of the other limiting acids are supplied. These levels were found to be 0.31, 0.19, and 0.21% of the diet for lysine, threonine, and methionine, respectively.

The lysine, threonine, and methionine content of raw and roasted peanut pastes, as determined by microbiological assay, is shown in table 5. The amino acid content is expressed as percentage of the protein and as percentage of the diet.

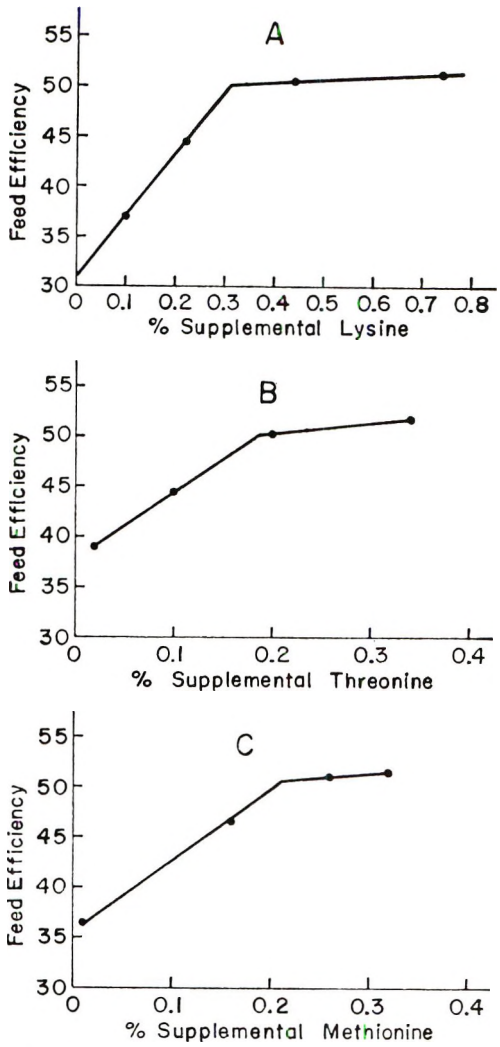


Fig. 1 Determination of minimal amounts of lysine, threonine, and methionine to produce optimal feed efficiency.

TABLE 5

*Lysine, threonine, and methionine content of raw and roasted peanut protein*

| | Raw peanut protein | | Roasted peanut protein | |
|---|---|---|---|---|
| | % of protein[1] | % of diet | % of protein[2] | % of diet |
| Lysine | 4.02 | 0.60 | 3.43 | 0.52 |
| Methionine | 1.30 | 0.20 | 1.16 | 0.17 |
| Threonine | 3.16 | 0.48 | 2.79 | 0.42 |

[1] Average of duplicate determinations on two different protein preparations.
[2] Average of duplicate determinations on 5 different protein preparations.

## DISCUSSION

The nutritive value and the limiting amino acids of the protein in peanuts roasted under conditions that approximate those of practical roasting has not been determined previously. Based on protein efficiency, the raw and roasted peanut preparations used in these experiments compare well with the boiled and 160°C roasted products studied by Buss and Goddard ('48). Their preparations, fed at a 10% protein level in a diet containing about 28% of fat, yielded a protein efficiency ratio of 1.84 for the boiled protein and 1.65 for the protein roasted 40 minutes at 160°C. Jones and Divine ('44) obtained a similar protein efficiency ratio (1.81) with unroasted peanut protein in a diet containing 15% of protein and 8% of fat.

It was unexpected that lysine, threonine, and methionine were equally limiting in the raw peanut protein, because methionine has been reported consistently as the most limiting with lysine the second most limiting. Two reasons are possible for this apparent discrepancy. First, there is the possibility that the amino acid requirements under these dietary conditions (high protein and high fat) are different from those previously published and, second, that the amino acids in peanut protein are not completely available to the rat. An indication that the amino acid requirements are not markedly increased by the high level of fat used in these diets was obtained by substituting whole-egg protein for peanut protein in these diets. It was found that a level of 10% of egg protein in the diet produced weight gains, feed efficiencies, and protein efficiencies equal to or better than those obtained with the amino acid supplemented peanut protein diets. The egg protein diet supplies 0.91% of lysine, 0.46% of threonine, and 0.32% of methionine. Assuming that the amino acids of whole egg are completely available and that whole egg is a well balanced protein, the lysine, threonine, and methionine content of the whole-egg protein diet may be used as an estimate of the requirement for these amino acids under these dietary conditions.

The roasted peanut diets supplemented at the optimal level contained 0.83 and

0.38% of lysine and of methionine, respectively — an amount that agrees quite well with the requirement estimated from the whole-egg protein diet. The peanut diets contained 0.61% of threonine which is considerably higher than the above estimated requirement of 0.46%. This, in conjunction with the demonstration that threonine must be added to the peanut protein diets, suggests that about 30% of the threonine in peanut protein is biologically unavailable to the rat. Rice is another example of a rather widely used protein which, based upon amino acid analyses, appears to contain adequate threonine. But Pecora and Hundley ('51) have reported threonine as second limiting after lysine. Rosenberg et al. ('59) also reported threonine as the second most limiting amino acid in rice protein.

Little effort has been directed towards explaining the low threonine availability. It is not known, for instance, whether the threonine exists in a peptide sequence which is resistant to enzymatic attack or as a threonine derivative, such as threonine phosphate, which the rat may not be able to utilize as threonine. This problem would seem to be growing in importance now that two rather widely used proteins, rice and peanut, are reported to contain a significant amount of biologically unavailable threonine.

The change in the sequence of limiting acids upon roasting is caused by either actual alteration of, or decreased availability of, lysine and threonine. Data in table 5 show a decrease of almost 15% in the lysine content of the roasted peanut protein as compared with the unroasted product, whereas the methionine and threonine levels are decreased by about 10 and 11%, respectively. Thus, it seems that a destruction of lysine as well as a probable lower biological availability are responsible for lysine becoming most limiting in the roasted peanuts.

Bressani and Mertz ('58) determined the lysine requirement of the rat at different dietary protein levels. At a level of 16% of protein they showed that 0.8 to 0.9% of lysine in the diet was required to produce maximal body weight gain. This compares favorably with the 0.8% determined in the present studies. However, in arriving at these values it has been assumed the lysine in the corn gluten used by Bressani and in the roasted peanut protein used in these experiments was 100% available. Such an assumption may not be justified.

Balasundaram et al. ('58) have reported on the effect of adding, as individual supplements, lysine, methionine, isoleucine, threonine, cysteine, or tryptophan to diets containing 15% of raw peanut protein and 9% of fat. These authors claim a significant increase in protein efficiency when lysine, methionine, isoleucine, and threonine are added; no effect from cysteine; and a significant decrease in protein efficiency ratio when tryptophan is added. The average growth rate of 1.58 gm per day obtained with their raw protein is considerably lower than the 2.71 gm per day obtained in the present study. The greatest improvement in weight gain shown by Balasundaram upon supplementation with a single amino acid (methionine) was only 0.17 gm per day. This is markedly less than the 2.90 gm per day obtained in these studies when the raw protein was supplemented with lysine, threonine, methionine, histidine, and tryptophan. It is impossible to accept their interpretation of their results without completely changing the present concept of limiting amino acids. It is probable that the animals used in their experiments were responding to the amino acid supplements in a nonspecific manner since the extremely poor growth rate is indicative of a poor diet.

## SUMMARY

The limiting amino acid sequence of blanched but unroasted peanut protein was found to be lysine equal to threonine equal to methionine. Tryptophan and histidine do not appear to be limiting in the unroasted protein.

In roasted peanut protein the limiting amino acid sequence was lysine, threonine, and methionine. Tryptophan and histidine sometimes produced an increase in feed efficiency when added in the presence of lysine, threonine, and methionine.

Roasting under the conditions described caused a decrease in the amount of lysine, threonine, and methionine equal to 15, 11,

and 10% of the total, respectively. This decrease was due to an actual destruction of the amino acids. No attempt was made to quantify the loss of biologically available amino acids.

Calculations of the limiting amino acids based upon amino acid content and upon the rat amino acid requirements suggest that methionine would be more limiting than lysine and that threonine should not be limiting. The discrepancy between the calculated and determined limiting sequence must be due to alteration of, or a decreased biological availability of, lysine and threonine, or to both.

A feed efficiency equal to or better than that of a 15% casein diet can be obtained by supplementing roasted peanut protein with at least 0.31% of L-lysine, 0.19% of DL-threonine, and 0.21% of DL-methionine.

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# Effects of Dietary Fat on Cholesterol Metabolism in the Diabetic Rat[1]

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The feeding of corn oil is reported to lower blood cholesterol levels in man (Ahrens et al., '57; Beveridge et al., '57a; Beveridge et al., '57b), an observation not always demonstrable in experimental animals (Avigan and Steinberg, '57, '58). Experimental animals, however, have shown an increased labeling (synthesis?) of cholesterol from acetate-$C^{14}$ (Avigan and Steinberg, '57; Wood and Migicovsky, '58) and an increase in liver cholesterol (Avigan and Steinberg, '58; Wood and Migicovsky, '58) while receiving diets containing corn oil. On the other hand, materials high in saturated fatty acids, namely, lard and hydrogenated oils, have been reported either as not affecting or as decreasing "synthesis" of cholesterol from acetate (Mukherjee and Alfin-Slater, '58; Wilson and Siperstein, '59a; Wood and Migicovsky, '58). Corn oil or lard, in the diets of rats, did not influence either the biliary excretion of total cholesterol end products or of cholesterol itself (Wilson and Siperstein, '59a), but there was a marked increase in the excretion of non-digitonin precipitable neutral sterols in rats fed corn oil (Wilson and Siperstein, '59b).

Other investigators (Hegsted et al., '57; Peifer et al., '60) have presented evidence and discussed the influence of a variety of fatty acids and oils upon nondiabetic, induced, hypercholesterolemia. Many previous dietary studies have been concerned only with blood lipid levels. In the present paper, liver cholesterol metabolism was studied, as well as blood cholesterol levels, since the liver is generally considered to exert the major influence on blood cholesterol levels. Data on skin, gut and carcass tissues were also obtained and are presented. It was the purpose, then, of the present study, to ascertain the effects of corn oil and lard diets upon blood and tissue cholesterol levels and upon the cholesterologenic activity of certain tissues of both normal and alloxan diabetic rats. The diets were found to influence tissue cholesterol levels, but were found not to influence cholesterol labeling from mevalonate.

## METHODS AND MATERIALS

Diets containing either 10 or 30% of corn oil or 10 or 30% of lard were fed to young adult, male Sprague-Dawley rats[2] for 28 days. Diets containing only 5% of fat (lard), or commercial rat chow diets,[3] were used as control diets. Equal numbers of animals were used with each diet. No statistical difference was found between the effects of these two diets in respect to tissue or plasma cholesterol levels or to cholesterologenesis. The composition of the diets is shown in table 1 and the supplements to the diets are listed as a footnote to the table.

The normal animals weighed about 160 gm when started on the diets and demonstrated a good weight gain. To restrict weight gain and to prevent excessive lipid storage, the animals were fed ad libitum for an initial 14 days and were then trained to feeding for the final 14 days. The trained feeding program consisted of two daily one-hour feeding periods during which the animals were allowed to eat 10 gm of food per period. The diabetic animals weighed between 149 gm and 270

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[2] Obtained from Northwest Rodent Company, Pullman, Washington.
[3] Ralston Purina Rat Chow, The Ralston Purina Company, St. Louis.

TABLE 1
*Composition of the diets*

| Ingredient[1] | Control % | 10% | 30% |
|---|---|---|---|
| Casein | 18 | 18 | 18 |
| Dextrin | 44 | 39 | 19 |
| Glucose | 25 | 25 | 25 |
| Yeast | 4 | 4 | 4 |
| Salt mix[2] | 4 | 4 | 4 |
| Lard or corn oil | 5 (lard) | 10 | 30 |

[1] Vitamin supplementation of the diets as mg/kg of diet: β-carotene, 5.4; calciferol, 0.03; 2-methyl-1, 4-naphthoquinone, 2.0; α-tocopherol, 60.0; pyridoxine, 4.0; thiamine, 4.0; riboflavin, 10.0; Ca pantothenate, 16.0; inositol, 1,000.0; p-aminobenzoic acid, 30.0; choline chloride, 2.0; biotin, 0.1. In addition, methyl linoleate, 15 mg/day/rat, was administered orally. The oil-soluble vitamins were dissolved in triproprionin and administered to the rats in daily doses, the other vitamins being mixed with the fat-free diet base.

[2] Mixture no. 3, Hawk and Oser ('31).

gm when started on the diets but were fed ad libitum throughout the 28 days.

Diabetic animals were prepared by an intramuscular injection of 0.06 ml/100 gm of rat of a 10% alloxan solution. Blood sugar determinations (Somogyi, '45) were performed three weeks after the injection of the alloxan and only the animals demonstrating fasting blood sugar levels of 200 mg/100 ml of blood or greater at that time were used as the diabetic animals. Fasting blood sugars were determined again 26 days after the commencement of the diet studies and animals exhibiting levels of less than 200 mg/100 ml of blood at this time were eliminated.

At the end of the 28-day feeding period, the animals were fasted for 24 hours, fed for an hour, fasted for another hour and injected with a tracer dose of mevalonic acid-2-$C^{14}$. Urine was collected for two hours,[4] the animals lightly anesthetized with chloroform, and decapitated. The animals were separated into 4 tissue fractions, liver, gut, carcass and skin. Blood was taken by heart puncture at the time of sacrifice. The tissues were saponified and separated into saponifiable and nonsaponifiable fractions as described below. After weighing, the tissues were immediately added to flasks containing 25% KOH in 95% ethanol. Liver was saponified in 50 ml of reagent, gut in 150 ml, carcass in 200 ml and skin in 100 ml. Following a two-hour reflux period, the flasks were removed while hot and the contents filtered through glass wool into graduated cylinders. The flasks were then rinsed twice with 5- to 10-ml volumes of water, twice with alcohol (10 to 20 ml); each time the flasks and washing solutions were heated over a hot plate. Washings were added to the original digest in the graduate cylinders, the digests were allowed to cool and were then diluted to convenient, minimal volumes. Small aliquots of the homogenous digest were transferred to 8-inch screw-top culture tubes (Emerson and Van Bruggen, '58), the tubes were heated in a steam bath and the entire alcohol phase removed under nitrogen. The digest was then reconstituted to 25 ml with 25% alcohol. The nonsaponifiable fraction was removed by four 25-ml extractions with redistilled low boiling petroleum ether. The petroleum ether phase was removed with the aid of a syringe fitted with a 6-inch blunt end needle. The extracts were combined, the petroleum ether removed on a steam bath and the nonsaponifiable material made to volume in 25-ml volumetric flasks with 95% ethanol. Cholesterol of these extracts was determined as the digitonide with the method of Zak et al. ('54), but the cholesterol digitonide was allowed a minimum of 5 hours to precipitate. Less time than this fails to yield a quantitative precipitation. Blood cholesterol determinations were made following essentially the procedure of Sobel and Mayer ('45), the digitonides being determined colorimetrically by the method of Zlatkis et al. ('53).

Mevalonic acid-2-$C^{14}$ was obtained as the N,N-dibenzylethylenediamine salt. After hydrolysis and extraction, the free acid was dissolved in water at a level of 0.67 μc/ml and all animals received 1 ml of this solution intraperitoneally.

Radioactive analyses were done with the aid of three G-M assemblies, a low background system (Nuclear-Chicago), a D47 Micromil gas flow system (Nuclear-Chicago), and a TGC-2 end-window assembly (Tracerlab). Lipids were counted as infinitely thin samples ($< 0.08$ mg $cm^{-2}$)

[4] Time course studies in our laboratory have shown that tracer levels of labeled mevalonic acid are maximally converted to sterol within an hour after injection (Elwood, J. C., 1960 Studies on pathways of lipid metabolism. Unpublished Doctor's Dissertation, University of Oregon Medical School).

and blood and urine samples as liquid samples (Van Bruggen et al., '59). Factors established here allowed the inter-conversion of radioassay results.

## RESULTS AND DISCUSSION

*Statistical analysis.* Two statistical tests were applied in the analysis of the data, the *t* test in comparing pairs of means, and the analysis of variance for the simultaneous comparison of more than two means. With each test, however, the interpretation is the same, that is, a *P* value of $< 0.05$ is considered to indicate a significance of the difference between sample means.

*Liver — normal animals.* The data on the concentration of cholesterol in the livers of normal rats fed the indicated diets are summarized in figure 1A. There is seen an increase in the liver cholesterol concentration as the amount of fat in the diet is increased. This is shown by the high levels of cholesterol ($P < 0.01$) in the livers of the animals fed the 30% corn oil and 30% lard diets. Although both kinds of fat showed an increase at the 30% feeding level, the corn oil diet caused liver cholesterol levels to be as high as those of the 30% lard diet even at a 10% level of feeding. The 30% corn oil in turn caused a higher level of cholesterol than did the 30% lard ($P < 0.02$).

*Liver — diabetic animals.* The values for the concentration of cholesterol in the livers of the diabetic animals are shown in figure 1B. The diabetic animals fed corn oil and lard show increases in liver cholesterol of a pattern similar to that seen with the normal animals, but the increases are not as pronounced.

These results for both normal and diabetic animals confirm and extend the reports (Avigan and Steinberg, '58; Wood

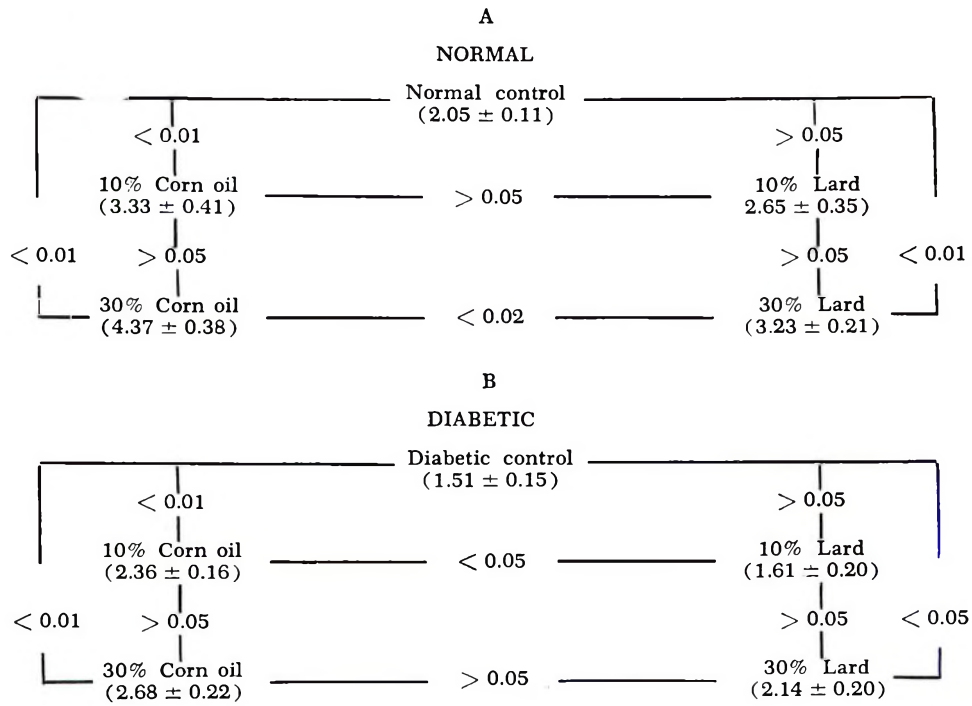


Fig. 1 Cholesterol levels in the livers of normal and diabetic rats fed the respective diets. The values in parentheses represent the means for that group as milligrams per gram ± the standard error. Statistical evaluation of differences between any two means was made by the *t* test. The *P* values obtained are shown as connecting figures between the means compared. (For example, normal animals fed the control diet had a level of 2.05 mg, whereas animals fed the 10% corn oil diet had 3.33 mg. These mean values are compared with a *P* value of $< 0.01$.)

TABLE 2

*Serum cholesterol levels in the normal and diabetic rats*

| Diet | Normal | Diabetic | P Value[1] |
|---|---|---|---|
| | *mg/100 ml* | *mg/100 ml* | |
| Control | 88 ± 5 (8)[2] | 124 ± 26 (6) | > 0.05 |
| Corn oil, 10% | 71 ± 12 (8) | 106 ± 16 (6) | > 0.05 |
| Corn oil, 30% | 67 ± 12 (7) | 121 ± 17 (7) | < 0.05 |
| Lard, 10% | 110 ± 13 (8) | 196 ± 23 (5) | < 0.01 |
| Lard, 30% | 100 ± 8 (10) | 225 ± 52 (5) | < 0.01 |
| *P* value[3] (variance) | > 0.05 | < 0.05 | |

[1] Results of the *t* test.

[2] Mean ± standard error; the numbers in the parentheses represent the number of animals in each group.

[3] Results of the analysis of variance.

and Migicovsky, '58) that ingestion of corn oil increases the amount of cholesterol in the liver. The cause of the increased cholesterol levels is, however, not clear. It does not appear that the oleic acid content of the diet could be responsible for the major part of the change, since the diets had comparable amounts of this fatty acid. Although the increase in the nonsaponifiable fraction is stated to be due to cholesterol, the possibility of the presence of β-sitosterol in this fraction must be considered because of the presence of this material in corn oil. β-Sitosterol is digitonin precipitable and reacts almost equivalently with color reagents. Gould ('55) reported that rats fed β-sitosterol containing tritium had tritium-labeled sterols in blood, liver and other tissues. β-Sitosterol was, however, found to be absorbed only about 10% as efficiently as cholesterol from the intestinal lumen. Preliminary paper and gas chromatography determinations on these tissue fractions failed to reveal the presence of plant sterol in liver fractions.

The corn oil used is reported to be free of cholesterol[5] but the lard fed did contain some 0.55 mg of cholesterol/gm of lard.

*Serum cholesterol — normal and diabetic animals.* The values for the serum cholesterol levels are presented in table 2. Within the normal group of animals fed the various diets, small apparent decreases in serum cholesterol can be observed with corn oil feeding, but these decreases with the different dietary regimens are not statistically significant (analysis of variance $P > 0.05$). From this observation, it is concluded that the serum cholesterol levels of normal rats are not significantly influenced by corn oil or by lard feeding under the described experimental conditions.

The *P* value of less than 0.05 for the analysis of variance among the diabetic animals indicates that an effect of diet upon serum cholesterol levels is present. Statistical comparisons by the *t* test of the mean levels of the serum cholesterols from the rats fed lard and corn oil revealed a significant elevation of the serum cholesterols of the lard-fed animals over the levels of the respective corn oil-fed animals. The serum cholesterol level of the diabetic animals receiving the 10% lard diet was significantly elevated over the controls.

An earlier publication from this laboratory (Wong and Van Bruggen, '60), concerned with large numbers of normal and diabetic animals fed chow diets, reported a hypercholesterolemia associated with the alloxan diabetic rats, as well as with pancreatectomized rats. The results reported in this paper, on a smaller number of control diet animals, fail to reveal a significant diabetic hypercholesterolemia but this appears to be due largely to the wide variations of the diabetic animals. Three of the diabetic diet groups did, however, have significantly higher blood cholesterol levels than the controls. Beveridge and Johnson ('50) found a hypercholesterolemia to occur in alloxan diabetic rats.

*Gut, carcass and skin.* The data for the cholesterol content of gut, carcass and skin fractions of the normal and diabetic

[5] Rathmann, D. M. 1957 Vegetable Oils in Nutrition. Corn Oil Refining Company, New York, p. 49.

TABLE 3

*Concentration of cholesterol in gut, carcass and skin tissue*

| Tissue | Diet | Normal | Diabetic | P Value (t test) |
|---|---|---|---|---|
| | | *mg/gm* | *mg/gm* | |
| Gut | Control | 1.09 ± 0.13 (8)[1] | 1.29 ± 0.18 (7) | > 0.05 |
| | Corn oil, 10% | 1.79 ± 0.21 (8) | 2.08 ± 0.19 (7) | > 0.05 |
| | Corn oil, 30% | 5.40 ± 0.60 (7) | 4.15 ± 0.31 (6) | > 0.05 |
| | Lard, 10% | 1.81 ± 0.27 (8) | 1.60 ± 0.10 (5) | > 0.05 |
| | Lard, 30% | 1.68 ± 0.15 (10) | 1.38 ± 0.13 (8) | > 0.05 |
| Carcass | Control | 1.45 ± 0.04 (8) | 1.78 ± 0.12 (7) | < 0.05 |
| | Corn oil, 10% | 1.50 ± 0.05 (8) | 1.85 ± 0.07 (7) | > 0.05 |
| | Corn oil, 30% | 1.53 ± 0.14 (7) | 1.54 ± 0.06 (6) | > 0.05 |
| | Lard, 10% | 1.33 ± 0.03 (8) | 1.81 ± 0.12 (5) | < 0.01 |
| | Lard, 30% | 1.38 ± 0.04 (10) | 1.65 ± 0.11 (8) | < 0.05 |
| | P Value[2] (variance) | > 0.05 | > 0.05 | |
| Skin | Control | 2.59 ± 0.07 (8) | 3.26 ± 0.17 (7) | < 0.01 |
| | Corn oil, 10% | 2.57 ± 0.23 (8) | 2.89 ± 0.08 (7) | > 0.05 |
| | Corn oil, 30% | 2.21 ± 0.10 (7) | 2.84 ± 0.20 (6) | < 0.05 |
| | Lard, 10% | 2.02 ± 0.06 (8) | 3.19 ± 0.11 (5) | < 0.01 |
| | Lard, 30% | 2.31 ± 0.07 (10) | 2.29 ± 0.19 (8) | > 0.05 |
| | P Value (variance) | < 0.05 | < 0.01 | |

[1] Mean ± standard error; the numbers in the parentheses represent the number of animals in each group.
[2] Results of the analysis of variance.

animals are presented in table 3. The gut fraction shows an unexpectedly large amount of sterol in both the diabetic and normal animals fed the high corn oil diets. Since the sitosterols are digitonin precipitable and develop a color similar to cholesterol when treated with the ferric chloride-sulfuric acid color reagent, there is a strong possibility that sterols present in the residual gut contents account for the increase in the gut sterols of the corn oil fed animals.

There are no significant differences between the means of the carcass fractions within the normal or within the diabetic groups of animals (variance for both, $P > 0.05$). These observations are interpreted to mean that the ingestion of high fat, corn oil or lard, diets does not influence carcass cholesterol levels to any significant extent.

With normal rats, diets containing 30% of corn oil or 30% of lard caused a slight decrease in the amount of skin cholesterol ($t$ test, $P < 0.05$ in both instances), as compared with the control value. Diabetic rats fed the 30% lard diet show a similar decrease in skin cholesterol concentrations ($P < 0.01$), no effect being seen on the skin cholesterol concentration with corn oil feeding. The significance of these observations is not clear.

A comparison of the control normal and diabetic values of the skin fraction confirms an earlier report of increased cholesterol concentrations in the skins of diabetic rats (Wong and Van Bruggen, '60). It would appear, then, that the diabetic condition is characterized by an accumulation of sterol in the skin tissue.

TABLE 4

*Incorporation[1] of mevalonate-2-$C^{14}$ into liver cholesterol*

| Diet | Normal | Diabetic |
|---|---|---|
| Control | 3.94 ± 0.15[2] (6) | 4.64 ± 0.34 (6) |
| Corn oil, 10% | 3.92 ± 0.18 (6) | 4.80 ± 0.32 (5) |
| Corn oil, 30% | 4.69 ± 0.41 (6) | 4.73 ± 0.27 (4) |
| Lard, 10% | 4.59 ± 0.21 (6) | 4.31 ± 0.60 (3) |
| Lard, 30% | 3.84 ± 0.13 (7) | 4.47 ± 0.95 (5) |
| P Value (variance) | > 0.05 | > 0.05 |

[1] Expressed as percentage of dose given.
[2] Mean ± standard error; the numbers in parentheses represent the number of animals in each group.

*Incorporation of label.* In table 4 are presented data for the incorporation of $C^{14}$ from mevalonic acid-2-$C^{14}$ into liver cholesterol in the normal and diabetic rats fed the various diets. Within either the normal or the diabetic dietary groups, no significant differences in label incorpora-

tion were found at any level of fat feeding, i.e., variances, $P > 0.05$. From the data presented here, it appears either that diet does not affect cholesterologenesis, or that any influence exerted by the diet is mediated at some point below mevalonic acid in the biosynthetic pathway of cholesterol. Elwood et al. ('60) previously observed a decreased incorporation of acetate, butyrate and acetoacetate, but a normal incorporation of mevalonate into cholesterol in alloxan diabetic rats. Siperstein ('60) and Siperstein and Guest ('60) suggested that cholesterologenesis was controlled by a specific homeostatic mechanism active at the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonic acid. It appears that the control of cholesterol synthesis at a metabolic step below mevalonate could be similar to the step at which the metabolic block occurs in alloxan diabetes.

The majority of the work concerning cholesterologenesis and diet, has been done using acetate as the cholesterol precursor. Avigan and Steinberg ('58) using *in vivo* preparations, found the rate of incorporation of acetate higher in rats fed corn oil, as did Wood and Migicovsky ('58) with *in vitro* as well as *in vivo* systems. Wilson and Siperstein ('59a) studied this incorporation *in vitro* in corn oil-fed rats with acetate and with mevalonate, and found no change in cholesterol biosynthesis with either precursor. The *in vivo* studies reported here, support the *in vitro* observation (Wilson and Siperstein, '59a) of a lack of change in cholesterologenesis from mevalonic acid in normal rats fed corn oil or lard diets. The present studies, however, extend these reports by presenting data which demonstrate normal cholesterol labeling from mevalonic acid when alloxan diabetic rats were fed the corn oil and lard diets.

## SUMMARY

1. The effects of corn oil and lard diets upon blood and tissue cholesterol in normal and alloxan diabetic rats are reported and discussed.

2. Dietary lard aggravated a trend towards hypercholesterolemia in the alloxan diabetic rat.

3. The corn oil diet increased the amount of sterol found in the livers of both the diabetic and normal animals.

4. Lard feeding increased the sterol content of the livers of normal animals as well as diabetic animals, but the increase was not as pronounced as with corn oil feeding.

5. Gut, carcass and skin data were presented and discussed.

6. The feeding of 10 or 30% of corn oil or lard diets to diabetic or normal animals resulted in no change in cholesterologenesis using mevalonic acid-2-$C^{14}$ as the cholesterol precursor.

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# Experimental Zinc Deficiency and Recovery of Calves[1,2]

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Zinc has been shown to be a required nutrient for many species of animals and is believed to be essential for all. Since most of the early work was carried out with laboratory animals, the value of supplemental zinc in practical rations for farm animals was not recognized until recently. Tucker and Salmon ('55) demonstrated that parakeratosis, which had been responsible for great economic losses in the swine industry, was due to zinc deficiency. Other studies have shown that zinc supplementation is of practical importance for poultry (Edwards et al., '59). Recent observations under field conditions (Legg and Sears, '60) suggest that zinc deficiency may occur in cattle, making a more fundamental knowledge of the subject of practical importance. Preliminary observations on the symptoms of an experimentally produced zinc deficiency in dairy calves were reported by Miller and Miller ('60). The present study was undertaken to produce a zinc deficiency in the dairy calf, describe the syndrome and study the effect of added zinc on deficient calves.

## EXPERIMENTAL PROCEDURE

Male Holstein calves were fed a low-zinc purified diet, with and without supplemental zinc in two experiments. The purified diet, based on those of Edwards et al. ('58) and Matrone et al. ('59), was composed of the following expressed in kg per 100 kg: egg albumin (autoclaved), 16.0; glucose, 60.0; stabilized fat,[5] 3.0; cellulose,[6] 5.0; $KHCO_3$, 4.5; $NaHCO_3$, 7.5; and the following as gm per 100 kg: $CaHPO_4$, 1861; KCl, 622; NaCl, 545; $MgSO_4 \cdot 7H_2O$, 463; $FeSO_4 \cdot 1H_2O$, 17.40; $MnSO_4 \cdot H_2O$, 3.17; $CuSO_4 \cdot 5H_2O$, 2.03; $CoCl_2 \cdot 6H_2O$, 0.020; KI, 0.018; choline chloride,[7] 262; cyanocobalamin (1 mg vitamin $B_{12}$ activity/gm), 10.76; Ca pantothenate, 3.24; niacin (80%), 2.58; riboflavin, 1.94; vitamin A palmitate (250,000 IU/gm), 1.76; vitamin $D_3$ (200,000 IU/gm), 1.76; *dl*-α-tocopheryl acetate (276 IU vitamin E activity/gm), 1.32; pyridoxine·HCl, 1.30; thiamine·HCl, 0.90; menadione, 0.26; folic acid, 0.13; biotin, 0.026; oxytetracycline·HCl, 11.0; and oleandomycin, (25%) 4.4.

The 4 calves in the first experiment were each given a total of 227 kg of whole milk over an 8-week period starting at one day of age. Beginning the fifth-week and continuing for 11 to 14 additional weeks, two of the calves were fed the low-zinc basal diet, and the other two received the basal diet supplemented with 43 ppm of zinc as ZnO. Eight calves in the second experiment were fed 145 kg of milk each over a 6-week period. The low-zinc diet, with the amounts of vitamin A and D supplements increased to 22.00 and 2.20 gm, respectively, was fed beginning the second week of age as follows. Three control animals (group 1) received the basal diet plus 40 ppm of supplemental zinc for 20 weeks. Five animals were fed the basal diet until 15 weeks of age at which time the ration of three (group 2) was supplemented with 260 ppm of zinc for 5 weeks. The remaining two (group 3) continued to be fed the

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[5] Tri-Co, Swift and Company, Chicago.

[6] Solka-Floc, Brown Company, Berlin, New Hampshire.

[7] Appreciation is expressed to the Chas. Pfizer Co., Terre Haute, Indiana, for the vitamins and antibiotics.

basal diet until death occurred at 18 and 22 weeks of age.

The calves were maintained in wooden pens with elevated slatted bottoms without bedding. Plastic buckets were used to feed both the milk and water. Stream water was obtained from the city supply through a system containing a minimum of galvanized pipe. The average zinc content of the milk, water, basal diet, and basal diet with zinc added were 3.9, 0.06, 2.7 and 46.0 ppm, respectively, in experiment 1. In experiment 2, the corresponding values were 3.3, 0.02, 3.6 and 45.0 ppm of zinc, respectively. The ration fed the calves in group 2 from the fifteenth through the twentieth week contained 265 ppm of zinc.

Clinical examinations of the calves were made periodically. All animals in experiment 1 were slaughtered for necropsy and histopathological studies after they had received the purified diet for 11 to 14 weeks. Samples of various tissues and internal organs were obtained at this time for zinc analysis. Zinc determinations were made by the method of Verdier et al. ('57). Blood was analyzed for carbonic anhydrase activity by the method of Philpot and Philpot ('36) as modified by Pincus et al. ('57). Statistical procedures as outlined by Snedecor ('56) were used.

## RESULTS

*Experiment 1.* The average total zinc intake of the control calves was 7.3 and for the deficient calves, 1.3 gm; of these amounts 0.9 gm was from milk. Deficiency symptoms in the two animals fed the basal diet with no supplemental zinc were first observed at 11 weeks of age, three weeks after milk feeding was discontinued. The symptoms appeared in the following order: inflammation of the nose and mouth with submucous hemorrhages; unthrifty appearance; rough hair coat; stiffness of the joints with soft edematous swelling of the feet in front of the fetlocks with an accumulation of fluid; breaks in the skin around the hoofs which later became deep fissures; dry scaly skin on the ears; thickening and cracking of skin around nostrils; appearance of horny overgrowths of the mucosa on the lips and dental pads; frequent gnashing of teeth; alopecia, beginning with the rear legs; red, scabby and shrunken skin on the scrotum; and bowing of the rear legs. Skin on the body and head tended to be hard and dry, and the legs were tender, easily injured, and often raw and bleeding. The skin on the neck of one calf became very rough and lumpy, making collection of blood samples difficult as bits of flesh usually plugged the needle. The two control calves that received the same purified diet supplemented with 43 ppm of zinc did not develop these symptoms.

Zinc content and carbonic anhydrase activity of the blood from calves fed the low-zinc diet were found to be statistically significantly lower ($P = 0.05$) than for the controls. For the last 7 weeks of the trial the average values of blood zinc in μg per ml and carbonic anhydrase activity in enzyme units per ml (Pincus et al., '57) were 2.9, 482, 1.7 and 398 for the control and low-zinc calves, respectively. Corresponding values for the previous 4-week period, when calves had been receiving the diets for only a short time, were 3.0, 469, 2.5 and 495.

On necropsy and histopathological examination, the skin of the two zinc-deficient calves exhibited parakeratosis. The stratum granulosum appeared reduced in a number of cells. There was acanthosis, elongation of the rete net with excessive keratin formation and retention of nuclei. Some edema appeared to have been present in these tissues. The papillae of the rumen showed excessive over-growth, a moderate keratin formation, and retention of nuclei. Although all of the calves exhibited some parakeratosis of rumen walls and of the esophageal mucosa, the two controls appeared normal in other respects. The testicles of the two zinc-deficient calves weighed 47.3 and 52.1 gm at 18 weeks of age. In contrast, the testicles of the controls weighed 65.6 and 53.1 gm at 19 and 16 weeks, respectively.

The zinc content of various organs and tissues from the deficient calves, with few exceptions, was lower than that of corresponding samples from the controls (table 1). Fecal excretion of zinc by the deficient calves was very low.

TABLE 1

*Zinc analysis of various organs and feces (exp. 1)*

| | 1 Control | 2 Control | 3 Low-Zn | 4 Low-Zn |
|---|---|---|---|---|
| | | *μg/gm dry matter* | | |
| Pancreas | 171.1 | 158.2 | 61.4 | 72.0 |
| Liver | 128.5 | 132.7 | 112.9 | 101.7 |
| Rumen mucosa | 124.3 | 105.4 | 104.7 | 100.0 |
| Spleen | 117.9 | 99.3 | 95.6 | 88.0 |
| Heart | 92.9 | 99.5 | 87.8 | 75.6 |
| Kidney | 92.7 | 90.4 | 78.7 | 82.1 |
| Fundus of stomach | 69.8 | 128.2 | 60.2 | 91.2 |
| Hoof | 122.2 | 128.9 | 107.8 | 113.4 |
| Bone | 92.1 | 104.9 | 60.6 | 78.0 |
| Teeth | 72.8 | 71.2 | 67.0 | 68.9 |
| Feces[1] | 310.0 | 249.0 | 18.1 | 14.8 |

[1] Average of two collections taken during last week of experiment.

*Experiment* 2. Average zinc intakes for groups 1, 2 and 3 were 5.9, 0.9 and 0.9 gm, respectively, for the first 15 weeks. Corresponding values for the following 5-week period were 4.5, 24.6 and 0.2 gm. Feed intake and weight gains, for calves receiving the deficient diet began to decline relative to the controls by the tenth week of age which was 4 weeks after milk feeding was discontinued. Deficiency symptoms then began to appear as in experiment 1 and were severe in the 5 deficient animals by the twelfth week of age (fig. 1). The control animals remained normal in appearance throughout the study. Feed consumption of groups 2 and 3 tended to reach a plateau by the ninth week (fig. 2), and growth was at a diminishing rate until the fifteenth week, whereas the controls continued to gain steadily (fig. 2). Blood zinc content and carbonic anhydrase activity began declining for these calves the seventh week, but remained essentially unchanged or increased

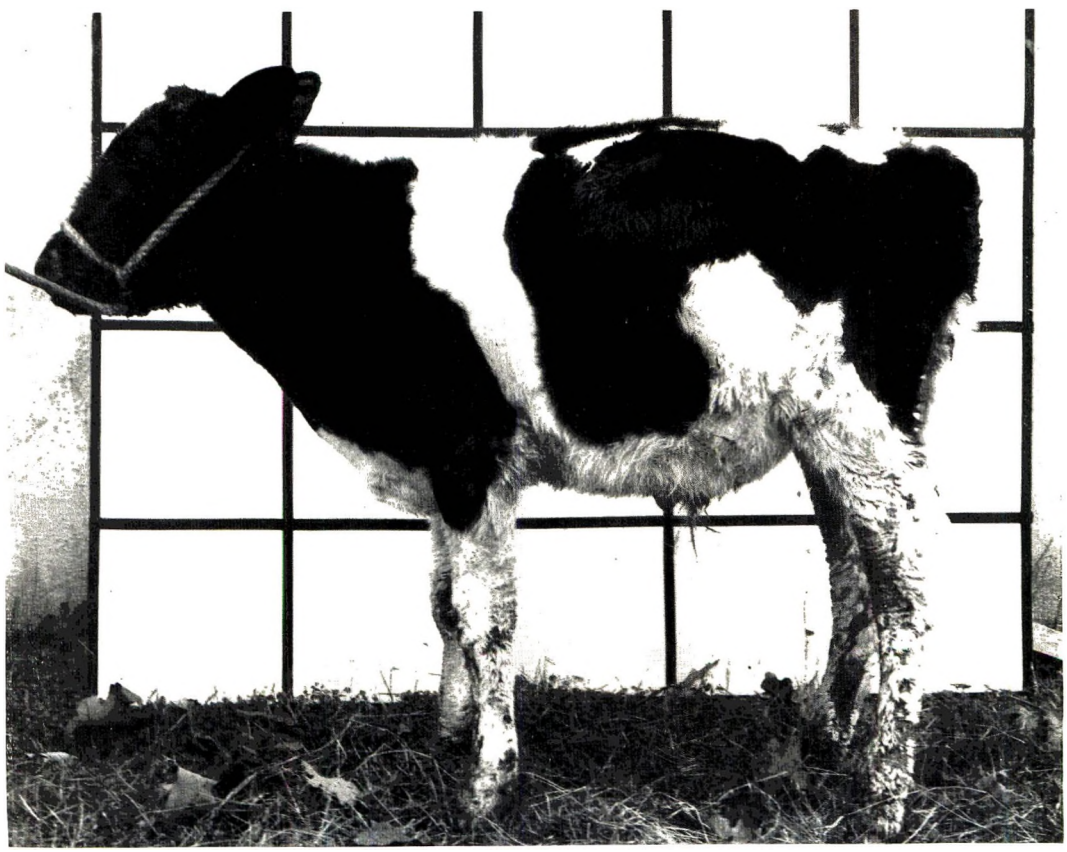

Fig. 1 Zinc-deficient calf, age 15 weeks.

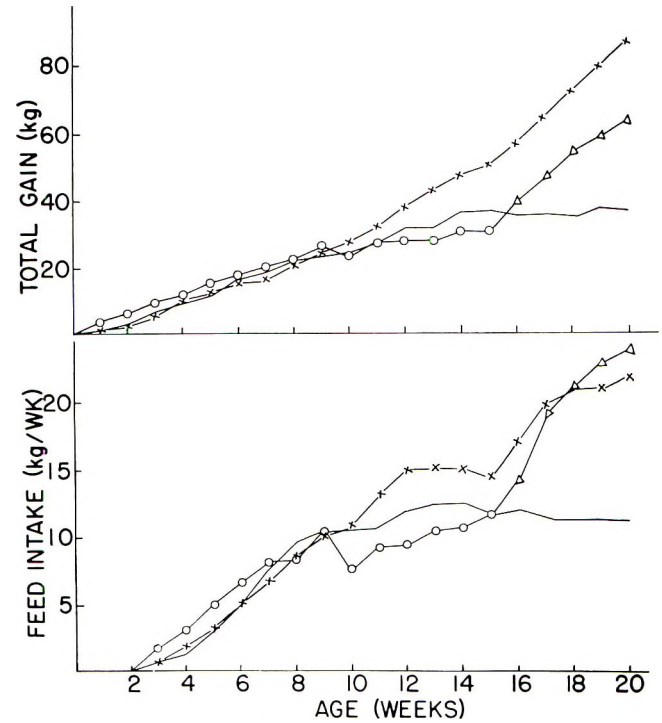


Fig. 2 Average weekly feed consumption and total gains of the calves as affected by zinc content of the diet (ppm): —, 3.6; ○—○, 3.6; x—x, 45; △—△, 265.

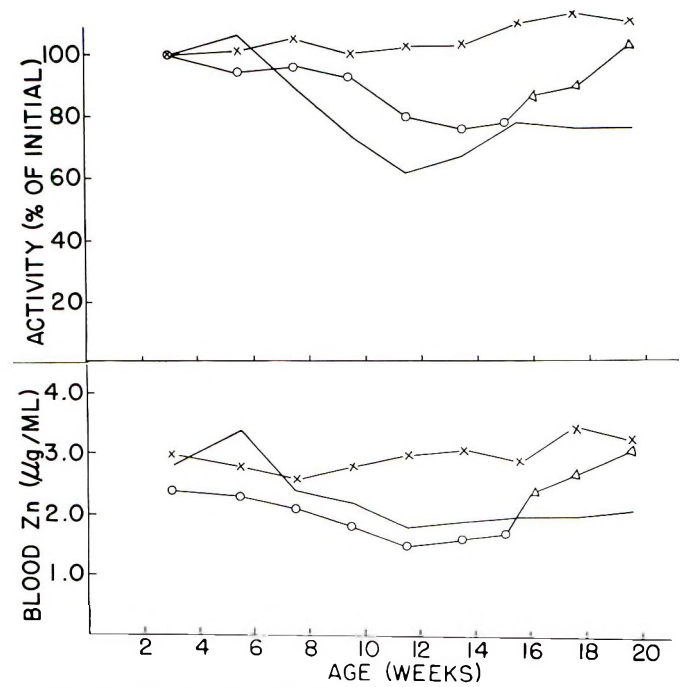


Fig. 3 Average levels of carbonic anhydrase activity and zinc in the blood as affected by zinc content of the diet (ppm): —, 3.6; ○—○, 3.6; x—x, 45; △—△, 265.

slightly for the controls (fig. 3). Zinc content was reduced significantly ($P = 0.05$) by the eleventh week, and the reduction in carbonic anhydrase activity approached significance by the thirteenth week.

After receiving an additional 260 ppm of zinc in the diet beginning with the fifteenth week, improvement of the calves in group 2 was very marked. The average weekly feed intake of these calves increased from 11.8 to 14.5 kg the first week after zinc supplementation was begun and continued to increase, whereas consumption by calves in group 3 remained low. The corresponding increase in rate of gain was from 0.0 to 9.1 kg the first week. After zinc was supplied to group 2, blood zinc content began to increase, and by the nineteenth week, approached the value exhibited by the controls (fig. 3). The blood zinc values of these in group 3 remained low. Blood carbonic anhydrase activity followed a pattern similar to that of blood zinc (fig. 3). The simple correlation coefficient between blood zinc content and carbonic anhydrase activity was 0.80 for all determinations.

The skin condition of calves in group 2 showed improvement within 4 days after they were given supplemental zinc. Dead hair and skin sloughed off in sheets, and the underlying tissue assumed a more healthy appearance. Within one week, the bowed rear legs began to straighten and the hard, dry skin on the backs and necks started to become soft and pliable. The skin continued to improve and within two weeks fine hair appeared in the bare spots. By the third week after zinc supplementation was begun the condition of the gums and dental pads had returned to approximately normal; the calves that had appeared dull and listless while receiving the deficient diet now frisked about in their pens and no longer acted as if their feet were sore; and hair almost completely covered the former bare spots. However, the skin of the calf that had exhibited the severest deficiency symptoms was still tender and easily injured. This animal also grew gray hair in many of the denuded areas where black hair had been.

Although skin on the scrotum had about healed at the end of the study, the testicles of calves in group 2 remained undersized (table 2). These measurements were made through the scrotum with a vernier caliper in order to follow development. When castrated at 29 weeks of age, the average testicle weight of the control group was 113 gm as compared with only 58 gm for group 2. The same deficient calf shown in figure 1, is shown again 5 weeks after 260 ppm of zinc were added to his feed (fig. 4).

TABLE 2

*Development of testicles as measured by length times greatest diameter measured through the scrotum (exp. 2)*

| Group | Average age (weeks) | | |
|---|---|---|---|
| | 17 | 20 | 22 |
| | *cm* | *cm* | *cm* |
| 1 Control | 6.6 ± 1.6[1] | 7.9 ± 1.4 | 9.2 ± 1.2 |
| 2 Deficient-revived | 3.3 ± 0.8 | 5.8 ± 0.2 | 6.4 ± 0.4 |
| 3 Deficient | 4.5 ± 0.1 | 5.1 ± 0.6 | 5.1 ± 0 |

[1] Mean ± standard error.

During the time the calves in group 2 were recovering, the condition of the calves in group 3 continued to become worse until the animals died at 18 and 22 weeks of age. Necropsy examinations revealed that death had resulted from acute indigestion in one case and perforated ulcer with peritonitis in the other. Other conditions observed included excessive overgrowth of rumen papillae; severe gastritis; edema of folds of the abomasum; congestion of the liver, intestinal serosa, and kidneys; cholecystitis; catarrhal enteritis; and enlarged and edematous lymph nodes. Extensive keratinization of thighs between legs and behind elbows and ulceration of the fetlock and pastern region were observed.

## DISCUSSION

The condition of the zinc-deficient calves on gross examination bore a striking resemblance to hyperkeratosis, which is also similar in many respects to severe vitamin A deficiency. Extremely low plasma vitamin A values have been reported in cattle with hypkerkeratosis (Maynard and Loosli, '56). However, in experiment 1 there were no abnormalities in plasma vitamin A

[8] Diamox, American Cyanamid Company, New York.

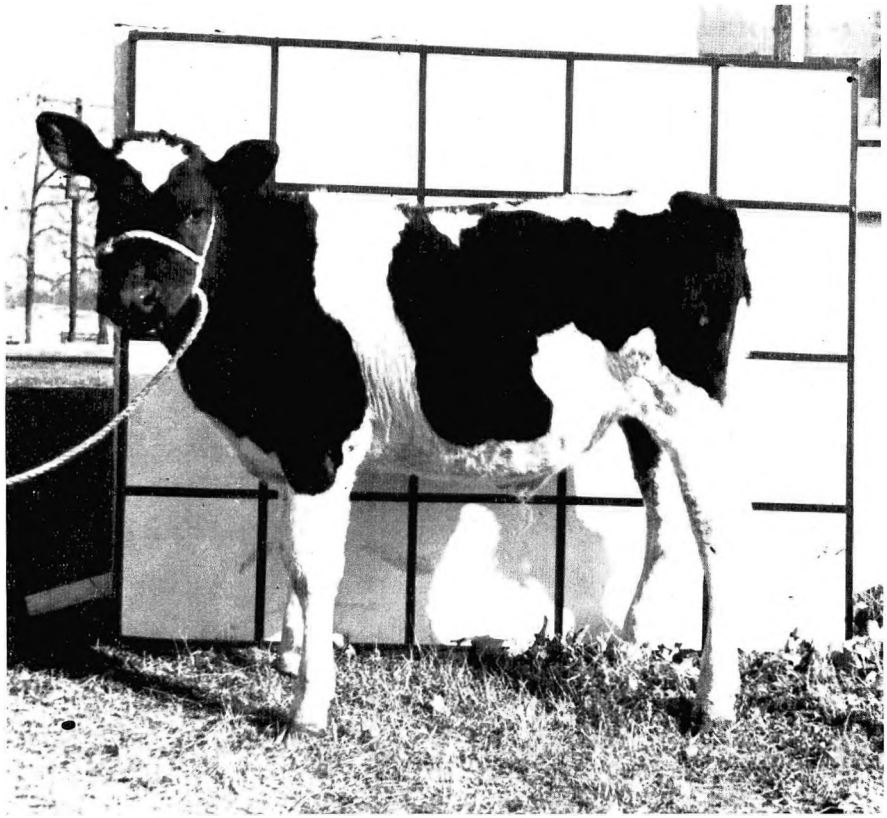

Fig. 4 Same calf as figure 1, 5 weeks after zinc was added to the ration.

and no significant difference was found between the control and zinc deficient animals.

The pancreas had the highest zinc content of the tissues analyzed in the controls and underwent the greatest reduction in the deficient animals. At least two zinc-containing enzymes, carboxypeptidase (Vallee et al., '60) and carbonic anhydrase (Birnbaum and Hollander, '53) occur in this gland. The liver had the second highest zinc content but the reduction in the deficient calves was not as marked as in the pancreas. Two enzymes, glutamic acid dehydrogenase (Vallee et al., '55) and alcohol dehydrogenase (Vallee and Hoch, '57), both containing zinc as a functional part, have been isolated from liver.

The high correlation between blood zinc content and carbonic anhydrase activity in the present study is of interest since this enzyme was not significantly lowered in zinc-deficient rats (Hove et al., '40). But Hove et al. ('40) stated that the deficiency was not as severe as that observed in an earlier study (Hove et al., '38), in which two deficient animals had an extremely rapid, gasping type of respiration for a week prior to death. Increased rates of respiration have also been observed in zinc-deficient chicks (Rahman et al., '61) as well as in dogs treated with a carbonic anhydrase inhibitor[8] (Tomashefski et al., '54). The significantly reduced blood zinc content and carbonic anhydrase activity in the deficient calves, as well as the recovery of these values following the addition of zinc to the ration, might be considered as additional evidence in support of the active involvement of zinc in the carbonic anhydrase molecule.

The marked improvement shown by the deficient calves when adequate zinc was made available has also been observed in pigs suffering from advanced parakeratosis (Beardsley and Forbes, '57; Smith et al.,

'58). Likewise the retarded testicle development of the deficient calves and failure to return to normal when zinc was fed has also been reported in rats (Millar et al., '58).

Since the calves in the present study were raised under controlled conditions where precautions were taken to isolate them from environmental zinc, the results do not necessarily apply to animals raised under practical conditions. However, it was shown that zinc is a necessary nutrient for the dairy calf and severe consequences result when it is present in inadequate amounts. It is possible that borderline deficiencies affecting calf performance may occur unrecognized under some practical conditions.

## SUMMARY

Seven Holstein calves, fed a low-zinc purified diet in two experiments, developed severe parakeratosis. Five comparable animals receiving the same diet supplemented with 40 ppm of zinc remained normal and made satisfactory weight gains. Zinc deficiency symptoms observed included: anorexia; dull and listless appearance; low weight gains, breaks in the skin with deep fissure formation around the hoofs; alopecia, especially on the rear legs with edematous soft swelling of the feet in front of the fetlocks with an accumulation of fluid; extensive dermatitis between the legs and behind the elbows; hard dehydrated skin on the body and head with that on the legs being tender and easily injured; red, scabby and shrunken skin on the scrotum; undersized testicles; inflammation of the nose and mouth with submucous hemorrhages; horny overgrowth of the mucosa on the lips and dental pad; and reduced blood zinc content and carbonic anhydrase activity. Addition of 260 ppm of zinc to the ration of three deficient calves in the second experiment beginning at 15 weeks of age resulted in rapid and dramatic recovery. All of the conditions listed above with the exception of the undersized testicles were corrected by zinc supplementation of the diet.

## ACKNOWLEDGMENTS

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# Growth and Liver Xanthine Dehydrogenase in Chicks and Poults Fed Casein or Soy Protein Diets[1]

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Reid et al. ('56, '57) reported that a part of the growth response by chicks and poults that resulted from the addition of unrefined supplements such as fish solubles to purified diets was due to molybdenum. This seemed improbable on the basis of our previous attempts to produce a Mo deficiency in both rats and chicks (Higgins et al., '56); in those studies the trace amounts of Mo still remaining in the highly purified diets were adequate for growth even though they were insufficient to maintain normal levels of tissue xanthine oxidase or dehydrogenase. Only when tungstate was used as a competitive inhibitor of molybdate could a Mo deficiency be produced of sufficient severity to have an effect on growth. The Mo content of the basal diets used by Reid et al. was well in excess of such minimal quantities.

In repeating these studies, the presence or absence of added Mo from the basal diet had no effect on growth rate, liver xanthine dehydrogenase activity, or the growth response from fish solubles. It soon became evident that a major factor governing all three of these responses was the kind and amount of protein supplied in the diet. The study therefore developed into a comparison of the utilization of soy protein and casein-gelatin diets by birds for growth and for the synthesis of liver xanthine dehydrogenase. Soy protein supported growth much better than casein-gelatin, and added fish solubles gave a growth response with either type diet. Since these differences in growth rate were not accompanied by corresponding differences in liver xanthine dehydrogenase and since the amino acid compositions of the various diets were quite similar, the growth effects from soy protein and fish solubles did not seem to be the result of the protein itself. Both soy protein and fish solubles appeared to contain unidentified factors which were missing from the casein-gelatin diets and which were required for a maximal growth rate by chicks and turkey poults.

## METHODS[2]

The initial experiments were carried out with the same diet described by Reid et al. ('56, '57). This was a 32.5% soy protein diet[3] supplemented with 0.75% of DL-methionine and 0.4% of glycine. In addition, it contained 3.5% of soy oil, 53% of starch, 8.3 to 9.5% of salts (depending upon the amount of water of crystallization in the salts), 0.1% of inositol, 0.2% of choline, and all the known vitamins as well as chlortetracyline[4] (50 mg per kg) and diphenyl-*p*-phenylenediamine (125 mg per kg). After the initial comparisons were completed, we also added 2 mg per kg of $Na_2MoO_4$ to eliminate any possibility of a Mo deficiency in the remaining studies. When the amount of soy protein in the diet was varied, the same supplementation with methionine and glycine

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[2] We are indebted to the following sources of supplies: aureomycin, Lederle Laboratories; fermentation residue (Omafac), E. R. Squibb & Sons; fish solubles, Philip R. Park, Inc., San Pedro, Cal.; vitamin D, Bowman Feed Products, Holland, Mich.; soybean oil, Procter and Gamble, Cincinnati; diphenyl-*p*-phenylenediamine, B. F. Goodrich Co.; distiller's dried solubles, Distillers Feed Research Council, Cincinnati, Ohio; Peebles M N C dried whey, Western Condensing Co., Appleton, Wis.; ADM C-1 Assay Protein, Archer-Daniels-Midland Co., Minneapolis, Minn. The chicks were obtained from Mr. Raymond Sachs, Camillus, New York and the turkeys from the River Valley Turkey Farms, Mannsville, New York.

[3] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.

[4] Aureomycin, American Cyanamid Company, New York.

was maintained. In all diet variations, appropriate adjustments were made in the starch content to keep the total solids at 100%.

The casein or casein-gelatin diets had the same composition listed previously except that 20 to 50% of casein, with or without 10% of gelatin, (or 15% of casein plus 5% of gelatin) was substituted for the soy protein. No methionine or glycine were added routinely to these diets. When fish solubles was added at a constant protein level, both the casein and gelatin concentrations were reduced proportionately.

After the first 9 out of 25 experiments, the pyridoxine, menadione, and $ZnCl_2$ content of the diets were routinely increased to 6, 1, and 40 mg per kg, respectively, and 100 mg per kg of mixed tocopherols were substituted for the 24 mg of α-tocopheryl acetate. In some of the experiments the entire vitamin mixture was doubled. In the last three experiments the Zn content of the diet was increased to 50 ppm (Dam et al., '59). Occasionally, Nutritional Biochemicals[5] casein was used instead of Labco[6] casein, and Wesson[7] or Mazola oil[8] was used instead of soybean oil. None of these precautionary adjustments, tests or substitutions had any apparent effect on the results. Both the vitamin and mineral compositions of these diets equaled or exceeded the minimal amounts recommended by the National Research Council (Bird et al., '54).

A mixture of unrefined materials commonly used for supplementing poultry feeds was made up of 6% of fish solubles, 6% of dried whey, 3% of distiller's solubles and 3% of antibiotic fermentation residue (as percentages of the final diet) in an attempt to reproduce the experimental conditions described by Reid et al. ('56, '57). On the basis of nitrogen analysis and multiplying by the factor 6.25, these materials contained 32, 15, 28 and 28% of protein, respectively. Similarly, on the basis of nitrogen content the casein, soy protein and gelatin contained a calculated 82.5, 80 and 96% of protein, respectively. The fish solubles also contained 6.4% of fat, 10.5% of ash and 50% of water; when added to the diet at the 20% level, it was substituted for 10% of the solids.

One-day-old male White Leghorn chicks or White Holland Broad Breasted turkeys were maintained in heated brooders with raised wire-mesh bottoms and fed the diets ad libitum. Groups of 10 to 15 birds were usually fed each diet, and body weights were recorded weekly. The values given with the results refer to the average body weight at 4 weeks. At the end of 4 weeks the birds were killed, and in some experiments the livers were analyzed for xanthine dehydrogenase by the manometric precedure previously described (Remy et al., '51); 1.7 ml of a 1:60 liver homogenate was tested in the presence of methylene blue and hypoxanthine substrate, and the net oxygen consumption was recorded in cubic millimeters per 20-minute reading period.

## RESULTS

*Turkey growth.* The basal 32.5% soy protein diet supplemented with 0.75% of methionine and 0.4% of glycine, as described by Reid et al. ('56), gave better growth (325 gm) than a commercial chick starter (255 gm). Maximal growth (450 gm) was obtained when the basal soy protein diet was supplemented with the mixture of unrefined materials; this response to the mixture could not be attributed to Mo since it was obtained when the basal diet contained added Mo. The mixture of unrefined materials contributed an additional 4.5% of protein to the diet, and part of the growth response to the mixture might have been due to the additional protein. Supplementing the basal diet with an additional 18% casein or soy protein (total protein = 50%) also gave a growth response (380 gm) but it was less than the maximal rate obtained with the mixture.

By comparison with the 32% soy protein diet, a 32% casein or a 22% casein-10% gelatin diet gave poor growth (185 gm and 155 gm). The addition of the mixture of unrefined materials to a 22 or 25% casein-10% gelatin diet had a marked effect and gave 80 to 90% of the maximal growth rate. The same response was ob-

[5] Nutritional Biochemicals Corporation, Cleveland.
[6] The Borden Company, New York.
[7] Refined cottonseed oil, The Wesson Oil Company, New Orleans, Louisiana.
[8] Refined corn oil, Corn Products Company, Argo, Illinois.

tained with 20% of fish solubles alone (400 gm), and no additional effect was obtained when the diet also contained 20% of whey. The magnitude of the growth response to fish solubles was obviously much greater when tested with a casein-gelatin than with a soy protein diet, but this difference appeared to be due to the "protein-inadequacy" of the casein-gelatin diet.

To determine the dietary protein requirement for poults, growth was measured with various diets containing different concentrations of soy protein or casein with or without gelatin, both in the presence and absence of 20% of fish solubles. The growth responses have been plotted in figure 1 as a function of the total protein content of the diet. Since figure 1 involves a direct comparison of growth rates in numerous diverse experiments, some scatter of the data could be anticipated because of the inherent differences in various groups of birds obtained and studied at different times. However, the same diet was often repeated with several different groups of birds to provide a cross-check, and the results were reasonably consistent. Approximately 35% total protein was adequate for maximal growth when soy protein was supplemented with fish solubles. Soy protein alone gave 70 to 80% of the maximal value when fed at protein levels of 32 to 50%. Purified casein or casein-gelatin diets gave increasing growth rates with increasing amounts of protein in the diet; at between 30 and 50% of dietary protein the growth rate increased from about 30% of the maximum to 70%. Thus, casein supported growth almost as well as soy protein when fed at sufficiently high levels. However, even when fish solubles was added to 30 to 35% of casein or casein-gelatin diets, the birds had an unthrifty appearance by comparison with the birds fed soy protein. When 20% of fish solubles was added to casein or casein-gelatin diets, no growth response was obtained at a total dietary protein of 20%. However, at 30 and 40% of total protein, the fish solubles gave good growth stimulation; at 50% of total protein the effect of fish solubles was somewhat less marked because the casein alone gave reasonably good growth. At 32% of total protein (casein plus fish solubles) 40% of fish solubles gave no greater response than did 20% of fish solubles; an excess of the fish solubles factor was apparently supplied at the 20% level. The maximal growth rate achieved with casein or casein-gelatin diets supplemented with fish solubles appeared to be 85 to 90% of the maximal rate achieved with soy protein plus fish solubles.

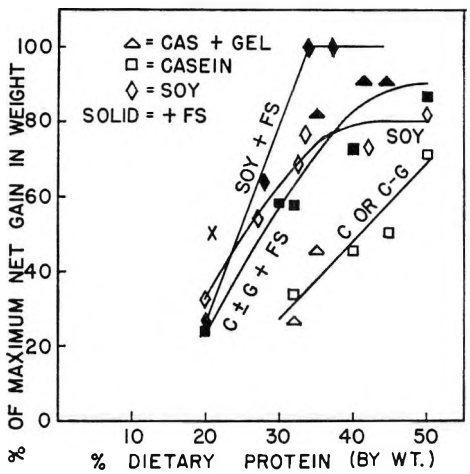


Fig. 1 The growth rate of turkeys in relation to the total protein content of the diet when the latter was supplied as casein (C), casein plus gelatin (CG), or ADM C-1 Assay Protein (soy) in the presence and absence of 20% fish solubles (FS). The growth rate was calculated as the net gain in body weight in 4 weeks. A maximal weight gain of approximately 400 gm (450 — 50) was achieved in these experiments with soy protein diets supplemented with fish solubles (17.5 gm/day during the last three weeks or an average of 14 gm/day throughout), and this value was arbitrarily rated 100% for comparison with the growth responses with other diets. The protein content of the diet was based on the weight of protein added; if based on nitrogen content (× 6.25), the values would be 80 to 85% of those listed.

*Chick growth.* Chicks responded to the various diets in a similar manner as the turkeys. A large number of growth rates plotted as a function of the total protein content of the diet are shown in figure 2. As with the turkeys, purified soy protein diets containing added methionine and glycine supported growth better than casein or casein-gelatin diets. Twenty per cent of soy protein was inadequate, but 30% allowed about 80% of maximal growth and 35 to 50% soy protein diets

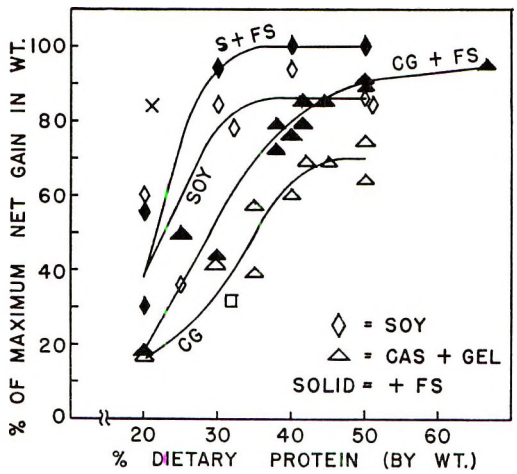


Fig. 2 The growth rate of chicks in relation to the total protein content of the diet. Other explanations are the same as in figure 1, except that 100% of the maximal weight gain in chicks was 320 gm in 4 weeks (357 — 37) (about 14 gm/day during the last two weeks); the square represents a casein diet and X represents a commercial chick starter.

gave a constant 85% of the maximal growth rate. The addition of 20% of fish solubles to the soy protein diets gave no growth response when the total dietary protein was 20%, but increased the growth rate to 100% of maximum when the total protein exceeded 35%. Clearly, the fish solubles supplied some factor other than protein which was missing from the purified soy protein diets and which was responsible for the last growth increment of about 15%, or the fish solubles "balanced" the diet in some unknown manner. A total protein content of about 35% was adequate for maximal growth when supplied as soy protein supplemented with methionine and glycine. Diets not supportng maximal growth during the first few weeks of life sometimes gave growth curves which were parallel to the maximal rate during the third or fourth week; in such cases the major effect of the better diet occurred in the first two or three weeks (Sunde and Bird, '59).

The casein-gelatin diets gave increasing growth rates with increasing amounts of protein in the diet up to a level of about 45% protein. The maximal growth rate achieved with casein-gelatin diets was only about 70% of that attainable, and was therefore significantly less than that achieved with purified soy protein diets. The addition of fish solubles to the casein-gelatin diets had no growth effect when the total protein was 20%. However, a growth stimulation was evident at higher protein levels, and the response curve with fish solubles was approximately parallel to the response curve of the unsupplemented diets. At 50% of total protein the casein-gelatin plus fish solubles diet gave 90% of the maximal growth rate attainable, and this was increased to 95% by the further addition of casein to a total protein of 66.5%.

Commercial chick starter allowed good growth (85%) of chicks on the basis of its protein content, but this is the only value in figure 2 for which the protein content of the diet was based on nitrogen analysis; the growth rate for the chick starter diet would be very close to the soy plus fish solubles curve if the protein content of the latter diets had also been calculated from their nitrogen contents rather than from the weights of purified protein added to the diets.

The addition of 20% of whey to the 25% casein-10% gelatin diet gave somewhat less growth stimulation than did the 20% of fish solubles. The addition of whey and fish solubles together gave no greater effect than the fish solubles alone. Twenty per cent of fish solubles supplied an excess of this factor since the addition of either 10 or 20% of fish solubles to a 25% casein-10% gelatin diet gave the same growth stimulation. Similarly, the presence of 10, 20 or 30% of fish solubles in casein-gelatin diets containing 50% of total protein all gave the same growth response. The ash from 20% of fish solubles added to a 40% casein-10% gelatin diet produced a slight stimulation of growth, and the same effect was obtained whether the basal diet contained added Mo or not. The omission of Mo from the this basal diet had no effect on the growth rate. The use of 10% of Amigen[9] as a form of hydrolyzed casein in a 35% casein-10% gelatin diet gave the same growth rate as the untreated casein.

[9] Amigen, Mead Johnson Company, Evansville, Indiana.

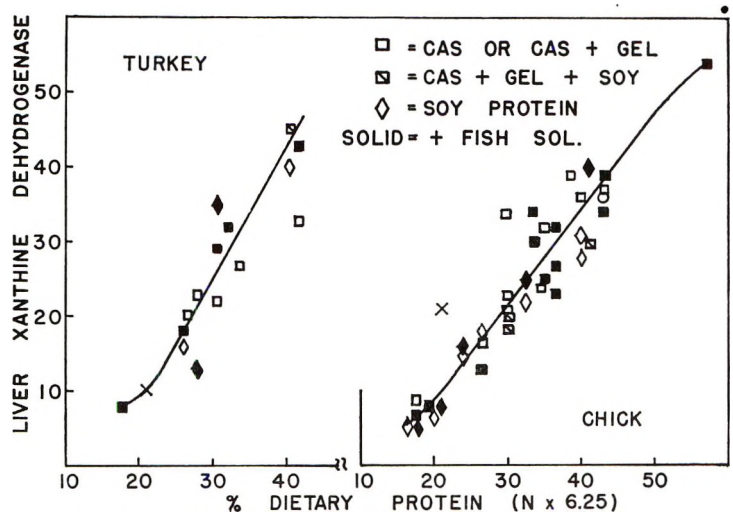


Fig. 3 The relationship between turkey or chicken liver xanthine dehydrogenase activity and the total protein content of the diet (based on N × 6.25). Liver xanthine dehydrogenase values are given as net $O_2$ consumption in cubic millimeters per 20 minutes per 1.7 ml of a 1:60 liver homogenate as determined manometrically in the presence of methylene blue. The circle represents a casein gelatin diet without added Mo, and the X represents a commerical chick starter. A total of 257 turkey and 447 chicken livers were analyzed to obtain the above curves. Individual groups have been plotted to illustrate the variability of the data in different experiments.

*Liver xanthine dehydrogenase (XD).* When liver XD was used as an index of the protein nutriture of turkeys or chicks, the curves in figure 3 were obtained. These curves represent a plot of liver XD activity as related to the total protein of the diet, irrespective of the kind of protein making up the total. In order to have all diets comparable, the protein content was calculated from the nitrogen analysis rather than from the weight of casein, gelatin or soy protein added.

In both turkeys and chicks the liver xanthine dehydrogenase activity increased in a relatively straight-line relationship with the total dietary protein; at 50% of dietary protein the liver XD was 5 to 6 times the value observed at 20%. The good growth obtained with soy protein diets as compared with casein diets was not accompanied by corresponding differences in liver XD; both proteins were equally effective in promoting the synthesis of this liver enzyme. Similarly the presence or absence of fish solubles in the diet at any protein level had no effect on liver XD.

When Mo was omitted from the 43% total protein diet (casein plus gelatin) fed to chicks, normal levels of liver XD were still obtained; all other diets contained added Mo. While the liver XD is not particularly sensitive to a border line Mo deficiency, these results indicate that the growth effect of fish solubles cannot be attributed to the presence of Mo, since there was more than enough Mo in all diets to allow for maximal synthesis of liver XD at all protein levels.

An increasing level of liver xanthine oxidase or dehydrogenase with increasing levels of dietary protein is not unique for birds. Campbell and Kosterlitz ('48) demonstrated that the protein content of rat liver increases continuously with increasing amounts of protein in the diet, and the amount of xanthine oxidase in rat liver parallels the total liver protein (Meikleham, et al., '51). Complete curves comparable to those in figure 3 have not been obtained for the rat, but the available data (Richert and Westerfeld, '53; Litwack et al., '52) show a similar trend for both rat liver and intestine. In the rat a saturation of the liver with xanthine oxidase is not correlated with growth rate; maximal growth rates can be achieved with diets containing less than 20% of pro-

tein, but such diets give relatively low levels of liver xanthine oxidase. Similarly in chicks and poults a maximal growth rate can be achieved with 35% of total protein in the diet even though such diets do not saturate the liver with xanthine dehydrogenase.

The principal contribution of figure 3 to a study of the growth of turkeys and chicks is the demonstration that (1) casein or casein-gelatin is utilized by these birds for the formation of liver XD just as effectively as soy protein; casein does not fail to support growth when present in high concentration because it is not digested or utilized, nor is it an inadequate protein for birds insofar as the synthesis of this liver enzyme is concerned; and (2) fish solubles does not seem to stimulate growth by supplying an unusually effective protein because no protein effect from fish solubles is evident on liver XD. It is possible that the proteins from fish solubles or soy could be unusually effective for growth without having any effect on liver XD, and it should be noted that there is no break in the liver XD curve which would indicate a protein deficiency for growth. However, an essential amino acid deficiency decreases chick liver XD inversely proportional to the amount of the limiting amino acid available (Litwack and Fisher, '57), and rat liver xanthine oxidase also reflects the quality of the dietary protein (Litwack et al., '54); on this basis the proteins from fish solubles or soy were no better than casein-gelatin.

The presence of inert fiber in a diet produced higher levels of liver XD than would have been predicted from the protein content of the diet, presumably because the birds ate more of the low-calorie diet and therefore had a higher daily protein intake. Turkeys fed a 20.5% casein plus 8% gelatin plus 20% fish solubles diet for 4 weeks had an average liver XD of 29; when 35% of cellulose was substituted for starch so that the total protein of the diet remained at 35%, the liver XD averaged 39. This relatively large amount of cellulose also decreased the growth rate so that at 4 weeks the control group averaged 375 gm, whereas the cellulose-fed poults weighed 300 gm. Similarly when 37% of cellulose was substituted for starch in a diet containing 26% soy protein plus 20% fish solubles (plus 0.75% methionine plus 0.4% glycine) the liver XD increased from 13 to 35 and the body weight at 4 weeks decreased from 450 to 245 gm. The addition of cellulose gave a higher protein: calorie ratio to the diet, and this was reflected by higher levels of liver XD.

## DISCUSSION

On the basis of growth rates and general appearance the casein or casein-gelatin diets were grossly deficient in at least two unidentified factors required by both turkey poults and chicks. One of these was present in soy protein and appeared to be responsible for the better growth and appearance achieved with soy diets. A different factor was present in fish solubles since purified soy protein diets alone did not support maximal growth rate at any protein level, and the plateau in the response curve between 35 and 50% of soy protein would not have been observed if the soy protein effect was due to the presence of small amounts of the fish solubles factor. For the same reasons the fish solubles factor was not a more effective protein; in addition, the fish solubles had no protein effect on liver xanthine dehydrogenase, and it had no effect on growth when the total protein in the diet was limiting at the 20% level.

The casein appeared to contain a small but inadequate amount of some growth factor, presumably the soy protein factor. Increasing amounts of casein or casein-gelatin in the diet between 35 and 50% gave an increasing growth rate, presumably because the additional protein supplied more of this factor; the latter was still inadequate since the maximal growth rate achieved with 50% casein-gelatin diets was less than the maximal rate achieved with 35 to 50% soy protein diets. The parallel but higher response curve obtained when fish solubles was added to the casein-gelatin diets reflected the limiting amount of "soy factor" in these diets; that is, the soy factor in the casein was being assayed by this response curve. Since less than maximal but increasing growth rates were obtained with increasing amounts of casein in the diet, the casein was supplying this additional factor in

small amounts. This type of response also indicated that the fish solubles was relatively free of the soy factor.

The soy protein factor could theoretically be protein itself; that is, the amino acid composition of soy protein could support the growth of birds better than the amino acid combination found in casein or casein plus gelatin. Although such a relationship was not reflected by corresponding differences in liver xanthine dehydrogenase, the latter criterion is not necessarily correlated with growth. The major reason for believing that the soy protein factor is not protein is that the amino acid composition of the 35% soy diet is very similar to the amino acid composition of the 25% casein-10% gelatin diet. Moreover, it is difficult to see how a protein that was inadequate because of an amino acid imbalance would support better growth when the total concentration of that same protein was increased.

Theoretically a 28% soy protein diet (22% on the basis of N content) supplemented with zero to 0.1% of glycine and 0.4 to 0.6% of methionine should meet all the minimal requirements for essential amino acids for chicks as listed by Bird et al. ('54) or by Klain et al. ('58, '60). Why a minimum of approximately 35% of soy protein was required to achieve maximal growth in these studies is not entirely clear. Supplementing the various diets with 20% of fish solubles had relatively little effect on the essential amino acid composition of the diet (Williams, '55). Diets containing less than 35% of casein alone are apt to be limiting in arginine and glycine (Greene et al., '60), but gelatin is rich in these amino acids, and the 25% casein-10% gelatin diet theoretically supplied more than enough of all the essential amino acids. Much less arginine appears to be required with a corn-soy diet than with a casein or casein-gelatin diet (Krautmann et al.,'57; Fluckiger and Anderson, '59) even though the arginine is absorbed completely from both types of diet (O'Dell et al., '58); this difference has been attributed to the presence of a factor in corn and soy meals which spared arginine (Krautman et al., '58), or to the amino acid composition of the casein (Klain et al., '59; Anderson and Dobson, '59, Fisher et al., '60). Hogan et al. ('57), Edwards et al. ('58) and Savage and O'Dell ('60) reported small growth responses from the addition of arginine or creatine to a 25% casein-10% gelatin diet, but we have not obtained any significant growth responses when 22 to 30% casein plus 10% gelatin diets were supplemented with 0.5 to 1% of arginine.

## CONCLUSIONS

1. Approximately 35% of total dietary protein (29% on the basis of N content) supported the maximal growth rate of both chicks and turkey poults when supplied as soy protein supplemented with methionine and glycine, and when the diet also contained fish solubles. In the absence of fish solubles, 35 to 50% soy protein diets allowed 80 to 85% of maximal growth.

2. Increasing concentrations of casein or casein-gelatin in purified diets as the sole source of protein gave increasing growth rates; at 50% of total protein the growth rate was 70 to 75% of maximum. Added fish solubles gave a growth stimulation at all levels of dietary protein except 20%, and gave 90 to 95% of the maximal growth rate when the total protein in the diet was 50 to 66%.

3. In both turkeys and chicks the liver xanthine dehydrogenase activity increased in a relatively straight-line relationship with the total dietary protein, and was 5 to 6 times higher at 50% of dietary protein than at 20%. Casein or casein-gelatin diets were just as good sources of protein as soy by this criterion. The presence or absence of fish solubles in the diet at any protein level had no effect on liver xanthine dehydrogenase.

4. These results were interpreted as indicating a deficiency of two unidentified factors in the casein-gelatin diets: (a) one carried by soy protein, and (b) another present in fish solubles.

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# Effect of Antibiotics, Sulfonamides, and a Nitrofuran on Development of Hepatic Cirrhosis in Choline-Deficient Rats[1,2]

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Continuous studies of choline deficiency and its effects on experimental animals have been in progress in this laboratory for many years. Hepatic cirrhosis has developed consistently in rats restricted to choline-deficient diets for 200 to 300 days. For more than 10 years a significant incidence of hepatocarcinoma was observed as a terminal lesion in AES[3]-strain rats subjected to prolonged periods of choline deficiency (Engel and Salmon, '41; Copeland and Salmon, '46; Schaefer et al., '50; Salmon and Copeland, '54; Salmon et al., '55). Beginning in 1955, however, it was noted that the incidence of hepatocarcinoma in this strain of rats was not as high as had previously been observed. Various steps of our procedures were studied in efforts to ascertain the cause of the decreased incidence, but no explanation was provided by the results of these studies. Coincidental with this period, however, certain antimicrobial additives had been included in the diet of our breeding colony of AES rats at various times in attempts to control chronic respiratory infections. These additives included oxytetracycline, sulfanilamide, sulfamerazine, and furazolidone. The remote possibility that the decreased incidence of hepatic lesions in our choline-deficient rats might be attributed to these materials in the stock diet was suggested by reports from two laboratories. György ('54) had reported that certain antibiotics afforded considerable protection against hepatic cirrhosis and hepatic necrosis. Rutenberg et al. ('57) reported that orally administered antibiotics markedly delayed the development of hepatic cirrhosis in rats fed choline-deficient diets.

An experimental examination of the effects of adding penicillin, oxytetracycline, neomycin sulfate, sulfanilamide, sulfaguanidine, and furazolidone to our standard choline-deficient diet for rats was therefore undertaken. Results of these experiments are presented in this paper.

## EXPERIMENTAL PROCEDURES AND RESULTS

Rats were housed individually in 0.5-inch mesh screen-bottom cages in air-conditioned rooms. Tap water was supplied ad libitum and the experimental diets were fed 6 days per week with a double portion fed on Saturday.

The same basal diet was used in all experiments. The percentage composition follows: extracted casein, 6; extracted peanut meal, 25; cod liver oil, 1; salts, 5; vitamin premix, 2; sucrose, 42; and lard, 19. The salt mixture supplied the following amounts of salts as per cent of diet: $CaHPO_4 \cdot 2H_2O$, 3.15; KCl, 0.6; $MgSO_4$, 0.4; $NaHCO_3$, 0.7; Fe citrate, 0.123; $MnSO_4 \cdot H_2O$, 0.010; $ZnCO_3$, 0.010; $CuSO_4$, 0.004; KI, 0.003. Vitamins contained in the premix with sucrose furnished the following amounts in milligrams per kilogram of diet: riboflavin, 2; thiamine·HCl, 4; pyridoxine·HCl, 1; Ca pantothenate, 10; niacin, 25; menadione, 5; folacin, 2; inositol, 200. Crystalline antibiotics and sulfonamides, when used, were incorporated into the diet at the expense of sucrose. Furazolidone was added to the total diet without adjustment of the sucrose. The casein

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[2] Supported in part by National Institutes of Health Grant C-1018. Donations of vitamins were made by Merck Sharp and Dohme Research Laboratories, and neomycin sulfate by The Upjohn Company.

[3] AES indicates Alabama Experiment Station strain of rats.

and peanut meal were extracted for 24 hours with 82% methanol and for 24 additional hours with 100% methanol in a steam-jacketed, continuous-type extractor. The basal diet with minor variations has been the standard diet for studies of choline deficiency in this laboratory since 1944.

*Effect of additives in long-term experiment.* A long-term test was conducted with AES-strain rats. They were 5 weeks of age and averaged 82 gm at the start of the experiment. The basal diet was supplemented with 0.10% of choline chloride for all groups during the first week of the experiment; the choline supplement was then omitted and the test additives were included in 8 treatment groups as shown in table 1. An additional group received the choline-deficient diet without any antimicrobial additive. Each treatment group consisted of 5 rats except the groups receiving 0.15% of penicillin and 0.15% of neomycin sulfate, which had only 4 rats each as a result of early death of one rat in each of these groups.

Of the additives tested, penicillin had the greatest effect on weight gains and average survival time (see table 1). Its effects were substantial at the 0.01% level, and very marked at the 0.15% level. The 0.15% penicillin treatment group was the only group in which all rats survived for the entire 630-day test period; these rats exhibited the physical appearance of normal rats at the end of the test. Oxytetracycline and neomycin sulfate, at a 0.15% level, the only level of these antibiotics tested, also increased weight gains and survival time. Sulfanilamide at a level of 0.50% and furazolidone at the low level of 0.0055% were almost equal to penicillin in their effects on average survival time. At the higher level of 0.022%, furazolidone was less beneficial. Average survival time of the rats receiving 0.50% of sulfaguanidine was substantially shorter than that of those receiving the same level of sulfanilamide. Neither of the sulfonamides nor furazolidone was very effective in improving average weight gains.

*Incidence and severity of hepatic cirrhosis.* The influence of the antimicrobial additives on hepatic cirrhosis was striking. A diagnosis of cirrhosis was recorded for any abnormal amount of fibrous connective tissue in the liver. Severity of the cirrhosis, if present, was coded over a 1+ to 4+ range and the average code for each group is shown in the last column of table 1. Typical histopathologic observations are illustrated by photomicrographs in figures 1 to 8. No rat receiving 0.15% of penicillin (fig. 1), 0.15% of neomycin sulfate (fig. 2), 0.50% of sulfanilamide, or 0.022% of furazolidone exhibited cirrhosis. Only one of 5 rats receiving 0.01% of penicillin, 0.50% of sulfaguanidine, or 0.0055% of furazolidone had a 1+ cirrhosis, and three of 5 rats given 0.15% of oxytetracycline (figs. 3 and 7) had a 1+ cirrhosis. In contrast, every rat fed the choline-deficient diet without additive had cirrhosis with an average severity code of 3.2+ (fig. 4). Most of the livers, irrespective of treatment, however, appeared en-

TABLE 1

*Effects of additives in long-term choline deficiency experiment*

| Additive | | Av. weight[1] gain | Av. days survival | Fatty liver | Hepatic cirrhosis |
|---|---|---|---|---|---|
| | *% of diet* | *gm* | | | |
| None | | 313 | 483 | 5/5[2] | 5/5(3.2+)[3] |
| Penicillin | 0.01 | 488 | 609 | 5/5 | 1/5(0.2+) |
| Penicillin | 0.15 | 546 | 630+ | 4/4 | 0/4(0) |
| Oxytetracycline | 0.15 | 405 | 567+ | 4/5 | 3/5(0.6+) |
| Neomycin sulfate | 0.15 | 411 | 567+ | 3/4 | 0/4(0) |
| Sulfanilamide | 0.50 | 364 | 602+ | 5/5 | 0/5(0) |
| Sulfaguanidine | 0.50 | 362 | 504+ | 5/5 | 1/5(0.2+) |
| Furazolidone | 0.0055 | 361 | 593+ | 5/5 | 1/5(0.2+) |
| Furazolidone | 0.022 | 317 | 520+ | 5/5 | 0/5(0) |

[1] To maximum weight attained.
[2] Number showing lesions/number in group.
[3] Cirrhosis, if present, was coded over a 1+ to 4+ range with increasing severity. Figures in parentheses represent average severity code for the group. The + signs in average days of survival column indicate that some rats were surviving at end of 630-day test period.

larged and fatty at autopsy and showed abnormal accumulations of fat when frozen sections were stained with oil red O and examined under the microscope. Such examination revealed considerable variation in the amounts and distribution of fat. There were intense accumulations of globules of fat within hyperplastic liver cells and in residual liver cells interspersed among fibrotic bands of connective tissue in the livers of rats fed the choline-deficient basal diet (fig. 5). The lightest accumulation of fat was in the livers of rats receiving penicillin, but some of these exhibited the centrolobular lipid accumulation that is typical of early choline deficiency (fig. 6); others showed a small amount of fat droplets diffusely distributed throughout the lobules. Throughout the livers of the rats receiving sulfanilamide or sulfaguanidine the cells were burdened with fat (fig. 8); despite this intense infiltration of fat, there was no cirrhosis in these livers.

*Short-term experiments.* A short-term experiment was conducted to determine whether penicillin or sulfanilamide would affect rate of mortality or survival time of rats subjected to acute choline deficiency. Charles River CD-strain rats were used in this experiment. The choline requirement of this strain is comparable to that of the AES strain. The rats were about 4 weeks of age and weighed 55 to 65 gm at the start of the experiment. No initial choline supplement was added to the diet in this experiment. The results are shown in table 2. Penicillin or sulfanilamide did not alter the mortality rate. These additives also failed to affect the survival time of the rats succumbing to acute choline deficiency.

A second short-term experiment was then conducted to determine the effect of penicillin or sulfanilamide on liver lipid levels in weanling rats subjected to acute choline deficiency. Charles River CD rats were also used in this experiment. These rats were 4 weeks of age and averaged 62 gm in weight at the start of the experiment. There were 4 treatment groups of 5 rats each. Three of these groups received 0.30% of choline chloride in their respective diets for the first 10 days, after which the choline chloride was omitted. This left one group receiving the choline-deficient basal diet, one group with this diet supplemented with 0.15% of penicillin G sodium, and one with the deficient diet supplemented with 0.50% of sulfanilamide; the additives were included in the latter two diets from the beginning of the experiment. The fourth group received 0.60% of choline chloride in the diet for the entire test. Average weights of each of the 4 groups ranged from 122 to 130 gm per rat on the tenth day of the test when the choline supplement was omitted from the diets of three treatment groups. All 4 groups were killed 8 days later. The average final body weights, liver dry weights, and liver total lipids are shown in table 3. Neither penicillin nor sulfanilamide prevented accumulation of abnormal levels of liver lipids or decreased

TABLE 2

*Effects of additives on mortality in acute choline deficiency experiment*

| Additive | | Mortality[1] | Days to death[2] |
|---|---|---|---|
| | *% of diet* | | |
| None | — | 12/20 | 8–11 |
| Penicillin | 0.10 | 6/10 | 7–12 |
| Sulfanilamide | 0.10 | 7/10 | 8–10 |
| Sulfanilamide | 0.50 | 7/10 | 7–11 |

[1] Rats dying/rats in group.
[2] Of rats succumbing within critical period.

TABLE 3

*Effects of additives on liver lipid level in acute choline deficiency*

| Additive | | Av. final body weight | Av. liver dry weight | Liver lipid average and range | Hemorrhagic kidneys |
|---|---|---|---|---|---|
| | *% of diet* | *gm* | *gm* | *% dry-weight basis* | |
| None | — | 153 | 3.75 | 46.9(44.9–52.0) | 4/5[1] |
| Penicillin | 0.15 | 138 | 3.58 | 50.4(44.5–56.6) | 5/5 |
| Sulfanilamide | 0.50 | 135 | 3.01 | 37.4(30.5–46.0) | 5/5 |
| Choline·Cl | 0.60 | 193 | 2.55 | 16.9(13.8–19.8) | 0/5 |

[1] Number of rats having hemorrhagic kidneys/number of rats in treatment group.

the incidence of hemorrhagic kidneys in rats subjected to acute choline deficiency.

## DISCUSSION

Results of the experiments presented here confirm and extend the observations of György ('54) and of Rutenberg et al. ('57). They indicate that hepatic cirrhosis in the choline-deficient rat is not a simple nutritional deficiency effect. The protection afforded by such diverse antimicrobial additives in choline-deficient diets favors the hypothesis that enteric microbial agents or their noxious products are essential to the production of the syndrome. The liver of the normal rat apparently is endowed with protective mechanisms that neutralize the effects of such noxious agents. These mechanisms are probably ineffective in the fat-laden liver of the choline-deficient rat.

Neither penicillin nor sulfanilamide substantially affected mortality rates or liver lipid levels of rats on short-term choline-deficiency experiments. In the long-term experiment, penicillin decreased liver lipids somewhat, but the sulfonamides appeared to increase them. Thus, it seems that the effects of the antimicrobials is not a choline-sparing action but a direct protective action against a harmful agent.

It is significant that all of the long-term control rats fed the choline-deficient diet without an antimicrobial additive exhibited hepatic cirrhosis. If an enteric agent(s) is involved in the pathogenesis of cirrhosis, it either was not eliminated from the AES strain of rats by the use of various antimicrobials in the stock colony diet, or reinfection occurred during the progress of the experiment. Despite the duration of the long-term experiment, not a single hepatocarcinoma was noted in any of the 43 experimental animals. However, in another experiment conducted during the same period, one of 10 rats fed the same choline-deficient control diet for 301 to 500 days developed a hepatocellular carcinoma. These results further demonstrate the marked change in response of the AES strain of rats to prolonged choline deficiency. Much more work will be required to establish the cause of such a change.

## SUMMARY

Penicillin G sodium, neomycin sulfate, oxytetracycline, sulfanilamide, sulfaguanidine, and furazolidone prevented or decreased the severity of hepatic cirrhosis in choline-deficient AES-strain rats over a 630-day test period. Penicillin or sulfanilamide did not prevent abnormal accumulation of lipids in livers of weanling rats subjected to acute choline deficiency; furthermore, they did not decrease incidence of hemorrhagic kidneys or mortality rates in such acutely deficient rats.

The protective action of these diverse antimicrobial diet additives indicates that noxious enteric agents are involved in the pathogenesis of hepatic cirrhosis in the choline-deficient rat. Inasmuch as none of the choline-deficient control rats developed neoplasms, no inference can be drawn regarding any possible effects of the diet additives on incidence of neoplasms in choline-deficient rats.

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PLATES

## PLATE 1

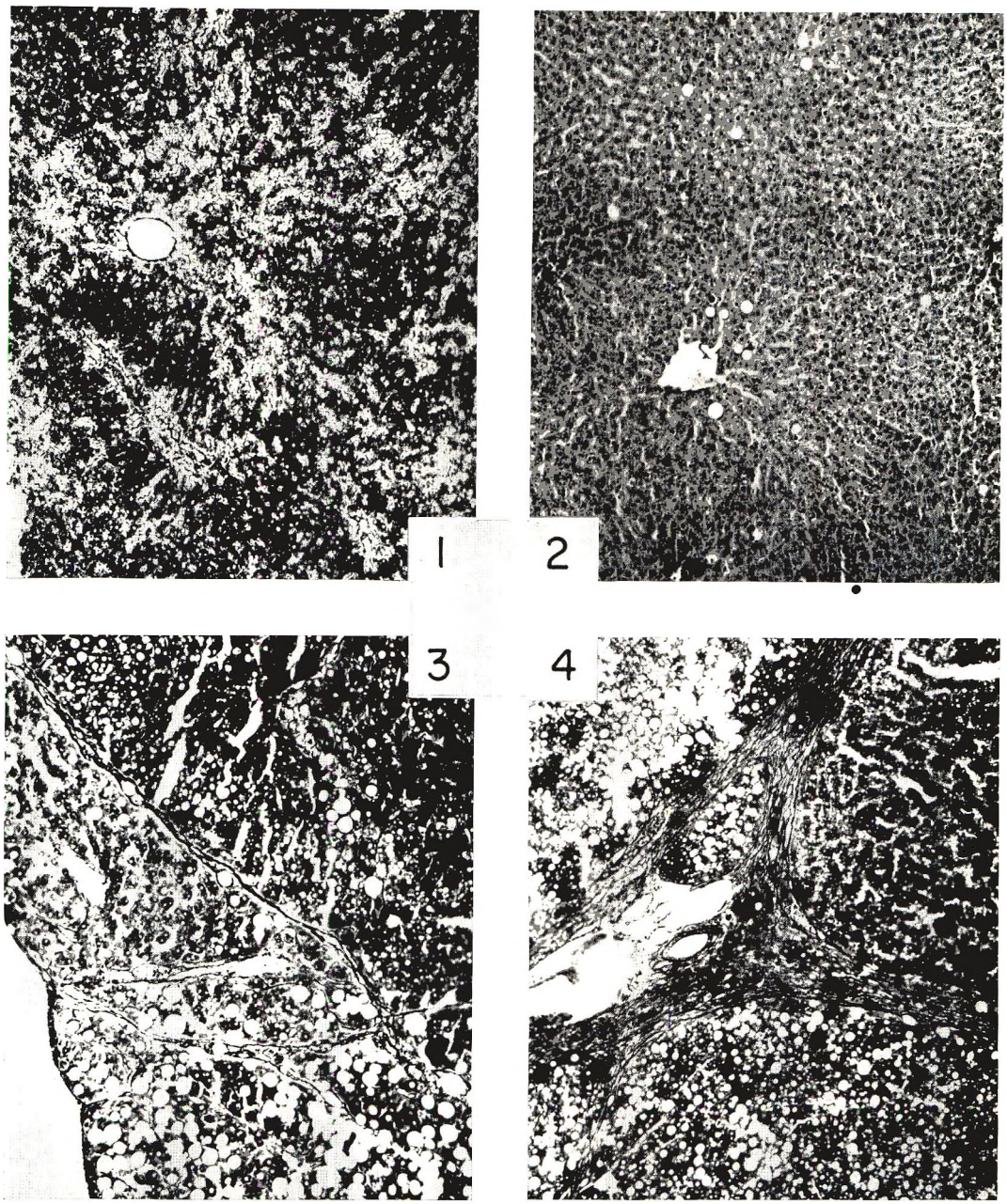
### EXPLANATION OF FIGURES

1 Liver from rat receiving choline-deficient diet with 0.15% of penicillin. There is no evidence of cirrhosis or any increase in reticular or fibrous tissue. Foot's modification of Bielschowsky's reticulum stain. × 100.

2 Liver from rat fed choline-deficient diet with 0.15% of neomycin sulfate. Results are comparable to those from a similar level of penicillin. A few large-droplet accumulations of lipid can be observed about a central vein. This was graded 1+ fat after confirmation with frozen sections and fat stains. Cirrhosis is absent. H. and E. × 100.

3 Liver from rat receiving choline-deficient diet with 0.15% of oxytetracycline. Note strands of reticulum and fibrous tissue coursing through the section. Foot's modification of Bielschowsky's reticulum stain. × 100.

4 Liver of rat fed choline-deficient diet without additive. Cirrhotic bands are shown separating hyperplastic nodules of hepatic cells that contain large quantities of fat. The nodule in upper right of photograph represents more recent hyperplasia. As nodules aged they increased in lipid content. Foot's modification of Bielschowsky's reticulum stain. × 100.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## PLATE 2

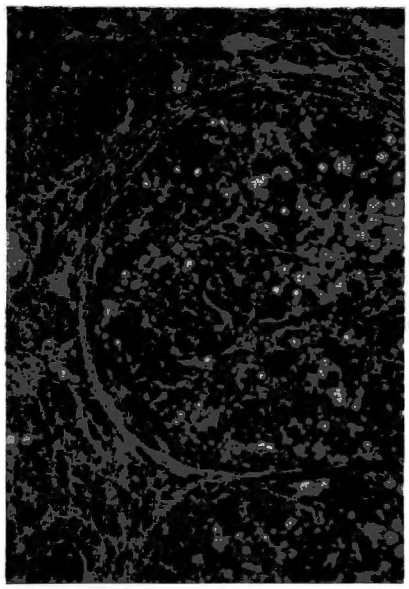
### EXPLANATION OF FIGURES

5 Liver of rat fed choline-deficient basal diet without additive, showing severe cirrhosis and lipid accumulation. Lipid has accumulated in zones of nodular hyperplasia and some lipid is present in residual liver cells between the fibrotic bands of connective tissue. Frozen section, oil red O stain, Harris' hematoxylin counterstain. × 40.

6 Liver of rat fed choline-deficient diet that contained 0.01% of penicillin. This section represents a typical 2+ centrolobular lipid accumulation of a choline-deficient liver. Cirrhosis is absent. Oil red O stain, Harris' hematoxylin counterstain. × 80.

7 Liver from rat consuming choline-deficient diet with 0.15% of oxytetracycline. There is scattered accumulation of lipid that was graded 1+. Stromal condensation and fibrous proliferation was graded 1+. Note atypical, hypertrophic liver cells and lack of cord formation. Oil red O stain, Harris' hematoxylin counterstain. × 80.

8 Liver from rat receiving choline-deficient diet with 0.5% of sulfanilamide. There is a large accumulation of fat graded 4+ but no cirrhosis. Oil red O stain, Harris' hematoxylin counterstain. × 80.



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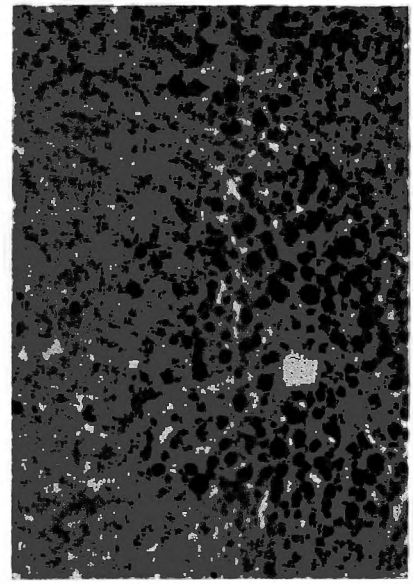

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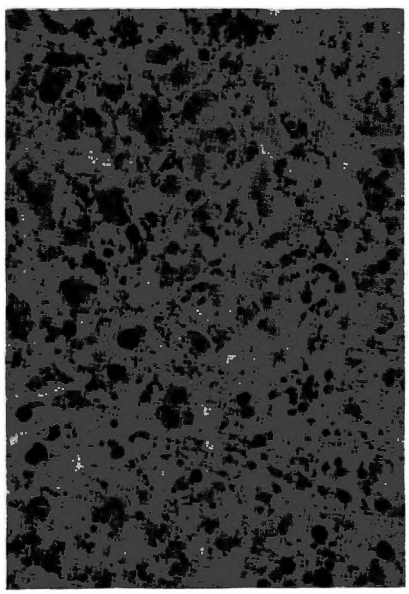

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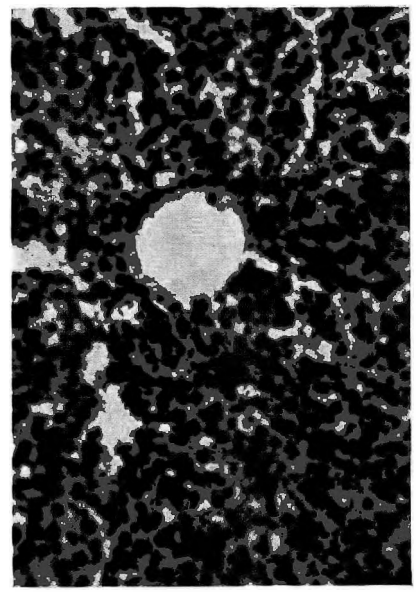

8

# Catabolism of L-Ascorbic Acid in Guinea Pigs[1]

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It appears certain that biological activity of vitamin C is dependent upon the integrity of the L-ascorbate molecule.[2] In tissues, ascorbic acid is considerably more stable than the product of its reversible oxidation, dehydroascorbic acid, and much more stable than diketogulonic acid (Curtin and King, '55), which results from hydrolysis of the lactone ring of dehydroascorbic acid. Although diketogulonic acid may be reduced to 2-keto- L-gulonate (Ashwell et al., '61), it has no appreciable antiscorbutic properties. This indicates that catabolism takes precedence over possible reconversion to ascorbic acid. Nothing has been reported regarding the stability *in vivo* of monodehydroascorbic acid, but the free-radical nature of the compound suggests that it should have limited life expectancy, that which escapes reduction to ascorbic acid soon being further oxidized to dehydroascorbic acid.

Aside from ascorbic acid and products of its oxidation with the intact 6-carbon chain, the bulk of the radioactivity derived from ascorbic-1-$C^{14}$ acid is excreted in oxalic acid and $CO_2$. Details of the mechanism by which these substances arise have recently been described (Ashwell et al., '61). Diketogulonic acid may apparently undergo two types of reactions. It is either decarboxylated, yielding $CO_2$ and pentonic acids, or it is cleaved between carbons 2 and 3, giving oxalic acid and threonic acid. Since neither $CO_2$ nor oxalic acid are long retained (Curtin and King, '55), it follows that destruction of ascorbic-1-$C^{14}$ acid must be reflected by excretion of radioactivity in these compounds without great delay.

It is consistent with these considerations that no appreciable radioactivity occurs in tissues other than that associated with ascorbic acid itself when ascorbic-1-$C^{14}$ acid is administered (Burns et al., '51).[3] The relationship between the rapid catabolism and rapid and nearly complete excretion of labeled substances from ascorbic-1-$C^{14}$ acid permits the use of relatively simple and straight-forward techniques in investigations of the catabolism of ascorbate *in vivo*.

The present work constitutes an extension of earlier observations (Salomon, '57; Salomon and Stubbs, '61). It was undertaken to gather evidence regarding the effect of changes in size of the body pool and of physiological stress upon the catabolism of ascorbic acid, to investigate the kinetics of the phase of catabolism immediately following the injection of labeled ascorbic acid, and to discover whether freshly introduced ascorbic acid led to sparing of that previously incorporated into the tissues. These aspects are kinetically interrelated.

## METHODS

*General.* Urinary and respiratory excretion of radioactivity derived from ascorbic-1-$C^{14}$ acid was determined under various conditions in guinea pigs. Ascorbic-1-$C^{14}$ acid was synthesized as described[4] and collection of radioactive specimens and assay of radioactivity followed established procedures, summarized elsewhere (Salomon, '57). Counts here reported are corrected for background and self-absorption (Calvin et al., '49), but are not absolute.[5]

*Effect of body pool on catabolism.* This series of experiments was performed ex-

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[1] This investigation was supported in part by U.S.P.H.S. Research Grant C-3994.
[2] This paper deals exclusively with the catabolism of L-ascorbic acid, the naturally occurring analog. The designation identifying the stereoisomer will be omitted in the subsequent discussions.
[3] Salomon, L. L. 1952 Studies on the metabolism under stress and the biosynthesis of ascorbic acid. Dissertation. Columbia University, New York.
[4] See footnote 3.
[5] The applicable statistical considerations have been discussed by Calvin et al. ('49). Self-absorption corrections by extrapolation to "infinite thinness" were determined substantially as described by these authors.

actly as described in a similar previous study (Salomon, '57), since a more recent report (von Schuching et al., '60) made confirmation of the results desirable. The animals were maintained with a scorbutigenic diet, aged commercial rabbit chow,[6] supplemented by 30 mg of ascorbic acid per kg of body weight per day. To induce scurvy, oral supplements were withheld for 21 days, at which time the weight was diminished. The first metabolism experiment was then performed, involving injection of ascorbic-1-$C^{14}$ acid and collection of urinary and respiratory radioactivity during the periods indicated in figure 1. Provided that contamination of feces with urine is avoided, as in this study, fecal radioactivity is negligible under these conditions (Burns et al., '51).

Following this, realimentation with 30 mg of ascorbic acid per kg of body weight per day by mouth was carried out until satisfactory gain in weight was observed. A second metabolism experiment was then conducted. For the third, and final, metabolism experiment, one minimal lethal dose of diphtheria toxin was administered subcutaneously 12 hours prior to the injection of ascorbic-1-$C^{14}$ acid and the start of the collection of radioactivity. Precautions in the spacing of successive experiments on the same animal are discussed later.

*Effect of graded and massive doses.* Guinea pigs were maintained as described (see above and Salomon, '57). Severe scurvy was produced by prolonged withdrawal of dietary ascorbic acid (39 and 51 days, respectively). This resulted in much emaciation, anorexia, great sensitivity to the touch, and diarrhea. The animals in this state presented a generally unkempt appearance and were visibly ill. The sequence of experiments summarized in table 1 was designed to eliminate effects of aging or gain in weight. Neither here nor previously (Salomon, '57), however, were there indications of effects attributable to changes in age or weight.

Successive runs were spaced sufficiently apart to insure virtual absence of interference from residual radioactivity. Because of the fairly short biological half-life of ascorbic acid in guinea pigs, this presented no difficulties. Approximately 25% of a physiological dose is generally lost during the 24 hours following injection, and considerably more when massive doses are used. Thus, with a biological half-life of 4 days (Salomon, '57), less than 2.5% of the dose remains after 21 days. To eliminate the possibility of error from carry-over of radioactivity, guinea pigs were placed in the metabolism chamber for several hours in all but the initial run, and excreted radioactivity was determined, just prior to the start of each metabolism experiment. In this way, the contribution of residual radioactivity to later measurements could be calculated by extrapolation of the decay curve for the particular animal. The corrections were always small, and most frequently within the error of measurements ($\pm$ 2%)[7] and negligible.

*Effect of intrahepatic injection and of nephrectomy.* For intrahepatic injections, guinea pigs were anesthesized with ether, and ascorbic-1-$C^{14}$ acid in 0.2 ml of physiological saline solution injected through a small incision in a shaved area. The incision was then closed by Michel wound clips.

To test the effect of nephrectomy, animals were anesthesized with 4 mg of pentobarbital sodium[8] per 100 gm of body weight, the back was shaved, two incisions were made just below the ribs permitting removal of tissue adhering to the kidneys and placement of tight ligatures around the pedicles. Kidneys were not actually removed, but failure to produce urine indicated success of the procedure. Wounds were closed with wound clips.

To insure comparability of the data, periods of measurement corresponded during the preliminary runs and after nephrectomy. The terminal periods, during which the animals expired (without noticeable distress), were discarded.

## RESULTS AND DISCUSSION

*Effect of body pool and physiological stress.* Retention of radioactivity, calculated as the difference between that administered as ascorbic-1-$C^{14}$ acid, and the total (respiratory plus urinary) excreted,

[6] Purina Rabbit Chow, Ralston Purina Company, St. Louis.
[7] See footnote 5.
[8] Nembutal, Abbott Laboratories, North Chicago, Illinois.

**TABLE 1**

*Urinary and respiratory excretion of radioactivity following injection of graded doses of ascorbic-1-$C^{14}$ acid*[1]

| Exp.[2] | Weight | Dose | | | | Excretion during indicated hours after injection[3] | | | | | | | Retention after | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 0–3 | 3–6 | 6–12 | 12–24 | 24–36 | 0–6 | 0–6 | 6 hrs. | 36 hrs. |
| | *gm* | *mg/kg* | *count/min × $10^6$* | *mg/kg corrected*[4] | | % *uncorrected* | | | | | *mg/kg* | *% corrected* | *mg/kg* | *mg/kg* |
| 23-A | 450 | 4.2 | 4.42 | 4.0 | Urine | 0.02 | 3.67 | 0.30 | 2.09 | 0.77 | 0.15 | | 3.7 | 2.8 |
| | | | | | $CO_2$ | 4.82 | 3.20 | 4.85 | 7.74 | 6.12 | 0.34 | 8.32 | | |
| D | 680 | 17.7 | 2.18 | 16.8 | Urine | — | 4.86 | 1.89 | 2.16 | 1.29 | 0.86 | | 15.3 | 9.6 |
| | | | | | $CO_2$ | 3.25 | 5.32 | 8.67 | 10.37 | 7.94 | 1.51 | 9.01 | | |
| C | 660 | 30.5 | 2.63 | 28.6 | Urine | 6.14 | — | 2.59 | 2.30 | 1.18 | 1.87 | | 24.5 | 13.5 |
| | | | | | $CO_2$ | 4.95 | 8.58 | 10.80 | 13.50 | 8.52 | 4.12 | 14.42 | | |
| G | 731 | 44.6 | 1.81 | 40.5 | Urine | — | 9.25 | 6.29 | | | 4.13 | | 36.8 | — |
| | | | | | $CO_2$ | 2.52 | 5.77 | 8.77 | | | 3.70 | 9.13 | | |
| E | 690 | 46.6 | 2.25 | 41.5 | Urine | 8.47 | 2.38 | 1.38 | 1.96 | 1.30 | 5.06 | | 35.9 | 15.4 |
| | | | | | $CO_2$ | 3.43 | 8.73 | 12.90 | 16.02 | 9.30 | 5.67 | 13.12 | | |
| F | 700 | 60.2 | 1.65 | 47.1 | Urine | — | 21.80 | | | | 13.12 | | 42.4 | — |
| | | | | | $CO_2$ | 2.16 | 5.54 | | | | 4.64 | 9.85 | | |
| B | 590 | 68.2 | 2.37 | 52.3 | Urine | — | 23.30 | 1.90 | 1.73 | 1.72 | 15.88 | | 48.0 | 26.8 |
| | | | | | $CO_2$ | 2.02 | 4.38 | 7.87 | 11.15 | 6.63 | 4.37 | 8.36 | | |
| I | 885 | 84.6 | 3.84 | 51.4 | Urine | 36.53 | 2.68 | — | | | 33.20 | | 46.8 | — |
| | | | | | $CO_2$ | 1.67 | 3.77 | 7.30 | | | 4.60 | 8.95 | | |
| H | 740 | 93.5 | 3.22 | 72.0 | Urine | 21.85 | 2.58 | 1.96 | 1.36 | 0.77 | 23.30 | | 65.7 | 49.2 |
| | | | | | $CO_2$ | 1.93 | 2.82 | 3.41 | 6.58 | 5.16 | 4.53 | 6.29 | | |
| 24-A | 450 | 4.0 | 4.25 | 3.9 | Urine | — | 2.70 | 1.07 | 2.26 | 1.52 | 0.11 | | 3.6 | 2.9 |
| | | | | | $CO_2$ | 3.98 | 2.86 | 3.73 | 5.62 | 5.12 | 0.28 | 7.04 | | |
| D | 720 | 15.8 | 2.07 | 15.1 | Urine | 3.28 | 1.50 | 1.47 | 2.32 | 1.32 | 0.76 | | 13.5 | 9.2 |
| | | | | | $CO_2$ | 5.54 | 4.12 | 5.92 | 9.13 | 7.06 | 1.53 | 10.14 | | |
| C | 665 | 28.8 | 2.50 | 26.9 | Urine | 5.92 | 0.82 | 1.52 | 2.03 | 1.00 | 1.94 | | 23.9 | 15.5 |
| | | | | | $CO_2$ | 4.51 | 5.78 | 8.32 | 9.96 | 6.72 | 2.97 | 11.03 | | |
| E | 740 | 44.6 | 2.35 | 37.5 | Urine | 14.00 | 1.82 | 1.47 | 2.26 | 1.70 | 7.06 | | 33.1 | 18.1 |
| | | | | | $CO_2$ | 3.50 | 6.40 | 7.62 | 11.79 | 8.76 | 4.42 | 11.76 | | |
| B | 630 | 63.8 | 2.37 | 52.9 | Urine | 16.35 | 0.73 | 1.53 | 1.61 | 1.35 | 10.90 | | 47.8 | 32.1 |
| | | | | | $CO_2$ | 3.60 | 4.34 | 6.65 | 7.61 | 5.96 | 5.06 | 9.58 | | |
| F | 785 | 87.0 | 1.94 | 62.1 | Urine | 27.22 | 1.47 | 2.17 | | | 24.95 | | 57.0 | — |
| | | | | | $CO_2$ | 2.22 | 3.60 | 6.16 | | | 5.06 | 8.16 | | |

[1] All injections were intraperitoneal. Note that the rate of urinary excretion of radioactivity is maximal during the first three hours after injection, whereas the rate of excretion of respiratory radioactivity is maximal during the 3 to 6 hour period, except at the lowest dosage, and that maximal absolute retention of ascorbic acid is not yet attained even at the highest dose employed. For further discussion, see text.

[2] Letters indicate sequence of experiments.

[3] Uncorrected values represent percentage of uncorrected dose in mg /kg. Corrected values represent percentage of corrected dose in mg/kg.

[4] Corrections are based upon the fact that excretion of urinary radioactivity immediately eliminates a large part of the dose, so that less than the total injected is available for conversion to $C^{14}O_2$. Calculations to make an approximate allowance for this were made as follows:

$$\frac{\text{Urinary radioactivity (count/min. in first 6 hrs.)}}{\text{Specific activity of dose (count/min./mg)}} = \text{ascorbic acid lost (mg)}.$$

This, subtracted from the total dose, gives the "corrected total dose," which is subsequently converted to "corrected dose per kg of body weight." Although some $C^{14}O_2$ is also excreted in urine (Salomon, '57), the addition of oxalic acid to urine collection flasks caused it to be evolved and appropriately collected together with respiratory $C^{14}O_2$.

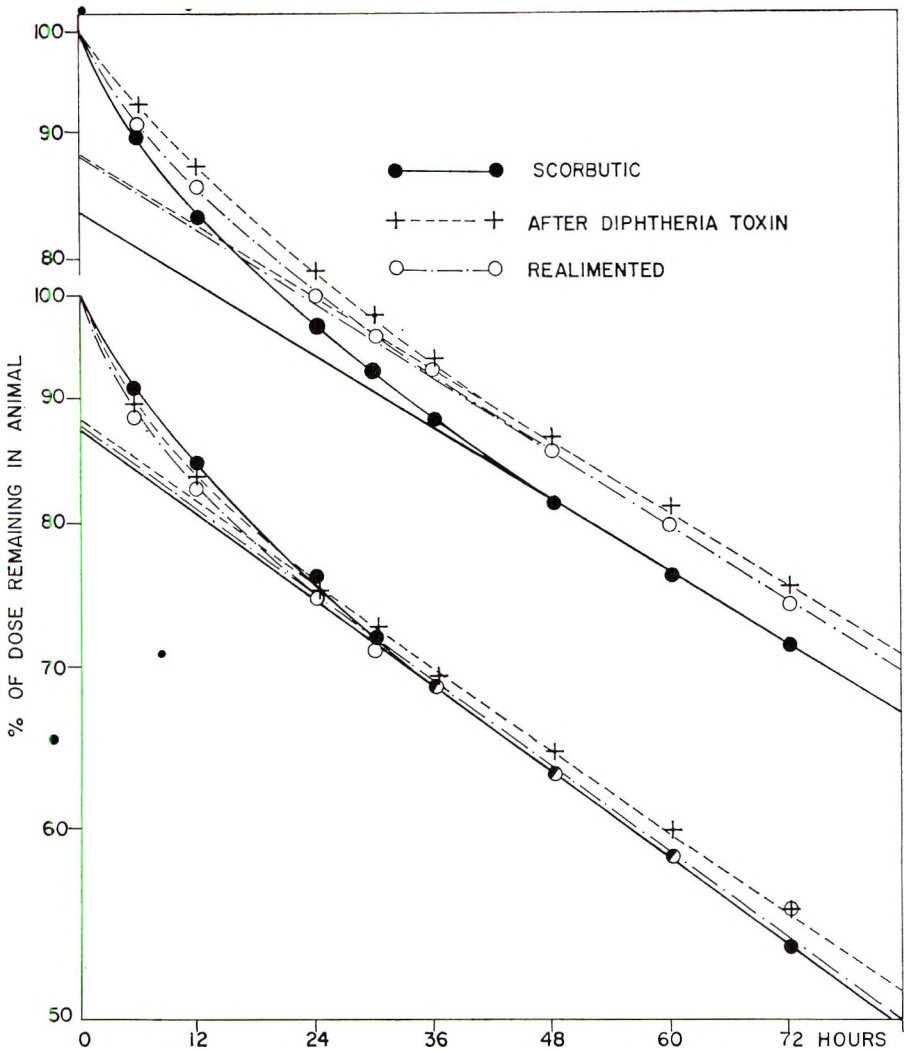


Fig. 1 Decay curves for ascorbic-1-$C^{14}$ acid in two guinea pigs. The biological half-life is not changed appreciably under the conditions of either set of internally controlled experiments. Curves extrapolated to zero time for better visualization of the linear second phase. Intraperitoneal doses close to 1 mg ($2 \times 10^6$ count/min). Second-phase half-life, upper set, about 115 hours; lower set, approximately 110 hours. Scurvy induced by withdrawal of ascorbic acid for 21 days.

is plotted on a logarithmic scale against time in figure 1. This shows the type of curve invariably obtained after injection of ascorbic-1-$C^{14}$ acid into guinea pigs, and is quite characteristic for decay curves. There is an initial phase during which there is rapid loss of ascorbic acid. The rate of catabolism gradually subsides until, after about 30 hours, the semilogarithmic plot becomes linear. Linearity of the curve provides substantial evidence that the rate-limiting reactions are of the first (or pseudo-first) order during the second phase.

Rather than cumulative excretion curves on rectangular coordinates (von Schuching et al., '60), a semilogarithmic presentation is used. This has the advantage of giving kinetic information, in particular, providing direct evidence for the first-order kinetics of the second phase. Moreover, a single transitory event that affects the

shape of the cumulative excretion curve will influence all subsequent segments of the curves, creating problems in interpretation that are not posed by semilogarithmic plots.

These curves permit a number of conclusions. Their shape indicates that ascorbic acid gradually becomes less subject to degradation with time, namely, as it is more widely distributed throughout the tissues. The environment to which it is exposed shortly after injection must be more conducive to catabolism than after penetration into peripheral tissues. If the reaction order and constants remained unchanged in all compartments, there would be no transition from a stage of rapid to slower catabolism. Since withdrawal of ascorbic acid of three weeks duration does not affect the slope of the curves, and apparently does not influence the initial phase, it seems that mere diminution of the body pool of ascorbic acid exerts no effect upon the kinetics of catabolism. In effect, this means that a constant fraction of the body pool is degraded per unit of time, regardless of the size of the body pool. This observation substantiates earlier data (Salomon, '57), but recent work by others was interpreted otherwise (von Schuching et al., '60). Similarly, the severe physiological stress of a lethal dose of diphtheria toxin fails to be reflected by changes in catabolism, leading to the conclusion that, at least under these circumstances, the often suspected increase in rate of loss of ascorbic acid does not materialize. This, too, supports earlier data (Salomon, '57).

Although it is possible to calculate turnover times (von Schuching et al., '60) from the data of figures 1 and 2, there is limited usefulness in this information. Turnover times and rates in animals incapable of synthesizing a metabolite are inextricably related to intake. Thus, the turnover time of ascorbic acid in a guinea pig fed a diet totally free of ascorbic acid theoretically should be "infinity," which is meaningless. Biological half-lives, as given in this paper, are independent of the dietary supply.

When ascorbic acid is withheld from the diet for 39 (animal 24) or 51 days (animal 23), there is an effect upon the kinetics of catabolism, as seen in figure 2, curves 23-S and 24-S. This is interpreted to be an indirect effect of the contraction of the body pool, caused by the debility of the animals at this stage, and the metabolic impairment which must have occurred by this time.[9] The body pool of ascorbic acid in this group of animals is not appreciably different from that of the scorbutic group of figure 1. The biological half-lives indicate that, even in the case of the slowest catabolism (upper curves of figure 1), only slightly more than 4% of the dose remains after 21 days, making allowance for a loss of about 25% during the first day after injection. This also means that little more than 4% of the body pool existing on the first day of the experiment will still remain in the animal after that period. Clearly, the major losses of ascorbic acid occur rapidly, this being reflected by the exponential nature of the decay curves. Subsequent losses can only be minor quantitatively (although this may involve a disproportionately great qualitative effect). The chief aspect is that no impairment of the catabolizing system becomes apparent during the time of most drastic depletion of the body pool. It is significant that this provides evidence against the existence of a response giving partial protection against the consequences of lowered intake of ascorbic acid. Furthermore, failure of scurvy to be immediately signaled by changes in the kinetics of catabolism makes the decay curves poor indicators of ascorbic acid requirements.

That prolonged scurvy may depress the catabolism of ascorbic acid also during the initial phase was noted when smaller, more physiological doses were administered (Salomon, '52). But a very analogous change appeared to occur in guinea pigs moribund from the injection of diphtheria toxin (Salomon, '52), and in these there was no evidence for a contracted body pool. Consequently, this furnishes additional evidence that it is probably the metabolic impairment under such condi-

[9] Metabolic derangements in scurvy have been recognized for years. Recent evidence of the specific defects have been published (e.g., Banerjee and Singh, '60; Ganguli and Banerjee, '61). Biochemical lesions are recognizable within a few days after feeding a deficient diet, even in adult guinea pigs (Gould, '58).

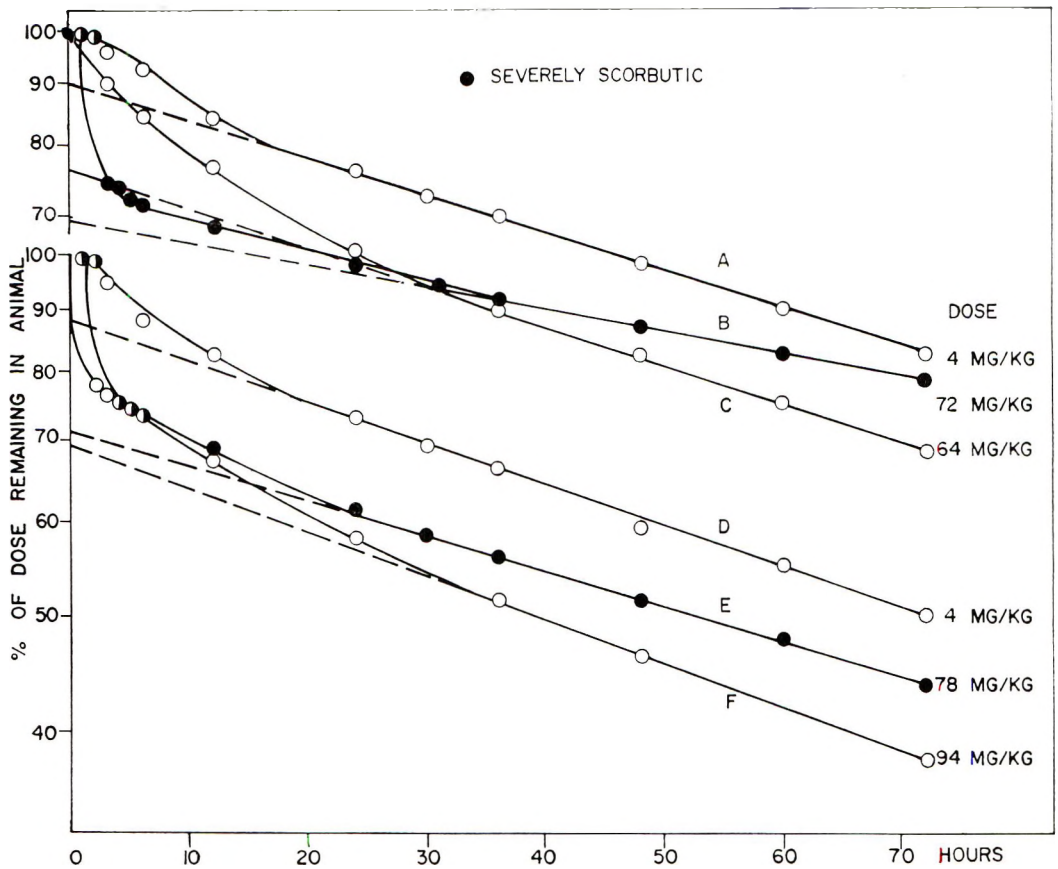


Fig. 2 Slopes of decay curves after massive doses of ascorbic-1-$C^{14}$ acid. The slopes in the second phase are unaffected. But they are diminished in severe scurvy (withdrawal of ascorbic acid for 51 and 39 days, respectively, in animals 23 and 24), analogous to changes seen in the initial phase with smaller doses (Salomon, '52). Curves A, S and B from animal 24, half-lives 89, 96 and 85 hours, respectively. Curves A, S and H from animal 23, half-lives 95, 113 and 92 hours, respectively. Doses administered intraperitoneally, containing close to $2 \times 10^6$ count/min as labeled ascorbic acid, except for curves 23-A and 24-A, close to $4 \times 10^6$ count/min. These animals are identical with those used to obtain data shown in table 1.

tions, rather than the specific result of the lack of adequate ascorbic acid, which causes the diminished catabolism.

*The initial phase of catabolism.* When massive doses of ascorbic-1-$C^{14}$ acid are administered to normal guinea pigs, no effect is noted on the slope of the second phase, but excretion of radioactivity during the initial phase is markedly increased, as shown in figure 2 and table 1. This further demonstrates that the kinetics of the second phase are independent of changes in the body pool. The effect during the initial phase is most clearly seen in table 1, where results from administration of graded doses of ascorbic-1-$C^{14}$ acid are shown. With increasing dosage, the amount converted to respiratory $C^{14}O_2$ increases until a plateau is attained. The reasonable proportionality between size of injected dose and amount excreted as $C^{14}O_2$ at the lower dosages indicates that the fraction of the dose so converted is approximately constant, and that the initial phase also appears to be governed by kinetics of the first order. With increasing dosage, there is also increasing loss of radioactivity via urine. This is the chief reason that a marked drop occurs in the amount retained during the initial phase

of curves 23-S and H, and 24-S and B in figure 2.

As the size of the doses increases, the *fraction* of the dose converted to respiratory $C^{14}O_2$ gradually falls off, but the *quantity* converted becomes constant. This phenomenon is not unexpected in processes depending upon enzymic catalysis, and may be tentatively explained by enzyme saturation. The difficulty in this specific instance is that rapidly increasing urinary excretion of radioactivity also occurs. The plateau thus might represent diminishing efficiency in renal tubular reabsorption of ascorbic acid so that, after a point, no increased amounts of ascorbic acid are made available to the catabolizing system despite larger injections. This is

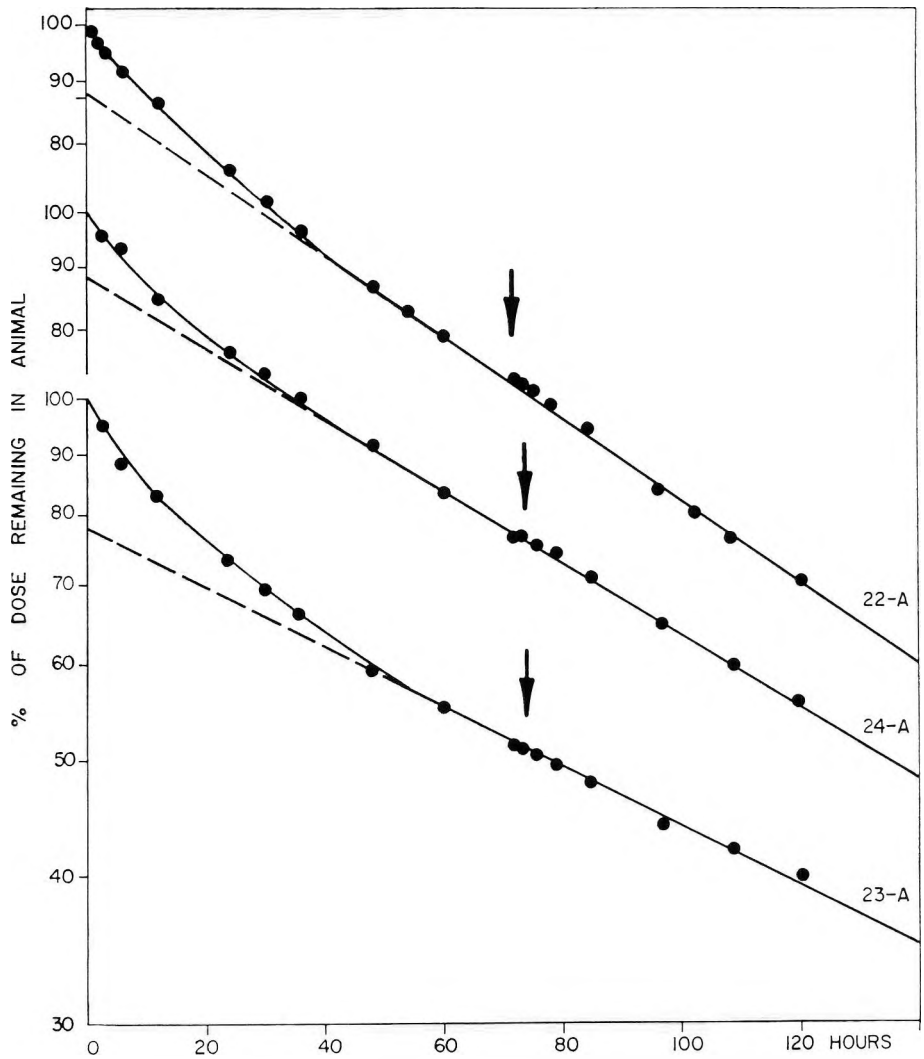


Fig. 3 Addition of unlabeled ascorbic acid to a labeled pool. Intraperitoneal administration of 25 mg of unlabeled ascorbic acid per kg of body weight at the time indicated by arrows has no apparent effect on the catabolism of a previously labeled pool. Dose at the start of the metabolism experiments, close to 4 mg of ascorbic-1-$C^{14}$ acid (about $4 \times 10^6$ count/min) per kg of body weight. Each curve represents data from a single guinea pig. Animal 24 is identical with that used to obtain data shown in table 1 and figure 2. All animals were maintained with the scorbutigenic diet plus 30 mg of ascorbic acid orally per day until the start of the metabolism experiments.

TABLE 2

*Anomalous catabolism of ascorbic-1-C¹⁴ acid*

| Exp. | A | | B | |
|---|---|---|---|---|
| | Scorbutic | Realimented[1] | Normal | After diphtheria toxin[2] |
| Weight, gm | 275 | 637 | 366 | 396 |
| Dose,[3] mg | 1.71 | 1.59 | 2.45 | 2.15 |
| Excretion during initial 24 hours after injection | | | | |
| In urine, % of dose | 5.8 | 2.7 | 3.7 | 2.8 |
| In respiratory $CO_2$ % of dose | 13.5 | 82.8 | 32.6 | 83.6 |
| Half-life (hours) in second phase | 132 | 122 | 97 | 91 |

[1] Supplements of 30 mg of ascorbic acid per kg of body weight orally per day for 52 days following termination of initial experiment.
[2] One MLD of toxin administered subcutaneously 12 hours before ascorbic-1-C¹⁴ acid and start of metabolism experiment.
[3] Ascorbic-1-C¹⁴ acid, $2.35 \times 10^6$ count/min./mg.

unlikely, however, because temporal differences exist in maximal excretion of urinary and respiratory radioactivity (see table 1), and because even at the highest dosages, retention of ascorbic acid does not appear to reach a maximum (although it becomes increasingly inefficient). This point does not constitute a critical consideration for this study.

If the initial process and the second phase are both reactions of the first order, it must be concluded that exogenous ascorbic acid cannot spare vitamin C already incorporated into the tissues. This can be experimentally confirmed, as shown in figure 3. Introduction of unlabeled ascorbic acid into a previously tagged pool is entirely without effect upon any phase. Thus, endogenous ascorbic acid is catabolized at a rate independent of the new dose, and products of excretion formed from the new dose are additive to those that would have been formed in the absence of fresh material.

Chance observations further reinforce the conclusion that there are two phases in the catabolism which are independent. It is occasionally seen that a very high fraction of the dose is converted to respiratory $CO_2$ during the first few hours after injection. This occurs in guinea pigs, replete or scorbutic, and in rats. It occurs more frequently in animals that may be thought to be less resistant to physiological stress, namely, guinea pigs that are, or have been scorbutic, and in hypophysectomized rats. The cause of this anomaly is unknown,[10] but it is suggestive that the

TABLE 3

*Excretion of radioactivity after intrahepatic injection of ascorbic-1-C¹⁴ acid*

| Exp. | H1 | | H2 | |
|---|---|---|---|---|
| Weight, gm | 411 | 450 | 405 | 437 |
| Dose,[1] mg | 0.88 | 0.78 | 0.80 | 0.63 |
| Route of injection[2] | IP | IH | IP | IH |
| Radioactivity % of dose[3] | | | | |
| In urine | 6.38 | 5.30 | 5.14 | 3.36 |
| In $CO_2$ | 12.04 | 12.18 | 12.67 | 12.05 |
| Total | 18.4 | 17.5 | 17.8 | 15.4 |
| Time, hours | 18.0 | 18.0 | 24.5 | 24.5 |

[1] Specific activity $2.35 \times 10^6$ count/min/mg.
[2] Abbreviations used are: IP, intraperitoneal; and IH, intrahepatic.
[3] Results of consecutive short periods in each run were summed to give the total radioactivity excreted during the indicated total experimental period.

phenomenon occurs sporadically and usually affects several members of a group of animals. The chief interest for the present purpose is that the effect is always confined to the initial period, but the slopes of the second phase fall within limits considered normal on the basis of experience with a large number of ani-

[10] The effect is not attributable to impurities in ascorbic-1-C¹⁴ acid, such as might arise from self-decomposition. This conclusion is based on the fact that the phenomenon has never occurred simultaneously in two animals that recived injections of the same solution virtually at the same time, although two metabolism experiments are routinely performed together. The extent of the rapid degradation indicates that the responsible contaminant, if any, would have to be the major component of the preparation of labeled ascorbic acid. Yet, radiochromatograms, developed as described by Mapson and Parsons ('49) and scanned with a 4-$\pi$ detector (Salomon, '60), failed to disclose components other than ascorbic acid in any sample used in these experiments. The small stock of labeled ascorbic acid is checked periodically and, when detectable decomposition products appear, the preparation is discarded.

TABLE 4

*Respiratory radioactivity after nephrectomy*

| Exp. | N1 | | N2 | | N3 | |
|---|---|---|---|---|---|---|
| | Intact | Nephrectomized | Intact | Nephrectomized | Intact | Nephrectomized |
| Weight, gm | 400 | 420 | 395 | 410 | 420 | 435 |
| Dose, mg[1] | 0.56 | 0.65 | 0.57 | 0.56 | 0.61 | 0.76 |
| Radioactivity, % of dose[2] | 28.1 | 32.8 | 21.6 | 68.2 | 25.6 | 36.7 |
| Time, hours | 42 | 42 | 30 | 30 | 48 | 48 |

[1] Specific activity $4.73 \times 10^6$ count/min./mg. Material injected intraperitoneally.
[2] Results of consecutive short periods in each run were summed to give the total radioactivity excreted during the indicated total experimental period.

mals. This is shown in table 2, where sets of measurements obtained prior to and after the onset of the defect allowed direct comparisons on the same animals.

Finally, as a means of localizing the site chiefly responsible for the rapid rate of catabolism during the initial phase, two types of experiments were performed. In one instance, guinea pigs received intrahepatic injections of labeled ascorbic acid. Conversion to radioactive products found in urine or to respiratory $CO_2$ does not appear to differ from prior assays. Such data are shown in table 3. A second type of experiment was suggested by the observations of Martin and Mecca ('61), who concluded that the kidneys of rats and dogs may play a role in the uptake of ascorbate by tissues. But, the conversion of ascorbic acid to $CO_2$ seems to be unimpaired, if not accelerated, in nephrectomized guinea pigs (see table 4), indicating that the function of the kidneys of guinea pigs may differ in this respect from those of rats and dogs. For this reason, it does not appear likely that the plateau in excretion of respiratory radioactivity, shown in table 1, is the consequence of limited conversion to a more penetrable form and, thus, limited decarboxylation of ascorbic-1-$C^{14}$ acid.

## SUMMARY

As an extension of earlier work on the catabolism of ascorbic acid in guinea pigs, investigations on the initial phase following administration of ascorbic-1-$C^{14}$ acid were made. Catabolism during this stage has the characteristics of first-order processes, was kinetically independent of the following slower phase, and could be "saturated" by injection of high levels of ascorbic acid. The rapid initial reaction did not appear to depend upon renal action and was unchanged by intrahepatic injection of ascorbic-1-$C^{14}$ acid.

Massive doses of labeled ascorbic acid in normal guinea pigs were catabolized similar to more physiological doses, except for higher levels of urinary excretion of radioactivity and conversion of a lower fraction of the dose to respiratory $C^{14}O_2$ during the initial phase. Contraction of the body pool of ascorbic acid exerted no direct effect on the catabolism of ascorbic acid. Such diminution in catabolism as was discovered after prolonged feeding of a scorbutigenic diet was presumably referable to general metabolic impairment, rather than to a specific response designed to protect, or coincidentally protecting, against the consequences of the scorbutigenic regimen.

As anticipated from the kinetics of the rate-limiting processes, newly-administered ascorbic acid did not cause sparing of that already incorporated into the tissues.

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# Studies on the Soy and Fish Solubles Growth Factors for Chicks[1]

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On the basis of previous studies (Westerfeld et al., '62), casein-gelatin diets containing 35% of more of total protein appeared to be deficient in two unidentified growth factors required by chicks and poults. One of these factors was present in fish solubles, and has been recognized by many investigators. The other "factor" was present in soy protein, but was relatively deficient in casein or gelatin; it could, therefore, be recognized as a growth factor by comparing the response to casein-gelatin vs. soy protein diets, or by determining the growth stimulation produced by the addition of soy protein to casein-gelatin diets containing the fish solubles factor.

To establish a reliable assay for the soy factor it was necessary to demonstrate that the basal diet was adequate in protein, contained an excess of the fish solubles factor, and produced a growth stimulation when supplemented with soy protein. Since casein appeared to contain a small amount of the soy factor, it seemed desirable to keep the dietary casein at a level only slightly in excess of that required to supply the protein requirement. On a theoretical basis a basal diet containing 20% casein plus 10% gelatin plus 20% fish solubles should supply 36.5% of protein and a 30% excess of all the essential amino acids with the possible exception of methionine. Such a diet met the above requirements for the assay of the soy factor. In the course of establishing its adequacy in all other respects, however, it was found that growth with a 20% casein plus 10% gelatin diet was markedly stimulated by the addition of 0.4 to 0.6% of DL-methionine; only a small additional response was obtained by further supplementation with other amino acids. When 20% of fish solubles was also present in the diet, the addition of methionine had relatively little effect. Hence, the addition of methionine to the basal diet did not appear to be essential, but since it was not inhibitory at the levels used, it was added to the basal diet in many experiments as a precaution.

The addition of 20% of soy protein to such a basal 20% casein plus 10% gelatin plus 20% fish solubles ± 0.6% of DL-methionine diet stimulated growth and allowed a maximal growth rate to be achieved. Under these test conditions the soy protein contributed some particular combination of amino acids or some unidentified factor which was responsible for its growth stimulating effect. For convenience, the growth response to soy protein will be attributed to a "soy factor" without any implication as to its chemical nature.

## METHODS

In addition to the protein and those factors mentioned specifically in the text,[2] all diets contained 3.5% of soy oil, 8.3% of salt mixture, 0.1% of inositol, 0.2% of choline, 125 mg per kg of diphenyl-*p*-phenylenediamine, 50 mg per kg of chlortetracycline,[3] a vitamin supplement, and sufficient starch to total 100% on a dry-weight basis. The vitamin and mineral supplements were identical with those de-

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[2] The diets are abbreviated to indicate the percentage composition of the variable constituents; a 20% casein plus 10% gelatin plus 20% soy protein diet is described as 20C + 10G + 20SP. All diets also contained the nonvariable constituents listed under methods. Fish solubles (FS) contained 50% water and was substituted for half as much starch.
[3] Aureomycin, American Cyanamid Company, New York.

scribed by Reid et al. ('56) except that the pyridoxine, menadione, and Zn concentrations were increased to provide 6 mg per kg, 1 mg per kg and 50 ppm, respectively. In addition, 2 mg per kg of $Na_2MoO_4$ were routinely added to all diets, and 100 mg per kg of mixed tocopherols replaced the α-tocopherol. When present, the 20% of fish solubles supplied 10 gm of solids and 6.4 gm of additional protein. All additions to the diet were made at the expense of the starch. Groups of 6 to 8 one-day-old White Leghorn chicks were fed the diets ad libitum for 4 weeks, and body weights were recorded weekly. The values given with the results refer to the average body weight at 4 weeks. Significant observations were repeated in at least two independent experiments.

## RESULTS

*Methionine diets compared with casein-gelatin diets.* The methionine in casein is either not completely available to chicks or the casein-gelatin diet creates a high methionine requirement. A 35% isolated soybean protein[4] diet, supplemented only with 0.4% of glycine, supplied a calculated 0.28% of methionine and 0.17% of cystine to the diet; this 0.45% of total sulfur amino acids was inadequate (215 gm at 4 weeks). When supplemented with 0.32% of DL-methionine to give a total of 0.6% of methionine and 0.77% of sulfur amino acids, a maximal growth response (295 gm) was obtained; no additional growth effect was noted from the addition of 0.52 or 0.72% of methionine. Therefore, 0.6% of methionine plus 0.17% of cystine in the diet was adequate for a maximal growth rate when supplied as such or as soy protein, and this agrees with the 0.5 to 0.8% level previously established as the methionine requirement for chicks (Klain et al., '58, '60; Bird et al., '54). However, a 20% casein plus 10% gelatin diet supplied a calculated 0.64% of methionine and 0.07% of cystine for a total sulfur amino acid content of 0.71%. This diet was grossly deficient (120 gm at 4 weeks) and did not support growth as well as the 0.45% of total sulfur amino acids in the soy protein diet. When supplemented with 0.16% of methionine, growth was slightly better (230 gm) than that obtained with the unsupplemented soy protein, whereas the addition of 0.36% of methionine to the casein-gelatin diet gave a maximal response (255 gm) and 0.56% had no further effect. From a comparison of these results it can be estimated that only 50% of the total sulfur amino acids present in the casein-gelatin diet was "available" to the chick. Attempts to improve the "availability" of methionine from casein by using an acid hydrolysate fortified with tryptophan and threonine or by using a commercial protein hydrolysate[5] instead of casein were inconclusive because of the poor growth obtained in the presence or absence of added methionine.

The major amino acid deficiency in the 20C plus 10G diet was methionine, as shown in figure 1, but additional amino acids may have been borderline. The addition of 0.5% of DL-methionine to such a diet increased the 4-week body weights from 145 gm (curve 1) to 245 gm (curve 2). The addition of 20% of soy protein or a mixture of amino acids equivalent to 20% of soy protein gave a further growth increment to 275 to 285 gm in different experiments (curve 3). The difference between curves 2 and 3 indicated a further small limitation of one or more amino acids in addition to the methionine. Although methionine was the most limiting amino acid theoretically and actually, a 20C plus 10G diet theoretically supplied no more than a 15 to 25% excess of tryptophan, leucine, histidine and arginine. When the diet containing 20C plus 10G plus 20SP (curve 3) was further supplemented with a mixture of all the essential amino acids (the supplement providing the minimal requirement of each essential amino acid per se), no further stimulation or inhibition of growth was obtained. Hence, the growth represented by curve 3 in figure 1 was the maximum possible rate that could be achieved with a purified diet obviously adequate in protein.

Curve 3 also represents the growth achieved when 20% of fish solubles was added to a 20C plus 10G diet supplemented

[4] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.

[5] Amigen, Mead Johnson Company, Evansville, Indiana.

with 0.25% of methionine, whereas curve 4 represents the maximal growth achieved when the basal diet was supplemented with both the soy factor (as 10 to 35% of soy protein or 10% of egg yolk) and 20% of fish solubles (355 to 365 gm at 4 weeks in different experiments). In this experiment, the addition of the soy factor or the fish solubles factor *alone* to the purified diet had no greater effect on growth than that obtained with adequate protein; however, a very significant growth stimulation was achieved when both were present in the diet simultaneously. In other similar experiments the results were not quite as marked, but in general the soy factor had to be present in the diet in order to demonstrate a good fish solubles growth response, and the fish solubles factor had to be present in the diet in order to demonstrate a good growth response to the soy factor.

When a mixture of essential amino acids was added to the 20C plus 10G diet, the growth stimulation obtained (curve 2A) was slightly less than that obtained with methionine alone (curve 2). Similarly, the addition of the mixed essential amino acids to a diet containing 20C plus 10G plus 20FS gave a slightly lower growth rate than was obtained in their absence (curve 3 vs. 3A). These minor growth inhibitions by this mixture of essential amino acids were not observed when soy protein was also present in the diet (curve 3) or when a complete mixture of all the amino acids was added (curve 3).

*Amino acids vs. 20C + 10G + 20FS diet.* When 20% of fish solubles was added to a 20% casein plus 10% gelatin diet, most, if not all, of the methionine deficiency of the latter was overcome. The fish solubles supplied only 0.14% of additional methionine plus an unknown amount of cystine to the casein-gelatin diet, but this diet usually gave body weights at 4 weeks of approximately 275 gm; the addition of 0.25 to 0.6% of DL-methionine to the 20C plus 10G plus 20FS diet sometimes gave a small growth increment (10 to 20 gm) and the unsupplemented diet was considered to be potentially borderline but not seriously deficient in methionine.

The growth response produced by the addition of 20% of soy protein to this diet could not be reproduced by any known amino acid present in the soy protein. When each amino acid was added individually to a basal diet containing 20C plus 10G plus 20FS plus 0.4 to 0.6 of methionine in an amount equivalent to its presence in 20% soy protein (see legend, fig. 1), there was no significant stimulation by any amino acid. A mixture of all the amino acids present in 20% soy protein or a mixture of the following nonessential amino acids gave an insignificant 6 to 7% increase: cystine, proline, glycine, alanine, aspartic and glutamic. Although small increases in growth might be attributed to the amino acids present in the soy protein, they alone or in the combinations tested could not reproduce the 20 to 30% increases consistently obtained with soy protein itself in a large number of experiments ($P = 0.01$).

The addition of excessive amounts of methionine to the diet produced inhibition of growth. The 4-week body weights were only 65 and 80% of that obtained with 0.6% of methionine when the latter was increased to 2.2 and 1.4%, respectively. As expected from the studies of Klain et al. ('60), the addition of 1.8% of DL-phenylalanine to the basal diet containing 0.6% of methionine produced a 20% inhibition, and a similar inhibition was also produced by a mixture of 1% of L-arginine, 1% of L-leucine and 0.5% of L-cystine. These inhibitions suggested that the poorer growth obtained with casein-gelatin as compared with soy protein diets might be due to a higher concentration of certain amino acids in the former. However, when a mixture of 1% of L-tyrosine, 0.3% of DL-tryptophan, 1% of L-leucine, and 1.5% of DL-valine was added to a 35% soy protein diet which was supplemented with 0.75% of methionine and 0.4% of glycine, only an insignificant 6% inhibition was observed.

*Further testing of the basal diet.* From the preceding studies a suitable basal diet for assaying the soy factor would be composed of 20C plus 10G plus 20FS plus 0.4 to 0.6 of methionine. The addition of soy protein to this diet gave a growth response

that could not be attributed to any individual amino acid or to a mixture of all the amino acids in the soy protein. However, the possibility that some properly selected combination of known amino acids would duplicate the effect of soy protein has not been eliminated.

An excess of the fish solubles factor in the basal diet was demonstrated by comparing the effect of adding 10 or 20% of fish solubles. When the fish solubles was added to the 20C plus 10G plus 20SP plus 0.6 of methionine diet, both concentrations gave the same maximal growth rate; the latter was identical with the rate achieved with a 35% soy protein diet supplemented with methionine, glycine and 20% of fish solubles. Hence, 10% of fish solubles supplied enough of this factor to allow maximal growth when present simultaneously with the soy factor, and 20% of fish solubles was clearly in excess.

The addition of 10, 20 and 35% of soy protein to a basal diet containing 20C plus 10G plus 20FS all gave the same maximal growth rate (365 gm at 4 weeks). This demonstrated that the deficiency in this basal diet could be corrected completely by soy protein, and that under these test conditions a concentration of 20% supplied an excess of this factor without producing any further growth increment. It has not been possible to increase the growth rate of the chicks used in these studies beyond the maximum of 355 to 365 gm at 4 weeks by any diet yet tested. This rate was achieved with a diet containing 20C plus 10G plus 0.6 of methionine plus 20FS plus 20SP, and was not increased by further additions of 10% of Wesson[6] oil or 10% of egg yolk.

There was no effect when the salt mixture in the basal diet of 20C plus 10G plus 20FS plus 0.6 of methionine was increased by 50% and the diet was further supplemented with one ppm of selenium. A comparison of the salt mixture used routinely in these studies with that devised by Fox and Briggs ('60) showed that the former gave a questionable 5% greater weight at 4 weeks with the basal diet both in the presence and absence of 20% of soy protein. The addition of 227 ppm of ethylenediaminetetraacetic acid or the deletion of the chlortetracycline and diphenyl-*p*-phenylenediamine from the complete diet containing 20% of soy protein had no effect.

*Soy protein fractionation.* Six hundred grams of soy protein were refluxed 24 hours with 3 liters of 8 N $H_2SO_4$, then diluted with 6 liters of $H_2O$, and the sulfate removed with $Ba(OH)_2$. After concentrating the filtrate to 5 liters, 600 gm of $Ba(OH)_2 \cdot 8H_2O$ and 15 liters of 95% ethanol were added, and the solution was chilled overnight. The insoluble barium salts were filtered off, washed with 75% alcohol, redissolved in 4 liters of warm water, and the barium was removed with $H_2SO_4$. The filtrate from the $BaSO_4$ precipitate contained about 120 gm of solids which, on the basis of two-dimensional paper chromatography, contained approximately 75% of glutamic, 10% of aspartic, 5% of glycine, 5% of serine, a trace of threonine, and no unidentified ninhydrin-positive component.

When this fraction was added to the 20C plus 10G plus 20FS plus 0.4 to 0.6 of methionine test diet at a level equivalent to the original 20% soy protein from which it was derived, it stimulated growth in each of three independent experiments by about 15% ($P = 0.01$), whereas the intact soy protein tested simultaneously gave a 30% increase ($P = 0.01$) (average basal = 265 gm; + Ba salts = 310 gm; + 20% SP = 345 gm). Although this fraction was only one-half as active as the original soy protein, its amino acid composition could be reproduced with identical amounts of free amino acids. When such a mixture of pure amino acids contributed 3.1% of L-glutamic, 0.4% of L-aspartic, 0.2% of glycine, 0.2% of L-serine and 0.13% of DL-threonine to the test diet, no growth response was obtained. The activity of the fraction could not be attributed to the amino acids present.

*Distribution of soy factor.* Reasonable approximation of the "soy factor" content of different foodstuffs was obtained from repeated tests. The assay range was relatively small, and the response to the basal diet varied somewhat in different experi-

[6] Refined cottonseed oil, The Wesson Oil Company, New Orleans, Louisiana.

TABLE 1

*Approximate soy factor effect of various supplements when added to a basal diet of 20C + 10G + 20FS ± 0.6 methionine and fed to chicks for 4 weeks*

| Test supplement[1] | 4-Week body weights | Test supplement | 4-Week body weights |
|---|---|---|---|
| Soy protein | 360 | | |
| Egg yolk | 350 | Oatmeal | 300 |
| Lactalbumin | 350 | Whole milk (dry) | 300 |
| Liver residue | 340 | Skim milk (dry) | 295 |
| Casein (20) | 335 | Whey | 290 |
| Bran | 330 | Cerophyl[2] | 285 |
| Soy flour (extracted) | 325 | Basal | 280 |
| Brewer's yeast | 320 | Basal or less | |
| Fibrin | 320 | Rice | |
| Wheat germ | 320 | Hemoglobin | |
| Fermentation residue[3] | 315 | Betaine and/or creatine | |
| Wheat gluten | 315 | Liver residue ash | |
| Yellow cornmeal | 310 | Soy protein ash | |
| Distiller's solubles | 310 | | |
| Liver (defatted) | 305 | | |
| Whole wheat | 305 | | |

[1] All substances were tested at 10% of the diet execpt for 20% casein, 1% betaine and/or 1% creatine, and the ash from 10% liver residue or 20% soy protein. The results are averages of 2 to 4 independent tests.
[2] Cerophyl Laboratories, Kansas City, Missouri.
[3] Omafac, E. R. Squibb and Sons, New York.

ments. The response to the test supplement was usually in the same range in replicate experiments, but occasionally there were unexplained failures; for example, egg yolk and liver residue gave good growth responses in two out of three and in 4 out of 5 tests, respectively, but failed to stimulate growth above the basal level in the remaining test. Because of the possibility of a methionine deficiency as well as a methionine excess, the test supplements were assayed with a basal diet of 20C plus 10G plus 20FS both in the presence and absence of an added 0.6% of methionine. The presence or absence of methionine made no difference in the results, and all of the assay values have been averaged in table 1.

In 9 different experiments the basal diet with and without methionine gave average 4-week body weights of 285 and 275 gm, respectively; supplementation with 20% of soy protein gave the same maximal weight of 355 to 365 gm for both diets. Egg yolk, lactalbumin and liver residue were also good growth stimulants under these test conditions and were, therefore, good sources of the soy factor; casein was also effective when tested at twice the usual level. All of these growth responses were significantly different from the basal ($P = 0.01$). Commonly used sources of chick growth factors, such as fermentation residue, distiller's solubles, and whey were relatively poor sources of the soy factor; such substances have usually been tested with a diet containing soy protein.

*Distribution of fish solubles factor.* The distribution of the fish solubles factor was determined by measuring the growth response of chicks when the foodstuff was added to a 35% soy protein diet supplemented with 0.75% of DL-methionine and 0.4% of glycine. Such a basal diet contained at least a 25% excess of all known essential amino acids. On the basis of previous studies (Westerfeld et al., '62) this diet supplied an adequate amount of both protein and "soy factor"; when supplemented with 10 or 20% of fish solubles, a maximal growth rate was obtained. Therefore, any growth stimulation obtained from the addition of a supplement to this diet could reasonably be attributed to the presence of the fish solubles factor, although there is no assurance that only a single factor is involved in the fish solubles itself.

In figure 1, curve 5, is shown the growth rate of chicks fed the basal 35% soy protein diet supplemented with methionine and glycine. This basal diet consistently

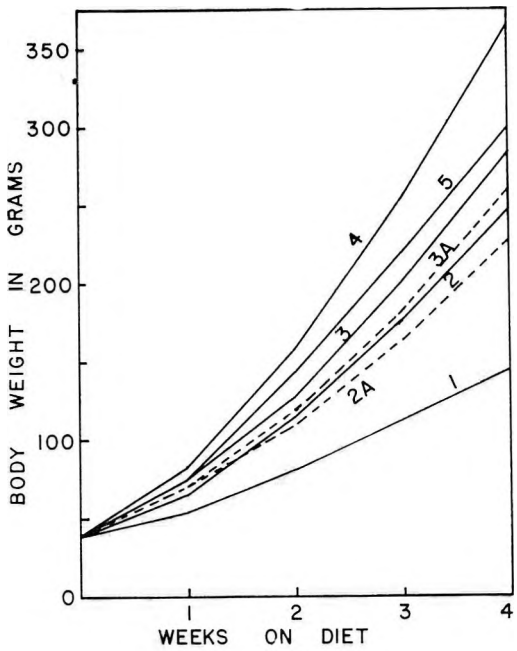


Figure 1

gave 4-week body weights of 280 to 300 gm, and the maximal growth achieved by the addition of 10 or 20% of fish solubles was 355 to 365 gm ($P = 0.01$). Since the same growth rate was obtained with 10 and 20% of fish solubles, this factor was in excess, and no further growth increment could be obtained by adding more fish solubles to the diet. The distribution of the fish solubles factor in a variety of products is shown in table 2. Fermentation residue, distiller's solubles, and egg yolk were good sources of the fish solubles factor, and were significantly different from the basal at $P = 0.02$ to 0.05.

*The soy and fish solubles factors in thyrotoxic chicks.* It was possible that the need for the soy and fish solubles factors by growing chicks could be intensified by the addition of thyroid substance to the diet. The results of such experiments are shown in table 3. When 0.35% of Protamone[7] was added to a basal diet containing 20C plus 10G plus 0.6 of methionine, all chicks died in less than two weeks. The addition of 20% of soy protein to this diet kept 5/6ths of the birds alive throughout the 4-week experiments; their growth rate was reasonably good, but appreciably below that of those birds not receiving Protamone. The addition of fish solubles to the same basal diet gave very little protection in terms of survival or growth rate. In fact the presence of fish solubles in the diet that also contained 20% of soy protein largely nullified the protective effect of the latter in the thyrotoxic chick.

The deterimental effect of fish solubles in the thyrotoxic state was also observed with the basal diet containing 35% of soy protein plus 0.75% of methionine and 0.4% of glycine. The high content of soy protein in this basal diet allowed good survival of the thyroid-treated birds, but when fish solubles was also present, this protective effect was largely nullified. Apparently soy protein exerted a protective

Fig. 1 Growth curves for chicks fed a basal 20% casein plus 10% gelatin diet supplemented with various amino acid mixtures and sources of the soy and fish solubles factors.

Curve 1 = Basal (20% casein + 10% gelatin)
2 = Basal + 0.5% DL-methionine
3 = Basal* + 0.5% DL-methionine + 20% soy protein
= Basal + 0.25% DL-methionine + 20% fish solubles
= Basal* + essential amino acids + 20% soy protein
= Basal + amino acids equivalent to 20% soy protein
4 = Basal + 20% fish solubles + 10 to 35% soy protein
= Basal + 20% fish solubles + 10% egg yolk + 0.25% DL-methionine
2A = Basal* + essential amino acids
3A = Basal* + essential amino acids + 20% fish solubles
5 = 35% soy protein + 0.75% DL-methionine + 0.4% glycine.

The amino acids added to be equivalent to 20% soy protein (gm/kg of final diet): L-amino acids: arginine, 13.3; aspartic, 9.9; cystine, 1.0; glutamic, 31.2; histidine, 4.2; leucine, 12.0; lysine, 10.9; phenylalanine, 8.0; proline, 4.0; serine, 11.1; tyrosine, 5.5. DL-Amino acids: alanine, 11.6; glycine, 6.6; isoleucine, 20.8; methionine, 3.2; threonine, 12.6; tryptophan, 3.2; valine, 17.6. The mixture of essential amino acids that supplied the minimal requirement of each, per se, included (gm/kg of final diet): L-Amino acids: arginine, 12; glycine, 5; histidine, 4; leucine, 16; lysine, 12; and tyrosine, 8. DL-Amino acids: isoleucine, 16; methionine, 16; phenylalanine, 18; threonine, 12; tryptophan, 4; and valine, 20. In those diets marked with an *, the vitamin supplement was doubled as a precautionary check. For curve 2 identical results were obtained with the usual as well as the doubled vitamin supplement.

[7] Thyroid-active iodinated casein, Agri-Tech, Inc., Kansas City, Missouri.

TABLE 2

*Fish solubles effect from various supplements, as tested with a basal 35% soy protein diet fortified with 0.75% of* DL*-methionine and 0.4% of glycine*

| Test supplement[1] | Body weights at 4 weeks | Negative supplements (Basal or less) |
|---|---|---|
| Fish solubles | 350 | |
| Fermentation residue[2] | 340 | Casein |
| Distiller's solubles | 340 | Defatted liver |
| Egg yolk | 335 | Liver residue |
| Yellow cornmeal | 335 | Soy flour |
| Brewer's yeast | 315 | Wheat germ |
| Bran | 310 | Whole wheat flour |
| Cerophyl[3] | 305 | Fish meal |
| Dry milk (whole or skim) | 305 | 1.5 × minerals + 1 ppm Se |
| Lactalbumin | 305 | Betaine |
| | | Fish solubles ash |
| Basal | 300 | |

[1] All substances listed were tested at 10% of the diet except for 1% betaine, and the ash from 20% fish solubles; increasing the usual salt mixture by 50% and adding 1 ppm of Se was negative. Ten per cent or 20% of fish solubles gave the same maximal growth rate.
[2] Omafac, E. R. Squibb and Sons, New York.
[3] Cerophyl Laboratories, Kansas City, Missouri.

TABLE 3

*Effect of soy protein and fish solubles on the growth and survival of chicks fed iodinated casein*

| Basal[1] diet no. | + 20% soy protein | + 20% fish solubles | Results at 4 weeks[2] | | | |
|---|---|---|---|---|---|---|
| | | | Without Protamone[3] | | + 0.35% Protamone | |
| | | | Body weight | Survival | Body weight | Survival |
| | | | *gm* | | *gm* | |
| 1 | − | − | 290 | 6/6 | — | 0/6 |
| 1 | + | − | 320 | 6/6 | 270 | 5/6 |
| 1 | − | + | 300 | 6/6 | 210 | 2/6 |
| 1 | + | + | 340 | 6/6 | 205 | 2/6 |
| 2 | − | − | 305 | 6/6 | 235 | 6/7 |
| 2 | − | + | 335 | 6/6 | 290 | 2/7 |

[1] Basal diet no. 1: 20% casein + 10% gelatin + 0.6% DL-methionine; other constituents as listed under methods. Basal diet no. 2: 35% soy protein + 0.75% DL-methionine + 0.4% glycine; other constituents as listed under methods.
[2] Starting body weights: Diet no. 1 = 31 to 35 gm; diet no. 2 = 35 to 38 gm.
[3] Obtainable from Agri-Tech, Inc., Kansas City, Missouri.

effect in thyrotoxic chicks without restoring a completely normal growth rate. Fish solubles had little protective effect by itself and largely nullified the beneficial effect of soy protein when fed together with the latter.

## DISCUSSION

No indication of a methionine deficiency in casein diets for chicks was detected in previous studies (Westerfeld et al., '62) of liver xanthine dehydrogenase. With practical-type diets Nelson et al., ('60) observed no effect of methionine on growth; the requirement for sulfur amino acids was 3.5% of the protein at all levels of dietary protein and energy. All of the methionine in casein and gelatin appeared to be available to chicks when added in relatively small amounts to a mixed grain type of diet (Grau and Almquist, '45). Ousterhaut et al. ('59) reported the methionine of soy flour to be readily available to chicks, and both methionine plus cystine appeared to be readily available from cottage cheese; of the amino acids studied, only arginine seemed to have a severely limited availability from this source. Fluckiger and Anderson ('59) reported that methionine, as well as the total sulfur amino acid requirement, was higher with a casein-gelatin than with a soy protein diet. The growth response to methionine when added to a 20% casein and 10%

gelatin diet could theoretically be due to a lack of availability or an increased requirement; the latter explanation seems more probable from the studies cited.

The literature is extensive concerning unidentified growth factors for chicks and poults. The earlier literature has been summarized by Rasmussen et al. ('57), Camp et al. ('56), Branion and Hill ('53), and by the other authors cited. Most of the recent studies have been concerned with one or more (Waibel, '58; Fry et al., '58; Combs et al., '57; Peterson et al., '55) of the following: fish solubles and fish meal (Wisman et al., '56; Sullivan et al., '60; Ritchey et al., '56), distiller's solubles (Tsang and Schaible, '60; Jaffe and Wakelam, '58), fermentation solubles (Russo and Heiman, '59), antibiotic fermentation residue, whey, alfalfa meal and egg yolk (Kratzer et al., '59; Arscott et al., '59; Menge et al., '57; Greene et al., '60; Liuzzo et al., '60). A part of the total response to these supplements has been obtained with the ash derived from the supplements (Morrison et al., '55, '56; Camp et al., '56), but the nature of this effect is still unknown. The activities observed in these various sources have been attributed to two or three factors (Camp et al., '55; Rasmussen et al., '57). Attempts to purify the fish solubles factor have so far been limited (Barnett and Bird, '56; Mason et al., '61). In nearly all cases these supplements have been added to a basal soy protein diet or to a practical-type corn-soy diet, and in some cases in relatively small amounts to basal diets of questionable protein adequacy. It is not unexpected that the activity of most of these supplements can be attributed to the fish solubles factor.

Such a large number of substances have been reported to be antithyrotoxic (Ershoff et al., '59) that a correlation of antithyrotoxic activity with some other biological response may have little significance. However, in a careful study of specific proteins by Overby et al. ('59), liver residue was the most protective and was followed in descending order of activity by fibrin, lactalbumin and whole egg; casein and egg albumin were essentially inactive. This bears some resemblance to the distribution of the soy factor in these same proteins.

SUMMARY

1. A 20% casein plus 10% gelatin diet was deficient in methionine for chick growth. When supplemented with a mixture of all the essential amino acids or all the amino acids in 20% soy protein, it was still deficient in two unidentified factors: the "soy" and the "fish solubles" factors. Both factors were required in the diet simultaneously to obtain the best growth response from either.

2. A 20% casein plus 10% gelatin plus 0.6% methionine plus 20% fish solubles diet provided a suitable basal diet for detecting the soy factor. It was adequate in protein, contained an excess of the fish solubles factor, and allowed a maximal growth rate when supplemented with soy protein. The growth stimulation produced by the addition of 20% of soy protein to this diet could not be duplicated by any amino acid or by a mixture of the amino acids in 20% soy protein. Egg yolk, lactalbumin and liver residue were also good sources of the soy factor.

3. A 35% soy protein diet supplemented with 0.75% of methionine and 0.4% of glycine provided a suitable basal diet for detecting the fish solubles factor. This diet contained adequate protein and soy factor, and it allowed a maximal growth rate of chicks when supplemented with 10 or 20% of fish solubles. Fermentation residue, distiller's solubles, and egg yolk were also good sources of the fish solubles factor.

4. Soy protein exerted a protective effect in thyrotoxic chicks. Fish solubles had little protective effect by itself and largely nullified the beneficial effect of soy protein when fed together with the latter.

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# Metabolism of $C^{14}$-Nicotinic Acid in Pig and Sheep

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In 1950, Perlzweig and associates, reporting on the metabolism of nicotinic acid in man and 8 other mammalian species (namely, rat, dog, pig, rabbit, guinea pig, sheep, goat, and calf), observed that in 24 hours only 7% of an oral dose of nicotinamide was excreted in the urine of the pig as N-methylnicotinamide, and only 10% as N-methyl-2-pyridone-5-carboxamide (2-PY). In the ruminant they found small amounts of N-methylnicotinamide and 2-PY in the urine; however, the amounts were not increased by giving large doses of nicotinamide or N-methyl-nicotinamide. Only 55% of a dose (1 mg per kg of body weight) of 2-PY given to a pig was recovered in the urine in 24 hours, and 11 to 58% for the ruminant. In our laboratory, after administration of $C^{14}$-2-PY it was found that 2-PY was one of the metabolic end products of nicotinic acid in the rat and only 2-PY was found in its urine (Chang and Johnson, '59).

In the present experiments carboxyl-$C^{14}$-nicotinic acid was administered to a pig and a sheep, and the radioactive metabolites were separated as in previous work with chicks, rats, and humans (Chang and Johnson, '57, '59, '61).

## EXPERIMENTAL

A healthy normal pig weighing about 30 pounds was injected intramuscularly with a saline solution of 2.5 mg of 7-$C^{14}$-nicotinic acid (92.5 μc) plus 118 mg of nonlabeled nicotinic acid. The animal was kept in a metabolism cage, food and water being given ad libitum, and urine was collected.

In each urine collection, the total radioactivity was determined, and the metabolites of nicotinic acid were separated. The animal was killed three days after injection, and muscle, blood, liver, heart, spleen, pancreas, lungs, and kidneys were stored frozen until analysis was performed.

A 78-pound sheep was injected intramuscularly with a saline solution of 2.6 mg of 7-$C^{14}$-nicotinic acid (96.2 μc) plus 298 mg of nonlabeled nicotinic acid. Since the sheep's urine was alkaline (pH9.6 to 10), 3 ml of glacial acetic acid were added to the collection bottle to prevent alkaline hydrolysis of metabolites. The collections were adjusted to pH6.5 to 6.8. The cage was washed after each collection. The animal was kept in the cage for 45 days, and the daily excretion of radioactivity was determined.

The methods of separation and identification of the metabolites have been reported previously (Chang and Johnson, '57).

## RESULTS AND DISCUSSION

*Pig data.* The distribution of urinary radioactivity among the different nicotinic acid metabolites in the urine during a 48-hour collection is shown in table 1. Six metabolites were observed in the second, third and fourth urine collections. They were identified as: N-methylnicotinamide; nicotinuric acid; nicotinic acid; N-methyl-4-pyridone-5-carboxamide (4-PY); 2-PY and nicotinamide. However, in the first urine collection, one and one-half hours after injection, only nicotinuric acid and a trace amount of nicotinic acid were noted. This indicated that the dose rate of 4 mg per pound of body weight was much too great and that nicotinuric acid was immediately excreted as a detoxification product. From the fifth and sixth collections, only three labeled components were separated, corresponding to N-methylnicotinamide, 4-PY and 2-PY. These three metabolites may then be considered to be the persistent metabolites of nicotinic acid. Of these three, 2-PY was found to be the major one,

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TABLE 1

*Distribution of radioactivity (%) in different metabolites of nicotinic acid in pig urine*

| Collection no. | Hours after injection of labeled compound | Radioactivity | | N-methyl-nicotinamide | Nicotinuric acid | Nicotinic acid | 4-PY | 2-PY | Nicotinamide |
|---|---|---|---|---|---|---|---|---|---|
| | | Total count/min $\times 10^{-3}$ | Count/min/hours $\times 10^{-3}$ | | | | | | |
| | | | | % | % | % | % | % | % |
| 1 | 0 – 1.5 | 7,368 | 4,912 | nil | 100 | trace | nil | nil | nil |
| 2 | 1.5 – 4.5 | 12,287 | 4,096 | 28.5 | 52.20 | 0.02 | 2.8 | 15.2 | 1.2 |
| 3 | 4.5 – 6.33 | 2,074 | 1,133 | 23.6 | 7.1 | 0.05 | 15.5 | 53.3 | 0.55 |
| 4 | 6.33–18.33 | 6,498 | 542 | 37.8 | 0.1 | 0.03 | 19.0 | 42.9 | 0.1 |
| 5 | 18.33–24 | 1,894 | 334 | 30.3 | nil | nil | 18.8 | 50.9 | nil |
| 6 | 24 –48 | 5,219 | 217 | 11.1 | nil | nil | 28.1 | 60.8 | nil |

TABLE 2

*Distribution of radioactivity (%) in different metabolites of nicotinic acid in sheep urine*

| Collection no. | Hours after injection of labeled compound | Total count/min $\times 10^{-3}$ | N-methyl-nicotinamide | Trigonelline | Nicotinuric acid | Nicotinic acid | Nicotinamide |
|---|---|---|---|---|---|---|---|
| | *hours* | | % | % | % | % | % |
| 1 | 0 – 1.25 | 10,490 | 0.3 | 0.3 | 61.9 | 36.2 | 1.3 |
| 2 | 1.25– 4.25 | 2,159 | 0.3 | 0.2 | 29.8 | 67.8 | 1.9 |
| 3 | 4.25– 8.25 | 1,609 | 3.0 | nil | 1.8 | 89.3 | 5.9 |
| 4 | 8.25–24 | 264 | nil | nil | nil | nil | 100 |
| 9 | 88 –96 | 62 | nil | nil | nil | nil | 100 |

accounting for 60% of the radioactivity in collection 6. In monkey urine, 2-PY was also found to be the major metabolite (Chang and Johnson, '61); however, 4-PY was the major metabolite in rat urine (Chang and Johnson, '59). This difference presumably reflects species differences in the catabolism of the nicotinamide portion of the pyridine nucleotides.

In all tissues examined, only pyridine nucleotides, nicotinamide, and traces of nicotinic acid were observed. The presence of free nicotinamide in tissues was probably due to postmortem breakdown of pyridine nucleotides, which are readily hydrolyzed after death of the animal (Handler and Klein, '42; Jandorf, '43). The present data, when nicotinic acid is used, are in complete agreement with those of Roth et al. ('48), who reported that endogenous nicotinic acid exists in the animal body in the coenzyme forms and that the breakdown products of these coenzyme forms are immediately excreted in urine.

*Sheep data.* The persistent metabolites of nicotinic acid in sheep urine can be seen (table 2) to be radically different from those observed for the rat, human and pig. Nicotinamide was the only labeled metabolite found after 9 hours from the time of injection of labeled nicotinic acid, and labeled nicotinamide continued to be detected in the urine as long as 45 days after the injection (fig. 1). The nicotinamide presumably comes from the metabolism of labeled pyridine nucleotides, indicating the

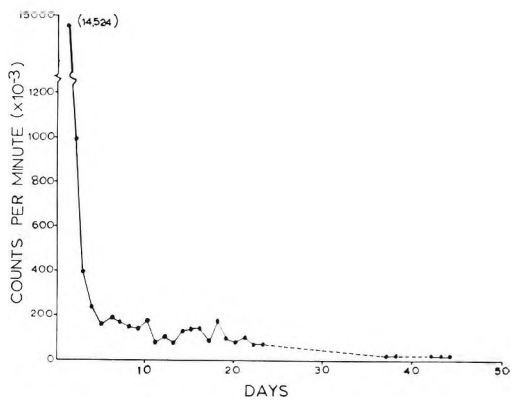


Fig. 1 Daily excretion of radioactive compounds in urine of sheep given $C^{14}$-nicotinic acid. The dotted lines indicate that no urine was collected.

limited capacity of the ruminant for methylation as compared with other species. This is in agreement with earlier less definitive studies (Johnson et al., '47; Hopper and Johnson, '55) with calves. It is further substantiated by the absence of methylated compounds (except for traces of N-methylnicotinamide in the urine, contrary to the report of Perlzweig et al. ('50).

On the other hand, the sheep behaves as other species do in detoxifying and excreting rapidly excess levels of nicotinic acid as nicotinuric acid. The trigonelline noted may be an artifact formed due to the alkaline conditions in the bladder.

## SUMMARY

Following the administration of 7-$C^{14}$-nicotinic acid intramuscularly into a pig, 6 metabolites were observed in the urine. These were N-methylnicotinamide, nicotinuric acid, nicotinic acid, 4-pyridone, 2-pyridone, and nicotinamide. Of these, the 2-pyridone and 4-pyridone were the major persistent metabolies coming from the breakdown of the pyridine nucleotides.

Three days after administering $C^{14}$-nicotinic acid, only pyridine nucleotides and nicotinamide were noted in the pig tissues.

In sheep urine, nicotinamide was the only persistent metabolite, whereas nicotinuric acid and nicotinic acid were the major excretion products during the first 8 hours after administration of the labeled nicotinic acid.

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