# Copper and Zinc Interrelationships in the Pig[1]

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In a series of three experiments conducted at the Michigan Station (Hoefer et al., '60),[2] it was observed that 125 ppm of dietary copper (0.05% copper sulfate) had a significant effect on swine growth, particularly at higher calcium levels. When both copper and zinc were added to a ration, the combination proved no more effective than either element alone; but 50 ppm of zinc caused a fivefold reduction in liver-copper stores. The latter results are similar to those reported by other workers (Allen et al., '58; Davis, '58; Barber et al., '60). Although less effective than zinc, copper reduced the incidence and severity of parakeratosis in all three trials. Wallace et al. ('60) also found a copper-zinc interrelationship with respect to parakeratosis. Bunch et al.[3] reported that supplemental zinc appeared necessary for maximal response to supplemental copper.

The purpose of the experiment reported here was to compare the effects of adding various levels and combinations of copper and zinc to a high calcium diet. It was designed to study a higher level of copper sulfate than had previously been investigated at this station. It was the hope of the authors to either confirm or refute the interrelationships between copper and zinc observed in earlier studies.

## EXPERIMENTAL

Sixty weanling pigs, averaging approximately 7 weeks of age, were divided uniformly into 6 lots and fed for a 15-week experimental period. The pigs were maintained in concrete-floor pens and had free access to water and feed at all times. They were vaccinated for hog cholera, sprayed for mange, and wormed during the early stages of the trial. Feed and growth data were collected at two-week and, in some cases, one-week intervals. Composition of the basal ration was essentially the same as that fed in experiment 2 of a previous study (Hoefer et al., '60). This was a rather typical growing ration except that it furnished twice as much calcium, 1.3%, as currently recommended by the National Research Council sub-committee on swine nutrition ('59). Protein level was 15.6%; phosphorus, 0.49%; zinc, 42.2 ppm; and copper, 13.1 ppm. Protein was reduced to 12.5% when the pigs averaged 125 pounds by reducing the soybean meal and increasing the corn. This change had no appreciable effect on the mineral content of the ration except for a 30% reduction in zinc.

Design of the ration treatments was as follows: lot 1, basal; lot 2, basal plus 0.05% copper sulfate (125 ppm Cu); lot 3, basal plus 0.1% copper sulfate (250 ppm Cu); lot 4, basal plus 0.0125% zinc oxide (100 ppm Zn); lot 5, basal plus zinc (100 ppm) and copper (125 ppm); lot 6, basal plus zinc (100 ppm) and copper (250 ppm).

When symptoms of parakeratosis in lot 1 (basal) appeared to be in the most severe stage, the pigs in this lot were divided equally according to weight and degree of dermatosis into two lots, which were referred to as lots 1A and 1B. Division of the control lot occurred approximately 10 weeks (71 days) after the experiment had started. Lot 1A continued to receive the unsupplemented basal ration while lot 1B was given dietary therapy in the form of

TABLE 1

*Effect of supplemental copper and zinc on pig performance, 0–10 weeks*

| Lot | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| | Basal (1.3% Ca) | Cu 125[1] | Cu 250 | Zn 100 | Zn 100 + Cu 125 | Zn 100 + Cu 250 |
| No. pigs[2] | 8 | 8 | 8 | 9 | 9 | 9 |
| Initial wt, pounds | 24.8 | 24.1 | 24.1 | 24.2 | 23.9 | 24.0 |
| 10-week wt, pounds | 59.0 | 101.6 | 109.7 | 107.7 | 113.1 | 112.3 |
| Daily gain, pounds[3] | 0.48 ± 0.07[4] | 1.09 ± 0.07 | 1.21 ± 0.07 | 1.18 ± 0.05 | 1.26 ± 0.04 | 1.24 ± 0.07 |
| Feed/gain | 3.33 | 2.87 | 2.66 | 2.76 | 2.82 | 2.79 |
| PK[5], 10 weeks, % | 88 | 0 | 0 | 0 | 0 | 0 |

[1] All supplemental additions of copper and zinc are in ppm.
[2] Two pigs removed from lots 1, 2 and 3, and one from lots 4, 5 and 6 as a result of pneumonia.
[3] Lot 1 significantly lower than all other lots (P < 0.01).
[4] SE of the mean.
[5] Parakeratosis.

copper sulfate at a level of 250 ppm of copper.

The lots fed the copper supplement were observed closely for symptoms of copper toxicity; autopsies were performed by the Department of Veterinary Pathology, Michigan State University.

After the fifteenth week, the pigs were slaughtered and their livers were obtained for colorimetric analyses of copper (AOAC, '55), zinc (Johnson et al., '59) and iron (Bandemer and Schaible, '44). Just prior to slaughter, a number of pigs on each treatment were bled for hemoglobin and hematocrit determinations.

The data were treated statistically by analysis of variance. Treatment means were compared by the multiple range test of Duncan ('55).

RESULTS AND DISCUSSION

Table 1 compares performance of the 6 treatments during the first stage of the trial, from zero to 10 weeks. It was necessary to remove one or two pigs from each lot shortly after the trial began, as a result of pneumonia. During the first 10 weeks, growth of the basal pigs was subnormal and they were significantly outgained by all 5 experimental lots. However, lots 2 through 6 were not significantly different from one another. The separate effects of copper and zinc were analyzed statistically and found to be highly significant (P < 0.01), as was their interaction. The pigs fed the basal ration required somewhat more feed per pound of gain than pigs in the supplemented lots, but there were no appreciable differences between the latter in feed conversion.

From 6 to 8 weeks after the experiment began, pigs in lot 1 began showing skin lesions typical of parakeratosis, as a result of the high calcium basal ration they were consuming. Seven out of 8 pigs, or 88% of this lot, developed the condition. Two of these were considered quite severe and the other 5 ranged from mild to severe. In general, there was a direct relationship between degree of dermatosis and retardation of growth. None of the pigs in the supplemented lots showed any symptoms of parakeratosis during the first 10 weeks. These observations are in good agreement with those of Wallace et al. ('60) who reported that parakeratosis was prevented in pigs receiving a corn-soybean meal diet supplemented with 150 ppm of copper for a 70-day experimental period. However, they are in direct contrast with the work of O'Hara et al. ('60), who observed the unexpected development of parakeratosis in pigs fed 0.1% copper sulfate for 10 weeks but found no occurrence of this condition in pigs fed a basal ration containing wheat and meat meal.

Table 2 shows results of the period from 10 to 15 weeks and summarizes the entire trial, from 0 to 15 weeks. The pigs, represented by lot 1A (basal), showed some spontaneous dermal improvement during the final 5 weeks but failed to recover completely; their average growth rate was considerably below normal. Although their progress was not as rapid or spectacular as is usually seen with zinc therapy, pigs in lot 1B experienced a marked recovery from the parakeratotic symptoms, and at the end of 15 weeks only one of the 4 pigs exhibited any skin le-

## TABLE 2

Effect of supplemental copper and zinc on pig performance from 10 to 15 weeks and for entire trial, 0–15 weeks

| Lot | 1A[1] | 1B[1] | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Treatment | Basal (1.3% Ca) | Cu 250[2] | Cu 125 | Cu 250 | Zn 100 | Zn 100+ Cu 125 | Zn 100+ Cu 250 |
| No. of pigs | 4 | 4 | 7[3] | 6[4] | 9 | 9 | 9 |
| 10-Week wt, pounds[5] | 60.3 | 57.8 | 98.4 | 110.2 | 107.7 | 113.1 | 112.3 |
| 15-Week wt, pounds | 86.3 | 102.0 | 140.9 | 152.8 | 158.1 | 160.7 | 164.4 |
| Daily gain, pounds[6] | 0.74±0.32[7] | 1.26±0.29 | 1.21±0.08 | 1.22±0.16 | 1.44±0.06 | 1.36±0.08 | 1.49±0.08 |
| Feed/gain | 3.78 | 2.74 | 3.90 | 4.52 | 4.27 | 3.89 | 3.90 |
| PK,[8] 10 weeks, % | 75 | 100 | 0 | 0 | 0 | 0 | 0 |
| PK, 15 weeks, % | 75 | 25 | 86 | 17 | 0 | 0 | 0 |
| Performance, 0–15 weeks | | | | | | | |
| Daily gain, pounds[9] | 0.58±0.18 | — | 1.10±0.06 | 1.21±0.06 | 1.26±0.04 | 1.29±0.05 | 1.32±0.06 |
| Feed/gain | — | — | 3.21 | 3.21 | 3.33 | 3.19 | 3.20 |

[1] Lot 1 (basal) was divided after 10 weeks into lots 1A and 1B.
[2] All supplemental additions of copper and zinc are in ppm.
[3] One pig removed for reasons unrelated to treatment. *Escherichia coli* isolated from kidney, spleen and bladder.
[4] Two pigs died as a result of copper poisoning. A third pig died from same cause after trial was terminated.
[5] Differences in 10-week weights were result of prior treatment differences.
[6] Lot 1A significantly lower than all other lots: 1B, 2, 3 (P < 0.05) and 4, 5, 6 (P < 0.01).
[7] SE of the mean.
[8] Parakeratosis.
[9] Lot1A significantly lower than all other lots (P < 0.01). Lot 6 significantly greater than lot 2 (P < 0.05).

sions. On the other hand, all three of the pigs in lot 1A that initially carried lesions still showed some evidence of them at the end of the trial.

In a recent experiment,[4] similar improvement in performance and skin condition was observed when pigs afflicted with parakeratosis were fed half the level of copper (125 ppm) used as therapy in the present study. These data will be reported in a subsequent paper.

Average daily gains of lots 1B through 6 were all significantly different from lot 1A but not from one another due to considerable variation within treatments.

The 125 ppm level of copper used in lot 2 was successful in delaying the onset of parakeratosis until the fourteenth week, when 6 out of 7, or 86%, of the pigs in that lot suddenly developed a dermatosis; two of these were considered quite severe. Rate of gain of lot 2 declined sharply during the final two weeks of the trial. The soybean meal content of the lot 2 ration had been reduced after the twelfth week. Because the low-protein ration contained less zinc than its high-protein counterpart (29.3 vs. 40.6), this change in diet may have been a factor influencing the development of parakeratosis in lot 2.

The higher copper level was apparently more effective in preventing parakeratosis since only one pig in lot 3 developed any parakeratotic lesions and they were very mild. However, several pigs in this lot exhibited symptoms of copper toxicity — severe anemia, internal hemorrhages, jaundice, yellow cirrhotic livers, gastric ulceration, loss of weight, weakness and incoordination. These symptoms resemble those reported by other workers (Allen and Harding, '62; Buntain, '61; Gordon and Luke, '57; O'Hara et al., '60; Wallace et al., '60). Two pigs in lot 3 died suddenly during the thirteenth week and another died after the fifteenth week. Although accurate diagnoses were difficult because of extensive postmortem changes, it appeared likely these three pigs died as a result of copper poisoning. Two other pigs in this lot were close to death when they were submitted for autopsy after the fifteenth week. There was little doubt these two pigs were suffering from copper toxicity. Dyspnea was

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[4] Unpublished data.

observed in one of these pigs after he was driven a short distance. A similar observation was made by O'Hara et al. ('60), who reported respiratory distress in pigs suffering from copper poisoning as a result of feeding 250 ppm of copper. In the latter investigation, the authors noted a sudden liberation of copper into the blood and proposed that this was associated with the development of a severe anemia, which in turn resulted in anoxia, circulatory failure and death.

One of the three remaining pigs in lot 3 had an enlarged cirrhotic liver and his carcass was condemned by the inspecting veterinarian due to a pronounced, generalized icterus. The other two pigs appeared normal in every respect except for the "muddy" color of their livers.

Studies at the Florida Station have demonstrated that copper toxicity in rats (McCall, '61) and swine (Wallace et al., '60) may be alleviated by increasing the protein content of the ration to relatively high levels. Perhaps some degree of protection against copper toxicosis was removed from lot 3 when their dietary protein was reduced to 12.5% after the twelfth week.

It was very apparent in this experiment that zinc offered considerable protection against copper toxicity since none of the pigs in lot 6 showed any signs of toxic effects. Furthermore, there was no evidence of toxicity in pigs receiving 125 ppm of copper, with or without zinc (lots 2 and 5). Had the experiment continued for an indefinite period of time, it would have been enlightening to observe if and when the pigs in lot 1B might have shown symptoms of copper poisoning. Results of this trial would seem to confirm the data of Wallace et al. ('60) which suggest that the margin between safe and toxic copper levels is relatively narrow. It is also apparent that effects of feeding high copper levels should not be evaluated on the basis of short-term trials. For example, copper toxicity in lot 3 and the onset of parakeratosis in lot 2 would not have been observed had the present experiment been terminated after the first 10-week period.

A summary of the entire trial shows there was a trend for improved average daily gain as dietary copper level was increased and as zinc was added to the ration. The animals in lot 6 gained significantly faster ($P < 0.05$) than those in lot 2 and the difference in gain between lots 5 and 2 approached significance. Feed conversion ratios for the treated lots were virtually identical.

The effect of copper thereapy on parakeratosis is illustrated in figure 1. These
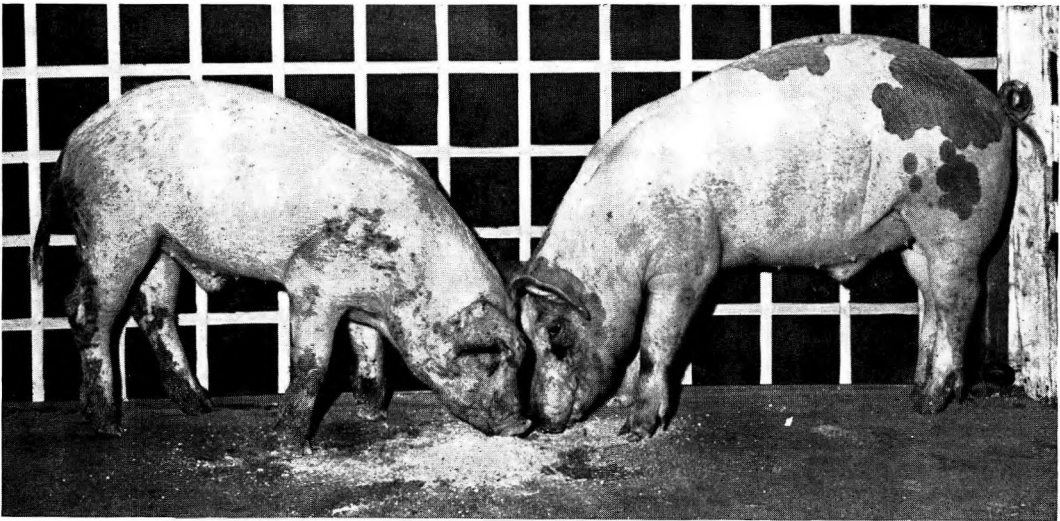


Fig. 1   Typical effect of copper therapy on parakeratosis. Both pigs weighed 60 pounds after receiving high-calcium basal diet for 10 weeks. Pig on left continued to be fed basal ration while pig on right was fed 250 ppm of supplemental copper for 5 weeks; difference in weight then was 38 pounds.

TABLE 3

*Hematology of pigs fed supplemental copper and zinc*

| Lot | 1A[1] | 1B[1] | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Treatment | Basal (1.3% Ca) | Cu 250[2] | Cu 125 | Cu 250 | Zn 100 | Zn 100+ Cu 125 | Zn 100+ Cu 250 |
| No. of pigs | 4 | 4 | 7 | 3 | 7 | 6 | 6 |
| Hemoglobin, gm %[3] | 11.8±0.7[4] | 10.8±1.1 | 12.4±0.6 | 8.4±2.8 | 13.2±0.4 | 12.8±0.5 | 12.3±0.9 |
| Hematocrit[5] | 35.5±1.6 | 37.5±1.5 | 39.0±2.0 | 27.7±7.4 | 40.7±1.0 | 39.5±1.3 | 37.6±2.7 |

[1] Lot 1 (basal) was divided after 10 weeks into lots 1A and 1B.
[2] All supplemental additions of copper and zinc are in ppm.
[3] Lot 3 significantly lower than lot 1A ($P < 0.05$) and 2, 4, 5, 6 ($P < 0.01$).
[4] SE of the mean.
[5] Lot 3 significantly lower than all other lots: 1A, 1B, 6 ($P < 0.05$) and 2, 4, 5, ($P < 0.01$).

two pigs were typical of their respective lots, both at 10 and at 15 weeks.

Hematological data of lots 1A through 6 may be compared in table 3. Hemoglobin and hematocrit values of pigs in lot 3 were significantly lower than those of the other treatments, which was undoubtedly a reflection of the copper toxicity prevalent in that lot. Although differences between the other lots were not statistically significant, there was a tendency for hemoglobin and hematocrit values to decline as dietary copper was increased, and to rise when the ration was supplemented with zinc.

Bunch et al. ('61) and Wallace et al. ('60) have also shown that hemoglobin concentrations are depressed with increased copper levels. However, the latter investigators found that supplemental zinc failed to alter the subnormal hemoglobin levels of pigs fed 200 ppm of copper. Šimek et al. ('61) reported that pigs receiving high levels of copper sulfate had lower hemoglobin concentrations but higher hematocrit values. This is contradictory to the present data except for lots 1A and 1B, where such a relationship exists.

Table 4 shows that by adding 125 or 250 ppm of copper to the basal ration, liver-copper levels were increased by 11- and 60-fold, respectively. Concentration of liver-copper in lot 3 was comparable to levels (684 to 1,800 ppm) observed by O'Hara et al. ('60) in the livers of pigs suffering from chronic copper poisoning. Buntain ('61) and Gordon and Luke ('57) have found liver-copper levels of 710 to 2,200 ppm and 2,160 to 2,500 ppm, respectively, in pigs that apparently died as a result of copper toxicity. Allcroft et al. ('61) reported a mean liver-copper level of 1,725 ppm in pigs fed a diet containing 0.1% copper sulfate for approximately 5 months. Unfortunately, in the present study, copper was not determined on livers from the three pigs that died of copper poisoning.

Liver-copper levels were dramatically reduced when zinc was added to the copper-supplemented rations. This is compatible with the observation that pigs receiving 250 ppm of copper with no added zinc developed symptoms of copper toxicity, whereas those receiving the same level of

TABLE 4

*Trace mineral content of livers from pigs fed diets supplemented with copper and zinc[1]*

| Lot | 1A[2] | 1B[2] | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Treatment | Basal (1.3% Ca) | Cu 250[3] | Cu 125 | Cu 250 | Zn 100 | Zn 100 + Cu 125 | Zn 100 + Cu 250 |
| No. of pigs | 4 | 4 | 7 | 5 | 9 | 9 | 9 |
| Liver wt, gm[4] | 1080 ± 134[5] | 1122 ± 192 | 1227 ± 87 | 1315 ± 88 | 1293 ± 72 | 1239 ± 34 | 1197 ± 35 |
| Copper, ppm[6] | 25 ± 13 | 437 ± 121 | 276 ± 57 | 1448 ± 101 | 19 ± 1 | 70 ± 22 | 419 ± 86 |
| Zinc, ppm[7] | 83 ± 12 | 88 ± 11 | 103 ± 15 | 126 ± 17 | 207 ± 14 | 272 ± 29 | 288 ± 36 |
| Iron, ppm[8] | 299 ± 92 | 125 ± 27 | 325 ± 32 | 96 ± 7 | 537 ± 42 | 420 ± 21 | 225 ± 40 |

[1] Expressed in ppm, dry weight basis.
[2] Lot 1 (basal) was divided after 10 weeks into lots 1A and 1B.
[3] All supplemental additions of copper and zinc are in ppm.
[4] No significant differences among lots.
[5] SE of the mean.
[6] Lot 3 significantly higher than all other lots ($P < 0.01$). Lots 1B, 2 and 6 significantly higher than 1A, 4 and 5 ($P < 0.01$).
[7] Lots 4, 5 and 6 significantly higher than all other lots ($P < 0.01$).
[8] Lot 4 significantly higher than 1A, 1B, 2, 3 and 6 ($P < 0.01$). Lot 5 significantly higher than 1B, 3 and 6 ($P < 0.01$). Lots 1A and 2 significantly higher than 1B and 3 ($P < 0.01$). Lot 6 significantly higher than lot 3 ($P < 0.05$).

copper with added zinc showed no evidence of toxicity.

Davis ('58) has reported that a high level of copper in the liver, such as may occur with copper toxicity, will result in an almost complete elimination of zinc from liver tissue. However, in the present trial, increased levels of copper resulted in no depression of liver-zinc; in fact there was a slight, but not significant, tendency for the storage of liver-zinc to increase as copper increased.

There was an inverse relationship between iron and copper concentrations in the liver (table 4). This is in agreement with the observations of Cassidy and Eva,[5] who reported that copper appeared to exert a depressing effect upon iron storage in the liver.

## SUMMARY

In summary, the following results were obtained in this study:

1. Copper sulfate, when added to a high calcium (1.3%) basal diet at the rate of 125 or 250 ppm of copper, stimulated pig growth and prevented parakeratosis during all but the last week of a 15-week trial. The 250 ppm level of copper was much more effective in preventing parakeratosis than the lower level.

2. Symptoms of copper toxicity were observed after 12 weeks in pigs receiving 250 ppm of copper without added zinc. These pigs exhibited significant reductions in hemoglobin and hematocrit and their livers were found to contain extremely high levels of copper.

3. When 100 ppm of zinc were added to copper-supplemented rations, there was an improvement in growth rate; however, combinations of zinc with copper were essentially no better than zinc alone. Zinc prevented parakeratosis in all lots to which it was fed and appeared to furnish complete protection against copper poisoning. Supplemental zinc markedly reduced liver-copper levels.

4. An inverse relationship was observed between concentrations of iron and copper in the liver.

5. After 10 weeks, one-half the pigs fed the basal diet began receiving the same ration plus 250 ppm of copper. A slow but marked recovery from parakeratosis occurred during the ensuing 5 weeks; there was no gross evidence of a toxicosis resulting from the copper therapy.

[5] Cassidy, J., and J. K. Eva 1958 Relationship between the copper and iron concentrations in pigs' livers. Proc. Nutrition Soc. (British), 17: XXXI (abstract).

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# Effect of a Low Dietary Level of Three Types of Fat on Reproductive Performance and Tissue Lipid Content of the Vitamin B₆-deficient Female Rat

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In a previous study, female rats of a mixed strain (BHE) were reared from weaning with a complete purified diet containing 15% fat, and deprived of pyridoxine at 80 days of age, a minimum of three weeks before mating. Under these conditions, animals fed hydrogenated shortening produced fewer than one-half as many young as animals fed corn oil (Brown, '60). The present study was undertaken to determine the effect of a low level of three dietary fats fed with and without pyridoxine supplementation on reproductive performance and tissue lipid content of female rats.

## EXPERIMENTAL

Weanling rats of the Sprague Dawley strain were fed synthetic diets containing 5% of either corn oil, a commercially hydrogenated vegetable oil (HVO), or olive oil as the source of dietary fat. A fourth group fed 15% HVO was included to compare with previous work using the BHE strain of rats, a mixed strain genetically susceptible to kidney damage. Each dietary fat was obtained from one commercial lot in order to minimize variation in intake.

The diet was similar to one used previously in this laboratory (Brown, '60) except that the level of fat was adjusted to 5% by replacement of an equal weight of sucrose.

When the animals were approximately 80 days old, roughly two-thirds of the animals in each group fed 5% fat and all animals receiving 15% fat were changed to the diet from which pyridoxine was omitted. Three weeks later animals were mated with stock males at the appropriate time in the estrous cycle as indicated by vaginal smears. Mating was confirmed by the presence of sperm in the vaginal smear. The day on which sperm was found was considered day one of pregnancy. On the twenty-second day of pregnancy just prior to delivery animals were anesthetized by sodium amytal injection and samples obtained for analyses. Approximately 8 ml of blood were removed by cardiac puncture and centrifuged to obtain serum for cholesterol, phospholipid, and transaminase determination. The contents of the uterus were removed and young were weighed. The liver, heart, brain, and kidney were weighed and frozen separately.

Lipid extracts of liver, heart and brain were prepared by the method of Folch et al. ('57). Total fat was obtained by evaporating an aliquot of the chloroform-methanol extract to dryness in a previously weighed flask, drying in an oven at 100°C for 30 minutes and allowing to stand at room temperature for 90 minutes before weighing. Additional aliquots of the extract were freed of solvent by evaporation for analysis of total cholesterol and phospholipid. For cholesterol determination, total lipid was dissolved in ethyl alcohol and the procedure taken from the point of saponification by the method described by Koval ('61). For phospholipid determination, extracts were ashed in micro-Kjeldahl flasks with a mixture of 10:1:1 nitric, sulfuric and perchloric acids, made up to volume with distilled water, and suitable aliquots analyzed for phosphorus according to the method of Chen et al. ('56). The usual factor of 25 was used for conversion of lipid phosphorous to phospholipid.

Serum glutamic oxalacetic transaminase (GOT) was analyzed by the method of Humoller et al. ('57). Serum cholesterol was determined by the micro-fluorometric method described by Koval ('61) and phospholipid by the Lowry method ('54) with minor adaptations for use with serum samples.

The data were subjected to an analysis of variance using the Duncan and Bonner test for significance (Duncan and Bonner, '54).

## RESULTS

*Growth.* After 8 weeks' maintenance with the complete diet, average body weights of animals fed the three fats were similar, ranging from 227 gm for animals fed 5% HVO to 238 for animals fed 5% corn oil. When the groups were divided at 8 weeks, animals given the diet without pyridoxine gained slowly, at a rate about one-third to one-half that of those fed the diet containing pyridoxine. There were no significant differences in growth rate associated with type of dietary fat.

*Total food and fatty acid intake.* Average daily food intakes and calculated fatty acid intakes for the three week gestation period are shown in table 1. Animals deprived of dietary pyridoxine ate about 30% less food than did animals fed the vitamin, but there were no significant differences associated with type of dietary fat.

Intakes of major component fatty acids were calculated from gas-liquid chromatographic analyses of the fats. The linoleic acid fraction of HVO included approximately 24% of an incompletely separated unknown component. Daily intakes of linoleic acid, therefore, more nearly approximated 62 mg for animals fed 5% HVO with pyridoxine, 42 mg for animals fed 5% HVO without pyridoxine, and 126 mg for those fed 15% HVO without pyridoxine.

*Reproductive performance.* Differences at the 1% level of statistical significance in the criteria for reproductive performance were associated only with the presence or absence of pyridoxine in the diet (table 2). Average maternal weight gains ($P = 0.01$) and average fetal weights ($P = 0.01$) were greater, and the average number of resorptions less ($P = 0.01$) in animals fed pyridoxine than in those fed the diets without pyridoxine. There were no significant differences in litter size among any of the groups. The difference of 0.6 gm between average fetal weight of groups fed 5% corn oil and 5% HVO without pyridoxine was significant only at the 5% level.

Despite the failure to find statistical differences, however, there was observed within groups without dietary pyridoxine a trend for most criteria to reveal a slight advantage in animals fed corn oil over

TABLE 1

*Average daily food intake and fatty acid intake of pregnant rats fed three types of fat with and without pyridoxine*

| Diet | Avg daily intake | | Avg daily fatty acid intake[1] | | | |
|---|---|---|---|---|---|---|
| | Food | Fat | Palmitic | Stearic | Oleic | Linoleic |
| | *gm* | *gm* | *mg* | *mg* | *mg* | *mg* |
| With pyridoxine | | | | | | |
| 5% Corn oil | 17.3 | 0.87 | 100 | 11 | 235 | 510 |
| 5% Olive oil | 17.5 | 0.88 | 164 | 14 | 548 | 131 |
| 5% HVO[2] | 18.2 | 0.91 | 117 | 77 | 632[3] | 81[4] |
| Without pyridoxine | | | | | | |
| 5% Corn oil | 13.0 | 0.65 | 75 | 8 | 177 | 384 |
| 5% Olive oil | 12.8 | 0.64 | 120 | 10 | 401 | 96 |
| 5% HVO | 12.3 | 0.62 | 79 | 52 | 427[3] | 55[4] |
| 15% HVO | 12.4 | 1.86 | 240 | 158 | 1290[3] | 166[4] |

[1] Calculated from area per cent fatty acids identified by gas-liquid chromatographic analysis using the Barber-Colman Model 10 and 15 instruments. Analyses obtained through courtesy of Dr. Katherine J. Hivon and Mrs. Susie N. Hagan, Food Composition Laboratory.

[2] Hydrogenated vegetable oil.

[3] Contained a trace of nonadecanoic acid.

[4] Contained approximately 24% of an incompletely separated unknown which may be an isomer of linoleic acid of unknown biological value.

## TABLE 2

Reproductive performance of female rats fed three types of fat with and without pyridoxine

| Diet | No. of animals | No. of successful pregnancies | Avg no. days depletion | Avg maternal wt gain | Body wt at term | Avg litter size | Avg fetal wt | Avg no. of resorptions |
|---|---|---|---|---|---|---|---|---|
| | | | | gm | gm | | gm | |
| With pyridoxine | | | | | | | | |
| 5% Corn oil | 12 | 9 | 0 | 130 ± 9[1] | 395 ± 13 | 10.4 ± 1.3 | 5.0 ± 0.1 | 0.6 ± 0.2 |
| 5% Olive oil | 9 | 9 | 0 | 126 ± 6 | 376 ± 10 | 9.2 ± 0.9 | 5.3 ± 0.1 | 0.9 ± 0.3 |
| 5% HVO[2] | 10 | 10 | 0 | 131 ± 11 | 386 ± 15 | 9.8 ± 1.5 | 4.7 ± 0.1 | 0.7 ± 0.2 |
| Without pyridoxine | | | | | | | | |
| 5% Corn oil | 20 | 15 | 37 ± 3 | 73 ± 6 | 319 ± 7 | 9.1 ± 1.0 | 4.1 ± 0.2 | 2.3 ± 0.7 |
| 5% Olive oil | 21 | 14 | 36 ± 4 | 63 ± 6 | 299 ± 6 | 8.2 ± 1.0 | 3.8 ± 0.2 | 2.8 ± 0.7 |
| 5% HVO | 23 | 15 | 38 ± 3 | 63 ± 6 | 296 ± 9 | 8.5 ± 1.0 | 3.5 ± 0.2 | 3.5 ± 0.8 |
| 15% HVO | 19 | 10 | 38 ± 3 | 67 ± 5 | 307 ± 6 | 8.9 ± 0.9 | 3.7 ± 0.2 | 2.2 ± 0.7 |

[1] SE of mean.
[2] Hydrogenated vegetable oil.

those fed the other two fats. This was most evident for the criterion of fetal weight; the proportion of animals in the group fed corn oil carrying young above the average weight for all animals deprived of pyridoxine was almost twice that for the other vitamin B₆-deficient groups.

Data presented on reproductive performance included only those animals in which at least one live fetus was present in the uterus at necropsy. Three out of 12 animals fed the complete diet containing corn oil mated as indicated by the presence of sperm in the vaginal smear, but showed no evidence of implantation at the end of the three-week period. In the vitamin B₆-deficient groups, in addition to the failure to implant following mating, resorption of entire litters also reduced the ratio of successful pregnancies in relation to the number of animals in the group (table 2). The lowest ratio of successful pregnancies was observed in the deficient group fed 15% HVO in which 4 animals failed to mate after repeated trials. Only in the two groups fed HVO without pyridoxine were there animals that failed to mate.

*Serum cholesterol, phospholipid and transaminase.* Serum cholesterol was significantly lower ($P = 0.01$) in animals fed either level of HVO as compared with those given the other two fats (table 3). Vitamin B₆ deprivation had no significant effect on serum cholesterol.

There was a tendency to lower concentrations of phospholipid in serum of animals fed HVO also, but the differences were not statistically significant.

The activity of glutamic oxalacetic transaminase in serum, expressed as spectrophotometric units, was approximately 40% lower in deficient animals than in animals fed pyridoxine, a statistically significant difference ($P = 0.01$). Type of dietary fat had no effect on serum transaminase activity.

*Liver, heart, and brain lipids.* Few differences were observed in the concentration of lipids in organs studied. Total lipid was similar for liver and heart, respectively, in all diet groups (table 4). Liver cholesterol concentration was significantly higher ($P = 0.01$) in animals fed HVO than in those fed the other two fats. In heart tissues, cholesterol concentration was

TABLE 3

*Average values of cholesterol, phospholipid and transaminase of maternal serum*

| Diet | No. of animals | Cholesterol | Phospholipid | Transaminase |
|---|---|---|---|---|
| | | mg/100 ml | mg/100 ml | units/ml |
| With pyridoxine | | | | |
| 5% Corn oil | 9 | 114 ± 4[1] | 227 ± 13 | 76 ± 4(8)[2] |
| 5% Olive oil | 8 | 113 ± 5 | 229 ± 12 | 85 ± 6(9) |
| 5% HVO[3] | 9 | 89 ± 6 | 187 ± 11 | 78 ± 6(10) |
| Without pyridoxine | | | | |
| 5% Corn oil | 14 | 103 ± 5 | 206 ± 8 | 45 ± 4(11) |
| 5% Olive oil | 12 | 98 ± 4 | 209 ± 8 | 47 ± 3(11) |
| 5% HVO | 14 | 87 ± 4 | 193 ± 9 | 49 ± 5(15) |
| 15% HVO | 8 | 86 ± 4 | 185 ± 11 | 44 ± 5(8) |

[1] SE of mean.
[2] Number of animals.
[3] Hydrogenated vegetable oil.

TABLE 4

*Average values of total fat, cholesterol and phospholipid of liver, heart and brain*

| Diet | No. of organs analyzed | Wt of organ | Total fat | Cholesterol | Phospholipid |
|---|---|---|---|---|---|
| | | gm | % wet tissue | mg/gm wet tissue | mg/gm wet tissue |
| Liver | | | | | |
| With pyridoxine | | | | | |
| 5% Corn oil | 9 | 13.5 ± 0.6[1] | 4.64 ± 0.07 | 2.67 ± 0.10 | 27.4 ± 0.7 |
| 5% Olive oil | 9 | 12.5 ± 0.4 | 4.59 ± 0.09 | 2.73 ± 0.13 | 27.5 ± 0.4 |
| 5% HVO[2] | 9 | 13.4 ± 0.6 | 4.74 ± 0.05 | 3.20 ± 0.16 | 28.0 ± 0.6 |
| Without pyridoxine | | | | | |
| 5% Corn oil | 15 | 10.6 ± 0.3 | 4.58 ± 0.08 | 2.64 ± 0.06 | 26.3 ± 0.4 |
| 5% Olive oil | 14 | 9.9 ± 0.4 | 4.75 ± 0.10 | 2.55 ± 0.07 | 27.1 ± 0.4 |
| 5% HVO | 13 | 10.4 ± 0.4 | 4.58 ± 0.15 | 2.98 ± 0.12 | 25.9 ± 1.2 |
| 15% HVO | 10 | 11.1 ± 0.5 | 4.68 ± 0.20 | 3.04 ± 0.16 | 27.0 ± 1.0 |
| Heart | | | | | |
| With pyridoxine | | | | | |
| 5% Corn oil | 9 | 0.94 ± 0.05 | 3.78 ± 0.12 | 1.39 ± 0.01 | 23.4 ± 0.4 |
| 5% Olive oil | 9 | 0.90 ± 0.02 | 3.69 ± 0.07 | 1.43 ± 0.02 | 23.9 ± 0.1 |
| 5% HVO | 10 | 0.92 ± 0.02 | 3.60 ± 0.05 | 1.37 ± 0.02 | 22.8 ± 0.4 |
| Without pyridoxine | | | | | |
| 5% Corn oil | 11 | 0.78 ± 0.02 | 3.62 ± 0.06 | 1.49 ± 0.03 | 23.2 ± 0.4 |
| 5% Olive oil | 12 | 0.76 ± 0.02 | 3.63 ± 0.04 | 1.56 ± 0.06 | 23.4 ± 0.3 |
| 5% HVO | 13 | 0.77 ± 0.02 | 3.61 ± 0.07 | 1.47 ± 0.06 | 22.8 ± 0.4 |
| 15% HVO | 8 | 0.80 ± 0.03 | 3.78 ± 0.05 | 1.44 ± 0.06 | 23.5 ± 0.2 |

[1] SE of mean.
[2] Hydrogenated vegetable oil.

significantly higher (P = 0.01) in animals deprived of pyridoxine than in those given the vitamin, but there were no differences related to type of dietary fat. No differences among groups were observed in brain lipid composition. The overall averages for this organ were total lipid, 8.8%, cholesterol, 17.2 mg/gm, and phospholipid, 45.2 mg/gm wet tissue.

*Comparison of Sprague Dawley and BHE animals fed 15% HVO without pyri-* *doxine.* A comparison was made of corresponding data on Sprague Dawley animals fed 15% HVO without pyridoxine in the present study, and previous work using BHE animals maintained under the same experimental conditions (Brown, '60). The data are shown in table 5.

The ratio of successful pregnancies to total number of animals was about one-half in Sprague Dawley rats and one-third in the BHE strain. Despite a slightly longer

TABLE 5

*Comparison of Sprague Dawley and mixed strain (BHE) pregnant rats fed 15% hydrogenated vegetable oil without pyridoxine*

| Average data | Sprague Dawley strain | BHE strain |
|---|---|---|
| No. animals | 19 | 26 |
| No. successful pregnancies | 10 | 9 |
| No. days depletion | 38 | 33 |
| Daily food intake, gm | 12.4 | 10.9 |
| Total maternal wt gain, gm | 67 | 45 |
| Weight gain/gm food, gm | 0.258 | 0.196 |
| Litter size | 8.9 | 7.3 |
| Fetal wt, gm | 3.7 | 3.8 |
| No. resorptions | 2.2 | 2.0 |
| Body wt at term, gm | 307 | 275 |
| Liver wt, gm | 11.1 | 10.4 |
| Liver fat, % | 4.7 | 3.5 |
| Liver cholesterol mg/gm tissue | 3.04 | 2.68 |
| mg/gm liver fat | 65.0 | 73.3 |
| Serum cholesterol, mg/100 ml | 86 | 77 |

average period of vitamin $B_6$ deprivation, Sprague Dawley animals gained more weight during gestation in relation to food intake than BHE animals and produced slightly larger litters. Sprague Dawley animals were about 30 gm heavier at term and had correspondingly heavier livers. Both liver fat and liver cholesterol concentration were greater in Sprague Dawley animals than in the BHE group, but liver cholesterol constituted a higher proportion of liver fat in the BHE strain. Serum cholesterol levels were slightly higher in the Sprague Dawley females.

### DISCUSSION

The slight advantage in reproductive performance observed in vitamin $B_6$-deficient Sprague Dawley females fed 5% corn oil as compared with those fed either olive oil or HVO in the present study is similar to the trend reported previously for the BHE strain fed a 15% dietary level of corn oil and HVO. Differences observed between animals fed corn oil and HVO were much less striking, however, in the

present study. Comparison of data on the two strains indicated that reproductive performance of Sprague Dawley strain rats fed 15% HVO without pyridoxine was superior to that of BHE strain animals under similar experimental conditions. Some of the differences between the two strains appear to be related to differences in the utilization of food, Sprague Dawley animals showing a more efficient utilization.

When young were carried to term, lowest birth weights occurred in the vitamin $B_6$-deficient group fed 5% HVO. In animals receiving an adequate supply of pyridoxine, the weight of young of mothers fed HVO was less than the 5 gm generally considered to be compatible with survival in the rat (Sica and Cerecedo, '48). Kummerow et al. ('52) found that young failed to survive for more than three days after birth when their mothers were reared with a fat-free diet and given a complete diet containing 5% HVO during gestation, and concluded that 5% HVO is inadequate for successful lactation. The results of the present study indicate that 5% HVO is barely adequate for satisfactory reproduction as well. This suggests that the low fetal weights and high incidence of resorptions of the group fed 5% HVO without pyridoxine were due to limited intake of linoleic acid as well as to the vitamin $B_6$ deficiency. Linoleic acid intakes of vitamin $B_6$-deficient rats fed the other fats were above the level of 80 mg/day suggested by Deuel et al. ('54) as the amount permitting optimal survival in the rat.

Differences in lipid composition of maternal organs were related in some instances to the type of dietary fat and in others to the vitamin $B_6$ deficiency. There was no evidence of interaction between the two. The lower serum cholesterol and concomitant higher liver cholesterol concentration of rats fed 5 and 15% HVO as compared with animals fed corn oil and olive oil suggest that the type of fat rather than the level of dietary linoleic acid per se is the more important factor. A similar conclusion was reached by Jagannathan ('62) from observations of serum cholesterol in male monkeys and in liver cholesterol of cholesterol-fed male rats fed diets containing different dietary fat mixtures

providing the same level of linoleic acid. A comparison between the two studies, however, cannot be made without reservation. Besides the well known differences in cholesterol metabolism associated with species, sex and with cholesterol feeding in the rat, serum cholesterol has been shown to increase by approximately 50% during pregnancy (Schwenk and Joachim, '61).

These factors may well account for the lack of agreement of the results obtained in the present study with those of Klein ('58). In male Holtzman rats fed 5 and 30% HVO and corn oil, Klein ('58) found that plasma cholesterol increased as dietary linoleic acid increased, but that liver cholesterol, chiefly the ester fraction, was increased in fat deficiency, low in normal ranges of dietary fat, and increased again at high levels of linoleic acid.

That serum and liver cholesterol were unaffected by vitamin B₆ deficiency is in agreement with the report of Shah et al. ('60). These workers report a greater incorporation of labeled acetate into liver cholesterol in vitamin B₆-deficient rats which was not reflected in serum or liver cholesterol levels or by a change in degradation of cholesterol via the bile. The increase observed in cholesterol concentration of heart of vitamin B₆-deficient animals in the present study may reflect in part increased cholesterol biosynthesis with distribution among tissues other than serum and liver, although under the conditions of this study, heart tissue obviously is a minor site of deposition. Pregnancy per se may be ruled out as a contributing factor since there appears to be no difference in rate of biosynthesis of cholesterol in pregnant and nonpregant rats (Schwenk and Joachim, '60).

The tendency of corn oil to improve reproductive response of the vitamin B₆-deficient pregnant animals in this study is consistent although it is not striking. The high linoleic acid content of corn oil as compared with the other two fats is an attractive explanation in view of previous studies suggesting a relationship between vitamin B₆ and EFA (Sherman, '50). This explanation, however, is difficult to reconcile with recent reports opposing a significant biochemical relationship between

vitamin B₆ and essential fatty acid metabolism (Johnston et al., '61; Kirschman and Coniglio, '61; Swell et al., '61) and with the lack of interaction of vitamin B₆ deficiency and type of dietary fat in affecting tissue lipid content and serum glutamic oxalacetic transaminase (GOT) of the animals in the present study.

Increasing the level of HVO from 5 to 15% improved reproductive performance by increasing maternal weight gain and average weight of young, and in reducing number of resorptions. However, the low ratio of successful pregnancies of rats fed 15% HVO suggests an adverse effect of some constituent of this fat. The HVO used in this study contained a high proportion of oleic acid and in animals fed 15% HVO, daily intake of oleic acid was at least three times that of other deficient animals. There is some indication of an antagonistic effect of oleic acid in vitamin B₆ deficiency (Sarma and Snell, '47). Although there is no direct evidence to support this suggestion, it appears that the character of dietary fat and the level at which it is fed influence the reproductive response of the vitamin B₆-deficient female rat.

SUMMARY AND CONCLUSIONS

Reproductive performance and tissue lipid composition were studied in Sprague Dawley strain female rats fed from weaning diets containing 5% of corn oil, olive oil or HVO or 15% HVO, and deprived of vitamin B₆ a minimum of three weeks prior to mating.

Although the differences were not statistically significant, animals fed corn oil tended to show an advantage over other deficient animals in reproductive performance, most importantly in the criterion of fetal weight. Mating performance of rats fed 15% HVO was somewhat less successful than animals fed 5% of either corn oil, olive oil or HVO, but not as poor as previously observed in another strain of animals.

Differences in organ lipid composition and serum glutamic oxalacetic transaminase (GOT) were related to type of dietary fat or to vitamin B₆ deprivation. Serum cholesterol concentration was lower and liver cholesterol concentration higher in animals fed either level of HVO than in

animals fed 5% of corn oil or olive oil. Cholesterol concentration of heart tissue was higher in vitamin $B_6$-deficient mothers than in those fed the vitamin. Serum GOT was lower in vitamin $B_6$-deficient animals than in animals fed pyridoxine, but there were no differences due to type of dietary fat.

The slight beneficial effect of corn oil on reproductive performance of vitamin $B_6$-deficient female rats was not reflected either in organ lipid content or in serum glutamic oxalacetic transaminase.

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# Effect of Certain Factors on Nitrogen Retention and Lysine Requirements of Adult Human Subjects

## III.  SOURCE OF SUPPLEMENTARY NITROGEN [1]

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Although major emphasis must be placed on adequate intakes of the essential amino acids, consideration also should be given to the nonessential amino acids because they may provide as much as two-thirds of the total nitrogen in self-selected diets. The importance of supplying compounds from which nonessential amino acids can be synthesized has been demonstrated in different species and under varying conditions. Men did not maintain nitrogen equilibrium when essential amino acids were administered in the absence of nonessential amino acids (Rose and Wixom, '55).

Investigators have used different sources of supplementary nitrogen when establishing amino acid requirements of human subjects, as glycine or urea, or both (Rose and Wixom, '55), glycine alone or with diammonium citrate (Leverton et al., '56), and a combination of glycine, diammonium citrate and glutamic acid (Clark et al., '57). Evidence that sources of nitrogen differ in their effectiveness for the growing rat (Rose et al., '49; Rechcigl et al., '57; Birnbaum et al., '57) indicated the necessity of determining whether the source of supplementary nitrogen would affect retention of nitrogen by human subjects. Nitrogen balances of men and women therefore were determined when a basal diet that provided adequate amounts of essential amino acids was supplemented with glutamic acid, glycine and diammonium citrate in varying proportions.

### PROCEDURE

The experimental approach was similar to that described earlier (Clark et al., '57)

except that L-isomers of all essential amino acids including isoleucine were fed and the source of supplementary nitrogen was varied as indicated hereafter.

*Basal diet.* The basal diet contained 159 gm of white wheat flour and 21 gm of cornmeal. These were served as baking powder biscuits, cornbread and shortbread. Each portion of shortbread contained 11.0 gm of flour, 4.0 gm of sugar and 7.5 gm of butter oil. Vitamins,[4] minerals and limited amounts of fruits and juices were provided. A mixture of essential amino acids was prepared that supplemented the basal diet in such a manner that total daily intakes of essential amino acids were similar to those in 20 gm of egg protein (Clark et al., '57). In experiment 1 the cereals, essential amino acid mixture and low-nitrogen foods contained, respectively, 3.13, 0.92 and 0.27 gm of nitrogen. To provide a total daily intake of 9.00 gm of nitrogen, the quantity of each supplementary source was adjusted to supply 4.68 gm of nitrogen. The distribution of nitrogen was similar in experiment 2.

*Lysine.* Biscuits, cornbread and shortbread contained 117, 115 and 102 mg of

lysine/gm of nitrogen, respectively, and supplied 265, 77 and 20 mg of lysine/day. The basal diet which provided 400 mg of lysine was supplemented with L-lysine monohydrochloride. Subjects consumed 1,500 mg of lysine in the initial adjustment periods, and either 900 mg (exp. 1) or 850 mg (exp. 2) during all experimental periods. These quantities were estimated from regression equations based on body size and creatinine excretion (Clark et al., '60) to provide a small but variable margin above the minimal requirements of the participants.

*Sources of supplementary nitrogen.* In preceding experiments (Clark et al., '57, '60, '62), the basal diet and essential amino acids were supplemented with a mixture of glycine, glutamic acid and diammonium citrate, each of which provided one-third of the nitrogen. Glycine was incorporated because it contains a high percentage of nitrogen, and glutamic acid because it participates actively in transamination reactions. In planning the present series, testing of other nonessential amino acids was considered. In foods such as milk, eggs, beef, legumes and wheat, glutamic acid usually is present in the highest concentration, aspartic acid or proline is next, then alanine or serine and finally glycine (Orr and Watt, '57). However, the interconversion of glycine and serine, the formation of alanine, aspartic acid and glutamic acid by transamination and the synthesis of proline from glutamic acid suggested that incorporation of all of these was unnecessary. Furthermore, young men maintained nitrogen equilibrium when limited quantities of essential amino acids were supplemented with glycine alone (Rose and Wixom, '55). Certain nonessential amino acids or their derivatives were excluded because present cost makes them unsuitable for long-term experiments with human subjects. For these reasons, only glycine, glutamic acid and diammonium citrate were evaluated as sources of supplementary nitrogen.

The advisability of using these compounds individually to provide the requisite quantity of nitrogen also was examined. Although the consequences of excess glycine in some species were known (Arnstein, '54), no report of adverse effects in man of either glycine or diammonium citrate was found in the literature when the first experiment was initiated. Glycine and diammonium citrate therefore were tested singly and together. The subsequent report by Swendseid et al. ('60) that glycine was not as well utilized for the maintenance of nitrogen equilibrium in human subjects as a mixture of glycine and diammonium citrate, suggested that the results obtained with glycine should be confirmed. Therefore this amino acid was retested as a single supplement in a pilot study and then in a second experiment. Glutamic acid was fed only in combination with glycine or diammonium citrate, or with both.

Six treatments were compared in experiment 1: Glycine only; diammonium citrate only; glycine plus diammonium citrate; glycine plus glutamic acid; glutamic acid plus diammonium citrate; glycine plus glutamic acid and diammonium citrate. In experiment 2, the standard mixture containing glycine, glutamic acid and diammonium citrate was compared with glycine alone and with a mixture in which glycine supplied twice as much nitrogen as glutamic acid. Each component of a mixture provided the same quantity of nitrogen except that in one mixture (exp. 2) glycine supplied twice as much as glutamic acid. The weight of each compound consumed daily is shown in table 1. One-third of the daily quota was served at each meal.

The amounts of glycine and glutamic acid in the basal diet were constant. Baked products contained 203 mg of glycine, cornbread 144 mg of glutamic acid and biscuits and shortbread 190 mg/gm of nitrogen. The amounts of these foods consumed daily in experiment 1 provided 0.63 gm of glycine and 5.62 gm of glutamic acid (table 1). It was estimated that the foods also supplied 0.65 gm of alanine, 0.90 gm of aspartic acid, 2.06 gm of proline and 0.92 gm of serine.

*Subjects.* Senior or graduate students between 20 and 23 years of age participated (table 2). Three male and three female subjects started in experiment 1 but subjects DC and CO withdrew because of severe respiratory infections.

TABLE 1

*Daily intakes of glycine, glutamic acid and diammonium citrate from foods and supplements[1]*

| Supplementary nitrogen source[2] | Glycine | | Glutamic acid | | Diammonium citrate supplement |
|---|---|---|---|---|---|
| | Supplement | Total | Supplement | Total | |
| | *gm* | *gm* | *gm* | *gm* | *gm* |
| | | Experiment 1 | | | |
| G, GA, DC | 8.35 | 8.98 | 16.38 | 22.0 | 12.59 |
| G, GA | 12.53 | 13.16 | 24.56 | 30.18 | |
| G, DC | 12.53 | 13.16 | | 5.62 | 18.89 |
| GA, DC | | 0.63 | 24.56 | 30.18 | 18.89 |
| G | 25.07 | 25.70 | | 5.62 | |
| DC | | 0.63 | | 5.62 | 37.76 |
| | | Experiment 2 | | | |
| G, GA, DC | 8.13 | 8.78 | 15.93 | 21.81 | 12.24 |
| G, GA | 16.26 | 16.91 | 15.93 | 21.81 | |
| G | 24.39 | 25.04 | | 5.88 | |

[1] To supply 4.68 gm of nitrogen in experiment 1 and 4.60 gm in experiment 2.
[2] Each component of a mixture provided the same proportion of nitrogen except in the mixture tested in experiment 2 in which glycine supplied twice as much nitrogen as gluatmic acid. G indicates glycine; GA, glutamic acid; DC, diammonium citrate.

TABLE 2

*Description of subjects*

| Subject | Sex | Mean weight | Height | Surface area | Caloric intake | Creatinine |
|---|---|---|---|---|---|---|
| | | *kg* | *cm* | *m²* | | *gm/day* |
| | | | Experiment 1 | | | |
| EV | F | 51.8 | 156 | 1.47 | 2500 | 1.03 |
| RDL | M | 83.8 | 169 | 1.59 | 3700 | 1.66 |
| MCM | F | 56.7 | 168 | 1.70 | 2400 | 1.03 |
| JY | M | 67.3 | 185 | 1.80 | 3100 | 1.60 |
| DC | M | 73.7 | 188 | 1.96 | 3600 | 1.89 |
| CO | F | 63.4 | 165 | 1.67 | 2550 | 1.13 |
| RJ | F | 62.4 | 169 | 1.70 | 2400 | 1.30 |
| AH | M | 71.4 | 174 | 1.84 | 3600 | 1.84 |
| | | | Experiment 2 | | | |
| FA | M | 62.5 | 168 | 1.68 | 3300 | 1.44 |
| KE | F | 52.7 | 161 | 1.51 | 2150 | 1.06 |
| JS | F | 58.6 | 165 | 1.63 | 2700 | 1.09 |
| TS | M | 76.8 | 180 | 1.93 | 3450 | 1.52 |
| PT | F | 62.0 | 160 | 1.61 | 2300 | 1.02 |

*Calories.* Caloric intakes were modified as necessary during the adjustment period and kept constant thereafter. The total caloric intakes stated in table 2 include a variable allowance for each supplementary nitrogen source.

*Plan of experiment.* All subjects attained nitrogen equilibrium during an adjustment period of 12 days. Intakes of foods, total nitrogen and lysine were constant during experimental periods which were 6 or 8 days in length. Because nitrogen retention tends to improve as an experiment progresses (Clark et al., '62), the sources of supplementary nitrogen were so distributed that all mixtures would be tested in each period and administered to each subject in a different sequence.

*Analyses.* Nitrogen in foods, supplements and excreta was determined by a macro-Kjeldahl method. Urinary nitrogen and creatinine values were determined daily and fecal nitrogen values for each period.

Lysine, glycine and glutamic acid in baked products and in urine composites

representing each period were assayed microbiologically. *Leuconostoc mesenteroides* P-60 (ATCC 8042) was the test organism in all assays. One-gram samples of ground ether-extracted baked products were hydrolyzed with 20 ml of 2.7 N HCl at 15 p.s.i. for 7 hours for lysine, and 5 hours for glycine and glutamic acid. Aliquots of 25 ml of urine were hydrolyzed with 5 ml of 11 N HCl for 5 hours for the assay of lysine and glutamic acid. Volumes were reduced to one-third in the glycine assay. Hydrolysates were adjusted to pH 6.8 for lysine and glycine and to pH 6.4 for glutamic acid. Titrations were carried to the same pH values as the hydrolysates. The basal medium described by Steele et al. ('49) for *L. mesenteroides* P-60 was used with minor modification. The amino acid being measured was omitted from the medium, and in the glutamic acid assay aspartic acid also was omitted.

The photometric ninhydrin method of Troll and Cannan ('53) was used for determination of α-amino nitrogen. Analytical and theoretical values for amino acids agreed closely and recoveries from amino acids were more satisfactory than with the copper method (Block and Weiss, '51). Free α-amino nitrogen was determined in all amino acid supplements, total amino nitrogen in foods and both free and total amino nitrogen in urine. Samples were hydrolyzed as for assay of amino acids.

### RESULTS

*Body weight.* The subjects maintained their initial weights or gained less than 0.4 kg except subject EV who gained 0.9 kg.

*Creatinine excretion.* Mean daily creatinine values were relatively constant for each individual regardless of treatment (table 2).

*Nitrogen retention in experiment 1.* Mean daily nitrogen balances of 4 subjects (table 3) are supported by data obtained for subjects DC and CO in certain periods. Mean nitrogen balances resulting from all treatments were positive, and ranked in descending order as follows: glycine plus glutamic acid, 0.62 gm; glycine, glutamic acid and diammonium citrate, 0.51 gm; glycine alone, 0.39 gm; glutamic acid plus diammonium citrate, 0.34 gm; glycine plus diammonium citrate, 0.27 gm; and diammonium citrate alone, 0.08 gm. When Duncan's new multiple range test (Duncan, '55) was applied at the 5% level, a significant difference was detected between the extreme values; that is, the balance resulting from the mixture of glycine and glutamic acid in contrast with diammonium citrate alone. Other values did not differ significantly from each other. Differences between nitrogen balances of individual subjects were highly significant ($P < 0.01$), the retention of subject RDL being greater than that of all others. Fecal nitrogen was not influenced by the nature of the supplement.

Nitrogen retention evidently was influenced by the source of supplementary nitrogen when the cereal-containing basal diet provided one-half of the daily intake of 9.0 gm of nitrogen and when lysine intake approximated minimal needs. Although the number of subjects who completed the first experiment was too limited to obtain definitive results, the following observations were made: (1) glycine, glutamic acid and diammonium citrate permitted satisfactory nitrogen retention when used together or in pairs; (2) diammonium citrate depressed nitrogen retention when it supplied as much as 4.68 gm of nitrogen but glycine did not; and (3) mixtures in which diammonium citrate supplied one-half of the supplementary nitrogen tended to be less satisfactory than the mixture in which it provided one-third of the nitrogen, although the difference was not statistically significant.

To test further the efficiency of glycine as a single supplement, subjects RJ and AH consumed for 30 days a basal diet like that used in experiment 1, supplemented with glycine to provide 4.60 gm of nitrogen. Mean fecal nitrogen of RJ was 0.59 gm and of AH 0.65 gm. Following a 12-day adjustment period in which nitrogen balance was positive, RJ retained 0.32 and 0.29 gm of nitrogen daily in two successive 9-day periods. No adverse effects of consuming this quantity of glycine were observed or reported by RJ or by AH who responded similarly.

TABLE 3

*Mean daily nitrogen balances and urinary excretion of certain amino acids in experiment 1[1]*

| Supplementary nitrogen source[2] | Subject | Nitrogen | | Lysine | Glycine | Glutamic acid | Free α-amino nitrogen |
|---|---|---|---|---|---|---|---|
| | | In urine | Balance[3] | | | | |
| | | *gm* | *gm* | *gm* | *gm* | *gm* | *gm* |
| G, GA, DC | EV | 8.12 | +0.20 | 0.040 | 0.58 | 0.22 | 0.35 |
| | RDL | 7.23 | +1.08 | 0.049 | 0.66 | 0.25 | 0.33 |
| | MCM | 7.65 | +0.45 | 0.038 | 0.67 | 0.16 | 0.41 |
| | JY | 8.09 | +0.31 | 0.036 | 0.53 | 0.23 | 0.40 |
| | Mean | 7.77 | +0.51 | 0.041 | 0.61 | 0.22 | 0.37 |
| G, GA | EV | 8.54 | −0.22 | 0.046 | 0.29 | 0.10 | 0.21 |
| | RDL | 6.69 | +1.62 | 0.044 | 0.87 | 0.27 | 0.44 |
| | MCM | 7.72 | +0.38 | 0.032 | 0.95 | 0.20 | 0.48 |
| | JY | 7.71 | +0.69 | 0.037 | 0.71 | 0.26 | 0.35 |
| | Mean | 7.66 | +0.62 | 0.040 | 0.71 | 0.21 | 0.37 |
| G, DC | EV | 8.29 | +0.03 | 0.048 | 0.74 | 0.22 | 0.38 |
| | RDL | 7.72 | +0.59 | 0.048 | 0.89 | 0.29 | 0.46 |
| | MCM | 7.85 | +0.25 | 0.030 | 0.87 | 0.19 | 0.43 |
| | JY | 8.17 | +0.23 | 0.041 | 0.63 | 0.25 | 0.38 |
| | Mean | 8.01 | +0.27 | 0.042 | 0.78 | 0.24 | 0.41 |
| GA, DC | EV | 8.15 | +0.17 | 0.022 | 0.24 | 0.24 | 0.19 |
| | RDL | 7.46 | +0.85 | 0.041 | 0.46 | 0.25 | 0.29 |
| | MCM | 7.74 | +0.36 | 0.031 | 0.40 | 0.16 | 0.34 |
| | JY | 8.41 | −0.01 | 0.033 | 0.27 | 0.17 | 0.27 |
| | Mean | 7.94 | +0.34 | 0.032 | 0.34 | 0.21 | 0.27 |
| G | EV | 7.87 | +0.45 | 0.045 | 1.50 | 0.28 | 0.61 |
| | RDL | 7.52 | +0.79 | 0.045 | 1.68 | 0.32 | 0.59 |
| | MCM | 7.80 | +0.30 | 0.035 | 1.78 | 0.26 | 0.65 |
| | JY | 8.36 | +0.04 | 0.041 | 1.51 | 0.29 | 0.64 |
| | Mean | 7.89 | +0.39 | 0.042 | 1.62 | 0.29 | 0.62 |
| DC | EV | 8.18 | +0.14 | 0.049 | 0.34 | 0.20 | 0.39 |
| | RDL | 8.29 | +0.02 | 0.044 | 0.39 | 0.22 | 0.38 |
| | MCM | 7.96 | +0.14 | 0.028 | 0.42 | 0.18 | 0.33 |
| | JY | 8.39 | +0.01 | 0.040 | 0.29 | 0.22 | 0.33 |
| | Mean | 8.20 | +0.08 | 0.040 | 0.36 | 0.20 | 0.36 |

[1] Total intake of nitrogen, 9.0 gm, and of lysine, 900 mg.
[2] G indicates glycine; GA, glutamic acid, and DC, diammonium citrate.
[3] Calculated with mean fecal nitrogen values, gm/day: subject EV, 0.68; RDL, 0.69; MCM, 0.90; and JY, 0.60.

*Nitrogen balances in experiment 2.* Mean nitrogen balances (table 4) were +0.49, +0.48 and +0.35 gm, respectively, when the following treatments were tested: glycine, glutamic acid and diammonium citrate; glycine to supply two-thirds and glutamic acid one-third of the nitrogen; and glycine alone. Nitrogen balances did not differ significantly from each other.

The mean balance obtained with the mixture of glycine, glutamic acid and diammonium citrate was almost identical with that observed in experiment 1 (table 3). The mean balances of the two groups that consumed supplementary glycine also were similar. Application of the *t* test to the pooled data for the 9 subjects in the two separate experiments revealed no significant difference between nitrogen balances obtained with glycine alone or with the standard mixture of glycine, glutamic acid and diammonium citrate.

*Urinary excretion of certain amino acids.* Since the excretion of essential amino acids might be altered if the nitrogenous supplement influenced their utilization, urinary lysine was measured in

TABLE 4

*Mean daily nitrogen balances and urinary excretion of glycine and*
*a-amino nitrogen in experiment 2[1]*

| Supplementary nitrogen source[2] | Subject | Nitrogen | | Glycine | Free a-amino nitrogen |
|---|---|---|---|---|---|
| | | In urine | Balance[3] | | |
| | | *gm* | *gm* | *gm* | *gm* |
| G, GA, DC | FA | 7.53 | +0.75 | 0.58 | 0.43 |
| | KE | 7.84 | +0.46 | 0.49 | 0.40 |
| | JS | 8.22 | +0.23 | 0.63 | 0.40 |
| | TS | 8.07 | +0.26 | 0.43 | 0.33 |
| | PT | 7.54 | +0.73 | 0.85 | 0.46 |
| | Mean | 7.84 | +0.49 | 0.60 | 0.40 |
| G, GA | FA | 7.94 | +0.34 | 0.84 | 0.53 |
| | KE | 7.67 | +0.64 | 1.10 | 0.55 |
| | JS | 7.64 | +0.81 | 1.09 | 0.54 |
| | TS | 7.92 | +0.42 | 0.68 | 0.38 |
| | PT | 8.06 | +0.21 | 1.45 | 0.58 |
| | Mean | 7.85 | +0.48 | 1.03 | 0.52 |
| G | FA | 8.00 | +0.28 | 1.60 | 0.75 |
| | KE | 8.01 | +0.30 | 2.04 | 0.72 |
| | JS | 7.79 | +0.66 | 1.64 | 0.57 |
| | TS | 8.14 | +0.20 | 1.03 | 0.63 |
| | PT | 7.95 | +0.32 | 2.34 | 0.73 |
| | Mean | 7.98 | +0.35 | 1.73 | 0.68 |

[1] Total intake of nitrogen, 9.0 gm, and of lysine, 850 mg.
[2] G indicates glycine; GA, glutamic acid, and DC, diammonium citrate.
[3] Calculated with mean fecal nitrogen, gm/day: subject FA, 0.72; KE, 0.69; JS, 0.55; TS, 0.66; and PT, 0.73.

experiment 1.[5] Mean urinary lysine values in experiment 1 (table 3) varied from 0.03 to 0.04 gm/day, i.e., from 3.6 to 4.7% of the 900 mg ingested, and were not influenced by the nitrogenous supplement being tested. Highly significant differences ($P < 0.01$) existed between subjects, RDL excreting more lysine than MCM or JY. Lysine excretion accounted for only 0.008 gm of urinary nitrogen per day. It was not related to body size or sex.

The total quantities of glycine consumed (table 1) and excreted (table 3) varied widely. When no supplementary glycine was added to the basal diet which contained 0.63 gm of this amino acid, urinary glycine values were 0.34 and 0.36 gm. Excretion increased to 0.61 gm when 8.98 gm of glycine were consumed, to 0.71 and 0.78 gm when 13.16 gm of glycine were ingested, and to 1.62 gm when 25.70 gm of glycine were present. Although urinary glycine was significantly higher ($P < 0.01$) when dietary glycine was 25.70 gm than during other treatments, only 6.3% of the ingested glycine appeared in the urine in comparison with 5.4 to

6.8% when glycine supplements were smaller. Differences between subjects in respect to urinary glycine were highly significant ($P < 0.01$), values of RDL and MCM being higher than others. Nitrogen present in urinary glycine increased from 0.06 gm when only the basal diet was consumed to 0.30 gm when 25.70 gm of glycine were ingested. Total urinary nitrogen, however, was approximately the same when glycine was the only supplement as when it was used in smaller amounts, and was significantly lower than when diammonium citrate was tested alone. There is therefore no evidence that provision of relatively large quantities of supplementary glycine decreased nitrogen retention.

Mean urinary excretion of glutamic acid (table 3) varied only from 0.20 to 0.29 gm despite a range in intake from 5.62 to 30.18 gm. There was, however, a significant difference ($P < 0.05$) between treatments. Unexpectedly, urinary glutamic acid was higher when glycine was the

[5] Vermillion, E. J. 1961 Urinary excretion of lysine, glycine and glutamic acid by human subjects. M.S. Thesis, Purdue University, Lafayette, Indiana.

only supplement than when any other treatment except glycine plus glutamic acid was tested. Subject RDL excreted more glutamic acid ($P < 0.05$) than EV or MCM. Nitrogen present in urinary glutamic acid did not exceed 0.03 gm.

In experiment 2, only glycine was determined (table 4). Again, excretion was related directly to intake, being 6.9% in all periods. When 8.78, 16.91 and 25.04 gm of glycine were consumed, subjects excreted 0.60, 1.03 and 1.73 gm, respectively, of glycine which contained 0.11, 0.19 and 0.32 gm of nitrogen. Nitrogen balances, however, did not differ significantly despite the increased glycine output which resulted from the highest intake of glycine.

*Alpha-amino nitrogen.* The basal diet contained 1.87 gm of total α-amino nitrogen.[6] The essential amino acid mixture contained 0.50 gm of free α-amino nitrogen, and the 6 supplementary sources tested in the first experiment varied from 2.33 to 4.38 gm. The total amounts of α-amino nitrogen consumed when different treatments were tested in experiment 1 were as follows, in gm:[7] G, GA, DC, 6.18; G, GA, 6.74; G, DC, 5.73; GA, DC, 5.71; G, 6.75; and DC, 4.70. In experiment 2, intakes of α-amino nitrogen differed little with treatment (6.15, 6.72 and 6.72 gm).

Free α-amino nitrogen in the urine (table 3) was significantly higher ($P < 0.01$) when glycine was the only supplement than when glutamic acid and diammonium citrate were fed together. The other sources produced intermediate values which did not differ from each other. These data are consistent with the increased intake of α-amino nitrogen and the higher excretion of glycine in certain periods. Free α-amino nitrogen values did not differ between subjects. The percentages of total urinary nitrogen present as free α-amino nitrogen in response to the various supplements were, in decreasing order: G, 7.8; G, DC, 5.3; G, GA, 5.0; G, GA, DC, 4.7; DC, 4.4 and GA, DC, 3.7.

In experiment 2, differences between free α-amino nitrogen values in the urine were highly significant ($P < 0.01$). Excretion was related directly to dietary glycine. Subject TS excreted significantly smaller quantities of α-amino nitrogen

than did other subjects. When glycine supplied one-third, two-thirds and all of the supplementary nitrogen, free α-amino nitrogen comprised 5.1, 6.7 and 8.4% of total urinary nitrogen.

*Acceptability of supplements.* In the first experiment, glycine alone was rated by all subjects as the most acceptable source, followed by the standard mixture containing equal amounts of nitrogen from glycine, glutamic acid and diammonium citrate. The combinations of two compounds were acceptable to some but unpleasant to others. The quantity of diammonium citrate required to provide 4.68 gm of nitrogen was described by all as bitter, irritating and undesirable for use throughout an experiment. All mixtures tested in the second experiment were acceptable, but the subjects ranked glycine first and the mixture of three components last. Mixtures that provided 2.34 gm of nitrogen as glutamic acid were difficult to keep in solution.

## DISCUSSION

The nitrogen balance data presented in tables 3 and 4 confirm earlier reports from this laboratory (Clark et al., '57, '60, '62) that a daily lysine intake of 900 mg not only maintains equilibrium but permits retention of nitrogen by most men and women. It is therefore an appropriate quantity to administer to groups during investigation of the influence of specific factors on nitrogen retention when time does not permit establishment of individual requirements.

The demonstration that nitrogen retention of human subjects may be influenced by the nature and quantity of a particular source of supplementary nitrogen is worthy of consideration in the planning of experiments and in the potential utilization of such sources for the improvement of dietaries. The effectiveness of glycine as the only source of nitrogen is in accord with the observation by Rose and Wixom ('55) that men attained nitrogen equilibrium when twice the minimal quantities of the 8 essential amino acids were supplemented

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[6] Goodwin, A. F. 1961 Urinary excretion of alpha-amino nitrogen by human subjects. M.S. Thesis, Purdue University, Lafayette, Indiana.
[7] G indicates glycine, GA glutamic acid, and DC, diammonium citrate.

with enough glycine to supply between 5.71 and 1.21 gm of nitrogen. In contrast, Swendseid et al. ('60) reported that glycine was not as well utilized as glycine and diammonium citrate or a mixture of nonessential amino acids. They supplied an amount of glycine comparable to that administered in this laboratory, but always tested glycine first. Under the conditions of their experiment a qualitative or quantitative interrelationship among the dietary components may have affected nitrogen retention unfavorably when only glycine was added. On the other hand, the generous amounts of arginine, methionine and choline provided by the cereals and supplements in the experiment reported herein may have facilitated the utilization of glycine.

Conclusions drawn from the present experiment should be applied only to healthy young men and women. Glycine is not entirely satisfactory for growth of the young. Rose et al. ('49) rated glycine as slightly inferior to diammonium citrate or glutamic acid for rat growth. Rechcigl and co-workers ('57) reported that glycine and diammonium citrate produced poor responses, whereas glutamic acid stimulated rapid growth. Birnbaum et al. ('57) concluded that ammonium acetate and ammonium glutamate were distinctly superior to glycine. Other deleterious effects of large amounts of glycine have been reported (Arnstein, '54). Differences in response between species emphasize the necessity of testing in human subjects those compounds which might serve as nitrogenous supplements or might influence nitrogen utilization. Moreover, demands for growth undoubtedly differ from those for maintenance.

The observation in this laboratory that human subjects retained less nitrogen when 4.68 gm of nitrogen were supplied by diammonium citrate than when this compound was omitted from the supplement is puzzling. Since total urinary nitrogen was higher but free $\alpha$-amino nitrogen was lower when diammonium citrate was fed alone than in other periods (table 3), production of urea may have exceeded that which occurred when glutamic acid or glycine replaced all or part of the diammonium citrate. Lardy and Feldott ('50) suggested that a quantitative relationship may exist between the essential amino acids and diammonium citrate. Rechcigl et al. ('57) stated that high levels of this compound may be toxic in growing animals.

The quantities of nitrogen present in the urine as lysine and glutamic acid were too small to influence nitrogen retention, and even the increased output of glycine, when it was the only supplement, did not depress nitrogen retention significantly. The limited amount of glutamic acid in the urine agreed with observations of Steele et al. ('50) who noted a direct relationship between intake and output of this amino acid and of glycine. Jagenburg ('59) in a comprehensive review stated that glycine is generally the predominant amino acid in urine. This is in contrast with its relatively low concentration in foods. Variability in the amount of each amino acid excreted by individuals is characteristic (Steele et al., '50; Jagenburg, '59). This may partially explain differences in nitrogen balances among a group of subjects.

Free $\alpha$-amino nitrogen excretion was definitely influenced by the source of supplementary nitrogen, especially when either glycine, which enters transamination reactions more slowly than does glutamic acid, or diammonium citrate, which is not a constituent of foods, was used alone. Nevertheless, not more than 5.3% of the total urinary nitrogen was present as free $\alpha$-amino nitrogen in most periods, the maximum being 8.7%. In most experiments reported in the literature, dietary nitrogen has been modified by altering the amounts of certain foods rather than supplements of amino acids. Steele[8] reported that under such conditions the excretion of $\alpha$-amino nitrogen declined as protein intake decreased. Jagenburg ('59) also concluded that food influenced urinary excretion of $\alpha$-amino nitrogen.

## SUMMARY

Glycine, glutamic acid and diammonium citrate were tested as sources of

[8] Steele, B. F. 1949 The Influence of diet on the amino acid content of biological fluids. Ph.D. Thesis, University of Wisconsin, Madison.

supplementary nitrogen for young adult human subjects who consumed a diet containing cereals and purified essential amino acids. Lysine intake was either 850 or 900 mg, and quantities of other essential amino acids also exceeded minimal requirements. The daily intake of nitrogen was 9.0 gm, of which approximately one-half was provided by a supplementary source. In the first experiment, glycine and diammonium citrate were tested alone and in combination with each other and with glutamic acid. In the second experiment, glycine was tested alone, with glutamic acid, and with glutamic acid and diammonium citrate.

Mean nitrogen balances varied from + 0.08 to + 0.62 gm. Nitrogen retention was significantly higher when glycine and glutamic acid were used together (+ 0.62 gm) than when diammonium citrate was administered alone (+ 0.08 gm). Other combinations of glycine, diammonium citrate and glutamic acid did not differ significantly from each other or from glycine alone. Urinary excretion of free α-amino nitrogen and of glycine was related directly to glycine intake. Urinary lysine was not influenced by the source of supplementary nitrogen.

## ACKNOWLEDGMENTS

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# Effect of Potassium Iodide and Duodenal Powder on the Growth and Organ Weights of Goitrogen-fed Rats[1]

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It has been reported that growth arrest induced by the ingestion of goitrogens, could be prevented or overcome by the addition to the diet of certain animal tissues (Ackerman, '59). An assay based on the reinstatement of growth of growth-arrested rats, was used to screen a variety of tissues for their growth-promoting activity, and it was observed that duodenal powder consistently evoked a growth response under these conditions (Ackerman and Tsou, '61). This suggested that duodenal powder and other tissues contained thyroid-active material since goitrogens are considered to exert their effect primarily by an inhibition of thyroid hormone synthesis (Mackenzie and Mackenzie, '43; Astwood et al., '43). Observations made in other laboratories suggest that thyroid-active material may be present in meat (Griesbach and Purves, '43; Goldberg and Chaikoff, '52), in liver (Ershoff, '54), and in casein (Van Middlesworth, '52). The thyroid-active material in these foods has not been characterized, and it appeared desirable to investigate the nature of the growth-promoting activity of duodenal tissue.

During the course of this investigation, it was observed that growth arrest could not be induced by thiouracil when it was fed in commercial laboratory chow.[2] Also, growth arrest could not be induced in sulfaguanidine-fed rats when the iodide level of the diet was less than 10 μg/gm and that prolonged feeding of either sulfaguanidine or thiouracil in purified diets was fatal to rats. When these drugs were fed in more natural-type diets, death did not occur.

The diet, therefore, had a profound effect on the response of rats to goitrogens and these experiments were initiated to determine the effect of KI and animal tissue on the response of rats to sulfaguanidine and thiouracil. As a representative of animal tissue, duodenal tissue was selected from those tissues that had been found capable of reinstating growth of sulfaguanidine-fed rats for further studies on its ability to maintain normalcy in goitrogen-fed rats. In addition, it was hoped to determine whether the unexplained fatal effects of goitrogens could be linked to the iodide level of the diet.

## EXPERIMENTAL AND RESULTS

*Materials and methods.* Weanling male Sprague-Dawley rats (35 to 45 gm) were housed in screen-wire cages and were fed food and water ad libitum. The basal diet for the experiments 1, 2, and 4 consisted of the following: (in grams) ground wheat, 55; crude casein, 8.5; alfalfa leaf meal, 2.0; peanut oil meal, 13.0; brewer's yeast, 0.5; refined hydrogenated cottonseed oil,[3] 10.0; sucrose, 8.75; sodium chloride, 0.75; calcium carbonate, 1.5; and crystalline vitamins (in mg); menadione, 0.5; biotin, 0.1; vitamin $B_{12}$, 0.004. This diet contained 19.3% of protein (Kjeldahl $N \times 6.25$) and 0.13 μg of iodine/gm of diet. The total iodine was determined by the method of Grossman and Grossman ('55) on three aliquots of the diet that had been prepared without the hydrogenated cottonseed oil.

Thiouracil and sulfaguanidine were added to the diet at the expense of sucrose. Duodenal powder[4] was added at

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the expense of the entire diet. Potassium iodide in aqueous solution (3.0 gm/liter) was added to the diets after they had been prepared and then thoroughly mixed.

Carrier-free NaI[131] was obtained from the Union Carbide Nuclear Company, Oak Ridge, Tennessee. Suitably diluted aliquots in sterile saline were injected intraperitoneally. Samples were counted in a Nuclear-Chicago, well-type scintillation counter and corrected for background and radioactive decay.

The standard error (SE) was calculated from $SE = \sqrt{\dfrac{\Sigma\ d^2}{n(n-1)}}$, where d = deviation from the mean and n = number of observations.

*Experiment 1*

*The effect of KI on growth and organ weights of sulfaguanidine and thiouracil-fed rats.* Three groups of 6 rats each were fed the basal diet containing 1% of sulfaguanidine and zero, 10, or 30 μg of additional KI/gm of diet. Three other groups of 6 rats each were fed diets containing 0.1% of thiouracil and zero, 10, or 30 μg of additional KI/gm of diet. Two additional groups of 6 rats each served as controls. One was fed the basal diet without further supplementation. The other was fed the basal diet containing 30 μg of additional KI/gm. When the experiment was terminated, the rats were exsanguinated. Certain organs were removed, cleaned of adventitious tissue and weighed to the nearest 0.1 mg on a Roller-Smith Torsion Balance.

The growth response of thiouracil and sulfaguanidine-fed rats to various levels of KI added to the basal diet is shown in figure 1. The final body weights and organ weights are summarized in table 1. In the absence of a goitrogen, KI (30 μg/gm of diet) had no effect on growth or organ weights. Sulfaguanidine, without additional KI had no effect on growth, and only the thyroid gland exhibited a moderate increase in weight (table 1, group 3). The addition of 10 μg of KI/gm to the sulfaguanidine diet resulted in growth inhibition and 30 μg of KI/gm resulted in growth arrest. The latter group weighed 120 gm at 28 days and gained 15 gm during the subsequent 13 weeks. As the iodide level increased, the relative weight of the thyroid, the adrenals, the testes, and pituitary increased (table 1, groups 4 and 5). The effect on the thymus was marked. The thymus glands from rats in group 3 were firm and well-formed. Those from group 4 were visibly more diffuse and fragile, requiring great care in handling. Those in group 5 were small, extremely diffuse, and fragile. This was true also of the thymus glands from rats in groups 7 and 8 (table 1). The reliability of this data was questionable and was, therefore, omitted from the table. The omission of these figures is intended to indicate an advanced stage of thymic involution.

Thiouracil alone was a more effective growth inhibitor than was sulfaguanidine, and growth arrest was observed in those rats receiving 10 μg of KI/gm. Those rats receiving 30 μg of KI/gm of diet gained only 2 gm between the fourth and seventeenth week (fig. 1). However, the pattern of response to additional dietary KI was similar to that observed with sulfaguanidine. As the iodide level increased, growth was progressively inhibited and the relative weights of the thyroid and the adrenal glands increased. The testes and the pituitary appeared to have been maximally affected by thiouracil alone (table 1, group 6) since the addition of 10 or 30 μg of KI/gm did not effect a further increase in the relative weight of these two glands (groups 7 and 8).

Thirty micrograms of KI/gm were fatal to rats fed both thiouracil and sulfaguanidine. On the ninety-seventh day, one rat in group 8 (table 1) developed tremors and was unusually sensitive when touched. It was moribund on the one-hundredth day and died on the one-hundred-first day. It had lost only 3 gm in body weight during this 4-day period. Its stomach and cecum were filled with food, and 6 firm and well-formed fecal pellets were in the intestinal tract indicating that inanition was not a factor contributing to its death. Only the adrenal glands appeared abnormal. These were almost black in color.

The rats in groups 5 and 8 were maintained for 17 weeks after these symptoms appeared. Identical toxic symptoms appeared in another rat of group 8 on the one-hundred-fifteenth day, and in two
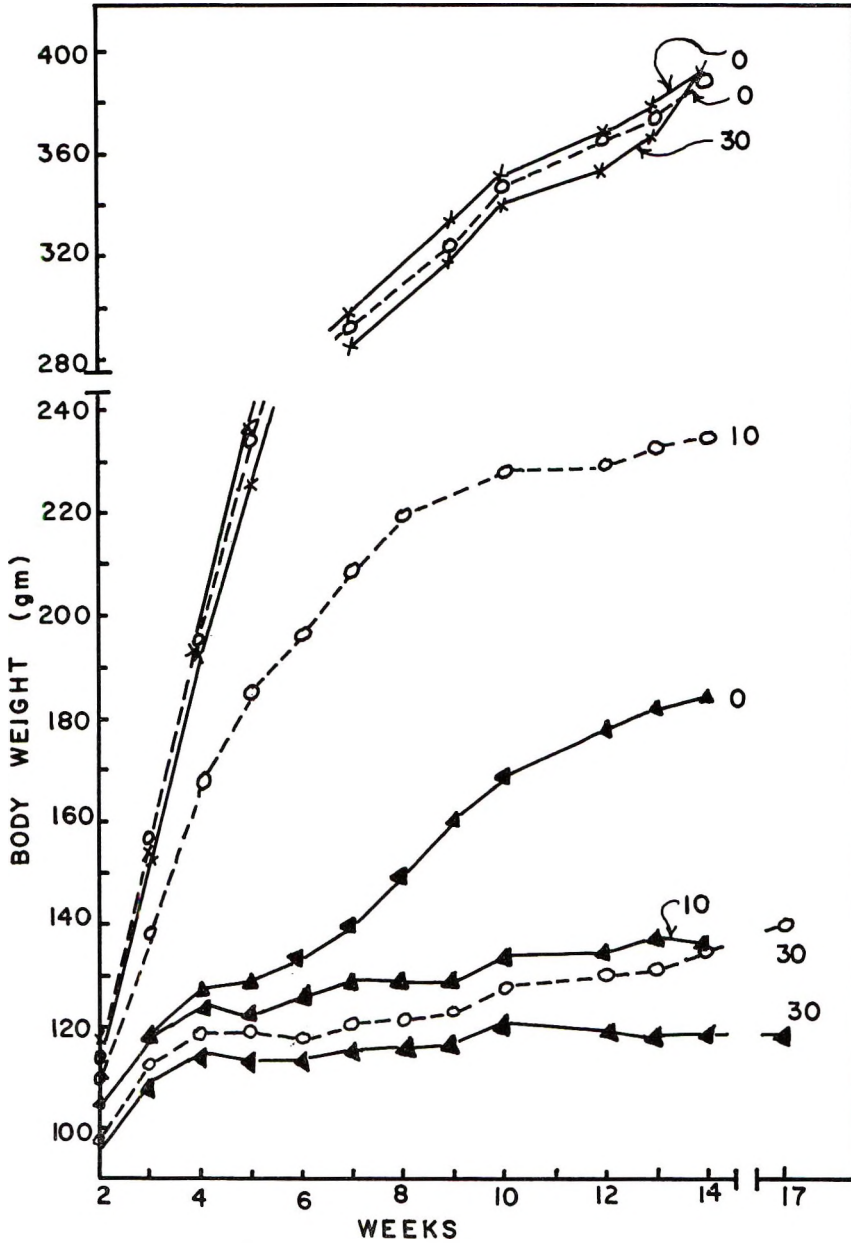
Fig. 1   Effect of zero, 10, and 30 μg of KI on growth of sulfaguanidine or thiouracil-fed rats.  Basal diet,  ×———×;  1% of sulfaguanidine  ——○———○—;  0.1% of thiouracil, ◄———◄.  The figures at the termination of each curve indicate the amount of KI added to the diet in micrograms per gram of diet.

rats of group 5 on the one-hundred-seventh and one-hundred-fourteenth day. Two of these were killed when they became moribund and the adrenal glands of these two rats were also dark in color indicating that this change in color was not due to postmortem changes. The remaining rats in groups 5 and 8 were alert and active exhibiting only severe exophthalmos. They were killed on the

TABLE 1

*Effect of potassium iodide on the growth and organ weights of goitrogen-fed rats*

| Group[1] | Additions to basal diet | | Final body wt[2] | Organ weights | | | | |
|---|---|---|---|---|---|---|---|---|
| | Drug | KI | | Thyroid | Thymus | Adrenal | Testes | Pituitary |
| | | $\mu g/gm$ | gm | mg/100 gm | mg/100 gm | mg/100 gm | mg/100 gm | mg/100 gm |
| 1 | 0 | 0 | $396 \pm 3$[3] | $6.09 \pm 0.2$ | $134 \pm 3.5$ | $12.0 \pm 0.8$ | $934 \pm 15$ | $2.6 \pm 0.2$ |
| 2 | 0 | 30 | $397 \pm 13$ | $7.02 \pm 0.4$ | $164 \pm 13$ | $11.8 \pm 0.5$ | $923 \pm 19$ | $2.5 \pm 0.2$ |
| 3 (5) | Sulfa-guani-dine, 1% | 0 | $391 \pm 9$ | $11.6 \pm 0.7$ | $149 \pm 9.7$ | $11.7 \pm 0.2$ | $958 \pm 36$ | $2.3 \pm 0.07$ |
| 4 | Sulfa-guani-dine, 1% | 10 | $235 \pm 19$ | $31.3 \pm 3.4$ | $120 \pm 7.2$ | $13.2 \pm 2.1$ | $1086 \pm 196$ | $4.3 \pm 0.3$ |
| 5 | Sulfa-guani-dine, 1% | 30 | $135 \pm 8$ | $54.3 \pm 6.6$ | — | $15.1 \pm 1.1$ | $1417 \pm 136$ | $4.5 \pm 0.2$ |
| 6 (5) | Thio-uracil, 0.1% | 0 | $184 \pm 18$ | $55.9 \pm 8.1$ | $181 \pm 32$ | $12.1 \pm 0.8$ | $1458 \pm 107$ | $4.8 \pm 0.1$ |
| 7 | Thio-uracil, 0.1% | 10 | $138 \pm 8$ | $77.5 \pm 10$ | — | $14.7 \pm 0.5$ | $1536 \pm 120$ | $6.3 \pm 0.1$ |
| 8 | Thio-uracil, 0.1% | 30 | $121 \pm 8$ | $87.6 \pm 16$ | — | $15.9 \pm 1.0$ | $1459 \pm 198$ | $4.7 \pm 0.2$ |

[1] Six male rats except where indicated by numbers in parentheses.
[2] Body weight at the end of the 14th week except for groups 5 and 8 which were maintained for 17 weeks. See text.
[3] Mean ± SE.

one-hundred-nineteenth day and their adrenal glands were normal in color. The organ weights of the rats that had died were included with the data of the surviving rats in their respective groups since no differences in their organ weights were noted.

### Experiment 2

*The ability of duodenal powder to maintain and reinstate growth of goitrogen-fed rats.* Nine groups of weanling rats were fed the basal diet supplemented with 30 µg of KI/gm, and the following: (1) none; (2) 4% of duodenal powder; (3) 1% of sulfaguanidine; (4) 1% of sulfaguanidine plus 4% of duodenal powder; (5) 0.1% of thiouracil; (6) 0.1% of thiouracil plus 4% of duodenal powder; (7) 1% of sulfaguanidine for 41 days at which time the drug was omitted from the diet; (8) 0.1% of thiouracil for 41 days at which time the drug was omitted from the diet; and (9)

0.1% of thiouracil. On the forty-first day, 2, 4, and 8% of duodenal powder was added to the diet of three rats each. All rats were weighed weekly but rats in groups 7 to 9 were weighed every two days from the forty-first to the fifty-fifth day. All rats were killed on the fifty-fifth day, and certain organs were removed and weighed.

Figure 2 illustrates that growth arrest again occurred after the twenty-eighth day in rats fed either thiouracil or sulfaguanidine with 30 µg of KI/gm, but 4% of duodenal powder in the diet was effective in maintaining growth of rats fed either goitrogen. Duodenal powder had no appreciable stimulatory effect on growth when it was included in the control diet (fig. 2). With the exception of the thyroid glands, whose increase in weight was inhibited but not prevented, duodenal powder also maintained organ weights at or near normal values when it was included
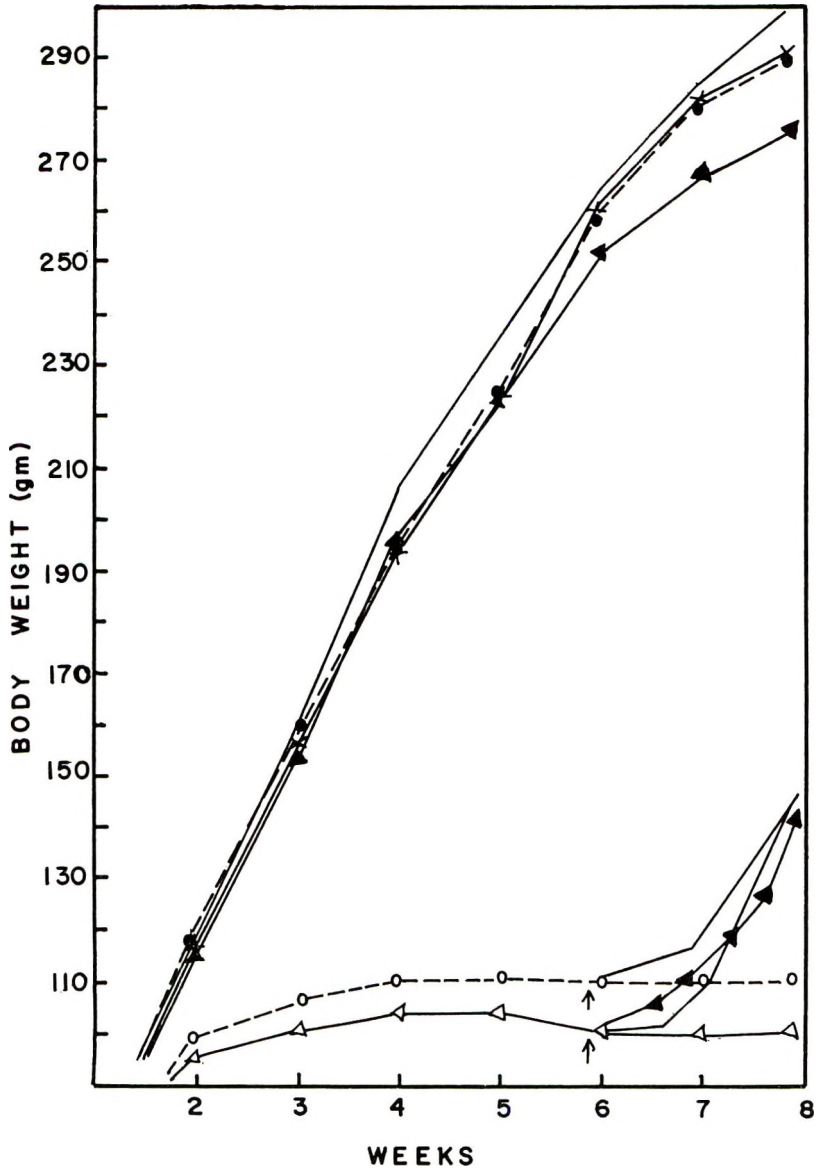
Fig. 2   The ability of duodenal powder to maintain and reinstate growth of thiouracil and sulfaguanidine-fed rats. All diets contained 30 µg of KI/gm. Normal, ————; normal + 4% of duodenal powder, ×————×; 1% of sulfaguanidine ◯-----◯; 0.1% of thiouracil, ◁------◁; solid symbols indicate that 4% of duodenal powder was added to the diet. Goitrogens were removed or duodenal powder was added on the forty-first day (arrow).

in the goitrogen-containing diets from the first day (table 2, groups 4 and 6).

Growth was reinstated when the goitrogens were removed from the diet on the forty-first day (fig. 2 and groups 7 and 8 of table 2). Normal thyroid function is restored under these conditions, and comparison of groups 7 and 8 with the corresponding groups 3 and 5, shows that growth resumption was accompanied by a decrease in the relative weight of the thyroid gland and an increase toward nor-

TABLE 2

*Effect of duodenal powder on growth of goitrogen-fed rats*

| Group (no. rats) | Diet[1] Drug | Diet[1] Duodenal powder | Final body wt. | Wt. gain 41–55th day | Organ weights Thyroid | Organ weights Thymus | Organ weights Adrenal | Organ weights Testes | Organ weights Pituitary |
|---|---|---|---|---|---|---|---|---|---|
| | | % | gm | gm | mg/100 gm | mg/100 gm | mg/100 gm | mg/100 gm | mg/100 gm |
| 1 (5) | 0 | 0 | 293 ± 9[3] | 32 | 4.4 ± 0.2 | 256 ± 22 | 11.6 ± 0.5 | 1116 ± 34 | 2.3 ± 0.3 |
| 2 (6) | 0 | 4 | 303 ± 8 | 38 | 4.2 ± 0.3 | 213 ± 9 | 15.4 ± 0.8 | 1146 ± 16 | 3.2 ± 0.1 |
| 3 (6) | Sulfa-guanidine, 1% | 0 | 110 ± 5 | −1 | 55.6 ± 2.7 | 133 ± 11 | 19.5 ± 0.6 | 1205 ± 309 | 4.6 ± 0.2 |
| 4 (7) | Sulfa-guanidine, 1% | 4 | 290 ± 6 | 31 | 10.2 ± 0.6 | 242 ± 14 | 11.7 ± 0.5 | 1146 ± 36 | 2.0 ± 0.2 |
| 5 (5) | Thiouracil, 0.1% | 0 | 100 ± 1 | 0 | 100 ± 11 | 126 ± 12 | 15.6 ± 0.5 | 1958 ± 216 | 5.1 ± 0.9 |
| 6 (7) | Thiouracil, 0.1% | 4 | 278 ± 11 | 25 | 17.1 ± 1.1 | 190 ± 12 | 11.3 ± 0.4 | 1154 ± 35 | 2.6 ± 0.2 |
| 7 (6) | Sulfa-guanidine, 1%[4] | 0 | 149 ± 2 | 35 ± 3 | 16.8 ± 0.8 | 263 ± 14 | 19.7 ± 0.7 | 1381 ± 81 | 3.4 ± 0.5 |
| 8 (6) | Thiouracil, 0.1%[4] | 0 | 149 ± 3 | 44 ± 2 | 24.4 ± 1.4 | 181 ± 11 | 17.3 ± 0.1 | 1037 ± 147 | 3.6 ± 0.3 |
| 9 (3) | Thiouracil, 0.1% | 2[5] | 143 ± 9 | 36 ± 4 | 73.4 ± 8 | 174 ± 26 | 16.2 ± 0.8 | 1752 ± 203 | 4.8 ± 1.2 |
| (3) | Thiouracil, 0.1% | 4[5] | 149 ± 2 | 43 ± 7 | 53.6 ± 11 | 185 ± 29 | 17.1 ± 1.0 | 1531 ± 150 | 3.6 ± 0.1 |
| (3) | Thiouracil, 0.1% | 8[5] | 164 ± 18 | 56 ± 3 | 23.3 ± 3 | 209 ± 32 | 16.2 ± 2.5 | 1508 ± 261 | 3.3 ± 0.3 |

[1] The diets of all rats contained 30 μg of KI/gm.
[2] Body weight on the 55th day.
[3] Mean ± SE.
[4] Sulfaguanidine and thiouracil were omitted from the diets on the 41st day.
[5] Duodenal powder was added to the thiouracil diet on the 41st day. The data for these groups is recorded as mean ± range.

mal of the thymus gland. The adrenal glands did not respond by a change in weight during this two-week period. A marked reduction in the relative weight of the testes was observed in those rats which had been fed thiouracil (group 8).

The addition of duodenal powder to the thiouracil diet on the forty-first day evoked a response similar to that observed in group 8. This is shown in group 9 (table 2) where the addition of 2, 4, and 8% of duodenal powder to the diet resulted in a progressive increase in body weight gain and a definite trend toward normal values

of the thyroid, thymus, and the pituitary. The adrenal glands and the testes did not respond by a change in weight during this two-week period. Figure 2 depicts the growth response of those rats in group 9 which were fed the thiouracil diet supplemented with 4% of duodenal powder.

### Experiment 3

*Growth of rats fed thiouracil in a commercial ration.* Commercial laboratory chow[5] was finely ground and then supple-
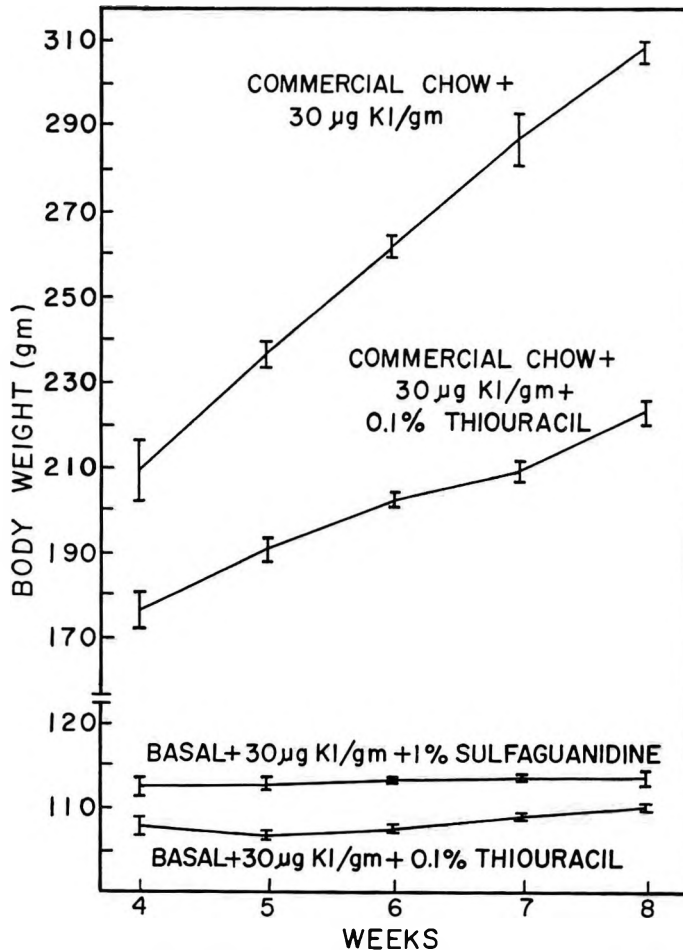
[5] See footnote 2.



Fig. 3   Growth of rats fed 0.1% of thiouracil in commercial laboratory chow compared with rats in experiments 1, 2, and 4 which were fed goitrogen plus 30 μg of KI/gm of diet. Vertical bars represent the standard error of the weight gain for the previous week. From the fourth to the eighth week, the points on the sulfaguanidine curve represent the average of 18, 18, 12, 12, and 12 rats, respectively. The points on the thiouracil curve represent the average of 24, 24, 12, 12, 12, and 6 rats, respectively.

mented with 30 μg of KI/gm. One group of 6 rats was fed this diet without further supplementation. Another group of 6 rats was fed the same diet supplemented with 0.1% of thiouracil. The experiment was terminated on the fifty-fifth day. Organ weights were not determined.

The laboratory chow, containing 0.1% of thiouracil and additional KI (30 μg/gm), did not produce the severe growth inhibition that was observed in experiments 1 and 2. This is illustrated in figure 3. For comparison, the growth data of those rats fed 1% of sulfaguanidine or 0.1% of thiouracil with 30 μg of KI/gm of diet in experiments 1, 2, and 4 were pooled and plotted in figure 3. The actual body weight attained in 28 days by an individual rat may vary from 90 to 140 gm, but the subsequent weight gain is negligible. This is illustrated in figure 3 by the vertical bars which represent the standard error of the average weight gain for each week. The various components of the commercial laboratory chow were not available for a more critical study, but it is inferred from these experiments that the meat meal component of this chow is responsible for the failure to observe growth arrest.

*Experiment 4*

*The effect of duodenal powder on the uptake and protein binding of radioiodide by goitrogen-fed rats.* Five groups of 5 rats each were fed diets indentical to those fed to groups 1, 3 and 5 of experiment 2. After growth arrest was established (35 days), 5 μc of NaI[131] was administered to each rat. Twenty-four hours later, blood was collected by heart puncture. The thyroid glands were removed, weighed, and homogenized with 5.0 ml of 0.001 M thiouracil in 0.01 N NaOH. An aliquot was removed and counted to determine total radioactivity in the gland. Four milliliters of the remaining thyroid homogenate were added to 4 ml of 20% trichloroacetic acid which contained 10 μg of KI/ml. The precipitate was washed three times with 5% trichloroacetic acid containining 5 μg of KI/ml. The precipitate was counted for radioactivity and this represented protein bound I[131] (PBI[131]). The protein from one milliliter of serum was precipitated and washed with trichloroacetic acid exactly as

TABLE 3

*Effect of duodenal powder on the incorporation of I[131] into thyroid and serum protein*

| Group[1] | Diet[2] Drug | Diet[2] Duodenal powder | Final body wt[3] | Thyroid weight | Total I[131]/ 100 mg | Thyroid I[131] % of dose | PBI[131] % of total[4] thyroid I[131] | Serum I[131] PBI[131]/ 100 ml % of dose |
|---|---|---|---|---|---|---|---|---|
| | | % | gm | mg | % of dose | % of dose | | % of dose |
| 1 | 0 | 0 | 267 ± 7[5] | 10.0 ± 0.25 | 23.1 ± 2.1 | 18.0 ± 2.1 | 68.9 ± 6.1 | 0.51 ± 0.04 |
| 2 | Sulfa-guanidine, 1% | 0 | 109 ± 7 | 32.5 ± 1.7 | 0.95 ± 0.09 | 0.006 ± 0.001 | 0.42 ± 0.02 | 0.53 ± 0.05 |
| 3 | Sulfa-guanidine, 1% | 4 | 248 ± 11 | 17.2 ± 1.7 | 0.63 ± 0.03 | 0.30 ± 0.02 | 54.8 ± 3.6 | 0.25 ± 0.03 |
| 4 | Thiouracil, 0.1% | 0 | 107 ± 4 | 55.7 ± 3.4 | 1.33 ± 0.12 | 0.0012 ± 0.0002 | 0.34 ± 0.01 | 0.48 ± 0.04 |
| 5 | Thiouracil, 0.1% | 4 | 217 ± 4 | 17.1 ± 0.40 | 0.10 ± 0.02 | 0.014 ± 0.003 | 15.9 ± 4.9 | 0.18 ± 0.01 |

[1] Five male rats per group.
[2] Thirty micrograms of KI/gm added to all diets.
[3] At 35 days.
[4] Percentage of total thyroid I[131] = $\frac{PBI^{131} \text{ count/min} \times 5/4}{\text{Thyroid } I^{131} \text{ count/min} \times 5} \times 100$.
[5] Mean ± SE.

described for the thyroid homogenate and then counted to determine the serum PBI[131].

The uptake of radioiodide and its incorporation into thyroid protein is markedly inhibited in rats fed sulfaguanidine or thiouracil with 30 μg of KI/ gm of diet (table 3, groups 2 and 4) which provides additional evidence that under the conditions used in these experiments, both goitrogens effectively block thyroid hormone synthesis. When 4% of duodenal powder was included in these diets, growth was maintained, thyroid weight decreased, and the total uptake of I[131] decreased.

Although the total uptake of I[131] decreased, the incorporation into thyroid protein increased 50-fold in sulfaguanidine-fed rats and 10-fold in thiouracil-fed rats. Only 0.42% of the total thyroid I[131] was protein bound in those rats fed sulfaguanidine alone, but the addition of duodenal powder to the diet increased this to 54.8%. In thiouracil-fed rats, the thyroid I[131] bound as protein increased from 0.34 to 15.9% when duodenal powder was added to the diet.

Whether this increase in thyroid PBI[131] could account for the growth-promoting effects of duodenal powder is not clear since this increase is not reflected in the serum PBI[131]. Duodenal powder resulted in a decrease in serum PBI[131].

## DISCUSSION

Growth arrest has not always been observed in rats fed goitrogens for prolonged periods of time (Mackenzie and Mackenzie, '43; Astwood et al., '43; Astwood and Bissell, '44) and inconsistencies with the expected response to goitrogens have been observed when other parameters of thyroid function were measured (Williams et al., '44; Mayer, '47; Stasili et al., '60). A consideration of these reports suggests that the diet may have been a primary factor responsible for these inconsistencies. For example, Welch and Wright ('43) reported that the unusually high level of 10% succinylsulfathiazole in commercial[6] diets had no effect on growth of rats, yet 1 and 2% of this drug in a purified diet resulted in growth failure. In contrast, 2% of sulfaguanidine in commercial fox chow[7] had only a slight inhibitory effect on growth (Astwood et al., '43).

The experiments reported here show that growth arrest may be induced in sulfaguanidine-fed rats when the dietary iodide level is between 10 and 30 μg/gm of diet, and that iodide (10 and 30 μg/gm) enhances the growth-inhibitory effect of 0.1% of thiouracil. Under these conditions, the synthesis of thyroid hormones by the thyroid gland was effectively inhibited. However, duodenal powder added to the diet maintained growth and organ weights of both thiouracil- and sulfaguanidine-fed rats. Thus, the failure to observe growth arrest or symptoms typical of a thyroid deficiency when thiouracil or sulfonamides are fed may be due either to insufficient dietary iodide or to the use of diets which contain animal tissues such as the commercial chows used, or to both.

The role played by iodide appears to be complex. Mackenzie ('47) reported that iodide inhibited the goitrogenic effect of thiouracil but enhanced the goitrogenicity of sulfaguanidine. Highley et al. ('54) noted that the growth-inhibitory and goitrogenic effects of low levels of thiouracil (0.025% or less) could be overcome by iodine, but that iodine did not prevent growth inhibition or thyroid hyperplasia caused by 0.1% thiouracil. High levels of iodide have been reported to promote growth of thyroidectomized rats (Evans et al., '60), but it had no effect on growth or other parameters of thyroid function when it was administered to normal rats fed normal diets (Pitt-Rivers, '60). It appears, therefore, that high levels of iodide function synergistically with a goitrogen such as thiouracil or sulfaguanidine to more effectively block thyroid hormone synthesis.

The fatal effects of sulfaguanidine and thiouracil (experiment 1) appear to be linked to the level of dietary iodide and this is now under investigation. That sulfaguanidine in purified diets is fatal to rats has been reported by Axelrod et al. ('44) and Tentori and Vivaldi ('50, '54). The former workers used diets containing 32 μg of KI/gm of diet, and the latter workers reported that sulfaguanidine was not fatal when it was fed in McCollum's stock diet. This diet is relatively low in iodide.

[6] Purina or Wayne Brand.
[7] Purina Fox Chow.

The effect of duodenal powder in these experiments suggests that this tissue contains thyroid hormones at physiologically effective concentrations. This is conceivable in view of the reports which indicate that the thyroxine requirement to maintain growth of thyroidectomized rats (Evans et al., '60a) or thiouracil-fed rats (Stasilli et al., '61) is 0.04 to 0.05 µg/day when it is administered parenterally.

The relative weights of the thyroid glands from rats fed sulfaguanidine plus 30 µg of KI/gm were consistently lower than those of rats fed thiouracil plus 30 µg of KI/gm (tables 1, 2, and 3). These rats had ceased to grow (fig. 3) due to a depletion of endogenous thyroid hormones. It would be expected then, that thyrotropin secretion would be maximal and that the compensatory response of the thyroid gland would be maximal in both thiouracil and sulfaguanidine-fed rats. This was not the case, indicating that the secretion of thyrotropin or the sensitivity of the thyroid gland to thyrotropin had been inhibited by sulfaguanidine in these experiments.

## SUMMARY

Two factors which modify the thyroid hormone deficiency produced by the ingestion of thiouracil or sulfaguanidine were studied and certain observations indicate that the mode of action of these two goitrogens are different.

1. The addition of 10 µg of KI/gm of diet markedly inhibited growth, and 30 µg of KI/gm of diet resulted in growth arrest of rats fed 1.0% sulfaguanidine. Growth arrest occurred after 28 days in thiouracil-fed rats when 10 µg of KI/gm were added to the diet. With either goitrogen, growth arrest was accompanied by an increase in the relative weights of the thyroid, adrenals, testes, and the pituitary, and a decrease in that of the thymus gland. The uptake of $I^{131}$ and its incorporation into protein by the thyroid gland was markedly reduced. Prolonged feeding of either goitrogen with 30 µg of KI/gm of diet was fatal to some of the rats.

2. Growth and organ weights were maintained at or near normal values when 4% duodenal powder was added to those diets capable of producing growth arrest. Under such conditions, duodenal powder decreased the uptake of $I^{131}$ by the thyroid gland but increased the incorporation of thyroidal $I^{131}$ into protein. The pattern of response to duodenal powder suggests that this tissue contains thyroid hormone-active material. Since growth arrest could not be induced in rats fed thiouracil plus 30 µg of KI/gm of commercial laboratory chow, it was inferred from these experiments that this diet contains thyroid hormone-active material in the meat meal which is a component of this chow.

3. The relative weights of the thyroid glands from growth-arrested, sulfaguanidine-fed rats were consistently less than that of growth-arrested, thiouracil-fed rats indicating that the mechanism which compensates for low levels of circulating thyroid hormones was inhibited by the sulfaguanidine under the conditions of these experiments.

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# Incorporation of Valine-1-C$^{14}$ into Serum and Tissue Proteins of Rats Fed Torula Yeast Diets

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Dietary necrotic liver degeneration in rats fed a 30% Torula yeast diet (Schwarz, '51) is characterized by a "latent phase" which precedes hepatic necrosis by 10 to 14 days. During this period, gross pathologic or microscopic alterations are not detected, but changes on the subcellular level can be seen by electron microscopy (Schwarz, '58). Aside from mitochondrial swelling, the degenerative alterations appear to involve primarily the microsomes, the major site of protein synthesis. The endoplasmic reticulum undergoes a change from rough to smooth (Piccardo and Schwarz, '58) and shows a conversion into cyst-like elements. To gain information on the functional status of these structures a study of the amino acid incorporation in rats fed Torula yeast diets was undertaken, using DL-valine-1-C$^{14}$ as a marker. The 30% Torula yeast ration, deficient mainly in sulfur amino acids, has a protein content of 12 to 14%, and produces suboptimal growth rates. Valine incorporation into the tissue proteins of rats fed Torula yeast diets was found to be highly elevated. The effects of necrosis-preventing factors and combinations of amino acids on this enhancement were studied.

## EXPERIMENTAL

Weanling, male rats of the Fischer 344 strain were individually caged and fed ad libitum the 30% Torula yeast diet used routinely in this laboratory (Schwarz, 61). For some groups of animals the basal diet was supplemented after 13 days with vitamin E (50 mg *dl-α*-tocopheryl acetate/100 gm diet), sodium selenite (15 µg/100 gm diet), DL-methionine (0.62%), L-cystine (0.5%), or chromium (III) hexaurea chloride (10 mg/100 gm diet), alone or in various combinations as indicated in the tables. Other groups received diets containing 10 or 20% casein as the source of protein, with sucrose making up the difference in weight, according to a scheme otherwise identical to that of the basal Torula ration.

In other instances mixtures of amino acids were added to the Torula diet. The amount of each amino acid in these mixtures was calculated to bring the amino acid supply of the Torula ration up to that of the 20% casein ration. In these calculations the 30% Torula diet was assumed to contain 12% "available" protein;[2] Block and Bolling's data ('47) on the amino acid composition of casein and those of the manufacturer[3] on the amino acid composition of Torula yeast were used. Double amounts of DL-amino acids were used. Mixtures were prepared of essential (A) and nonessential (B) amino acids. Composition of mixture A in grams, without methionine, was as follows: DL-threonine, 0.212; DL-valine, 1.232; L-tryptophan, 0.162; L-tyrosine, 0.662; L-leucine, 1.266; DL-isoleucine, 0.856; L-arginine·HCl, 0.350; L-histidine·HCl, 0.411. Mixture B (nonessential amino acids) contained: L-glutamic acid, 2.454; L-aspartic acid, 0.726; glycine, 0.392; DL-serine, 1.648; L-tyrosine, 0.662; L-proline, 1.448. The mixtures A (essential complement

minus methionine) or B (nonessential amino acid complement), or a 50/50 combination of the two, were incorporated in the Torula diet at 10% levels, with or without addition of 0.62% DL-methionine (equivalent to 0.5% cystine).

Rats fed the optimal wheat-casein diet of McCollum (Mertz and Schwarz, '59) were used as controls. The animals were fed the various rations for 10 to 14 days after weaning. In two experiments (D and G), all rats received the Torula diet for the first week, followed by the supplemented diets or the wheat-casein diet for 4 to 5 more days prior to sacrifice.

The rats were injected intraperitoneally with the indicated amounts of DL-valine-1-$C^{14}$ [4] dissolved in 0.5 ml saline/100 gm body weight. The injections were given between 8 and 10 AM. Unless otherwise stated, the animals were killed after a 4-hour interval during which food had been withdrawn. The blood was collected from the jugular vein, and the serum separated by centrifugation after clotting. The liver and the other organs were quickly excised, washed in ice-cold 0.25 M sucrose, weighed after blotting on filter paper, and homogenized in 0.25 M sucrose with a Potter-type tissue homogenizer. Necrotic areas in the liver, when present, were carefully eliminated. The subcellular liver fractions ("nuclei" contaminated with red cells and cellular debris, mitochondria, microsomes and supernatant) were separated by centrifugation (Corwin and Schwarz, '59). The particles were resuspended in 0.25 M sucrose.

Proteins were precipitated from aliquots of blood serum, homogenates and liver fractions by addition of an equal volume of 10% (w/v) trichloroacetic acid, and washed as described by Rabinovitz et al. ('54), but with an additional 5% trichloroacetic acid washing at room temperature after the incubation at 90°, and two ethanol washings instead of one. The washed proteins were resuspended in ether; and 4- to 5-mg aliquots, made approximately equal to avoid corrections for thickness, were plated on weighed filter paper discs. After reweighing, these were mounted with cement on copper planchets. Radioactivity was measured in a thin-window Geiger

flow counter; the results were expressed as specific activity (count/min/mg protein/μc injected). Since absolute values differed somewhat from experiment to experiment, each experiment is presented separately in order to insure comparison with its own control. The results have been corrected for the number of counts injected (8.5 × $10^5$ count/min/μc) per 100 gm body weight.

## RESULTS

*Effect of diets on incorporation of valine into serum and liver proteins.* Throughout consecutive experiments the animals fed the basal Torula diet incorporated valine into serum protein faster than the controls fed the optimal wheat-casein ration (table 1). The higher rate of valine incorporation in Torula animals was even more pronounced in liver proteins. Investigations were carried out to determine which ingredients were responsible for this difference in incorporation. The basal Torula diet is lacking in Factor 3, vitamin E and sulfur amino acids. Addition of selenite, vitamin E, or a combination of both (exp. A, B and C) failed to affect significantly the incorporation in serum and liver.

The protein content of the basal Torula diet is quantitatively and qualitatively inferior to that of McCollum's wheat-casein ration. It became evident from experiments on rats fed 10 and 20% casein diets that the enhancement of valine incorporation in animals fed the basal Torula diet appeared related to the amino acid supply. The results showed that with the 20% casein diet valine incorporation into liver proteins was comparable to that of the normal controls on the optimal wheat-casein ration, whereas it was doubled when the casein level in the diet was reduced to 10%. This level is similar to the content of "available" protein in the basal Torula diet (exp. E and F).

Sulfur amino acid supplementation alone did not restore normal rates of valine incorporation. With 0.62% methionine, incorporation in the liver is not affected, whereas that in serum is enhanced (table

[4] Nuclear Chicago, Chicago, Illinois, and Volk Radiochemical Company, Chicago, Illinois.

TABLE 1

*Incorporation of valine-1-C¹⁴ into serum and liver proteins*

| Exp. | Diet | No. rats | Body wt | Liver wt | Valine injected | Specific activity of proteins | |
|---|---|---|---|---|---|---|---|
| | | | | | | Serum | Liver |
| | | | gm | gm | $\mu c/100\ gm$ body wt[1] | count/min/mg protein/$\mu c$ injected | |
| A | Wheat-casein | 4 | $80 \pm 3$[3] | $3.7 \pm 1.4$[3] | 5.060 | $72.9 \pm 3.9$[2,3] | $36.9 \pm 2.4$[3] |
| | Torula | 4 | $67 \pm 6$ | $3.2 \pm 0.3$ | | $87.1 \pm 4.7$ | $54.3 \pm 3.0$ |
| | Torula + Se | 4 | $53 \pm 3$ | $2.6 \pm 0.2$ | | $85.9 \pm 3.7$ | $56.1 \pm 1.6$ |
| | Torula + vitamin E | 3 | $51 \pm 2$ | $2.6 \pm 0.1$ | | $82.5 \pm 8.1$ | $56.5 \pm 6.3$ |
| B | Wheat-casein | 4 | $92 \pm 3$ | $3.6 \pm 0.3$ | 4.910 | $66.1 \pm 2.1$ | $29.5 \pm 0.6$ |
| | Torula | 4 | $64 \pm 2$ | $2.9 \pm 0.1$ | | $92.5 \pm 13.6$ | $62.5 \pm 7.9$ |
| | Torula + Se + vitamin E | 4 | $71 \pm 3$ | $3.2 \pm 0.2$ | | $83.9 \pm 5.5$ | $53.1 \pm 2.4$ |
| C | Wheat-casein | 4 | $85 \pm 6$ | $3.6 \pm 0.2$ | 0.873 | $74.5 \pm 3.4$ | $34.8 \pm 2.3$ |
| | Torula | 8 | $57 \pm 2$ | $2.6 \pm 0.1$ | | $97.2 \pm 2.3$ | $65.0 \pm 2.3$ |
| | Torula + Se + vitamin E | 8 | $54 \pm 2$ | $2.5 \pm 0.1$ | | $107.3 \pm 4.6$ | $70.4 \pm 3.4$ |
| D | Wheat-casein + methionine | 3 | $99 \pm 4$ | $4.7 \pm 0.2$ | 4.910 | $76.4 \pm 1.6$ | $36.2 \pm 4.3$ |
| | Torula + methionine | 2 | $94 \pm 5$ | $4.5 \pm 0.3$ | | $123.6 \pm 1.4$ | $67.6 \pm 0.2$ |
| | Torula + methionine + Se | 2 | $95 \pm 0$ | $4.3 \pm 0.1$ | | $134.8 \pm 22.2$ | $60.0 \pm 2.6$ |
| | Torula + methione + vitamin E | 2 | $87 \pm 2$ | $4.2 \pm 0.1$ | | $122.8 \pm 16.7$ | $64.3 \pm 6.5$ |
| E | Wheat-casein | 4 | $82 \pm 2$ | $3.9 \pm 0.3$ | 0.800 | $86.9 \pm 11.2$ | $35.2 \pm 2.5$ |
| | Torula | 4 | $55 \pm 4$ | $2.6 \pm 0.3$ | | $91.0 \pm 5.0$ | $61.5 \pm 3.7$ |
| | Casein, 10% | 5 | $44 \pm 1$ | $1.8 \pm 0.1$ | | $106.5 \pm 6.2$ | $72.8 \pm 5.0$ |
| F | Wheat-casein | 3 | $71 \pm 2$ | $3.0 \pm 0.5$ | 0.852 | $87.1 \pm 5.9$ | $39.6 \pm 3.5$ |
| | Torula | 4 | $32 \pm 3$ | $1.7 \pm 0.2$ | | $115.5 \pm 2.3$ | $75.4 \pm 5.9$ |
| | Torula + methionine | 4 | $66 \pm 6$ | $3.1 \pm 0.3$ | | $149.0 \pm 15.3$ | $74.2 \pm 2.3$ |
| | Casein, 20% | 6 | $63 \pm 3$ | $2.6 \pm 0.1$ | | $91.4 \pm 2.3$ | $47.2 \pm 1.2$ |
| | Casein, 20% + Se + vitamin E | 6 | $63 \pm 5$ | $2.9 \pm 0.2$ | | $88.0 \pm 2.3$ | $48.4 \pm 3.5$ |
| H | Wheat-casein | 4 | $73 \pm 5$ | $3.0 \pm 0.2$ | 4.910 | $63.0 \pm 2.6$ | $27.6 \pm 0.4$ |
| | Torula | 3 | $53 \pm 4$ | $2.4 \pm 0.3$ | | $73.2 \pm 5.5$ | $50.3 \pm 0.6$ |
| | Torula + methionine + Se + vitamin E | 3 | $67 \pm 6$ | $3.2 \pm 0.3$ | | $80.5 \pm 8.1$ | $38.7 \pm 2.2$ |
| I | Wheat-casein | 4 | $73 \pm 9$ | $3.0 \pm 0.4$ | 0.945 | $70.9 \pm 3.2$[2] | $36.4 \pm 1.1$ |
| | Torula | 4 | $62 \pm 3$ | $2.9 \pm 0.2$ | | $96.8 \pm 2.1$[2] | $59.0 \pm 4.2$ |
| | Torula + methionine + Se + vitamin E + Cr(III) | 3 | $73 \pm 8$ | $4.0 \pm 0.5$ | | $99.8 \pm 10.6$ | $50.0 \pm 4.2$ |

[1] $8.5 \times 10^5$ count/min/$\mu c$.
[2] One experiment less than indicated.
[3] SE of the mean.

1, exp. F, and fig. 1, exp. G). Placing the values of the wheat-casein fed animals at 100%, the specific activities of the serum proteins are: Torula, $125 \pm 16\%$; and Torula plus methionine, $171 \pm 8\%$. The corresponding values for liver amounted to $167 \pm 14\%$, and $177 \pm 7\%$, respectively. Supplementation of 0.5% L-cystine, equivalent to 0.62% methionine in terms of sulfur, decreased the specific activity of the liver proteins slightly (fig. 1, exp. G). When methionine was given together with
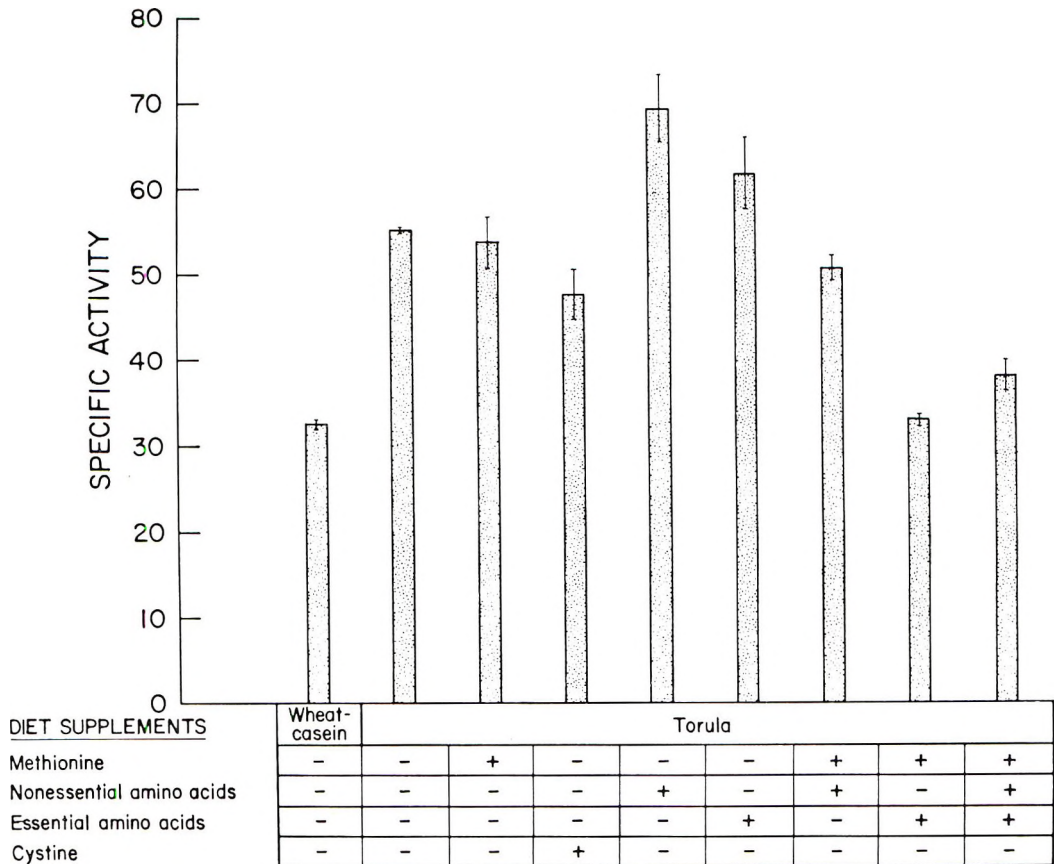
| DIET SUPPLEMENTS | Wheat-casein | Torula | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Methionine | − | − | + | − | − | − | + | + | + |
| Nonessential amino acids | − | − | − | − | + | − | + | − | + |
| Essential amino acids | − | − | − | − | − | + | − | + | + |
| Cystine | − | − | − | + | − | − | − | − | − |

Fig. 1   Effect of diets on the incorporation of DL-valine-1-$C^{14}$ into liver proteins (exp. G). Values are averages of three separate assays. For amounts supplemented, see text.

selenite or vitamin E, the incorporation into serum and liver proteins appeared unaffected (table 1, exp. D). Incorporation into serum proteins was also unaffected by a combination of both selenite and vitamin E with methionine; however, incorporation in liver declined (table 1, exp. H). The addition of Cr (III) to this diet had no effect (table 1, exp. 1). The addition to the Torula diet of 10% of the mixture of nonessential amino acids appeared to cause a relative increase of the incorporation (fig. 1, exp. G). A similar effect was given by a mixture of the essential amino acids minus methionine. Addition of 0.62% methionine to the nonessential mixture was without effect; but when the mixture of essential amino acids was made complete by the addition of methionine, it established normal rates of valine in-

corporation both in liver and serum. A similar result was obtained by the combination of 5% of the essential mixture with methionine plus 5% of the nonessential mixture.

*Distribution of valine in subcellular fractions of liver.* In normal rats the incorporation of labeled valine into the protein of the subcellular fractions of the liver was in the following, decreasing order: microsomes, supernatant, "nuclei" and mitochondria (table 2, exp. A, D and H). However, this sequence might be illusory, since the livers were not perfused. Thus, protein of the nuclei is contaminated by that of some red cells and tissue debris, and the protein of the supernatant by that of plasma. This difficulty is apparently unavoidable, since it has been shown that perfusion removes some liver proteins (Lee et al., '57).

In the Torula-fed rats the order was maintained, as far as absolute values were concerned; however, the maximal *increase* occurred in the mitochondria (average increase, 96%), followed by the supernatant

(74%), microsomes (58%), and nuclear fraction (55%) (table 2).

Dietary supplements that did not affect the incorporation in the whole homogenate did not alter the distribution pattern in

TABLE 2

*Incorporation of valine-1-C$^{14}$ into protein of subcellular fractions of the liver*

| Exp. | Diet | No. rats | Specific activity of proteins (count/min/mg/protein/μc injected) | | | | |
|------|------|----------|----------------------|--------|--------------|------------|-------------|
| | | | Whole homogenate | Nuclei | Mitochondria | Microsomes | Supernatant |
| A | Wheat-casein | 4 | 36.9 ± 2.4 | 33.2 ± 1.8 | 23.5 ± 1.6 | 50.0 ± 1.4 | 37.3 ± 2.8 |
| | Torula | 4 | 51.4 ± 3.0 | 47.2 ± 3.0 | 42.0 ± 2.2 | 67.1 ± 3.8 | 60.4 ± 4.1 |
| | Torula + Se | 4 | 56.0 ± 1.6 | 49.1 ± 2.4 | 43.9 ± 0.8 | 70.9 ± 3.0 | 62.1 ± 1.8 |
| | Torula + vitamin E | 3 | 56.5 ± 6.3 | 49.5 ± 6.1 | 40.6 ± 5.9 | 70.0 ± 7.5 | 61.0 ± 8.3 |
| D | Wheat-casein + methionine | 3 | 36.2 ± 4.1 | 34.2 ± 4.9 | 24.8 ± 3.7 | 50.9 ± 6.5 | 41.4 ± 6.3 |
| | Torula + methionine | 2 | 67.5 ± 0.2 | 61.1 ± 3.3 | 48.0 ± 3.0 | 84.9 ± 15.2 | 74.2 ± 6.1 |
| | Torula + methionine + Se | 2 | 60.0 ± 2.6 | 60.6 ± 5.1 | 53.7 ± 6.1 | 87.8 ± 12.2 | 73.9 ± 7.5 |
| | Torula + methionine + vitamin E | 2 | 64.3 ± 6.5 | 63.2 ± 7.5 | 48.5 ± 5.7 | 89.6 ± 17.3 | 75.1 ± 6.1 |
| H | Wheat-casein | 4 | 27.7 ± 0.3 | 25.0 ± 0.6 | 17.5 ± 0.6 | 41.5 ± 3.7 | 30.4 ± 0.3 |
| | Torula | 3 | 50.3 ± 0.6 | 41.1 ± 1.8 | 34.6 ± 1.6 | 64.3 ± 4.1 | 56.1 ± 5.7 |
| | Torula + methionine + Se + vitamin E | 3 | 38.7 ± 2.2 | 34.8 ± 3.5 | 27.7 ± 3.1 | 50.3 ± 6.3 | 40.4 ± 3.5 |
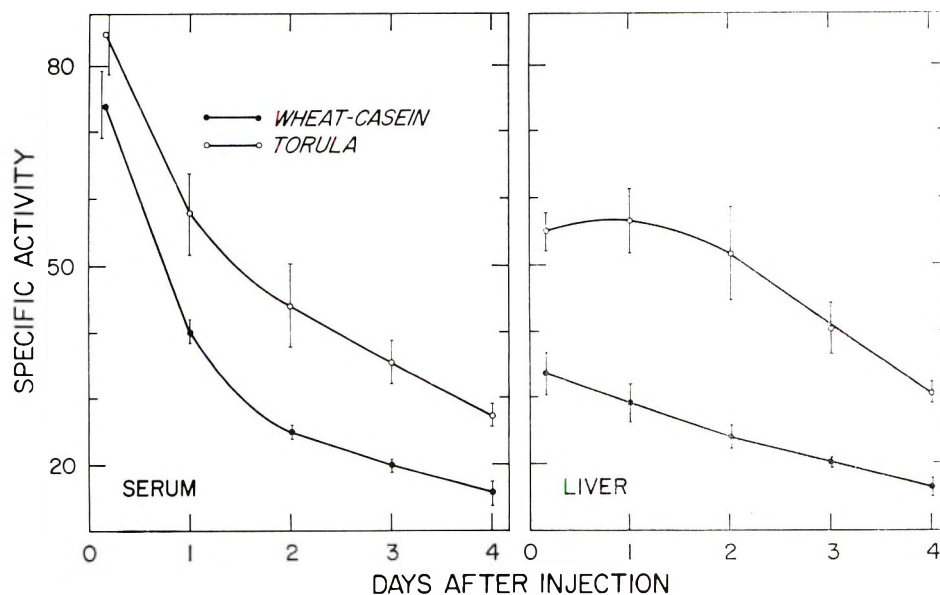


Fig. 2   Disappearance of DL-valine-1-C$^{14}$ from serum and liver proteins (exps. J, K, M and N).

the subcellular fractions. The combined supplementation of methionine, selenite and vitamin E to the Torula regimen lowered the incorporation in the homogenate. The individual subcellular fractions were affected to the same extent, so that the ratios between the various fractions were unchanged. Thus, incorporation in mitochondria is still increased more than in the other fractions.

*Disappearance of labeled valine from serum and liver proteins.* The specific activity of serum and liver proteins in control and Torula-fed rats at various time intervals after the injection of labeled valine is shown in figure 2. The initial rate of disappearance of radioactivity from serum proteins is higher in the wheat-casein fed rats, but it diminishes sooner, so that after two days, disappearance is faster in the Torula-fed group. The same pattern is observed in liver, but here the initial difference is more pronounced. In the basal Torula group the specific activity of liver stays rather constant for the first two days; it shows a slight but insignificant increase between the fourth and the twenty-fourth hour. In the normal animals this phenomenon does not appear; their livers show a rather steady decline of activity over the entire 4-day observation period.

In a study on the turnover of serum albumin in growing rats, Jeffay ('60) introduced a correction for the dilution of the radioactive material due to increases in body weight during the experiment. Corrections calculated according to Jeffay's formula — but not shown here — do not cause any significant changes in our results, since our rats grew considerably less than those of Jeffay, which gained 50% of their body weight in 8 days.

*Incorporation of valine in various organs.* The specific activities of proteins of serum and various organs determined in animals of experiment E, 4 hours after application of labeled valine, are shown in table 3. In normal rats they were in the following, decreasing order: serum, spleen, kidney, liver, lung, testes, heart, brain and skeletal muscle (psoas). In Torula-fed animals the increased incorporation was observed in all these organs, except for mus-

TABLE 3

Incorporation of valine-1-C14 into protein of serum and various organs

| Diet | No. rats | Specific activity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Serum[1] | Liver[1] | Kidney | Muscle | Testes | Brain | Heart | Lung | Spleen |
| | | count/min/mg protein/μc injected | | | | | | | | |
| Wheat-casein | 4 | 86.7 ± 11.0 | 35.1 ± 2.0 | 35.9 ± 1.5 | 13.5 ± 0.1 | 29.4 ± 2.6 | 14.6 ± 1.2 | 21.4 ± 0.1 | 30.0 ± 0 | 56.6 ± 5.4 |
| Torula | 4 | 91.0 ± 5.4 | 63.0 ± 2.9 | 53.5 ± 2.1 | 12.7 ± 0 | 36.1 ± 1.4 | 19.5 ± 0.1 | 33.3 ± 1.9 | 35.5 ± 1.2 | 57.3 ± 3.7 |

[1] Table 1, experiment E.

cle and spleen. Maximal enhancement occurs in the liver (+ 80%), heart (+ 55%) and kidney (+ 49%).

### DISCUSSION

The main result of these studies is the observation that in rats fed a 30% Torula diet, as well as in those receiving a 10% casein ration, the incorporation of valine into the proteins of serum, liver and a variety of other organs, but not muscle or spleen, is considerably enhanced, as compared with animals fed an optimal stock diet (McCollum's wheat-casein ration) or a 20% casein regimen. Experiments with various supplements to the Torula diet lead to the conclusion that the cause of this enhancement is the lack of protein, more exactly, of essential amino acids. The first evidence for this conclusion is the fact that the elevated incorporation is also seen with a 10% casein diet. Increasing the dietary casein to 20% brings the elevated rate of incorporation down to that of normal controls. Further evidence is furnished by the addition to the Torula diet of a mixture of amino acids calculated to make the dietary supply of amino acids approximately equal to that of the 20% casein diet. Only a slight difference remains when the casein-simulating mixture is given, probably due to differences between the analytical data used, and the actual amino acid composition of our casein and Torula yeast.

The enhancement of the incorporation appears to be quickly established and quickly reversed; it is noted after a few days of feeding the deficient diets and is abolished after 4 days of feeding of adequate diets, if not earlier. The enhancement is not due to lack of amino acid nitrogen as such, since it is not reversed, but further increased, when the nonessential amino acids are supplied by themselves. When all essential amino acids are given, on the other hand, the rate of incorporation becomes normal. The fact that the omission of methionine alone from the essential amino acids suffices to annul their beneficial effect shows that deficiency of a single essential amino acid may induce the alteration. Supplementation of methionine or cystine alone at levels equiv-

alent to 20% casein does not reverse the condition. On the contrary, in serum proteins it appears to further enhance valine incorporation.

Vitamin E and selenite selenium do not affect the enhancement when added to the Torula diet alone or in combination. Lack of these factors has no effect when the amino acid supply is adequate. However, when both of them are given together with methionine, a lowering of the incorporation of valine into the protein of the liver results, indicating a metabolic relationship between these three factors, probably identical in nature to that observed in the prevention of liver necrosis.[5]

Other investigators who have studied the incorporation of amino acids in animals fed diets inadequate in protein reported somewhat conflicting results. Solomon and Tarver ('52) noted an increased incorporation of methionine-S³⁵ in several organs, including muscle; and Lee and Williams ('54) reported an increase with cystine-S³⁵. Kean ('59), on the other hand, found a slower incorporation of methionine in short-term experiments; and Rutman et al. ('55) reported a slower methionine incorporation *in vitro* by liver slices.

An enhanced incorporation similar to that in our results was observed by Farber and Sidransky ('58) in rats force-fed diets deficient in a single amino acid. In protein-deficient rats, enhanced incorporation was also found by Waterlow ('59). More recently, Wannemacher ('61) reported an enhanced incorporation of S³⁵ in protein-depleted animals, using S³⁵-labeled methionine; and Nimni and Bavetta ('61) observed an increased incorporation of glycine-1-C¹⁴ in tryptophan-deficient rats.

Although Lee and Williams ('54) reported the maximal increase of incorporation in protein-depleted animals in the microsomal fraction, it is a peculiar feature of our results that the maximal enhancement is obtained in the mitochondria, i.e., in that fraction which has the lowest activity in normal animals, and which is believed to synthesize only certain proteins, such as cytochrome c. Possibly this may

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[5] The fact that the improvement so induced is comparable to that caused by the addition of excess methionine may indicate a better utilization of methionine when vitamin E and selenite are both supplied.

be related to the reconstitution of normal levels of certain respiratory enzymes in mitochondria. We have reported (Stirpe and Schwarz, '62) that the same dietary conditions shown here to produce the enhancement of amino acid incorporation cause greatly diminished activities of the DPNH-cytochrome c reductase system in mitochondria and microsomes. Addition of the essential amino acids, or of 10% casein, leads to a rapid reconstitution of normal enzyme activity.

Studies with injected amounts of labeled amino acids do not give an exact idea of the rate of protein turnover (Tarver, '58). Our experiments, however, appear to indicate that the enhancement of valine incorporation is associated with a decreased protein turnover in animals fed deficient or imbalanced amino acid levels, since in such rats the radioactive valine disappears more slowly from serum proteins, and even more so from liver, than in normal controls. These results are in agreement with those of others (Steinbock and Tarver, '54; Jeffay and Winzler, '58) who found a lowered protein turnover in protein-deficient rats by using labeled plasma, or purified, labeled plasma proteins.

The extent of the increase of valine incorporation in various organs is independent of the normal rate of incorporation. For example, no changes were found in the spleen, which has a high normal rate. The Torula diets used in the present study were applied for the production of dietary necrotic liver degeneration and other, related disease entities. Although it is well known that amino acid imbalance, especially in the form of a low sulfur amino acid content, plays a role in the etiology of these diseases, it is evident that the increased rate of amino acid incorporation is independent of and unrelated to the specific metabolic lesion which leads to necrotic degeneration, for the following reasons. (a) The enhancement is not influenced by vitamin E or selenite selenium. (b) Methionine causes a further increase in incorporation while decreasing the development of necrotic degeneration. (c) No difference in the rate of incorporation is observed, in animals from the same group, between nonnecrotic livers and the intact areas of livers showing massive necrosis. (d) No correlation is found between the rate of enhancement in different organs and their involvement in necrotic, pathological changes. Muscle, for example, shows no enhancement, although tending to undergo severe changes in the form of muscular dystrophy in a high percentage of animals fed the basal Torula diet.

Variation in the pool size does not appear to be the cause of the enhancement of valine incorporation. Although no direct measurements were made, indirect evidence is provided by the following considerations: (a) The enhancement is different in various organs, and even in the subcellular fractions of the same organ. (b) The alterations are independent of the growth rate of the animals. Methionine-supplemented rats grow twice as fast as those supplied with the unsupplemented basal diet, but the enhancement is the same, if not more pronounced. (c) The incorporation is further enhanced in methionine-deficient rats given the 10% essential amino acid supplements inclusive of relatively large amounts of valine. (d) In rats of similar dietary background, injected with different amounts of labeled valine, the extent of the increase is approximately the same.

Of all organs examined, muscle and spleen did not participate in the acceleration of amino acid incorporation. It is tempting to theorize that the enhancement is in the nature of an adaptive, physiological mechanism, and that these two tissues do not participate in it since they are primary sources of endogenous amino acids during protein depletion.

## SUMMARY

The incorporation of intraperitoneally injected valine-1-$C^{14}$ into proteins of serum and various tissues was measured in rats fed vitamin E-deficient Torula yeast diets during the latent, prenecrotic phase of dietary necrotic liver degeneration. As compared with controls fed on McCollum's wheat-casein diet or on a 20% casein regimen, incorporation of valine into liver and serum protein was found considerably enhanced.

The accelerated valine incorporation was not directly related to the prenecrotic condition of the animal: The necrosis-preventing supplements, vitamin E or selenite, with or without addition of DL-methionine, were without effect; L-cystine also had no influence. However, a combination of both vitamin E and selenite with methionine significantly reduced the incorporation of valine into liver protein. The observed changes are independent of the growth rate of the animals.

When a mixture of essential amino acids, with methionine, was fed at a 10% level, normal rates of valine incorporation were established in liver and serum protein. The same normalization was obtained by a combination of 5% essential amino acids, with methionine, and 5% nonessential amino acids. In the absence of methionine, the mixture of the essential amino acids did not reverse the enhanced rate of incorporation. Supplementation of 10% of a mixture of nonessential amino acids, with or without methionine, did not reverse the condition, but caused a further increase of the rate of valine incorporation. Thus, the enhancement of valine incorporation appears to be a sign of an insufficient or imbalanced supply of essential amino acids.

In subcellular fractions of liver, the maximal increase of the rate of incorporation was noted in mitochondria, followed by that in the supernatant, microsomes and nuclear fraction.

The increase in valine incorporation was observed in all organs except muscle and spleen. Maximal enhancement occurred in the liver (+ 80%), heart (+ 55%) and kidney (+ 49%).

A characteristic difference occurred in the rates at which the radioactivity disappeared from serum and especially liver protein. During the first two days following the injection, the disappearance of valine was delayed in animals fed the basal Torula yeast diet.

## ACKNOWLEDGMENTS

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# Effect of Pyridoxine Deficiency upon Delayed Hypersensitivity in Guinea Pigs[1]

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The inhibitory effects of a pyridoxine deficiency upon formation of circulating antibodies in response to various antigenic stimuli have been demonstrated in the rat (Stoerk and Eisen, '46; Axelrod and Pruzansky, '55; Axelrod, '60) and the requirement for this vitamin in various phases of the anamnestic response has been established in this species (Stoerk, '50; Axelrod, '58). Pyridoxine deficiency also depressed formation of circulating antibodies and the early Arthus-type skin hypersensitivity reaction to diphtheria toxoid in guinea pigs (Axelrod et al., '61). Therefore, it becomes important to know whether this deficiency can also affect delayed hypersensitivity. Accordingly, in the present investigation we have studied the effect of a pyridoxine deficiency on delayed hypersensitivity of the tuberculin type in guinea pigs.

## MATERIALS AND METHODS

*Animals.* Male, albino guinea pigs, 3 to 5 days old, of the Hartley strain, were kept in individual cages with wide-mesh screen bottoms and weighed twice weekly throughout the experiment.

*Diets and supplements.* Compositions of the highly purified diet used to produce pyridoxine deficiency and the corresponding highly purified control diet (4 mg of pyridoxine·HCl/kg) have been described previously (Axelrod et al., '61). To prevent early death, each animal receiving the pyridoxine-deficient diet from the beginning of the experiment was given a daily intraperitoneal injection of 1 mg of pyridoxine for the first 4 days. In some instances a laboratory chow diet[3] was fed. All diets were fed ad libitum.

Daily, intraperitoneal injections of 10 mg of deoxypyridoxine·HCl ($DB_6$)/100 gm of body weight induced a pyridoxine deficiency in more mature animals previously fed the purified control diet (Axelrod et al., '61). These animals, designated as subgroups $A_2$ and $B_2$ in figure 1 and table 1, received the purified pyridoxine-deficient diet during $DB_6$ administration.

*Production of hypersensitivity.* For sensitization purposes, 0.5 ml of a 10-day culture of *Mycobacterium tuberculosis*, BCG, in albumin medium (Dubos and Middlebrook, '47) was administered intraperitoneally to each animal.

*Skin tests.* Delayed hypersensitivity reactions were observed in skin following intradermal injection of 25 test units of purified protein derivative (PPD)[4] dissolved in 0.1 ml of the diluent recommended by Magnusson et al. ('58). One test unit is 0.02 µg of PPD. Control sites on the same animal were injected only with diluent. Criteria for rating skin reactions were degree of erythema and changes in skin thickness. Two diameters of the area of erythema were measured at right angles and erythema was classified as negative to 4 plus depending on area and intensity of redness. Special calipers were used to measure thickness of a double skin layer (Axelrod et al., '61). Good correlation usually existed between degree of erythema and skin thickness changes. Occasionally, however, some change in
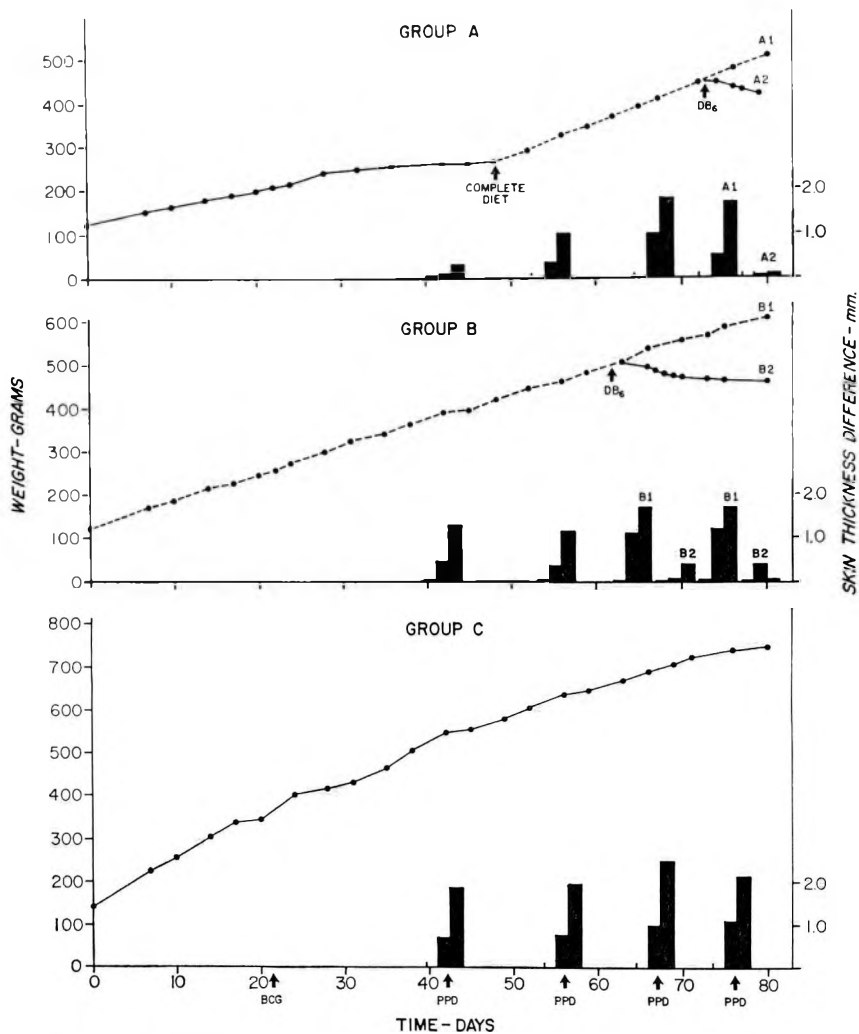
Fig. 1  Growth curves of guinea pigs correlated with skin reactions to purified protein derivative (PPD). Differences between mean skin thickness at control and PPD sites were determined at 0, 4 and 24 hours following injection of PPD and are indicated by the bars. Sensitization and skin testing was performed at times indicated by BCG (*Mycobacterium tuberculosis*) and PPD, respectively. In groups A and B, broken lines represent periods during which the purified, control diet was fed and unbroken lines periods during which the purified, pyridoxine-deficient diet was fed. Animals of group C received the laboratory chow diet. Deoxypyridoxine·HCl (DB$_6$) administration was initiated in animals of sub-groups A$_2$ and B$_2$ at times indicated by arrows. Animals of sub-groups A$_1$ and B$_1$ were not treated with DB$_6$ and continued to receive the purified, control diet.

skin thickness could be detected in the absence of erythema. Nonreacting animals exhibited no erythema and skin thickness decreased after 4 hours. Sites injected with diluent alone reacted similarly. Skin testing was performed at the times indicated in figure 1. To test general reactiv-

ity of skin, 275 µg of histamine phosphate in 0.1 ml of normal saline buffered at pH 7.2 were injected intradermally.

*In vitro sensitivity tests.* In addition to skin reactions, it is possible to determine sensitization by investigating *in vitro* the response of certain cells to the respec-

TABLE 1

*Distribution of skin reactions of individual animals within groups*

| Group | Day tested with PPD[1] | Erythema | | | | | Changes in skin thickness[2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | − | ± | + | ++ | +++ to ++++ | 0 to 0.40 | 0.50 to 0.90 | 1.0 to 1.50 | > 1.50 |
| A | | 13 | 7 | 14 | 0 | 0 | 30 | 4 | 0 | 0 |
| B | 42 | 0 | 0 | 2 | 13 | 14 | 2 | 4 | 11 | 12 |
| C | | 0 | 0 | 1 | 4 | 15 | 0 | 2 | 7 | 11 |
| A | | 0 | 0 | 7 | 13 | 4 | 0 | 9 | 10 | 5 |
| B | 56 | 0 | 0 | 2 | 7 | 13 | 0 | 3 | 13 | 6 |
| C | | 0 | 0 | 1 | 0 | 15 | 0 | 0 | 2 | 14 |
| A | | 0 | 0 | 3 | 12 | 8 | 0 | 1 | 5 | 17 |
| B₁ | | 0 | 0 | 0 | 2 | 5 | 0 | 0 | 2 | 5 |
| B₂ | 67 | 2 | 8 | 4 | 0 | 0 | 10 | 2 | 2 | 0 |
| C | | 0 | 0 | 1 | 0 | 14 | 0 | 0 | 1 | 14 |
| A₁ | | 0 | 0 | 5 | 4 | 3 | 0 | 2 | 3 | 7 |
| A₂ | | 6 | 2 | 3 | 0 | 0 | 7 | 4 | 0 | 0 |
| B₁ | 76 | 0 | 0 | 2 | 2 | 3 | 0 | 0 | 2 | 5 |
| B₂ | | 5 | 6 | 1 | 0 | 0 | 10 | 2 | 0 | 0 |
| C | | 0 | 0 | 2 | 2 | 9 | 0 | 0 | 2 | 11 |

[1] Purified protein dervative; days after begnning of experiment. *Mycobacterium tuberculosis*, BCG, given at day 21.
[2] Differences in millimeters between control and PPD sites; 24-hour readings.

tive allergen. In the present test, we studied the response of splenic mononuclear cells to the allergen, PPD. Migration of normal, nonsensitized, splenic mononuclear cells is unaffected by concentrations of PPD capable of inhibiting migration of cells from animals sensitized with *Mycobacterium tuberculosis*, BCG. Inhibition of migration can, therefore, be utilized to determine sensitivity.

Preparation of reagents used in this test has been described by Merchant et al. ('60), and the method discussed by Boyd ('56). Fresh heparinized chicken plasma (0.25 ml) was added to each Leighton tube. Spleen fragments obtained by mincing spleen with scalpel blades were washed twice in Hanks salt solution (no bicarbonate). Two fragments were suspended in 0.25 ml of chick embryo extract and deposited in the plasma of each tube. A single animal supplied spleen fragments for 6 to 10 culture tubes used in each of the PPD and control series. After the clots had become firm, 1.0 ml of medium consisting of 10% chick embryo extract, 10% fresh normal guinea pig serum, 80% Hanks solution buffered at pH 7.4, and 25 μg of PPD was added. The PPD was omitted from medium added to control tubes. Tubes were incubated at 37°C for 3 to 5 days and then read for extent and density of migration of large wandering cells. To eliminate subjective bias, all tubes were originally coded and randomized. In reading, all tubes were placed in a rank order and the numbers were decoded. Statistical analysis was performed by the method of White ('52).

*Passive transfer experiments.* It is well established that delayed hypersensitivity can be induced in normal recipient animals by transfer of cells from sensitized donors. The present experiments were conducted to determine whether peritoneal cells of pyridoxine-deficient guinea pigs injected with *Mycobacterium tuberculosis*, BCG, were capable of transferring delayed skin hypersensitivity to PPD. Successful passive transfer of this type would indicate that such cells from deficient donors had indeed been sensitized to the allergen.

Guinea pigs that had received the purified, pyridoxine-deficient or control diets for 4 weeks were inoculated with *Mycobacterium tuberculosis*, BCG. Each animal was injected intraperitoneally with 10 ml of a glycogen solution (1 mg/ml) 18 and 27 days later in experiments 1 and 2, respectively. At this time, skin reactivity to

PPD was diminished markedly in deficient animals. Controls exhibited typical skin responses. After 5 days, peritoneal cells from each group were harvested, pooled and injected intraperitoneally into a non-immunized, normal, adult guinea pig fed the laboratory chow diet. These recipients were skin-tested with PPD on the following day. Each animal was fed the same diet during the entire experiment.

## EXPERIMENTAL AND RESULTS

The experimental plan of studies designed to determine the effect of pyridoxine deficiency upon delayed, hypersensitivity reactions of skin is presented in figure 1. This figure also illustrates graphically the correlation between pyridoxine deficiency and skin reactivity at various times as well as the growth-depressant effect of a pyridoxine deficiency induced either by prolonged omission of pyridoxine from the diet or by administration of $DB_6$. Table 1 records results of skin sensitivity tests with individual animals comprising groups described in figure 1.

Pyridoxine-deficient guinea pigs inoculated with *Mycobacterium tuberculosis*, BCG, consistently displayed decreased skin sensitivity to PPD (group A). On the forty-ninth experimental day, these animals received the purified, control diet and 7 days later a marked increase in skin sensitivity was noted. On the sixty-seventh day, the skin reactivity of this group was equivalent to that of the group receiving the purified, control diet from the beginning of the experiment (group $B_1$). Pyri-

TABLE 2

*Distribution of skin reactions of individual animals to histamine*

| Group | Day tested | Erythema and blanching[1] | | | | |
|---|---|---|---|---|---|---|
| | | − | ± | + | ++ | +++ to ++++ |
| A | 48 | 0 | 2 | 5 | 6 | 3 |
| B | 48 | 0 | 0 | 2 | 4 | 4 |
| C | 48 | 0 | 0 | 2 | 3 | 5 |
| A | 60 | 0 | 0 | 3 | 5 | 4 |
| B | 60 | 0 | 0 | 2 | 4 | 4 |
| C | 60 | 0 | 0 | 2 | 3 | 5 |
| $B_1$ | 70 | 0 | 0 | 0 | 2 | 4 |
| $B_2$ | 70 | 0 | 0 | 0 | 4 | 10 |

[1] Graded on basis of area of erythema and blanching.

TABLE 3

*In vitro sensitivity of spleen cells of individual animals[1]*

| Day tested | Treatment | Control[2] | | | | Purified protein derivative (PPD)[2] | | | | P[3] | Skin reaction to PPD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Excellent | Good | Fair | Very poor | Excellent | Good | Poor | Very poor | | |
| — | Nonimmunized, laboratory chow diet | 4 | 2 | 0 | 0 | 7 | 2 | 0 | 0 | $> 0.05$ | negative |
| — | Nonimmunized, laboratory chow diet | 9 | 0 | 0 | 0 | 8 | 1 | 0 | 0 | $> 0.05$ | negative |
| 52 | Immunized, purified control diet[4] | 4 | 4 | 0 | 1 | 0 | 0 | 0 | 9 | $< 0.01$ | strongly positive |
| 55 | Immunized, purified control diet | 5 | 1 | 1 | 0 | 0 | 0 | 1 | 7 | $< 0.001$ | strongly positive |
| 55 | Immunized, laboratory chow diet | 7 | 1 | 0 | 0 | 0 | 0 | 0 | 9 | $< 0.001$ | strongly positive |
| 52 | Immunized, pyridoxine deficient diet[5] | 8 | 1 | 0 | 0 | 0 | 0 | 0 | 9 | $< 0.001$ | negative |
| 52 | Immunized, pyridoxine deficient diet[5] | 4 | 3 | 2 | 0 | 0 | 0 | 0 | 7 | $< 0.001$ | negative |
| 55 | Immunized, pyridoxine deficient diet[5] | 8 | 2 | 0 | 0 | 0 | 0 | 1 | 8 | $< 0.001$ | negative |
| 55 | Immunized, pyridoxine deficient diet[5] | 5 | 4 | 0 | 0 | 0 | 0 | 4 | 4 | $< 0.001$ | negative |

[1] Individual tubes of each animal tested (see text). Usually 9 replicates of each treatment were employed.
[2] The ratings excellent to very poor refer to cellular migration and vary inversely with degree of sensitization.
[3] Calculated by ranking method. P > 0.05 indicates no significant difference between control and PPD-treated replicates.
[4] Group B.
[5] Animals of group A which were fed the purified, pyridoxine-deficient diet during the entire experiment.

TABLE 4

*Passive transfer of skin sensitivity to purified protein derivative (PPD) by peritoneal cells*

| Experiment | Donors | No. of peritoneal cells injected | Skin reactions of recipients[1] | |
| --- | --- | --- | --- | --- |
| | | | Erythema | Change in skin thickness[2] |
| 1 | Control[3] | $2.7 \times 10^8$ | + | 0.4 |
| | Control[3] | $2.7 \times 10^8$ | + + | 0.8 |
| | Pyridoxine-deficient[4] | $2.9 \times 10^8$ | + + | 1.4 |
| 2 | Control[5] | $1.4 \times 10^8$ | + | 0.3 |
| | Pyridoxine-deficient[6] | $1.4 \times 10^8$ | + | 0.4 |

[1] Observations made 24 hours after injection of PPD.
[2] Difference in millimeters between control and PPD sites.
[3] $5.3 \times 10^8$ cells were obtained from 6 controls and one-half of these pooled cells injected into each of two recipients.
[4] Cells pooled from 5 animals.
[5] Cells pooled from 4 animals.
[6] Cells pooled from 6 animals.

doxine deficiency induced by administering DB₆ to animals of sub-groups $A_2$ and $B_2$ produced a marked decrease in skin reactivity in comparison with controls (sub-groups $A_1$ and $B_1$). Since these animals had previously exhibited a high degree of skin sensitivity, it is apparent that the effect of a pyridoxine deficiency is not limited to a depression of initial sensitization. The purified, control diet allowed good skin reactivity which, however, was not as marked as that established in animals receiving the laboratory chow diet (group C). An incidental finding was that growth of *Mycobacterium tuberculosis*, BCG, as evidenced by gross lesions, primarily of the omentum, was less in pyridoxine-deficient animals.

Skin reactions of individual animals to histamine are recorded in table 2. On day 48, reactions of pyridoxine-deficient animals (group A) were marked although somewhat less pronounced than those of controls (groups B and C). On day 60, after receiving the purified, control diet for 11 days, animals of group A exhibited histamine sensitivities comparable to those of controls. Histamine reactivity in animals treated for 8 days with DB₆ (subgroup $B_2$) did not differ from that of controls (sub-group $B_1$) although marked differences in skin reactivity to PPD had already been observed a few days earlier.

*In vitro* sensitivity tests of spleen cells are recorded in table 3. There is no doubt that spleen cells of pyridoxine-deficient animals with diminished skin reactivity to PPD were still sensitive to this allergen. Although no actual comparative determinations of levels of sensitivity were made, it was obvious that in all cases the splenic cells were highly sensitized. In further support of the existence of cellular sensitivity, it should be noted that peritoneal cells from pyridoxine-deficient animals inoculated with *Mycobacterium tuberculosis* were capable of transferring sensitivity to PPD (table 4).

DISCUSSION

Data reported in this paper indicate clearly that the delayed, tuberculin-hypersensitivity reaction in skin was depressed in pyridoxine-deficient guinea pigs. Earlier investigations of the relationship between specific nutritive factors to the hypersensitivity state were discussed fully in a previous paper (Axelrod et al., '61). Mueller et al. ('62) have since reported on the prevention of experimental allergic encephalomyelitis by vitamin C-deprivation. It is, therefore, becoming increasingly evident that host nutrition is a factor of crucial significance in the development of hypersensitivity phenomena.

The depressant effect of a pyridoxine deficiency upon the tuberculin skin reaction cannot be attributed to the concomitant state of inanition since no inhibition of this reaction in inanition control guinea

pigs has been observed either in our laboratory or by Mueller et al. ('62).[5] The possibility that the depressed skin reaction to PPD resulted from nonspecific harmful effects of the deficiency state upon skin condition is rendered unlikely by the observation that skin reactivity to histamine was unaffected in $DB_6$-treated animals.

The decreased number of focal lesions in the omentum of immunized, pyridoxine-deficient animals might indicate a lessened production of sensitivity. However, results of *in vitro* tests of cellular sensitivity and passive transfer experiments indicated that cellular sensitivity had actually been achieved in the pyridoxine-deficient animals. Although growth of the *Mycobacterium tuberculosis* may have been depressed in the deficient guinea pigs, sensitization of the cells was not affected. Whether adrenal hyperfunction resulting from the deficiency state can inhibit the skin tuberculin reaction requires further investigation. In view of the experiments of Mueller et al., ('62), however, this does not appear likely.

An interesting comparison of our observations with those previously reported on the effects of pyridoxine deficiency on circulating antibody formation can be made. Stoerk ('50) and Axelrod ('58) have both reported that administration of antigen to pyridoxine-deficient rats did not prepare animals for the anamnestic response when pyridoxine deficiency of these animals was corrected. Similar data on the anamnestic response in guinea pigs have not been published. However, data reported in this paper demonstrated that the sensitization mechanism had not been inhibited at the cellular level even though ability to respond to the allergen might be affected. One can speculate that pyridoxine or its coenzyme is an essential component in the sequence of reactions between sensitized cell and antigen. Normal serum was utilized in the *in vitro* tests of cellular sensitivity and a normal animal served as the recipient in passive transfer experiments. Thus, in both instances, requirement for pyridoxine during reaction between sensitized cells and antigen was fulfilled. However, both tests demonstrated that initial sensitization of the cells occurred during

pyridoxine deficiency. Further investigations of the cellular mechanisms involved are in progress.

## SUMMARY

Pyridoxine-deficient guinea pigs inoculated with *Mycobacterium tuberculosis*, BCG exhibited depressed delayed-hypersensitivity skin reactions to the allergen, purified protein derivative (PPD). Deoxypyridoxine treatment of previously sensitized animals also depressed skin reactivity. However, *in vitro* tests and passive transfer experiments demonstrated that cells of the pyridoxine-deficient animals were sensitive to PPD. Altered skin sensitivity may be explained not by a lack of cellular sensitivity, skin tissue changes, or inanition but by a depressed ability of sensitized cells to react with PPD.

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# Feeding Value of beta-Carotene following Treatment with $N_2O_4$[1]

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Feeds containing high levels of nitrate have been implicated in a lowering of the vitamin A status of animals (Garner, '58).[3,4,5] Nitrite, a reduction product of nitrate, appears to be the principal causative agent in this relationship (Emerick et al., '62).[6] Olson et al. ('62) demonstrated that nitrite, under acid conditions such as occur in the abomasum, or gaseous oxides of nitrogen similar to those produced in some silages (NO and $N_2O_4$), are capable of causing a rapid bleaching of carotene. The experiment reported herein was conducted to determine whether the product obtained from treatment of β-carotene with $N_2O_4$ retains any vitamin A activity or is toxic.

## EXPERIMENTAL

*Preparation of the reaction products.* A gas consisting principally of $N_2O_4$, prepared as described by Olson et al. ('62), was passed through a solution containing 192 mg of crystalline β-carotene dissolved in approximately 400 ml of *n*-hexane. Treatment was continued until bleaching of the carotene reached a maximum and the solution began to show color due to an excess of the gas. The $N_2O_4$-treated carotene, which was less soluble than β-carotene in hexane, began to precipitate toward the end of the treatment period. A treatment period of approximately 2 minutes was required for completion of the reaction when a fairly high rate of gas flow was used. The reaction mixture was finally taken to dryness under reduced pressure, and immediately dissolved in 400 gm of corn oil.

A treatment flask containing 400 ml of hexane, but no carotene, was treated in a manner identical to the treatment of the carotene solution. The small amount of residue which remained after drying was taken up in 400 gm of corn oil, and is henceforth called the $N_2O_4$-treated hexane product.

*Feeding trials.* Replicated trials, each involving 9 groups of 8 Sprague-Dawley male rats, were conducted over periods of 39 days (trial 1) and 40 days (trial 2). Although the trials were conducted at different times, the treatments and procedures were the same in both instances.

The basal diet was designed to be deficient in vitamin A and consisted of the following in percentages: Corn starch, 65; vitamin-free casein, 18; brewer's yeast, 8; salts mixture, 4 (Phillips and Hart, '35); corn oil, 5. An oil solution of irradiated ergosterol and α-tocopheryl acetate was administered once weekly in an amount equivalent to 10 IU vitamin D and 4 mg of α-tocopherol/rat.

Treatments, consisting of additions to the basal diet, were as follows: Group 1, none; group 2, 10 IU vitamin A/gm; group 3, 6 μg β-carotene/gm; group 4, $N_2O_4$-treated carotene; group 5, $N_2O_4$-treated carotene plus 10 IU vitamin A/gm; group

6, $N_2O_4$-treated carotene plus 6 µg β-carotene/gm; group 7, $N_2O_4$-treated hexane product; group 8, $N_2O_4$-treated hexane product plus 10 IU vitamin A/gm; group 9, $N_2O_4$-treated hexane product plus 6 µg β-carotene/gm. The oil solutions of the reaction products, when used, replaced one-fourth of the corn oil in the diet. In the case of the $N_2O_4$-treated carotene, this was equivalent to 6 µg of the original carotene/gm of diet.

At the termination of the experiment the rats were decapitated, and the livers were obtained and immediately frozen. Liver vitamin A and carotene were determined by the method of Johnson and Baumann ('47).

## RESULTS AND DISCUSSION

Average weight gain and liver analysis data are shown in table 1. Greater weight gains were made by rats receiving the basal diet supplemented with vitamin A (group 2) or carotene (group 3) than were made by the corresponding group (group 1) not receiving vitamin A or carotene. This difference in growth attests to the vitamin A-deficient state of the group 1 rats receiving only the basal diet.

No apparent benefit in weight gain was derived from the feeding of $N_2O_4$-treated carotene in group 4. That the $N_2O_4$-treated carotene retained no apparent vitamin A activity is further demonstrated by the failure of this material to contribute to

TABLE 1

*Weight gains and liver vitamin A storage in rats receiving various treatments*

| Treatment | | Trial | Avg wt gain[1] | Avg liver carotene[2] | Avg liver vitamin A |
|---|---|---|---|---|---|
| | | | *gm* | *µg/liver* | *µg/liver* |
| 1 | Control | 1 | 98 | 2 | 4 |
| | | 2 | 104 | 10 | 10 |
| | | Avg | 101 | 6 | 7 |
| 2 | Vitamin A[3] | 1 | 137 | 3 | 308 |
| | | 2 | 171 | 4 | 546 |
| | | Avg | 154 | 4 | 427 |
| 3 | β-Carotene[4] | 1 | 157 | 5 | 265 |
| | | 2 | 160 | 5 | 306 |
| | | Avg | 158 | 5 | 286 |
| 4 | $N_2O_4$-treated carotene[4] | 1 | 107 | 5 | 5 |
| | | 2 | 106 | 6 | 8 |
| | | Avg | 106 | 5 | 6 |
| 5 | $N_2O_4$-treated carotene + vitamin A | 1 | 123 | 2 | 386 |
| | | 2 | 135 | 4 | 513 |
| | | Avg | 129 | 3 | 450 |
| 6 | $N_2O_4$-treated carotene + β-carotene | 1 | 121 | 3 | 203 |
| | | 2 | 161 | 4 | 228 |
| | | Avg | 141 | 4 | 216 |
| 7 | $N_2O_4$-hexane product | 1 | 103 | 5 | 6 |
| | | 2 | 123 | 5 | 4 |
| | | Avg | 113 | 5 | 5 |
| 8 | $N_2O_4$-hexane product + vitamin A | 1 | 123 | 2 | 272 |
| | | 2 | 166 | 3 | 562 |
| | | Avg | 144 | 2 | 417 |
| 9 | $N_2O_4$-hexane product + β-carotene | 1 | 153 | 5 | 249 |
| | | 2 | 157 | 4 | 288 |
| | | Avg | 155 | 4 | 268 |

[1] Average initial weights for trials 1 and 2 were 76 and 59 gm, respectively.
[2] Error due to the presence of substances that absorb light at 440 mµ is probably high.
[3] Vitamin A palmitate equivalent to 3 µg vitamin A alcohol/gm of diet.
[4] β-Carotene, 6 µg/gm of diet; $N_2O_4$-treated carotene equivalent to 6 µg of original β-carotene.

liver vitamin A stores of these animals (table 1). This observation verifies the assumption made in the work of Olson et al. ('62) that the destruction of carotene by gaseous oxides of nitrogen does not yield compounds having vitamin A activity.

A toxic effect was not obtained by the feeding of either the $N_2O_4$-treated carotene or the $N_2O_4$-treated hexane product. This is indicated by the lack of significant differences between the final weights of groups 1, 4 and 7 which received the respective treatments of control, the $N_2O_4$-treated carotene, and the $N_2O_4$-treated hexane product. Further, both vitamin A and β-carotene supplementation gave fairly consistent responses in terms of weight gain and liver vitamin A storage regardless of the presence or absence of the reaction products. A somewhat smaller average weight gain made by the rats receiving the $N_2O_4$-treated carotene and vitamin A (group 5), does not appear to indicate a toxicity of the $N_2O_4$-treated carotene since no reduction in weight gains was observed in the other two groups (groups 4 and 6) receiving this material. This indicates that the similarly low growth obtained with the basal diet, the $N_2O_4$-treated carotene or $N_2O_4$-treated hexane product treatments was due only to the vitamin A inadequacy

of these diets. These data show that $N_2O_4$-treated carotene neither retains vitamin A activity nor is toxic.

## SUMMARY

To further clarify the relationship between feeds of high nitrate content and the vitamin A status of animals, β-carotene treated with $N_2O_4$ was fed under various dietary conditions to albino rats to determine whether this material retained any vitamin A activity or was toxic. Weight gains and liver vitamin A storage indicate that the material was neither toxic nor had any vitamin A activity.

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# Effects of Dietary Nitrite on the Chick: Growth, Liver Vitamin A Stores and Thyroid Weight

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Experimental evidence has been presented showing that dietary nitrate accelerates the depletion of vitamin A from body stores of ruminants (Hatfield et al., '61)[1] and that dietary nitrite or nitrate exerts similar effects on the rat (Smith et al., '61).[2] Welsch et al.[3] observed enlarged thyroid glands in rats consuming a ration containing 2.5% $KNO_3$. The data reported herein describe the effects of dietary nitrite on growth, vitamin A status and thyroid gland weight of the chick when vitamin A and carotene were fed or when vitamin A was administered by intramuscular injection.

## METHODS AND MATERIAL

*Experiment 1.* One-day-old, White Leghorn cockerels obtained from a commercial hatchery were fed a practical, vitamin A-free ration for one week. After the depletion period, 25 chicks were randomly allotted to each of 12 pens in electrically heated chick batteries, and 6 treatment groups were randomly assigned pens in a replicated block design.

As shown in table 1, treatments were divided into groups A and B. The treatments in group A were applied to 20 chicks per experimental pen. Group B was established by randomly selecting 5 chicks per experimental pen and applying the treatments listed under group B, table 1, for the entire experimental period. The only difference between groups A and B was that chicks used in group B received a high level of vitamin A by intramuscular injection in addition to vitamin A supplied in ration.

The basal ration contained 510 IU of vitamin A/kg and consisted of the following: (expressed as percentage of the total ration), ground wheat, 67.0; soybean meal (45% protein), 22.0; meat scrap (50% protein), 5.5; rapeseed oil, 3.0; defluorinated rock phosphate, 1.0; vitamin premix, 1.0; salt premix, 0.5.

The vitamin premix contributed the following per kilogram of ration: vitamin A, 510 IU; vitamin $D_3$, 818 ICU; vitamin E, 22 IU; and the following expressed as milligrams per kilogram of ration: riboflavin, 5.5; niacin, 16.5; pantothenic acid, 11; choline, 275; menadione, 1.1; vitamin $B_{12}$, 0.01; the salt premix supplied the following (expressed as milligrams per kilogram of ration), sodium chloride (iodized), 4,600; manganese, 120; iron, 17; zinc, 11; copper, 6.6.

The vitamin A used in the rations was an accurately assayed gelatin encapsuled material.[4] Nitrite, as potassium nitrite, was premixed with finely ground wheat and a sufficient quantity of this premix was substituted for an equal amount of wheat contained in the basal ration to give the desired level of nitrite.

Feed and water were supplied ad libitum, with feed consumption and body weights being recorded weekly.

A commercial preparation of vitamin A in oil[5] was diluted with corn oil immediately prior to injection. Chicks were injected intramuscularly every second day with 0.1 ml of the corn oil solution. The quantity of vitamin A injected per chick

TABLE 1

*Outline of treatments used in experiment 1*

| Treatment no. | Dietary nitrite, 0.4% | Source of vitamin A[1] | | Total vitamin A[1] (oral or oral plus injected) |
|---|---|---|---|---|
| | | Ration | Injected | |
| | | Group A | | |
| 1 | — | 510 | 0 | 510 |
| 1+N | + | 510 | 0 | 510 |
| 2 | — | 4080 | 0 | 4080 |
| 2+N | + | 4080 | 0 | 4080 |
| 3 | — | 510 | 3570 | 4080 |
| 3+N | + | 510 | 3570 | 4080 |
| | | Group B | | |
| 1 | — | 510 | 10,720 | 11,230 |
| 1+N | + | 510 | 10,720 | 11,230 |
| 2 | — | 4080 | 10,720 | 14,800 |
| 2+N | + | 4080 | 10,720 | 14,800 |
| 3 | — | 510 | 10,720 | 11,230 |
| 3+N | + | 510 | 10,720 | 11,230 |

[1] Vitamin A values expressed as international units per kilogram of ration or the equivalent thereof.

TABLE 2

*Outline of treatments used in experiment 2*

| Treatment no. | Dietary nitrite, 0.4% | Source of vitamin A[1] | | | Total vitamin A[1] (oral or oral plus injected) |
|---|---|---|---|---|---|
| | | Ration | | Injected | |
| | | Vitamin A | Carotene | | |
| 1 | — | 0 | 0 | 0 | 0 |
| 1+N | + | 0 | 0 | 0 | 0 |
| 2ₒ | — | 4080 | 0 | 0 | 4080 |
| 2ₒ+N | + | 4080 | 0 | 0 | 4080 |
| 3ₒ | — | 16,320 | 0 | 0 | 16,320 |
| 3ₒ+N | + | 16,320 | 0 | 0 | 16,320 |
| 2ᵢ | — | 0 | 0 | 4080 | 4080 |
| 2ᵢ+N | + | 0 | 0 | 4080 | 4080 |
| 3ᵢ | — | 0 | 0 | 16,320 | 16,320 |
| 3ᵢ+N | + | 0 | 0 | 16,320 | 16,320 |
| 2꜀ | — | 0 | 4080 | 0 | 4080 |
| 2꜀+N | + | 0 | 4080 | 0 | 4080 |
| 3꜀ | — | 0 | 16,320 | 0 | 16,320 |
| 3꜀+N | + | 0 | 16,320 | 0 | 16,320 |

[1] Vitamin A values expressed as international units per kilogram of ration or the equivalent thereof.

in group A was computed on the basis of the difference between the vitamin A intake of chicks consuming rations containing 4,080 IU of vitamin A/kg (treatments 2 and 2+N) and those consuming the same ration with respect to nitrite but containing only 510 IU of vitamin A/kg (treatments 3 and 3+N). These computations were made at two-day intervals between injections. Sufficient vitamin A was injected into chicks consuming the low vitamin A ration (group A) to equalize vitamin A intake (oral vs. oral plus injected) between each respective treatment pair (2 vs. 3 and

2+N vs. 3+N). The quantity of vitamin A injected into chicks of group B was similarly calculated, except that all chicks were injected with vitamin A equivalent to 10,720 IU/kg of ration consumed.

All chicks not receiving vitamin A by injection were injected with 0.1 ml of corn oil carrier every second day.

*Experiment 2.* In this trial, chicks of the same type and from the same source as those in experiment 1 were used. Fifteen chicks were randomly allotted to each of 42 experimental pens at one day of age and 14 treatments, as outlined in table 2,

were randomly assigned to the pens according to a triplicated block design. The basal ration listed under experiment 1 was used and was identical in composition except vitamin A was not included in the vitamin premix. Feed was supplied ad libitum for the first week of the experiment. A depression in feed intake of nitrite-fed chicks was observed; consequently feed consumption was restricted for the remaining three weeks of experiment 2. The chicks in all treatments were restricted to the quantity of feed consumed per chick in the treatment consuming the least feed. Chicks consuming the nitrite, vitamin A-free ration (1+N) ate the least feed. The quantity of feed allowed the remaining chicks was calculated daily on a per chick basis. Feed restriction was initiated to: (1) study the influence of nitrite on chick performance when feed intake was equalized, and (2) more precisely control the vitamin A intake among treatments to facilitate more valid comparison of the influence of nitrite on vitamin A status of the chick. Body weights were recorded weekly.

The quantities of vitamin A to be injected were computed as described for experiment 1. Carotene levels used in the rations were computed on the basis of a 2:1 conversion ratio by weight of β-carotene to vitamin A. From these computations, levels of dietary β-carotene were derived which theoretically would be approximately equivalent to the two levels of dietary vitamin A. Stabilized synthetic β-carotene[6] was used in this study.

Liver samples were taken from three chicks per experimental pen after 4 weeks of the experiment for analysis as described under experiment 1. At the same time, thyroid glands were removed from the sacrificed birds and weighed.

## RESULTS

The data from experiment 1 (table 3) show that dietary nitrite significantly decreased weight gain ($P \leqslant 0.05$) and generally reduced efficiency of feed utilization by the chicks regardless of vitamin A level or method of administration. Accompanying this reduction in weight gain was a marked drop in feed consumption. An attempt to relate these observations more precisely by covariance analysis was precluded because the regression coefficients of weight gain on feed consumption for the nitrite and non-nitrite treatments were significantly different.

[6] β-Carotene kindly supplied by Hoffmann La Roche, Limited.

TABLE 3

*Influence of nitrite on weight gain, feed conversion, liver vitamin A and thyroid gland weight of chicks, experiment 1*

| Treatment no. | Weight[1] gain | Feed[2] conversion | Liver vitamin A | Thyroid gland wt | Mortality |
|---|---|---|---|---|---|
| | *gm* | | *μg/gm fresh wt* | *mg/100 gm body wt* | *%* |
| | | | Group A | | |
| 1 | 194 ± 13[3] | 2.10 ± 0.04 | 2.96 ± 0.08 | 8.80 ± 1.16 | 10 |
| 1 + N | 116 ± 2 | 2.76 ± 0.18 | 1.14 ± 0.49 | 15.70 ± 0.30 | 44 |
| 2 | 223 ± 4 | 1.86 ± 0.03 | 31.58 ± 1.55 | 6.86 ± 1.25 | 2 |
| 2 + N | 125 ± 2 | 2.32 ± 0.06 | 2.00 ± 0.27 | 13.88 ± 1.40 | 8 |
| 3 | 184 ± 22 | 1.89 ± 0.02 | 13.68 ± 0.17 | 7.90 ± 0.22 | 8 |
| 3 + N | 126 ± 6 | 2.17 ± 0.07 | 5.90 ± 2.18 | 9.62 ± 0.47 | 10 |
| | | | Group B | | |
| 1 | 207 ± 17 | — | 36.32 ± 6.28 | 8.66 ± 1.53 | 0 |
| 1 + N | 123 ± 13 | — | 34.32 ± 4.13 | 10.91 ± 2.16 | 0 |
| 2 | 228 ± 11 | — | 49.52 ± 4.86 | 7.30 ± 0.97 | 0 |
| 2 + N | 132 ± 8 | — | 42.30 ± 8.21 | 8.12 ± 0.86 | 0 |
| 3 | 197 ± 5 | — | 46.85 ± 4.55 | 8.33 ± 0.56 | 0 |
| 3 + N | 121 ± 14 | — | 39.76 ± 2.14 | 9.47 ± 0.97 | 0 |

[1] Weight gain per cockerel from zero to 4 weeks of age.
[2] Grams of feed required per gram of gain. Weight gain of cockerels from groups A and B were pooled to arrive at total weight gain per experimental pen. Total gain was used to compute feed conversion.
[3] Mean ± SE.

A marked influence of dietary nitrite on liver level of vitamin A of chicks in group A occurred. Whether the higher level of vitamin A was fed (treatment 2+N) or intramuscularly injected (treatment 3+N) nitrite significantly reduced liver vitamin A level ($P \leqslant 0.05$) as compared with that of chicks not receiving nitrite (treatments 2 and 3).

The liver vitamin A data of treatments 2 and 3, group A, also indicate that chicks receiving vitamin A by injection did not utilize the vitamin as well, in terms of liver storage, as chicks receiving vitamin A as part of the ration. However, this had little effect on interpretation of these data since nitrite reduced liver vitamin A levels of chicks receiving the vitamin by either method to essentially the same level (treatments 2+N and 3+N).

In contrast with the liver vitamin A data of group A, the liver vitamin A level of chicks in group B, which received injections of massive doses of vitamin A, was not markedly influenced by dietary nitrite. Evidently this level of injected vitamin A greatly surpassed the quantity of vitamin A that was being lost for liver storage under the influence of nitrite, and as a result, vitamin A levels of the liver were increased.

The continued growth depression caused by nitrite in group B, even though liver vitamin A levels were high, suggests that the low liver vitamin A levels induced by nitrite in group A were a secondary effect of nitrite and not necessarily the cause of or correlated with reduced growth. It was obvious that the depression in weight gain induced by nitrite in groups A and B was of similar magnitude, and this depression in growth persisted even though liver vitamin A levels were high (group B). Exact comparison of liver vitamin A levels between chicks consuming nitrite and those not receiving nitrite may be questioned because of differences in feed intake, which led to differences in oral vitamin A intake. However, these differences were small compared with the differences observed in liver vitamin A levels of chicks receiving appreciable quantities of vitamin A either orally or *per os* plus injection.

The feeding of nitrite significantly increased thyroid gland weight of chicks in group A ($P \leqslant 0.05$) except when vitamin A was injected. Nitrite-fed chicks receiving vitamin A by injection (treatment 3+N) exhibited enlarged thyroid glands compared with non-nitrite fed chicks (treatments 1, 2 and 3). However, these were significantly smaller than the thyroid glands of nitrite fed chicks receiving vitamin A orally (treatment 1+N and 2+N) ($P \leqslant 0.05$). This indicates that vitamin A administered by injection partially overcame the effect of nitrite on the thyroid gland whereby hypertrophy was induced. On the other hand, an equivalent level of oral vitamin A failed to counteract this effect. This phenomenon was further substantiated by the weight of the thyroid glands of chicks in group B. These chicks received massive doses of vitamin A by injection and exhibited thyroid glands which had undergone little, if any, hypertrophy.

The percentage of mortality (table 3) was high when nitrite accompanied the low vitamin A level (treatment 1+N) but was reduced when 4,080 IU of vitamin A/ kg of ration or the equivalent thereof accompanied dietary nitrite. Mortality was most severe during the first 10 days of the experiment with relatively few losses encountered thereafter. All chicks receiving nitrite, and especially those simultaneously receiving the low level of vitamin A, exhibited anorexia, mild ataxia and dypsnea. These chicks were pale and quickly became exhausted when excited. At no time during the experiment were any specific symptoms of vitamin A deficiency observed during antemortem or postmortem examination. Supplemental vitamin A partially overcame the acute toxicity of dietary nitrite in group A and massive doses of vitamin A completely prevented mortality in group B.

In experiment 2, when daily feed consumption by all groups was equalized, differences between weight gain (1 to 4 weeks of age) of chicks fed nitrite and those receiving no nitrite were not observed except where vitamin A-free rations were fed (table 4). Chicks consuming the nitrite, vitamin A-free ration (treatment

TABLE 4

*Influence of nitrite on weight gain, liver vitamin A and carotene, and thyroid gland weight of chicks, experiment 2*

| Treatment[1] no. | Weight[2] gain | Liver vitamin A | Liver carotene | Thyroid gland wt | Mortality |
|---|---|---|---|---|---|
| | $gm$ | $\mu g/gm$ fresh wt | $\mu g/gm$ fresh wt | $mg/100\ gm$ body wt | $\%$ |
| 1 | $72 \pm 3$[3] | $0.88 \pm 0.27$ | $1.05 \pm 0.19$ | $7.67 \pm 0.65$ | 7 |
| $1 + N$ | $51 \pm 3$ | $0.19 \pm 0.14$ | $1.14 \pm 0.07$ | $16.97 \pm 2.62$ | 33 |
| $2_o$ | $88 \pm 2$ | $24.13 \pm 7.20$ | $1.03 \pm 0.07$ | $6.66 \pm 0.89$ | 0 |
| $2_o + N$ | $86 \pm 2$ | $1.26 \pm 0.34$ | $1.14 \pm 0.15$ | $13.59 \pm 2.83$ | 7 |
| $3_o$ | $94 \pm 6$ | $87.25 \pm 4.67$ | $1.16 \pm 0.21$ | $5.67 \pm 0.88$ | 0 |
| $3_o + N$ | $80 \pm 3$ | $15.87 \pm 5.98$ | $1.05 \pm 0.50$ | $10.21 \pm 0.78$ | 16 |
| $2_i$ | $91 \pm 1$ | $35.39 \pm 7.70$ | $1.47 \pm 0.10$ | $8.13 \pm 0.59$ | 9 |
| $2_i + N$ | $85 \pm 2$ | $8.20 \pm 1.78$ | $1.27 \pm 0.13$ | $12.98 \pm 1.20$ | 2 |
| $3_i$ | $85 \pm 4$ | $69.80 \pm 4.27$ | $1.54 \pm 0.14$ | $6.74 \pm 0.91$ | 11 |
| $3_i + N$ | $86 \pm 1$ | $55.58 \pm 5.78$ | $1.16 \pm 0.10$ | $10.92 \pm 0.92$ | 2 |
| $2_c$ | $92 \pm 3$ | $4.55 \pm 2.03$ | $1.41 \pm 0.17$ | $5.93 \pm 1.03$ | 4 |
| $2_c + N$ | $79 \pm 6$ | $1.35 \pm 1.27$ | $1.05 \pm 0.27$ | $12.31 \pm 2.55$ | 13 |
| $3_c$ | $92 \pm 6$ | $36.66 \pm 4.31$ | $3.05 \pm 0.25$ | $6.32 \pm 1.08$ | 4 |
| $3_c + N$ | $85 \pm 6$ | $2.55 \pm 0.49$ | $1.04 \pm 0.15$ | $10.01 \pm 2.55$ | 2 |

[1] The subscript used after each treatment number denotes the following: (o) vitamin A-fed, (i) vitamin A-injected and (c) carotene fed and was the vitamin A source.
[2] Weight gain per cockerel from 1 to 4 weeks of age.
[3] Mean $\pm$ SE.

$1 + N$) gained significantly less ($P \leq 0.05$) than all other treatment groups although average feed consumed per chick was the same. The consistent improvement in weight gain when a source of vitamin A was administered to chicks fed nitrite as compared with chicks fed the nitrite, vitamin A-free ration, indicates that vitamin A or β-carotene largely overcame the growth depressing effect of nitrite when feed intake was equalized. Although nitrite generally depressed weight gain regardless of level or source of vitamin A, the magnitude of depression was not large in most instances and within treatment pairs ($3_i + N$ vs. $3_i$, etc.) was not consistent. The data show that vitamin A, given as part of the ration or injected, and ration β-carotene were essentially equal in overcoming the growth depressing effect of nitrite under the conditions of this experiment.

Liver vitamin A data (table 4) demonstrate the influence of dietary nitrite, whereby storage of vitamin A was markedly reduced, with the exception of where the high level of vitamin A was injected ($3_i + N$). These data support those of experiment 1. It was also demonstrated that nitrite exerted nearly the same effect on liver vitamin A level when β-carotene was the source of vitamin A for the chick as when vitamin A was administered. The

results of experiment 2 also show that a high level of injected vitamin A would markedly increase liver vitamin A in the presence of dietary nitrite, and that this occurred only with injected vitamin A. On the other hand, an equivalent quantity of vitamin A administered in the ration failed to increase liver vitamin A to the same extent. This would suggest an increase in destruction of ration vitamin A prior to absorption due to nitrite or to interference by nitrite in absorption of vitamin A from the digestive tract, or both.

Although only obvious when high levels of β-carotene were fed ($3_c$ and $3_c + N$), the effect of dietary nitrite on storage of carotene in the liver was parallel to its effect on vitamin A storage. Nitrite significantly decreased ($P \leq 0.05$) liver carotene level of chicks in treatment $3_c + N$ as compared with $3_c$.

Dietary nitrite caused a consistent enlargement of the thyroid gland (table 4). As in the case with weight gain, this effect was most severe with the vitamin A-free ration (treatment $1 + N$). With each successively higher level of vitamin A or β-carotene consumed, thyroid gland enlargement became less. Again vitamin A, fed or injected, and ration β-carotene were essentially equal in this respect. Degree of thyroid enlargement, although not completely consistent, was generally inversely related

to weight gain during the three-week experimental period.

Early mortality due to feeding nitrite was observed in experiment 2. Nitrite induced the most severe losses when chicks consumed the vitamin A-free ration (1+N). As in experiment 1, vitamin A or β-carotene partially alleviated acute nitrite toxicity although this influence was not completely consistent in all treatments. Specific vitamin A deficiency symptoms were not observed in any treatment.

### DISCUSSION

Inhibition of growth by dietary nitrite or nitrate has been reported with cattle,[7] swine[8] and rats (Smith et al., '61).[9] The data reported herein describe similar effects of dietary nitrite on the chick under ad libitum feeding conditions. However, when feed intake was equalized among nitrite-fed and chicks not fed nitrite, growth was not greatly influenced by nitrite, except in chicks that did not receive vitamin A or vitamin A precursor. This suggests that nitrite depressed growth primarily by reducing feed consumption of chicks receiving supplemental vitamin A. Weinchethal et al.[10] reported reduced feed consumption by cattle receiving 1% of $NaNO_3$ in the ration and Hale et al. ('62) observed similar results in cattle when 1% $KNO_3$ was fed.

Muhrer et al. ('55) reported symptoms of vitamin A deficiency in cattle consuming nitrate and O'Dell et al.[11] reported that a diet containing 0.3% of $KNO_2$ caused xerophthalmia and reduced liver vitamin A level in rats. Smith et al. ('61) presented data showing that a ration containing 0.8% of $KNO_2$ reduced liver vitamin A levels and growth of rats, and this "chronic nitrite toxicity" could be largely overcome by supplementing the ration with vitamin A or carotene. In the present study the chicks did not exhibit any specific vitamin A deficiency symptoms when fed 0.4% of nitrite for 4 weeks. However, giving supplemental vitamin A to nitrite-fed chicks largely overcame the mortality caused by nitrite when low levels of vitamin A were fed, but failed to alleviate the growth depressing effect of nitrite under ad libitum feeding conditions. In contrast, when feed intake was equalized, supplemental vitamin A or β-carotene almost completely overcame the growth depressing effect of nitrite. These results strongly suggest that nitrite inhibits growth of the chick primarily by decreasing feed consumption.

Nitrite also accelerated the loss of injected vitamin A from the chicks as determined by the quantity stored in liver. However, chick growth and liver vitamin A data of both experiments failed to show that reduction in liver vitamin A levels, caused by nitrite, was the major reason for growth depression. In the case where nitrite accompanied the vitamin A-free ration, growth was most severely depressed. On the other hand, marked increases in liver stores induced by massive doses of vitamin A injected intramuscularly failed to overcome the growth depressing effect of nitrite in experiment 1.

The liver vitamin A levels in experiment 2 are of interest from the standpoint of the effect of dietary nitrite on utilization of ration vitamin A. In both instances where equivalent levels of vitamin A were administered either as part of the ration or by injection, liver vitamin A levels of nitrite-fed chicks receiving ration vitamin A were appreciably lower than in chicks receiving vitamin A by injection. This was particularly true of chicks receiving high levels of vitamin A. These results suggest that nitrite either accelerated the destruction of vitamin A in the ration or decreased absorption of vitamin A from the digestive tract, or both, thereby, decreasing utilization of ration vitamin A as compared with that given by injection. Under practical circumstances this would be an important mechanism by which dietary nitrite could adversely affect the vitamin A status of animals. Nitrite also decreased liver vitamin A level by either interfering with the storage mechanisms or accelerating loss of vitamin A from the

[7] Weichenthal, B. A., R. J. Emerick, L. B. Embry and F. W. Whetzal 1961 Influence of nitrate on performance and vitamin A status of fattening cattle. J. Animal Sci., 20: 955 (abstract).

[8] Tollett, J. T., D. E. Becker, A. H. Jensen and S. W. Terrill 1960 Effect of dietary nitrate on growth and reproductive performance of swine. J. Animal Sci., 19: 1297 (abstract).

[9] See footnote 2.
[10] See footnote 7.
[11] See footnote 2.

liver, or both. This was shown by the relative liver vitamin A levels of chicks receiving vitamin A by injection with or without dietary nitrite. Nitrite appreciably decreased liver vitamin A level of the injected chicks as compared with chicks not receiving nitrite. However, in the case of high levels of injected vitamin A, nitrite did not decrease liver levels as much as in chicks fed a comparable level of vitamin A.

Nitrite consistently caused enlargement of the thyroid gland when low levels of vitamin A (510 IU/kg of ration or less) were given. Bloomfield et al. ('61) reported that dietary nitrate adversely influenced iodine metabolism by the rat and sheep, thereby, suggesting interference with thyroid gland function. Welsch et al.[12] observed enlarged thyroid glands in rats consuming a ration containing 2.5% of $KNO_3$. The results of the present investigation indicate that nitrite exerted a goitrogen-like influence on the thyroid gland of the chick. The present data on chicks also illustrate that the highest percentage of mortality and marked growth depression caused by nitrite was accompanied by the most severe thyroid gland hypertrophy. However, under ad libitum feeding conditions, the same degree of growth depression was apparent even though massive doses of vitamin A partially overcame the thyroid gland hypertrophy induced by nitrite. Under conditions of equalized feed intake, degree of thyroid gland enlargement was inversely related to the level of vitamin A or carotene supplementation. This illustrates that thyroid gland hypertrophy due to dietary nitrite was alleviated by vitamin A supplementation, but that degree of thyroid enlargement was not necessarily related to the growth of nitrite-fed chicks.

The data presented suggest that dietary nitrite depresses growth of chicks primarily by reducing feed consumption. The effect of nitrite on liver vitamin A depletion and thyroid gland hypertrophy can be partially alleviated by supplementing the ration with high levels of vitamin A or carotene, or by injecting vitamin A intramuscularly. However, overcoming these effects of dietary nitrite will not alleviate growth inhibition when chicks are supplied with feed ad libitum. This suggests that the underlying mechanism of the influence of nitrite on feed intake and consequent growth of the chick is located in some other physiological processes than those in which vitamin A or the thyroid gland or both, are critically involved.

## SUMMARY

Experiments were conducted to determine the effect of 0.4% dietary nitrite (as potassium nitrite) on growth, vitamin A status and thyroid gland weight of chicks. Nitrite depressed growth under ad libitum feeding conditions regardless of whether vitamin A was administered orally or by intramuscular injection. However, when feed intake between nitrite and non-nitrite fed chicks was equalized a depression in growth was observed only in chicks receiving the vitamin A-free ration containing nitrite. Liver vitamin A levels were low in chicks fed the various levels of vitamin A or carotene along with nitrite. However, when high levels of vitamin A were injected, which were equivalent to levels fed, liver vitamin A concentration was relatively high in nitrite-fed chicks. Thyroid gland hypertrophy was observed in chicks receiving nitrite and was largely overcome by intramuscular injection of massive does of vitamin A. Nitrite increased chick mortality rate but specific vitamin A deficiency symptoms were not observed. The data suggest that nitrite depresses growth of chicks primarily by reducing feed consumption. In addition, these data indicate that the influence of nitrite on feed intake and consequent growth of the chick resides in some other physiological processes than those in which either vitamin A or the thyroid gland, or both, are critically involved.

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[12] See footnote 3.

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# Biochemical and Morphological Observations on Choline-deficient Rats.[1] Effects of Various Levels of Beef Fat and Cholesterol, and of Injections of Sulfamerazine and Parathyroid Hormone

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During the course of previous investigations (Salmon and Newberne, '62), it was observed that saturated fats such as beef tallow, lard or hydrogenated vegetable oils were especially injurious to the cardiovascular and renal systems of the choline-deficient rat. Morphological changes in tissues and alteration in plasma and liver lipid levels were more prominent when cholesterol was added to these diets. Other investigators have observed an effect of beef fat on plasma lipids and cardiovascular damage. Barnes et al. ('59) reported a rapid increase in serum cholesterol in young swine maintained with diets containing beef fat. Fisher et al. ('59) observed increased levels of cholesterol in plasma and aorta and more severe aortic lesions when hens were given beef fat than when given no fat or linseed oil. Cox and Hale ('60) found that dietary beef fat resulted in elevated levels of serum cholesterol in swine.

One of the characteristic lesions of acute choline deficiency in the rat is the hemorrhagic kidney syndrome (Griffith and Wade, '39). A satisfactory method of separating the cardiovascular injury from the renal damage of choline deficiency has not been devised. Thus, it is not possible to say with certainty just what relationship, if any, exists between injury to the two systems in the choline-deficient rat. Lehr and Martin ('56) reported that severe arteriosclerosis and myocardial necrosis, induced by means of renal injury in the rat, could be prevented by prior thyroparathyroidectomy. These investigators related cardiovascular injury to excess function of the parathyroid glands following renal damage resulting in autointoxication with parathyroid hormone and certain sequelae attendant upon this condition.

Experiments were designed using rats maintained with choline-deficient diets to test the specific effects of beef fat on a number of parameters. In addition, the influence of sulfa-induced kidney damage and the effects of administered parathyroid hormone on cardiovascular injury to choline-supplemented rats were investigated. The results of these studies form the basis for this report.

## EXPERIMENTAL MATERIALS AND METHODS

Groups of weanling rats of the Charles River CD strain from 4 to 6 weeks of age were used in this experiment. Each group contained approximately equal numbers of males and females. The basal ingredients for diets 701, 702, 980, 1011 and 1012 consisted of the following in percentage: casein, 6; peanut meal, 25; salts mix,[3] 5; cod liver oil, 1; vitamin mix,[4] 2; sufficient sucrose to make a total of 100% including either beef fat or cholesterol and choline additions, or both, as shown in the tables. The casein and peanut meal were exhaustively extracted with hot methanol and were essentially free of fat and phospholipid.

The animals injected with sodium sulfamerazine and parathyroid hormone were maintained with diet 544/3 that consisted of the following ingredients in percentage: peanut meal, 34.3; lard, 34; cholesterol, 5; salts (as above) 5; cod liver oil, 1; B-complex vitamins (as above), 2; choline chloride, 0.3; sucrose to 100. The amounts of sodium sulfamerazine and parathyroid hormone administered are shown in the appropriate tables. The sulfa compound was dissolved in sterile physiological saline and administered intraperitoneally. The parathyroid hormone was given intramuscularly.

All animals were given feed and water daily ad libitum. Weights were recorded daily or weekly as conditions of the specific experiment indicated. Samples were taken at autopsy for the various determinations. Histopathologic studies were made on organs and tissues fixed in Bouin's fixative or in 10% neutral buffered formalin. Routine Technicon methods were used for paraffin sections. Tissues were cut at 6 μ and stained with hematoxylin and eosin. Frozen, formalin-fixed sections were cut at 15 μ and stained with oil red O for lipid studies. Durrum cells and the Spinco analytrol were used for the separation and measurement of serum protein fractions. Total serum protein (N × 6.25) was determined on representative samples with the micro-Kjeldahl apparatus. Total lipids were extracted from plasma and from samples of dried liver with 1:2 mixture of methanol and chloroform, dried and determined gravimetrically. The lipid was then dissolved in chloroform. Cholesterol was determined directly on the chloroform solution, without saponification or precipitation, by use of the Liebermann-Burchard reagent. Lipid phosphorus was determined on another aliquot, after evaporation of the chloroform, by oxidation with perchloric acid and determination of the phosphorus by the method of Fiske and Subbarow ('25).

TABLE 1

*Influence of choline on weight gains of rats consuming beef fat with and without cholesterol*

| Diet | No. of animals | Additions to diet | | | Avg initial wt | Avg wt gain in 7 days |
|------|------|------|------|------|------|------|
| | | Beef fat[1] | Choles-terol | Choline chloride | | |
| | | % | % | % | gm | gm |
| Experiment A, animals fed diet at approximately 4 weeks of age[2] | | | | | | |
| 701 | 15 | 49 | 0 | 0.00 | 54 | 21 |
| 701 | 15 | 49 | 0 | 0.12 | 55 | 27 |
| 701 | 15 | 49 | 0 | 0.20 | 53 | 27 |
| 701 | 15 | 49 | 0 | 0.60 | 55 | 26 |
| 702 | 15 | 44 | 5 | 0.00 | 54 | 2 |
| 702 | 15 | 44 | 5 | 0.12 | 52 | 13 |
| 702 | 15 | 44 | 5 | 0.20 | 52 | 14 |
| 702 | 15 | 44 | 5 | 0.60 | 49 | 18 |
| Experiment B, animals fed diet at approximately 4 weeks of age[3] | | | | | | |
| 701 | 5 | 49 | 0 | 0.00 | 81 | 10 |
| 701 | 5 | 49 | 0 | 0.60 | 81 | 33 |
| 702 | 5 | 44 | 5 | 0.00 | 71 | −1 |
| 702 | 5 | 44 | 5 | 0.60 | 70 | 19 |
| 980 | 5 | 19 | 0 | 0.00 | 91 | 10 |
| 980 | 5 | 19 | 0 | 0.60 | 93 | 39 |
| 1011 | 10 | 14 | 5 | 0.00 | 77 | 7 |
| 1011 | 10 | 14 | 5 | 0.60 | 80 | 24 |
| 1012 | 10 | 0 | 0 | 0.00 | 90 | 19 |
| 1012 | 10 | 0 | 0 | 0.60 | 87 | 30 |

[1] All diets contained 1% of cod liver oil in addition to the beef fat and cholsterol shown in table.
[2] Rats in experiment A started to be fed the respective diets, with the choline supplements as shown, at 4 weeks of age and were fed these diets for 7 days.
[3] Rats in experiment B started to be fed the respective diets at 4 weeks of age. All diets were supplemented with 0.60% of choline chloride for 7 days; after this the choline supplement was omitted from one group fed each diet and the feeding was continued for 7 more days. The weights at the end of the first 7 days are given as the initial weights and the average weight gains are given for the last 7 days.

## RESULTS AND DISCUSSION

*Choline – deficiency effects.* Table 1 shows the effects of beef fat and cholesterol on the weight gain of rats fed choline-deficient diets for one week. Animals in experiment A were fed the respective diets and choline supplements as indicated in table 1 from the start of test. Diet 701 contained 49% beef fat but no added cholesterol. When 0.12% of choline chloride was included in this diet, the average weight gain increased during the one-week period; further increments of choline up to and including 0.6% of the diet failed to produce greater increases in average weight gain. When 5.0% of cholesterol replaced an equal amount of beef fat in the choline-deficient diet (diet 702), average weight gain decreased. Choline supplements partially restored average weight gain. The increase in choline requirement when cholesterol was included at this level in a diet containing a high level of beef fat is evident.

All animals in experiment B received 0.60% of choline chloride in the diets for one week. The choline supplement was then omitted for one-half of the animals fed each diet and the feeding was contin-

ued for one week. Weight gains are given in table 1 for the final week only. When the level of beef fat was reduced to 19% (diet 980) there was no significant increase in average weight gain as compared with the gain produced by the diet with 49% of beef fat (diet 701). When 5% of cholesterol replaced an equal amount of beef fat in the 19% beef-fat diet (diet 1011) the average weight gain was about the same as with 49% of beef fat (diet 701). Choline supplementation to the cholesterol-containing diet 1011 improved weight gain. Choline supplementation to the 1% fat diet (1012) and the 19% beef-fat diet (980) also resulted in an increase in weight gain. A significant feature of these experiments was the depression in weight gain caused by the addition of cholesterol to the diet, an effect that was only partially corrected by choline supplementation.

Table 2 shows the effects of choline supplementation on plasma and liver lipids of rats consuming diets containing beef fat at levels from zero to 49% over a period of 8 days. In most cases choline supplements to the diets resulted in elevations of plasma lipid concentration and in lower-

TABLE 2

*Effect of choline on plasma and liver lipid of rats consuming beef fat with and without cholesterol*

| Diet | No. of animals | Additions to diet | | | Liver[1] | | | Plasma | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Beef fat[2] | Choles-terol | Choline chloride | Total lipid | Choles-terol | Lipid phos-phorus | Total lipid | Choles-terol | Lipid phos-phorus |
| | | % | % | % | % | % | % | *mg/100 ml* | | |
| Animals 4 weeks of age at start — fed diet 8 days | | | | | | | | | | |
| 701 | 5 | 49 | 0 | 0.0 | 47.0 | 1.8 | 0.14 | 568 | 113 | 5.10 |
| 701 | 5 | 49 | 0 | 0.6 | 23.0 | 1.1 | 0.32 | 622 | 149 | 5.85 |
| 702 | 5 | 44 | 5 | 0.0 | 47.5 | 2.8 | 0.14 | 619 | 114 | 5.58 |
| 702 | 5 | 44 | 5 | 0.6 | 36.4 | 1.5 | 0.34 | 674 | 143 | 5.95 |
| 980 | 5 | 19 | 0 | 0.0 | 41.8 | 1.2 | 0.17 | 688 | 100 | 4.39 |
| 980 | 5 | 19 | 0 | 0.6 | 16.8 | 1.8 | 0.28 | 567 | 206 | 8.66 |
| Animals 6 weeks of age at start — fed diet 8 days | | | | | | | | | | |
| 701 | 5 | 49 | 0 | 0.0 | 59.9 | 2.6 | 0.19 | 388 | 69 | 3.47 |
| 701 | 5 | 49 | 0 | 0.6 | 23.4 | 1.2 | 0.36 | 335 | 120 | 4.28 |
| 702 | 5 | 44 | 5 | 0.0 | 51.2 | 3.6 | 0.24 | 322 | 68 | 5.98 |
| 702 | 5 | 44 | 5 | 0.6 | 25.1 | 2.9 | 0.39 | 533 | 161 | 7.10 |
| 980 | 5 | 19 | 0 | 0.0 | 51.6 | 1.3 | 0.24 | 369 | 114 | 3.49 |
| 980 | 5 | 19 | 0 | 0.6 | 17.7 | 1.3 | 0.33 | 377 | 89 | 3.31 |
| 1011 | 5 | 14 | 5 | 0.0 | 45.5 | 2.4 | 0.19 | 212 | 72 | 2.47 |
| 1011 | 5 | 14 | 5 | 0.6 | 22.1 | 3.0 | 0.39 | 585 | 116 | 4.44 |
| 1012 | 5 | 0 | 0 | 0.0 | 55.8 | 1.2 | 0.17 | 307 | 88 | 3.28 |
| 1012 | 5 | 0 | 0 | 0.6 | 19.6 | 1.0 | 0.42 | 357 | 56 | 1.30 |

[1] Values for liver expressed on dry-weight basis.
[2] All diets contained 1% cod liver oil.

ing of total liver lipid and liver cholesterol levels. Choline supplements lowered total liver lipid levels of animals receiving all diets. Liver total cholesterol was variable with no marked differences among diets except in those groups where cholesterol was added to the diet. When cholesterol was added, choline supplements were less effective in lowering liver cholesterol. Plasma lipid levels were more erratic than liver lipid levels. In most cases choline supplementation increased plasma cholesterol levels. However, liver and plasma cholesterol levels in all groups were within the normal range for the rat. An interesting observation was the relatively high level of liver and plasma total lipid in the animals receiving diet 1012 that contained 1% of cod liver oil as the only source of dietary fat. Choline supplementation to this diet lowered liver total lipid substantially but had little influence on plasma total lipid levels. It was obvious that the animals fed this diet were synthesizing significant quantities of fat and cholesterol when these constituents were omitted from the diet. The amounts of synthesized lipid were in the range of those observed with high dietary fat levels.

Table 3 indicates damage observed in the cardiovascular system and kidneys of rats consuming diets with beef fat, with and without cholesterol. High levels of beef fat in choline-free diets with cholesterol or without cholesterol inflicted damage to the cardiovascular system and kidneys of rats. Moderate levels also caused damage to the kidneys. The cardiovascular system and the kidneys were protected by 0.60% of choline chloride when cholesterol was omitted from the diet. When cholesterol was included in the high beef-fat diet (diet 702), 0.60% of choline chloride failed to protect coronary arteries and kidneys completely. In the latter diet more choline was required to protect the heart valves and myocardium. Choline protected the cardiovascular system and kidneys from necrosis when the dietary fat was at a moderate level and cholesterol was not included (diet 980) but the myocardium was not afforded complete protection. When cholesterol was included in the diet with a moderate level of beef fat (diet 1011), choline supplementation failed to protect the heart valves and coronary arteries but did protect the myocardium from damage. It will be recalled that the animals con-

TABLE 3

*Effect of choline on cardiovascular and renal damage in rats receiving diets containing beef fat with and without cholesterol[1]*

| Diet | No. of animals | Additions to diet | | | Average damage grade[2] | | | | |
|------|------|------|------|------|------|------|------|------|------|
| | | Beef fat | Choles- terol | Choline chloride | Heart valves | Myo- cardium | Coronary vessels | Aorta | Kidney |
| | | % | % | % | | | | | |
| Experiment A, animals fed diet at approximately 4 weeks of age[3] |
| 701 | 15 | 49 | 0 | 0.00 | 0.8 | 1.8 | 1.4 | 0.6 | 2.4 |
| 701 | 15 | 49 | 0 | 0.12 | 0.2 | 0.2 | 0.2 | 0.4 | 0.6 |
| 701 | 15 | 49 | 0 | 0.20 | 0.0 | 0.0 | 0.0 | 0.6 | 0.4 |
| 701 | 15 | 49 | 0 | 0.60 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 702 | 15 | 44 | 5 | 0.00 | 0.6 | 1.6 | 2.0 | 0.6 | 3.4 |
| 702 | 15 | 44 | 5 | 0.12 | 0.0 | 1.4 | 1.8 | 0.6 | 1.2 |
| 702 | 15 | 44 | 5 | 0.20 | 0.2 | 0.4 | 0.8 | 0.0 | 1.0 |
| 702 | 15 | 44 | 5 | 0.60 | 0.0 | 0.0 | 0.6 | 0.0 | 0.6 |
| Experiment B, animals fed diet at approximately 4 weeks of age[4] |
| 980 | 5 | 19 | 0 | 0.00 | 0.6 | 0.8 | 1.2 | 0.2 | 3.0 |
| 980 | 5 | 19 | 0 | 0.60 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 |
| 1011 | 10 | 14 | 5 | 0.00 | 0.2 | 0.8 | 0.2 | 0.2 | 2.4 |
| 1011 | 10 | 14 | 5 | 0.60 | 0.2 | 0.0 | 0.2 | 0.0 | 0.0 |
| 1012 | 10 | 0 | 0 | 0.00 | 0.0 | 0.0 | 0.0 | 0.0 | 0.8 |
| 1012 | 10 | 0 | 0 | 0.60 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

[1] All diets contained 1% cod liver oil.
[2] Graded on a basis of 1 to 4 with slight amount of damage graded 1 and severe damage graded 4. Table figures are averages for animals in each group.
[3] See footnote 2, table 1.
[4] See footnote 3, table 1.

TABLE 4

*Effect of choline on serum protein of rats consuming beef fat with and without cholesterol[1]*

| Diet | No. of animals | Additions to diet | | Average serum proteins | | | | | | |
|------|------|------|------|------|------|------|------|------|------|
| | | Choline chloride | Choles-terol | Total | Albumin | Globulin | | | |
| | | | | | | Alpha₁ | Alpha₂ | Beta | Gamma |
| | | % | % | gm/100 ml | gm | gm | gm | gm | gm |
| 701 | 5 | 0.0 | 0 | 4.7 | 2.14 | 0.573 | 0.721 | 0.946 | 0.304 |
| 701 | 5 | 0.12 | 0 | 5.8 | 3.104 | 0.784 | 0.532 | 0.976 | 0.332 |
| 701 | 5 | 0.20 | 0 | 6.5 | 3.615 | 0.682 | 0.637 | 1.202 | 0.364 |
| 701 | 5 | 0.60 | 0 | 6.3 | 3.402 | 0.617 | 0.762 | 1.197 | 0.321 |
| 702 | 5 | 0.0 | 5 | 4.0 | 1.872 | 0.564 | 0.576 | 0.752 | 0.236 |
| 702 | 5 | 0.12 | 5 | 4.9 | 2.714 | 0.553 | 0.553 | 0.862 | 0.215 |
| 702 | 5 | 0.20 | 5 | 5.9 | 3.357 | 0.654 | 0.660 | 0.979 | 0.247 |
| 702 | 5 | 0.60 | 5 | 6.0 | 3.540 | 0.564 | 0.774 | 0.930 | 0.192 |

[1] Animals 4 weeks of age at start, maintained with diet 8 days.

suming diets with 1% cod liver oil as the only source of dietary lipid had high levels of liver total lipid and moderate levels of plasma cholesterol when choline was omitted from the diet. Despite this, no damage to the cardiovascular system was observed whether or not choline was included in the diet (diet 1012).

Serum proteins, specifically the beta-lipoproteins, are thought to be related to cardiovascular disease. Table 4 shows the effect of choline on synthesis of total serum proteins and the amounts in the various fractions. Choline supplementation to high fat diets increased total serum protein. This occurred with (diet 702) and without cholesterol (diet 701) (table 4). Dietary choline at 0.12% sharply increased serum albumin; there was no further effect of higher concentrations of choline. A slight decrease in serum albumin content was noted when cholesterol was added to the diet. The alpha₁, alpha₂, and beta globulin fractions tended to increase slightly in absolute amounts with increases in choline content of both diets. The gamma globulin fraction of animals fed both diets varied, but no appreciable change in this fraction occurred with increments of choline. It was lower in animals fed cholesterol. Total serum proteins were restored to a normal range with choline supplements but they were restored to normal at a slightly slower rate when cholesterol was included in the diet.

The effects of choline on organ weights are shown in table 5. Measured in percentage of body weight, the heart size increased as choline was added to the diet,

with or without cholesterol. The animals that received cholesterol had larger hearts on a body weight basis than those not receiving cholesterol; however, a part of this difference was due to the decreased total body weight in the former. Choline supplementation reduced the size of the kidney, but the animals that received dietary cholesterol had larger kidneys than those without it. This indicated that there was increased damage to the kidney when cholesterol was a dietary constituent, and supported the results of histopathologic studies shown in table 3. Table 5 again illustrates the depressing effect of cholesterol on body weight gain, a condition not entirely corrected by choline.

Abnormal increase in liver size as a percentage of body weight was essentially prevented when 0.12% of choline chloride was added to the high beef-fat diet (diet 701). Further increments of choline to this diet had little effect on liver size. Supplements up to and including 0.6% of choline chloride were progressively effective in preventing increase in liver size as a percentage of body weight when the high beef-fat diet contained cholesterol (diet 702).

Spleen and thymus weights increased in size as the choline content increased up to and including 0.6% of choline chloride in the diet. The addition of cholesterol to the diet had no appreciable influence on size of these organs. The absolute increase in spleen size, as well as an increase relative to body weight, with choline supplementation, was not reflected in an increased production of gamma globulin

**TABLE 5**

Effect of choline on weight of organs of animals receiving beef fat with and without cholesterol[1]

| Diet | No. of animals | Additions to diet | | Final body wt | Weight of organs | | | | | | | | | |
| | | Choline chloride | Choles-terol | | Heart | | Kidney | | Liver | | Spleen | | Thymus | |
| | | % | % | gm | gm | % body wt | gm | % body wt | gm | % body wt | gm | % body wt | gm | % body wt |
| 701 | 5 | 0.0 | 0 | 72 | 0.23 | 0.32 | 0.49 | 0.69 | 5.1 | 7.0 | 0.26 | 0.38 | 0.14 | 0.22 |
| 701 | 5 | 0.12 | 0 | 91 | 0.29 | 0.32 | 0.36 | 0.40 | 4.2 | 4.6 | 0.35 | 0.41 | 0.24 | 0.29 |
| 701 | 5 | 0.20 | 0 | 97 | 0.34 | 0.36 | 0.35 | 0.37 | 4.3 | 4.5 | 0.50 | 0.58 | 0.31 | 0.36 |
| 701 | 5 | 0.60 | 0 | 89 | 0.33 | 0.37 | 0.32 | 0.35 | 3.8 | 4.3 | 0.51 | 0.62 | 0.33 | 0.40 |
| 702 | 5 | 0.0 | 5 | 62 | 0.19 | 0.31 | 0.68 | 1.10 | 4.7 | 7.6 | 0.20 | 0.36 | 0.13 | 0.23 |
| 702 | 5 | 0.12 | 5 | 68 | 0.25 | 0.37 | 0.41 | 0.60 | 4.6 | 6.8 | 0.30 | 0.45 | 0.21 | 0.31 |
| 702 | 5 | 0.20 | 5 | 78 | 0.30 | 0.39 | 0.35 | 0.45 | 4.0 | 5.1 | 0.44 | 0.59 | 0.27 | 0.36 |
| 702 | 5 | 0.60 | 5 | 77 | 0.37 | 0.49 | 0.32 | 0.42 | 3.7 | 4.8 | 0.50 | 0.71 | 0.29 | 0.40 |

[1] Animals 4 weeks of age at start, maintained on diet for 8 days.

(table 4). These observations confirm histopathologic studies of the spleen and thymus where it has been observed that a choline deficiency results in pronounced atrophy of these organs.

Table 6 shows the effect of choline deficiency on the accumulation of stainable lipid in the various organs of the rat. Lipid accumulation was greatest when the diet contained added cholesterol (diet 702). There was a progressive decrease in the amount of stainable fat with increments of choline.

*Effects of injections of sulfamerazine and parathyroid hormone.* Cardiovascular damage is often associated with pathological changes in the kidney of choline-deficient rats. Attempts were made to produce similar damage by administration of sulfamerazine or parathormone to rats receiving adequate choline in the diet. In one experiment 10 rats were fed diet 544/3 which contained 0.30% of choline chloride. Five of these animals received intraperitoneal injections of sodium sulfamerazine at a rate of 500 mg/kg of body weight three times per week. Each rat received approximately 1,900 mg over a 7-week period. There was no effect on weight gain until after the fourth week. At the end of 7 weeks, however, the injected rats averaged 198 gm in weight as compared with an average weight of 275 gm for the controls. At the close of the experiment, the parathyroid glands of the injected rats exhibited pronounced follicular hyperplasia. Some kidney damage was evident, but there was no appreciable cardiovascular injury.

In another experiment, two groups of rats were injected with large doses of parathyroid hormone over a period of several weeks. One group of 8 rats received a total of 1,200 IU/rat over a period of 4 weeks. There was little effect on body weight and no detectable histologic evidence of cytotoxic effect on the cardiovascular system. Another group of 5 rats received a total of 4,000 IU of parathormone/rat over a period of 8 weeks. The average body weight was not appreciably affected as compared with that of control rats fed the same diet, (controls, 270 gm; hormone-injected, 250 gm). The coronary vessels and myocardium of the hor-

TABLE 6

*Effect of choline on the affinity of tissues for lipid stains*

| Diet | No. of animals | Additions to diet | | Staining affinity[1] | | |
|------|------|------|------|------|------|------|
| | | Choline chloride | Choles- terol | Heart | Aorta | Kidney |
| | | % | % | | | |
| 701 | 15 | 0.0 | 0 | 1.0 | 0.6 | 2.2 |
| 701 | 15 | 0.12 | 0 | 0.6 | 0.6 | 1.2 |
| 701 | 15 | 0.20 | 0 | 0.0 | 0.0 | 0.2 |
| 701 | 15 | 0.60 | 0 | 0.0 | 0.0 | 0.0 |
| 702 | 15 | 0.0 | 5 | 2.4 | 0.8 | 3.8 |
| 702 | 15 | 0.12 | 5 | 1.8 | 0.8 | 2.2 |
| 702 | 15 | 0.20 | 5 | 0.8 | 0.2 | 1.6 |
| 702 | 15 | 0.60 | 5 | 0.0 | 0.0 | 0.4 |

[1] Graded on a basis of 1 to 4 with slight amount graded 1 and large amount graded 4. Table figures are averages for all animals in each treatment group. Rats were approximately 4 weeks of age when started to be fed respective diets and were fed for 8 days.

mone-treated rats, however, exhibited mild degenerative changes. The results indicate that excess hormone of the parathyroid gland may result in damage to the coronary vessels and myocardium. It appears that damage to the heart and blood vessels by parathormone, however, develops at a much slower rate than damage by choline-deficient diets containing high levels of saturated fat and cholesterol.

*Histologic studies.* The lesions observed in this study were characteristic of the acute choline-deficiency state in the growing rat. The typical fatty liver of choline deficiency was present in all deficient animals.

Figure 1 shows a coronary artery with a grade 2 injury to the wall and a 1 + myocarditis. The arterial wall was unevenly thickened in many cases and a small amount of intimal fibrosis was present in the arteries of some of the animals. This observation was unexpected as the animals had been fed the diet (702, no choline) for only two weeks. This type of lesion has usually been associated with the more chronic choline deficiency syndrome.

Mural thrombi (fig. 2) were observed in coronary arteries of three animals fed the choline-deficient diet high in beef fat with added cholesterol (diet 702). These animals had received the deficient diet for only 7 days. An additional animal fed the choline deficient diet 702 for 7 days had an occlusive coronary thrombus with infarction (fig. 3).

Lipid accumulations were present in the myocardium and the walls of coronary vessels in varying amounts. Figure 4 illustrates the fat accumulation typical of the sections graded 2 + (table 6). Previous studies (Salmon and Newberne, '62) have shown that lipid accumulation precedes a disturbance in mucopolysaccharides and tissue necrosis. Aortic injury varied from lipid accumulation between elastic laminae to swelling and fragmentation of the laminae.

The hemorrhagic kidney syndrome and sequelae of choline deficiency, as described by Hartroft ('48) were observed in all the deficient rats of this series of experiments. The most severe injury was present in animals fed diet 702 that contained high levels of beef fat and cholesterol.

SUMMARY

The effects of choline deficiency in young rats fed diets containing various levels of beef fat with or without added cholesterol for 7 to 8 days were studied. Liver lipid levels always decreased and plasma lipid levels generally increased following choline supplementation. Serum-protein levels increased with choline supplementation.

Injury to the kidney, coronary vessels, myocardium and aorta were produced by this short period of choline deprivation; the injury and the level of choline required for protection were increased by the substitution of 5% of cholesterol for 5% of fat in the diet.

Injections of sodium sulfamerazine over a period of 7 weeks resulted in kidney damage and hyperplasia of the parathy-

roids but in no appreciable cardiovascular injury. Injections of a total of 4,000 IU of parathyroid hormone/rat over a period of 8 weeks induced minimal damage to the coronary vessels and myocardium. Injections of a total of 1,200 IU/rat over a period of 4 weeks produced no detectable damage. All rats receiving injections were fed diets containing adequate choline.

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## PLATE 1

### EXPLANATION OF FIGURES

1 Myocardium and coronary artery. The walls of the artery are irregularly thickened and minimal intimal fibrosis is present in upper area of vessel. This section was taken from an animal fed choline-deficient diet 702 (high beef fat plus cholesterol) for one week. H & E stain, × 100.

2 Coronary artery and myocardium. The artery contains a mural thrombus covered by endothelium. Minimal perivascular infiltration is present. This section was taken from a rat fed choline-deficient diet 702 (high beef fat plus cholesterol) for one week. H & E stain, × 100.

3 Myocardial infarct. The pale zone extending from upper left to lower right is the ischemic area undergoing necrosis as a result of occlusive thrombosis of a coronary vessel proximal to this level of the myocardium. Note cellular infiltration at junction of normal and ischemic zones. This animal was fed choline-deficient diet 702 (high beef fat plus cholesterol) for one week. H & E stain, × 100.

4 Myocardium and coronary arteriole (upper right) from rat fed choline-deficient diet 702 for one week. The darkly stained myocardial fibers and the wall of the vessel are areas of lipid accumulation. Frozen section, oil red O stain, × 400.
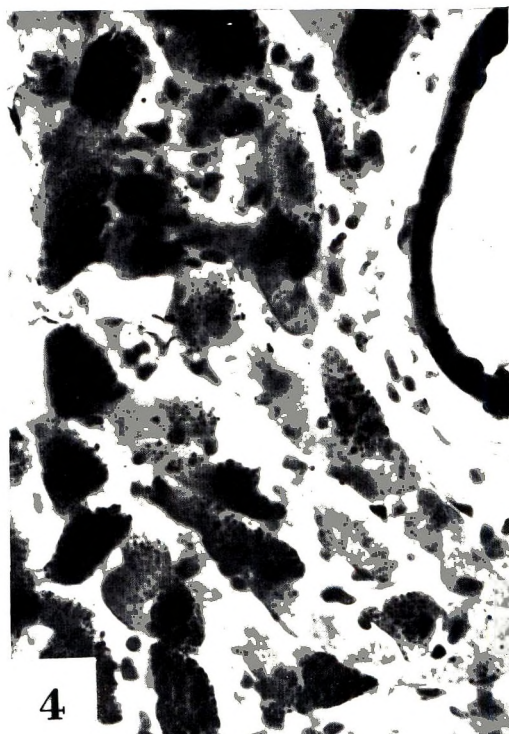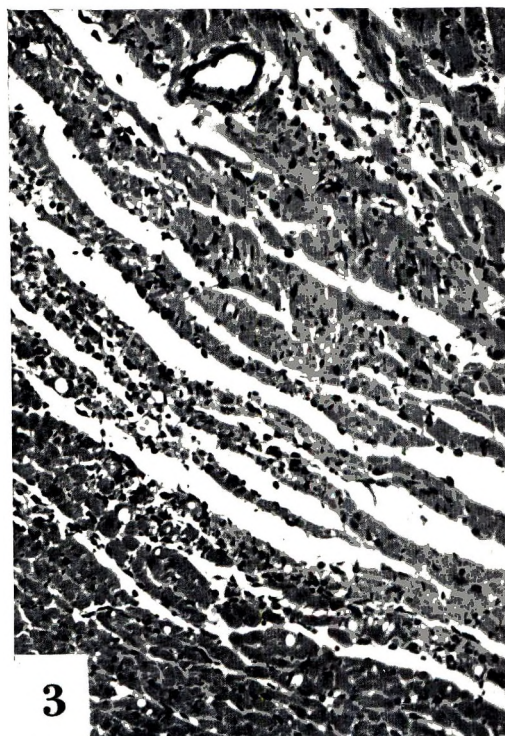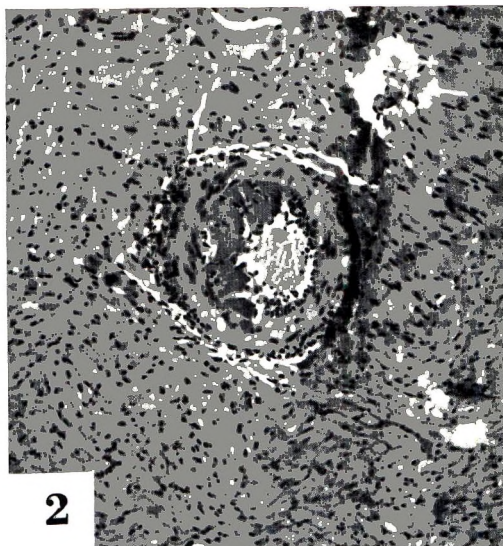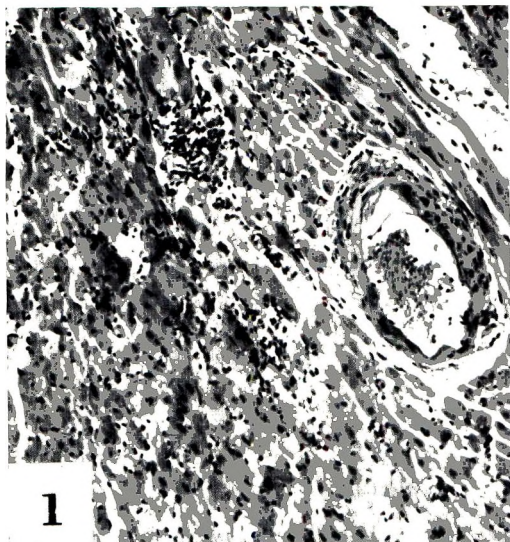
# Enzyme Studies in Thiamine-deficient Pigeons[1]

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Thiamine pyrophosphate is known to function as a specific coenzyme for a number of enzyme systems involving decarboxylation and transketolation (Peters, '36; Racker et al., '53). Several such systems have been studied in thiamine-deficient animals, but unequivocal evidence for a causal relationship between the decreased activity of any of the enzyme systems studied and the actual appearance of deficiency signs has not been demonstrated. Furthermore, the various thiamine-requiring enzyme systems do not appear to be affected similarly in different animal species by a deficiency of this vitamin. For example, in contrast with observations in the present study with pigeons, Gubler ('61) reported that the rate of oxidation of $\alpha$-ketoglutarate was not depressed in the brains of thiamine-deficient rats.

In the course of other studies at this institution, it was observed that the atherosclerosis-susceptible White Carneau (WC) pigeon was also more susceptible to thiamine deficiency than were pigeons of the resistant Show Racer (SR) breed (Lofland et al., '62). Although breed differences in susceptibility to thiamine deficiency have been described in chickens (Payne et al., '32; Nichita and Iftimesco, '34; Howes and Hutt, '56), the biochemical basis for such differences has not been demonstrated. We have compared, therefore, the activities of certain thiamine-requiring enzymes during vitamin $B_1$ deficiency in these two breeds of pigeons, in an attempt to gain information regarding this apparent genetic control of thiamine utilization.

## MATERIALS AND METHODS

The WC and SR pigeons were obtained from our stock colony. The genetic origin of these birds has been described elsewhere (Clarkson et al., '59). Since significant quantities of thiamine are excreted into the egg, only male pigeons were used in these studies, and birds of the two breeds were matched for age. The birds were housed in growing batteries $29 \times 24 \times 15$ inches (5 pigeons/cage). To avoid differences in food consumption due to anorexia associated with vitamin deficiency, the birds were force-fed with a special plexiglass syringe developed for this purpose. The diet was fed as a slurry in water. In all experiments, each bird received 30 gm/500-gm pigeon a day of a synthetic diet (table 1) supplemented with a vitamin mixture complete except for thiamine.

For the determination of the levels of pyruvate in blood, a 4-ml sample was drawn from the alar vein and was added quickly to a tube containing 25 mg of iodoacetic acid. Pyruvate was determined by the method of Lu ('39a), based on the color of the 2,4-dinitrophenylhydrazones in alkaline solution.

For the determinations of transketolase activity in blood, hemolysates of washed erythrocytes were first preincubated for 30 minutes with no substrate, followed by the addition of ribose-5-phosphate (R-5-P) and further incubation for one hour, as described by Brin et al. ('60). For brain transketolase, the pigeons were decapitated, and the brains were removed, chilled rapidly, and homogenized with a Potter-Elvehjem homogenizer in the "B" buffer described by Brin. The final volume of homogenate was 5 times the weight of the brain. The exact conditions used for both blood and brain transketolase activity are shown with figure 1. The reaction was

TABLE 1

*Time of development of deficiency signs in two breeds of pigeons*[1]

| Breed of pigeon | No. | Days fed deficient diet to appearance of signs[2,3] | Body weight | |
|---|---|---|---|---|
| | | | Initial | At time of sign |
| | | | gm | gm |
| White Carneau | 30 | 14.1 ± 0.4 | 525 ± 10 | 456 ± 9 |
| Show Racer | 29 | 21.7 ± 0.8 | 521 ± 12 | 494 ± 11 |

[1] Mean values and SE of the means.
[2] Difference between the two breeds significant ($P < 0.01$ by the Fisher-Behrens test).
[3] The diet consisted of vitamin-free casein, 25%; salt mixture, USP XIV, 4%; corn oil, 10%; dextrin, 57%; nonnutritive bulk (Alphacel, Nutritional Biochemicals Corporation, Cleveland), 2%; thiamine-free Vitamin Diet Fortification Mixture (Nutritional Biochemicals Corporation) containing vitamins in the following amounts (grams per 45.36 kg of diet): vitamin A concentrate, 4.5; vitamin D concentrate, 0.25; $\alpha$-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; Ca pantothenate, 3.0; biotin, 20 mg; folic acid, 90 mg; vitamin $B_{12}$, 1.35 mg.
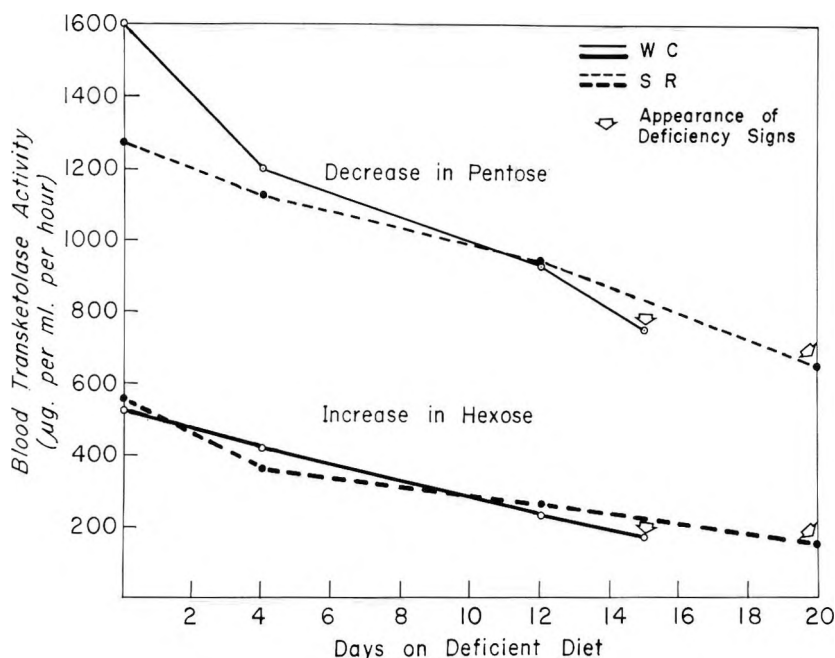


Fig. 1 Transketolase activity in erythrocyte hemolysates from two breeds of pigeons. The experimental reaction mixture consisted of hemolysate (1:3) of washed erythrocytes, 0.5 ml; "B" buffer, pH 7.4, 0.45 ml; R-5-P, 5.2 $\mu$moles in 0.2 ml. The control mixture was similar but had 6.0 ml 7.5% TCA added before incubation. Pentose and hexose determinations were carried out on both experimental and control mixtures (Brin, '60). Similar conditions were used for brain transketolase, except that 0.5 ml of 1:5 homogenate of brain was added to each reaction vessel instead of the erythrocyte hemolysate.

stopped by the addition of trichloroacetic acid, and in the filtrate, the decrease in pentose was measured by the orcinol reaction, and the increase in hexose was measured by the anthrone method. The addition of thiamine pyrophosphate (40-µg/ flask) to hemolysates prepared from the blood of thiamine-deficient pigeons resulted in the stimulation of transketolase activity to values approaching those obtained from nondeficient birds.

The activity of $\alpha$-ketoglutaric oxidase was measured by the method of Gubler ('61). In this procedure, the whole brain,

or a sample of liver (approximately 2.0 gm) was homogenized in 0.25 M sucrose, and brought to a volume of 10 × (brain) or 15 × (liver) the weight of the tissue. The oxidation of α-ketoglutarate was measured by following spectrophotometrically the reduction in color of ferricyanide, which acts as the final electron acceptor. The readings were made at 420 mμ in a Beckman Model DU spectrophotometer, and the results expressed as change in optical density/0.1 mg N in 30 minutes. Nitrogen was determined by the direct nesslerization method of Koch and McMeekin ('24).

Thiamine determinations were carried out by the thiochrome method of Hennessy and Cerecedo ('39), except that the steps involving adsorption and elution of thiamine from a base-exchange silicate were found to be unnecessary.

### RESULTS AND DISCUSSION

The results of feeding the thiamine-deficient diet to the two breeds of pigeons are shown in table 1. The White Carneau breed developed deficiency signs (opisthotonus and ataxia) significantly earlier than the Show Racer breed. The pigeons of the Show Racer breed were significantly more variable, which is probably to be expected, since they are more heterogeneous genetically than are White Carneau pigeons. However this difference has been observed consistently, regardless of whether we compared young birds (6 weeks of age) or older, sexually-mature pigeons (> 1 year of age). Table 1 also shows the changes in body weight that accompanied the development of the deficiency. The Show Racer breed appeared to lose weight less rapidly than the White Carneau breed. Pigeons force-fed a similar diet but which contained thiamine, maintained their body weight and did not develop deficiency signs.

As mentioned previously, the White Carneau breed develops atherosclerosis spontaneously whereas the Show Racers are more resistant to this disease. We were interested in the possibility that susceptibility to atherosclerosis and to thiamine deficiency might be regulated by some genetic factors common to both systems. To study this point, matings between WC males and SR females, and the reciprocal cross were established. The $F_1$ resulting from these types of matings were allowed to mate naturally to obtain the $F_2$ birds. We do not have sufficient data to indicate whether, in the $F_1$ generation, the susceptibility to thiamine deficiency or to atherosclerosis, is related to the parental mating type. The $F_1$ and $F_2$ generations resulting from these crosses were fed thiamine-deficient diets and maintained until deficiency signs appeared. At this time, thiamine was administered parenterally, and after a recovery period of one week, the birds were fed for six months a diet previously shown to be atherogenic for pigeons (Clarkson and Lofland, '61), in order to hasten the onset of atherosclerosis. The results are shown in table 2. Extremely low correlation coefficients were found between the two conditions, indicating that there is probably no close association between susceptibility to atherosclerosis and to thiamine deficiency.

Table 3 depicts the progressive increase in the levels of blood pyruvate as the vitamin deficiency progresses. Although this does not constitute a direct measure of pyruvate oxidase, previous studies have indicated that tissues of thiamine-deficient animals oxidize pyruvate at a reduced rate, and that the decrease in enzyme activity is the result of a deficiency in the coen-

TABLE 2

*Relationship between susceptibility to thiamine deficiency and to atherosclerosis in pigeons[1]*

| Generation | No. | Days to deficiency signs | Atherosclerotic index[2] | Correlation coefficient | Probability[3] |
|---|---|---|---|---|---|
| $F_1$ | 45 | 16.8 ± 0.4 | 7.3 ± 1.4 | − 0.209 | > 0.10 |
| $F_2$ | 33 | 16.5 ± 0.6 | 4.8 ± 1.3 | − 0.088 | > 0.10 |

[1] Mean values, and SE of the means.
[2] Percentage of the surface of thoracic aorta which was occupied by plaques.
[3] Probability that correlation coefficient obtained would be as large or larger if correlation in population sampled equalled zero.

TABLE 3

*Changes in levels of blood pyruvate in two breeds of pigeons during thiamine deficiency[1]*

| Breed of pigeon | No. | Blood pyruvate level[2] | | | | |
|---|---|---|---|---|---|---|
| | | Mean day of sign | Day 0 | Day 4 | Day 12 | Day of sign |
| White Carneau | 5 | 13.2 | $2.6 \pm 0.2$ | $4.3 \pm 0.3$ | $4.8 \pm 0.9$ | $8.8 \pm 0.4$ |
| Show Racer | 5 | 18.4 | $2.8 \pm 0.3$ | $3.8 \pm 0.2$ | $3.9 \pm 0.6$ | $7.2 \pm 0.4$ |

[1] Mean values and SE of the means.
[2] Pyruvate values in mg/100 ml whole blood.

zyme rather than of an inability to synthesize the apoenzyme (Gubler, '61). Therefore it seems logical to assume that the elevation in blood pyruvate is due to the decreased activity of pyruvate oxidase. Initial values for the two breeds of pigeons were very similar, and remained so during the course of the deficiency. Similar results were obtained for the activity of transketolase in the blood of the two breeds, as shown in figure 1. In agreement with the work of Brin et al. ('60), we find that blood transketolase activity shows a significant decrease early in thiamine deficiency (in some experiments we were able to detect significant decreases from initial activity as early as three days after the removal of thiamine from the diet). It should, of course, be recognized that the reactions being measured here as "transketolase" actually represent a complex system consisting of several enzymatic reactions.

These observations with respect to pyruvate oxidase and transketolase activity indicate that these functions are depressed as thiamine deficiency progresses. Since, however, the two breeds behave similarly, it is doubtful that the difference between the breeds in the time of appearance of deficiency signs could be due directly to either the increased level of pyruvate, or the decreased activity of transketolase in the blood. However, the possibility exists that there is a breed difference in the sensitivity of some target organ to a product (or products) of these enzyme systems. Furthermore, since the curves for pyruvate or transketolase in the two breeds do not at any time diverge, it appears unlikely that the breed difference could be explained by one breed's having a higher rate of utilization of thiamine, or a higher rate of destruction or excretion of this vitamin. It seemed logical, there-

fore, to study the metabolism of thiamine in the brains of these birds.

Figure 2 shows a typical experiment in which the rate of α-ketoglutarate oxidation was compared in the brains of normal and thiamine-deficient White Carneau and Show Racer pigeons, and in table 4 the summary of the results of several such experiments are tabulated for both brain and liver. The activity of the enzyme de-
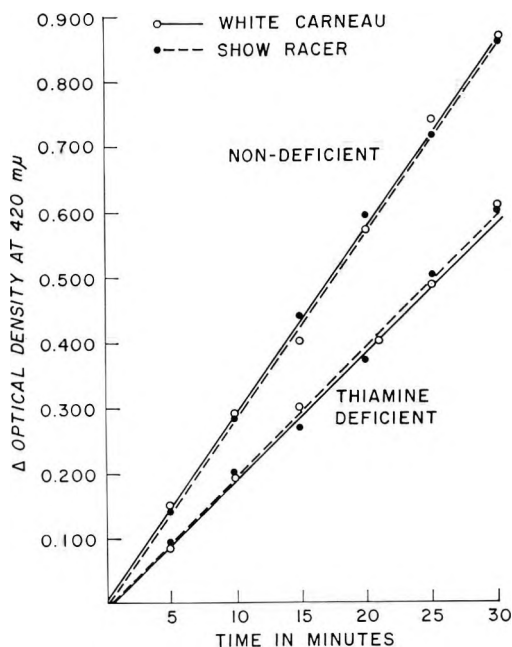


Fig. 2 Change in optical density at 420 mμ versus time when α-ketoglutarate is incubated with pigeon brain homogenate, with $Fe(CN)_6^{\equiv}$ as electron acceptor. The reaction mixture consisted of 2.6 ml phosphate buffer (0.15 M, pH 7.4), 75 μmoles, containing in addition $MgSO_4$, 20 μmoles; versene, 2 μmoles; ATP, 6 μmoles; α-ketoglutarate substrate, 40 μmoles; 1:10 homogenate of brain (in 0.25 M sucrose), 0.2 ml. Final volume made to 3.0 ml with 0.25 M sucrose; 0.7 ml of 0.00666 M $K_3Fe(CN)_6$ added. Incubated at room temperature.

creased in both organs, again at remarkably similar rates in the two breeds. The failure of the decrease in White Carneau pigeons to reach statistical significance is probably due to the small number of animals. It should be pointed out that at the time the birds were killed, all of the White Carneau pigeons were exhibiting deficiency signs, whereas all of the Show Racer breed were free of signs. Despite this fact, the two breeds had similar levels of α-ketoglutarate oxidase activity. The decrease in the activity of this enzyme parallels the decrease in the amounts of thiamine determined by a chemical method in the brains of the deficient pigeons (table 5).

Measurements of transketolase activity in the brains of these pigeons (table 6), indicate that neither the rate of pentose disappearance nor of hexose accumulation is affected as the pigeons become thiamine-deficient. Similar findings were reported for pigeon brain transketolase by Mochanacka ('56). On the other hand, our observation of a decreased α-ketoglutarate activity in the brains of deficient pigeons is in contrast with those of Gubler ('61), who reported that this enzyme system is *not* depressed in the brains of rats, even when the animals are exhibiting frank deficiency signs. It appears, therefore, that (a) in different animal species, thiamine-requiring enzyme systems are affected to a

TABLE 4

*Oxidation of α-ketoglutarate by homogenates of brains and livers from thiamine-deficient pigeons[1]*

| Breed of pigeon | No. | Δ OD[420]/0.1 mg N/30 minutes | | | |
|---|---|---|---|---|---|
| | | Organ | Initial | At day 14[2] | Probability[3] |
| White Carneau | 4 | brain | $0.268 \pm 0.05$ | $0.188 \pm 0.01$ | $< 0.20$ |
| | | liver | $0.257 \pm 0.04$ | $0.185 \pm 0.01$ | $< 0.20$ |
| Show Racer | 5 | brain | $0.269 \pm 0.05$ | $0.201 \pm 0.02$ | $< 0.05$ |
| | | liver | $0.303 \pm 0.04$ | $0.188 \pm 0.01$ | $< 0.05$ |

[1] Mean values and SE of the means.
[2] All White Carneau pigeons were exhibiting deficiency signs; none of the Show Racer pigeons exhibited signs at this time.
[3] Probability that the difference between the means would be as large or larger due to chance alone.

TABLE 5

*Thiamine levels in brains and livers of pigeons[1]*

| Breed of pigeon | Organ | No. | Thiamine level | |
|---|---|---|---|---|
| | | | Initial | Day 14 |
| White Carneau | brain | 5 | $3.3 \pm 0.1$ | $1.3 \pm 0.1$ |
| | liver | 5 | $6.9 \pm 0.8$ | $1.0 \pm 0.2$ |
| Show Racer | brain | 5 | $3.0 \pm 0.3$ | $1.4 \pm 0.2$ |
| | liver | 5 | $4.0 \pm 0.8$ | $1.0 \pm 0.1$ |

[1] Mean values (and SE of the means) in $\mu g/gm$ of fresh tissue.

TABLE 6

*Transketolase activity in the brains of two breeds of pigeons[1]*

| Breed of pigeon | No. | Value measured | Transketolase activity | |
|---|---|---|---|---|
| | | | Initial value | Day 14 |
| | | | *μg/gm tissue/hour* | |
| White Carneau | 6 | Increase in hexose | $5,345 \pm 116$ | $5,372 \pm 152$ |
| | | Decrease in pentose | $6,917 \pm 234$ | $6,953 \pm 116$ |
| Show Racer | 6 | Increase in hexose | $5,840 \pm 241$ | $5,535 \pm 144$ |
| | | Decrease in pentose | $6,854 \pm 240$ | $7,277 \pm 145$ |

[1] Mean values and SE of the means.

different degree by a deficiency of the vitamin; and (b) that some enzymes, such as transketolase, decline rapidly in blood, although their level remains high in brain. These observations suggest that some portion of the brain transketolase system might bind thiamine, in some of its forms, more tightly than does blood transketolase, or brain or liver $\alpha$-ketoglutarate oxidase.

At any rate, in none of the enzyme systems studied could differences between the breeds of pigeons be demonstrated. These results suggest that the actual appearance of the neurological signs of thiamine deficiency is related to factors other than the enzyme systems studied here. A likely possibility appears to be the system described by Nakada and Sund ('58), which oxidizes glyoxylate via $CO_2$ and formate, and which is stimulated by thiamine pyrophosphate in vitro. If thiamine is, indeed, required for this system, the accumulation of a toxic end-product (glyoxylate) would result. Actually, Liang ('62) has shown that glyoxylate does accumulate in the brain and other tissues of thiamine-deficient rats. We are attempting at present to extend this observation to our two breeds of pigeons.

## SUMMARY

White Carneau pigeons are significantly more susceptible to thiamine-deficiency than are pigeons of the Show Racer breed. When the two breeds were force-fed a thiamine-deficient diet, the levels of thiamine in the brains and livers of both breeds decreased, but to about the same extent.

After feeding the deficient diet for approximately 14 days, the White Carneau pigeons exhibited deficiency signs, whereas those of the Show Racer breed did not. Nonetheless, neither the level of pyruvate oxidase (as measured by increases in blood pyruvate), nor of blood transketolase differs significantly in the two breeds. Likewise, the activity of $\alpha$-ketoglutarate oxidase decreases to the same extent in the brains and livers of both breeds of pigeons. On the other hand, the activity of brain transketolase in either breed did not appear to change as the deficiency progresses.

It is postulated that the appearance of the neurological signs of thiamine deficiency is related to factors other than the decreased activity of the enzyme systems studied here.

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# Fluoridated Water and the Skeletal Uptake of Sr⁸⁵ and Ca⁴⁵ by Young Rats[1]

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In comparing the uptake of radiostrontium to that of radiocalcium by embryonic chick bone cultured *in vitro*, it was noted that the inclusion of $10^{-4}$ M NaF in the culturing medium markedly increased the accumulation of these radioisotopes by the bones; no significant alteration was noted in the comparative handling of the strontium and calcium (Lengemann, '60a). These data have been cited in several recent public debates as evidence that fluoridation of drinking water might well increase the amount of fallout radiostrontium deposited in the bones of growing children. The fallacy of such an extension and interpretation of the data is readily apparent. Yet it seemed worthwhile to investigate whether an effect of fluoride on strontium deposition could be demonstrated in animals.

Muhler et al. ('59) have reported that fluoride given daily to rats did not increase the uptake of radiostrontium given as a single dose. It is imprudent, however, to conclude from such data that there would be no effects from the long term ingestion of both fluoride and strontium, which is the situation of practical interest. Important is that fluoride would have an effect on the radiotoxicity of Sr⁹⁰ only if it changed the concentration of Sr⁹⁰ in bone; that is, if fluoride increased or decreased the amount of Sr⁹⁰ deposited in bone and at the same time caused similar change in the amount of bone mineral, there would be no effect on the radiation dose delivered per unit of bone tissue. Therefore, emphasis was given to the use of both radiostrontium and radiocalcium to take advantage of the predictions possible from such double tracer studies.

## METHODS

Briefly, the experimental approach was to give young growing rats various levels of fluoride in drinking water to which had been added Sr⁸⁵ and Ca⁴⁵. This drinking water was the only fluid available for consumption by the rats for test periods of from 10 to 21 days; at the end of this period samples of bone were obtained to estimate the effect of fluoride on the retention of both radionuclides. The experimental design is shown in table 1.

TABLE 1

*Experimental design[1]*

| Exp. | No./ group | Initial body wt | F⁻ in diet | Pretreatment | | Experimental (on Sr⁸⁵ and Ca⁴⁵) | |
|---|---|---|---|---|---|---|---|
| | | | | Days | F⁻ added | Days | F⁻ added |
| | | *gm* | *ppm* | | *ppm* | | *ppm* |
| 1 | 6 | 50 | 9 | 0 | 0 | 21 | 0, 1, 10, 100 |
| 2 | 6 | 50 | 9 | 21 | 0, 1, 10, 100 | 21 | 0, 1, 10, 100 |
| 3 | 12 | 40 | 3 | 0 | 0 | 10 | 0, 1 |
| 4[1] | 6 | 40 | 3 | 12 | 0, 1[2] | 10 | 0, 1[2] |
| 5 | 6 | | 3 | 12 | 0, 1 | 10 | 0, 1 |

[1] Experiment 4 and 5 were identical except that rats in experiment 5 were 12 days older than those in experiment 4.
[2] See table 2 for combinations of F⁻ levels used in pretreatment and experimental periods.

In the first experiment 50 male weanling rats weighing about 50 gm each were obtained from Carworth Farms and were given glass-distilled water and a normal protein diet[3] which contained 9.2 ppm of fluorine. On the third day, 24 of these rats (exp. 1) were put into individual metabolism cages and then given measured amounts of the normal protein diet and glass-distilled water for a three-week period. Fluoride was added to the glass-distilled water as NaF at levels of zero, 1, 10 and 100 ppm; radioactivity was added to the water at levels of 0.5 μc of $Ca^{45}$ and 0.2 μc of $Sr^{85}$/6 liters of solution.

The remaining rats (exp. 2) were divided into 4 groups and given ad libitum the normal protein diet and redistilled water containing zero, 1, 10 and 100 ppm of added NaF for three weeks. No radioactivity was given at this time. At the end of the three weeks these rats were put into the metabolism units and the intakes of food and water were measured. The fluoride level of the drinking water was maintained at the levels used during the preparative period and $Sr^{85}$ and $Ca^{45}$ was added as noted previously.

All rats were killed after the three-week period in the metabolism cages and the femurs were removed for assay by gamma and beta counting techniques customarily used in this laboratory (Lengemann, '60b). The femur content of $Sr^{85}$ and $Ca^{45}$ was expressed as a percentage of the total amount of each isotope ingested by each rat during the entire experiment.

Experiments 3, 4 and 5 were planned to clarify some of the issues raised by the results from the first two studies. In the third experiment 24 weanling rats (Carworth Farms) averaging 40 gm in weight were divided into two groups and put into the metabolism cages immediately upon arrival. One group received no $F^-$, and the other received 1 ppm $F^-$ in the labeled water. Other conditions were the same as in the preceding experiment except that the normal protein diet contained 3 ppm of fluorine and the animals were killed at the end of a 10-day test period.

In the fourth experiment (which can be visualized from tables 1 and 2) weanling rats were subjected to a conditioning period of 12 days before going on experiment. In this period some of the rats

[3] The diet was obtained from Nutritional Biochemicals Corporation, Cleveland. The composition was vitamin-free casein, 27%; starch, 59%; vegetable oil, 10%; salt mixture, U.S.P. XIV, 4%; plus vitamin diet forfication mixture. For experiment 1 this diet was used as such and analyzed as 9.2 ppm F. For experiment 3, 4, and 5 the NaF was omitted from the salt mixture and the diet then analyzed 2.9 ppm F.

TABLE 2

*Effect of fluoride in the drinking water upon the ash weight, the $Sr^{85}$ and $Ca^{45}$ content of femurs of young rats given labeled water for 9 days*

| Exp. | Fluoride in water | | Femur ash wt | $Sr^{85}$ | $Ca^{45}$ | $OR^1_{bone/diet}$ |
| | Pretreatment period | Experimental period | | | | |
| | ppm | ppm | gm | % of intake | % of intake | |
| 3 | — | 0 | 0.11 | 2.9 | 3.7 | 0.78 |
| | — | 1 | 0.11 | 2.7 | 3.5 | 0.77 |
| | SE of mean | | 0.09 | 0.2 | 0.1 | 0.08 |
| 4 | 0 | 0 | 0.20 | 3.5 | 4.0 | 0.86 |
| | 1 | 0 | 0.22 | 2.7 | 4.0 | 0.66 |
| | 0 | 1 | 0.21 | 2.7 | 4.3 | 0.65 |
| | 1 | 1 | 0.23 | 2.8 | 3.6 | 0.77 |
| | SE of mean | | 0.01 | 0.3 | 0.3 | 0.05 |
| 5 | 0 | 0 | 0.31 | 2.5 | 3.0 | 0.82 |
| | 1 | 0 | 0.31 | 2.3 | 3.0 | 0.76 |
| | 0 | 1 | 0.30 | 2.3 | 3.0 | 0.78 |
| | 1 | 1 | 0.31 | 2.2 | 2.8 | 0.77 |
| | SE of mean | | 0.003 | 0.2 | 0.1 | 0.04 |

[1] Observed ratio: $OR_{bone/diet} = \dfrac{Sr/Ca \text{ of bone}}{Sr/Ca \text{ of diet}}$.

received no $F^-$ in the drinking water, and others received 1 ppm $F^-$. For the experimental period, in which the water contained $Sr^{85}$ and $Ca^{45}$, 6 rats pretreated with no $F^-$ in the water were continued on this regimen, while 6 similar rats were switched to water containing 1 ppm $F^-$; similarly, 6 rats conditioned to 1 ppm of $F^-$ were changed to no $F^-$, and 6 other rats pretreated with 1 ppm $F^-$ in the water were continued at the 1 ppm $F^-$ level. The conditions of the test period were then the same as for the third experiment. In the fifth experiment weanling rats, obtained at the same time as those for experiment 4, were maintained with the normal protein diet (3 ppm $F^-$) and redistilled water (no ppm $F^-$) for 12 days before being subjected to a pretreatment period. The experimental treatment after the 12-day holding period was then identical to that of the fourth experiment except that the rats were 12 days older at each step.

## RESULTS

It was observed that during the period of radionuclide administration, the rats of experiment 1 gained about 66 gm and those of experiment 2, about 60 gm; no effect of the level of added fluoride was noted. The feed consumption was similar for all the rats of the two experiments except that the rats receiving 100 ppm $F^-$ in the water consumed 10% more feed than those on the "zero" level ($P < 0.05$); these high fluoride rats drank about 25% less water than did the other rats ($P < 0.05$). The animals receiving no added fluoride in the water ingested 2.1 mg $F^-$ from the diet in the three-week test period. For the other rats 2.4, 5.1, and 25.0 mg of $F^-$ were consumed by the groups given 1, 10 and 100 ppm $F^-$, respectively. Of these amounts of fluorine, 12, 56 and 91% came from the drinking water for the groups given 1, 10 and 100 ppm, respectively. Since feed consumption was relatively the same for all groups, any differences within an experiment must have been the result of the fluoride of the water.

The data for the femurs of the rats are shown in table 3. The effect of age is readily apparent in that the rats of experiment 1 showed less total bone ash, a greater accumulation of radioactivity, and lesser selection against strontium, i.e., a higher observed ratio (OR) value, than was observed for the older rats of experiment 2 ($P < 0.01$).

The fluoride in the water had no effect on the ash weight of the femurs of the rats. The $Sr^{85}$ content of the femurs of the rats of experiment 1 receiving added $F^-$ was elevated over that of the controls; the semiconfidence interval method of testing indicated that the 1 ppm group was just barely significantly different ($P < 0.05$)

TABLE 3

*Effect of various levels of fluoride in the drinking water on the femur retention of radiostrontium and radiocalcium by growing rats*

| Exp. | Fluoride in $H_2O$ | Femur ash wt | Sr⁸⁵ | Ca⁴⁵ | OR[1]$_{bone/diet}$ |
|---|---|---|---|---|---|
| | *ppm* | *gm* | *% of intake* | *% of intake* | |
| 1 | 0 | 0.23 | 2.7 | 3.9 | 0.70 |
| | 1 | 0.23 | 3.2 | 3.9 | 0.80 |
| | 10 | 0.23 | 3.1 | 3.5 | 0.89 |
| | 100 | 0.23 | 3.5 | 4.1 | 0.85 |
| 2 | 0 | 0.33 | 1.3 | 2.6 | 0.50 |
| | 1 | 0.33 | 1.4 | 3.4 | 0.41 |
| | 10 | 0.31 | 1.1 | 3.0 | 0.37 |
| | 100 | 0.34 | 1.7 | 3.0 | 0.56 |
| | SE of the mean[2] | 0.007 | 0.12 | 0.13 | 0.03 |

[1] Observed ratio: $OR_{bone/diet} = \dfrac{Sr/Ca \text{ of bone}}{Sr/Ca \text{ of diet}}$.

[2] Standard error of the mean: $s\bar{x} = \sqrt{\dfrac{\Sigma x^2 - \dfrac{(\Sigma x)^2}{n}}{n(n-1)}}$

from the control group, whereas the 10 ppm $F^-$ group was not. In experiment 2 neither the 1 or 10 ppm group differed from the control.

In both experiments 1 and 2 the 100 ppm $F^-$ group showed a statistically significant greater uptake of $Sr^{85}$ than the control group. With $Ca^{45}$ only the 1 ppm group of experiment 2 took up more radiocalcium than did its control group ($P < 0.05$).

The OR values are of particular interest since they give a reliable measure of the long-term deposition of dietary calcium and strontium. In the 1 and 10 ppm groups there was a higher relative deposition of strontium as shown by a higher OR value ($P < 0.05$) in the younger rats but with little change evident in the older animals. In the 100 ppm groups of both experiments there was a higher relative deposition of strontium to calcium, but this was significant only in the younger rats ($P < 0.05$).

Since the results with the 1 ppm groups appeared to be at variance it was essential to investigate further possible effects at this level. The apparent difference in behavior could have been the result of the difference in age between rats in experiments 1 and 2 or because the animals in experiment 1 received no pretreatment, whereas those in experiment 2 were given the $F^-$ treatment for 21 days before the experimental period. The remaining studies were planned to show whether the observed differences were real and whether the above two factors were involved.

To study the effect of age, experiment 3 was carried out with weanling rats of about 40-gm body weight. The results, table 2, experiment 3, show that there was no difference in the uptake of $Sr^{85}$ and $Ca^{45}$ when rats received zero or 1 ppm of $F^-$ in the drinking water. The OR values were close to those of the group receiving 1 ppm $F^-$ of experiment 1.

Experiments 4 and 5 were designed to check the results of experiment 1 and in addition to determine the effect of previous fluoride status on the accumulation of the radioelements in the bone of the animals.

In experiment 4 it was observed that pretreatment with fluoride appeared to in-crease slightly the femur ash weight ($P < 0.05$); this, however, was not detected in the older rats of experiment 5. In neither experiment was the uptake of $Sr^{85}$ and $Ca^{45}$ changed by the level of $F^-$ in diet. Moreover, the OR values indicate no differences in relative strontium and calcium deposition whether $F^-$ was given in pretreatment period, in the experimental period, or during both periods. The control groups, especially in experiment 4, showed an apparent higher OR value as a result of a higher $Sr^{85}$ content, but there are not enough data to indicate whether this is a meaningful difference.

The results of experiments 3, 4, and 5 thus failed to confirm that observation of experiment 1 that 1 ppm of fluoride could increase the uptake of radiostrontium by bone. The result shown in table 1 must then be regarded as the one chance in twenty that a false statement will be made.

In addition to the studies using long-term ingestion of $Sr^{85}$, experiments were also carried out using a single dosage of $Sr^{85}$. These animals underwent the same pretreatment as the rats in experiments 3, 4, and 5. In no case was there a significant effect upon the skeletal retention of $Sr^{85}$ and $Ca^{45}$ as determined by killing the animal at 24 hours after dosing.

## DISCUSSION

Throughout these studies low levels of fluoride were emphasized in order to reproduce situations where water destined for human consumption is fluoridated or where the drinking water is moderately high in fluoride. The results of such experiments are more appropriate than other types of studies thus far reported but are still not ideal for predicting the effect of fluoridated drinking water upon the retention of $Sr^{90}$ in the bones of children.

The data reported here indicate that the addition of fluoride to the water of rats at levels of 1 ppm did not significantly affect the degree of bone mineralization, the uptake of $Sr^{85}$ or $Ca^{45}$, or the discrimination between the isotopes of strontium and calcium. At 10 ppm the results on $Sr^{85}$ uptake were equivocal, there being an increase in one study and a decrease in another. A positive effect of 100 ppm of fluoride upon

the uptake of radiostrontium was noted in two experiments using two different ages of rats. The increase, however, was quite small being only 30% above that of the control animals even though the total fluoride intake was more than 10 times greater.

The age of the rat seemed to be unimportant since rats of approximately 4, 7, and 9 weeks of age at the start of the experiments all showed essentially the same pattern. The pretreatment experiments showed that animals receiving fluoridated water for some time previous to exposure to radiostrontium would not take up more Sr[85] than animals that had not received fluoridated water. Finally, the addition of fluoridated water and radiostrontium simultaneously to the diet did not lead to a greater deposition of radiostrontium than for the other groups.

As is true in all research aimed at predicting events that would occur in man, a rat experiment can only provide suggestive evidence. From data reported here, however, it can be implied that the levels of fluoride customarily added to drinking water will not lead to an increase in the Sr[90] concentration in the bones of growing children.

## SUMMARY

This investigation dealt with the effect of various levels of fluoride in the drinking water upon the accumulation of Sr[85] and Ca[45] in the skeleton of young rats. The results showed that fluoride at 1 ppm in the water had no effect upon the retention of Sr[85] and Ca[45] in the skeleton of the rats. A level of 100 ppm $F^-$ in the water, which increased the total fluoride intake by a factor of about 10, would be expected to increase the bone concentration of ingested radiostrontium by about 30% in rats under the conditions of this study.

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# Response of Rural Guatemalan Indian Children with Hypocholesterolemia to Increased Crystalline Cholesterol Intake[1,2]

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Until recently, the idea that dietary cholesterol had little influence upon human serum cholesterol levels has been supported by epidemiological and experimental evidence (Keys et al., '56; Mattil). Dietary cholesterol as a factor contributing to the pathogenesis of atherosclerosis in man, therefore, was given no importance. Recent publications, however, appear to indicate that dietary cholesterol increases serum cholesterol levels (Beveridge et al., '59, '60).[4] The design of the experiments supporting this position depends, in most cases, primarily upon a cholesterol-free period to decrease serum cholesterol levels before cholesterol supplementation is begun. Beveridge et al. ('60), using this procedure, obtained a remarkably good correlation between cholesterol increments, up to 800 mg/950 Cal., and cholesterol concentration in the serum, but no further significant increases in cholesterol concentration have been observed even with daily intakes of as high as 1300 to 4500 mg. Similar increases have also been obtained using diets high in egg yolk or in other sources of dietary cholesterol (Connor et al., '61a, b; Taylor et al., '60). These results have supported the possible role of dietary cholesterol as an important factor influencing serum cholesterol levels in man, and, possibly, in the pathogenesis of atherosclerosis.

It has been shown that members of the rural, lower socio-economic population of Guatemala, who have a low dietary intake of fat, also have low serum cholesterol levels and show very low prevalence of severe aortic atherosclerosis and myocardial infarction. On the other hand, those in the urban upper socio-economic population of Guatemala have a high dietary intake of fat, show high serum cholesterol levels, and exhibit a high prevalence of severe aortic atherosclerosis and myocardial infarction (Mann et al., '55; Scrimshaw et al., '57; Tejada et al., '58). Previous dietary surveys (Méndez et al., '62; Scrimshaw et al., '57) showed that although there is no difference in total protein intake between the groups, the urban, upper socio-economic group consumes 6 times as much animal protein as the rural group. The daily fat intake furnishes 37 and 7% of the caloric intake of the urban and rural group, respectively. The ratio of polyunsaturated to saturated fatty acid is 0.25 in the urban and 1.43 in the rural diet. Also, only 52% of the total caloric intake of the urban diet is furnished by carbohydrates, whereas the percentage in the rural diet is 80. The estimated daily cholesterol intake of the urban group is 626 mg and of the rural population 61 mg. The average serum cholesterol level is 200 mg/100 ml for the urban, and 135 for the rural populations (Mann et al., '55; Méndez et al., '62).

The conditions prevalent among rural Guatemalans are almost ideal for a study of the effect of dietary cholesterol on serum lipid levels. Because of the very low cholesterol intake of these rural people, diets need not be manipulated before

testing the effect of increased cholesterol intake, as usually necessary in this type of experiment. Consequently, experiments were carried out to determine the effect of crystalline cholesterol on the low serum cholesterol level of rural Guatemalan children.

## MATERIAL AND METHODS

In the rural village of Magdalena, Guatemala, 60 school-age Mayan Indian children were divided into two groups. Group 1, called the "cholesterol group," included 25 boys and 9 girls whose average age was 11.8 years. Group 2, or the "control group," included 13 boys and 13 girls whose average age was 9.3 years.

During two experimental periods the children in the "cholesterol group" received a daily mid-morning snack of a glass of Incaparina, INCAP's all-vegetable protein mixture for human consumption (Scrimshaw et al., '61). The cholesterol was dissolved in the heated oil, incorporated into the Incaparina gruel, seasoned with sugar and cinnamon. This made a palatable preparation that was well accepted. During the first 30 days, 600 mg of crystalline cholesterol and 15 ml of cottonseed oil were given, and during the second 30-day period this amount was increased to 1200 mg/day.

Group 2, the "control group," received a daily glass of Incaparina with only the 15 ml of cottonseed oil added, during both 30-day periods. Table 1 shows the daily intake of the children under study, as well as the total composition of the supplement.

The usual daily caloric intake of 1163 was increased to 1493, and the protein intake was increased from 32 to 39 gm. Carbohydrates were also increased from 221 to 260 gm, 26 gm of which were furnished by the sucrose added as flavoring. Total fat was increased from 17 to 33 gm and the calories thus derived amounted to 20%. Saturated fatty acids were increased from 5 to 9 gm, oleic acid from 7 to 11 gm, and polyunsaturated fatty acids from 3 to 11 gm.

None of the subjects studied evidenced severe diarrhea, a few of them experienced a small degree of gastrointestinal discomfort when the supplementation was initiated, but this disappeared within few days.

Height, weight, and fasting finger-tip blood samples were taken before the experiment started and at the end of both the first and second 30-day periods. Total serum lipids were determined by the Bragdon method (Bragdon, '51), lipid phosphorus by the Chen et al. ('56) method, and total cholesterol by the method of Abell et al. ('52) adapted for micromethods.

## RESULTS

The height and weight changes of the children throughout the two experimental periods are shown in table 2. The cholesterol group had an average increase in height of 0.8 cm, whereas the height of the control group increased 1.2 cm. On the other hand, the weight changes were 1.3 and 0.6 kg, respectively. The differences in height and weight gain could be

TABLE 1

*Estimated daily dietary intake during the cholesterol study*

|  | Home | Supplement | Total | Percentage of calories |
|---|---|---|---|---|
| Calories | 1163 | 330 | 1493 | — |
| Total protein, gm | 32 | 7 | 39 | 10 |
| Carbohydrate, gm | 221 | 39 | 260 | 70 |
| Fat, gm | 17 | 16 | 33 | 20 |
| Fatty acids | | | | |
| Saturated, gm | 5 | 4 | 9 | 6 |
| Oleic, gm | 7 | 4 | 11 | 7 |
| Polyunsaturated, gm | 3 | 8 | 11 | 7 |
| Cholesterol, mg | 45 | 600–1200 | 645–1245 | — |

accounted for by the differences in the mean ages of the two groups.

Table 3 shows the total serum lipids, lipid phosphorus, and cholesterol levels at the different periods of observation. The initial level of 598 mg serum total lipids did not change significantly at the end of the first 30-day period of cholesterol treatment, when it was 577 mg, or at the end of the second period, when it was 581 mg. The children in the control group showed a similar response. The initial serum level was 609 mg, and at the end of both the first and second 30-day periods, these values were 616 and 599 mg, respectively.

There also was no effect on serum lipid phosphorus. The initial values in the group receiving cholesterol were 6.8 mg, and the values at the end of the first and second 30-day treatment periods, 6.7 and 6.7, respectively, were not significantly different. The control group reacted similarly

with an initial value of 7.2, ending with 7.1 and 6.9 mg, respectively.

No significant change in total serum cholesterol levels occurred in the children receiving crystalline cholesterol. They had 133 mg initially and 134 mg at the end of the first 30 days during which they had received 600 mg daily. After they had received 1200 mg for another 30 days, the value was 132 mg. The control group responded similarly with values of 136, 135 and 135 for the same periods, respectively.

## DISCUSSION

The observations presented in this paper agree with those of some investigators who have used crystalline cholesterol (Keys et al., '56; Mattil), although they are at variance with the results of Beveridge et al. ('60), Connor et al. ('61a, b) and Anderson et al.[5] ('62).

[5] See footnote 4.

TABLE 2

*Height and weight changes during the dietary cholesterol study*

| | Age | Height | | | Weight | | |
|---|---|---|---|---|---|---|---|
| | | Initial | Period 1 | Period 2 | Initial | Period 1 | Period 2 |
| | | cm | cm | cm | kg | kg | kg |
| | | Cholesterol group (N = 30) | | | | | |
| Mean | 11.8 | 130.8 | 130.3 | 131.6 | 29.2 | 29.9 | 30.5 |
| Standard deviation | 1.0 | 6.3 | 6.7 | 6.6 | 3.9 | 3.7 | 4.2 |
| | | Control group (N = 22) | | | | | |
| Mean | 9.3 | 118.6 | 119.4 | 119.8 | 23.6 | 23.9 | 24.2 |
| Standard deviation | 1.8 | 7.3 | 7.3 | 7.4 | 3.1 | 3.4 | 3.3 |

TABLE 3

*Effect of high cholesterol intake on serum lipid levels[1]*

| | Cholesterol group (N = 34) | | | Control group (N = 26) | | |
|---|---|---|---|---|---|---|
| | Initial | Period 1 | Period 2 | Initial | Period 1 | Period 2 |
| | Total lipids | | | | | |
| Mean | 598 | 577 | 581 | 609 | 616 | 599 |
| Standard deviation | 76 | 98 | 111 | 114 | 106 | 117 |
| | Lipid phosphorus | | | | | |
| Mean | 6.8 | 6.7 | 6.7 | 7.2 | 7.1 | 6.9 |
| Standard deviation | 0.9 | 1.1 | 1.0 | 1.4 | 1.2 | 1.1 |
| | Total cholesterol | | | | | |
| Mean | 133 | 134 | 132 | 136 | 135 | 135 |
| Standard deviation | 21 | 26 | 26 | 23 | 22 | 28 |

[1] All values are expressed in mg/100 ml of serum.

Since these studies were performed with children, the results may not be applicable to adults. However, the marked differences in serum cholesterol levels observed between adults of lower and upper socio-economic groups in Guatemala, are also found between school children of the same population groups (Scrimshaw et al., '57).

If dietary cholesterol per se were responsible in part for the high serum cholesterol levels encountered among urban, upper socio-economic population groups in Guatemala (Mann et al., '55; Scrimshaw et al., '57), then the increase in daily cholesterol intake from 45 mg to 645 mg in the first period, and to 1245 in the second period would have elevated the serum levels. This, however, was not the case. Ahrens et al. ('57) failed to increase serum cholesterol levels of a hypercholesterolemic child after giving a corn oil formula diet supplemented daily with 0.6 to 2 gm of crystalline cholesterol; but small increases, although significant, were obtained when 4 to 8 gm of cholesterol were given. A possible explanation for the lack of effect of dietary cholesterol reported here is that the increase of polyethenoid fatty acid content in the diet, with the concomitant decrease in the saturated fatty acid, could have overcome the effect of the crystalline cholesterol. The expected change in serum cholesterol due to the change in fatty acid intake calculated from a prediction equation (Keys et al., '59) did not support this possibility. Connor et al. ('61a, b) failed to lower the serum cholesterol level in subjects given a high cholesterol diet containing increased polyethenoid fatty acids simultaneously.

Finally, there is some evidence to support the idea that the physical state of cholesterol in foods, such as the lipoprotein complexes found in egg yolk, and the levels and kind of dietary fat in the diet (Cook et al., '56) result in greater intestinal absorption and might, therefore, increase the serum cholesterol levels in man. This will be the subject of the next field experiment.

## SUMMARY

In the rural village of Magdalena, Guatemala, 60 school-age Mayan Indian children, consuming diets low in fat and cholesterol, were divided into two groups: Group 1 received a 600-mg crystalline cholesterol and 15-ml cottonseed oil supplement daily in a glass of Incaparina for 30 days. The cholesterol intake was then increased to 1200 mg and given for another 30 days. Group 2, the control group, received daily a glass of Incaparina with only 15 ml of cottonseed oil for the same two periods. The results showed that exogenous cholesterol did not increase the low serum lipid and cholesterol levels of these children.

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# Growth and Development of Chick Embryos Supplied with Various Concentrations of Yolk[1]

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The initial work of Grau et al. ('57) showed that direct studies of the nutrition of the chick embryo, *in ovo*, are feasible. After much of the yolk of three-day chick embryos was displaced by a balanced salts solution, which was, in turn, replaced by large amounts of yolk from donor eggs, the embryos developed normally to the hatching stage. Attempts to replace yolk by defined or simplified media perfused through the yolk-sac failed, when survival or growth were the criteria, and survival was increased only slightly by addition of yolk to the perfusion fluid (Klein et al., '58).

This lack of response led to a separate series of studies designed to determine the yolk requirements of young embryos[2] during early incubation. To preserve the normal environment as nearly as possible following very thorough removal of the original yolk, nutrients were introduced in a single dose rather than by perfusion, as in previous studies.

In this paper we wish to report the effects on embryos, and on extra-embryonic membranes and their fluids, of returning various amounts of yolk to the yolk-sacs of 4-day embryos, after very thorough yolk removal. A preliminary report has been published.[3]

## EXPERIMENTAL

The electro-coagulation technique of Grau et al. (Grau et al., '62) was used to connect the interior of the yolk-sac of a three-day incubated egg with a hole drilled in the shell opposite the embryo. This technique permitted formation of a ring of coagulated egg white, bonded at one end to the shell membrane around the hole, and at the other to the yolk membranes, and allowed displacement of yolk without loss of albumen. The egg was then transferred, hole down, to a small ring stand through the center of which a 20-gauge hypodermic needle extended upward 12 mm into the canal. A reservoir connected to the needle by latex tubing allowed sterile salts-glucose solution (Salts 25) to flow into the egg under pressure of about 30 cm of water. Salts 25 used contained the following, in grams per liter: sodium chloride, 4.46; potassium chloride, 1.70; calcium chloride, 0.20; magnesium chloride hexahydrate, 0.34; magnesium sulfate heptahydrate, 0.20; sodium dihydrogen phosphate monohydrate, 0.28; *d*-glucose, 5.0; sodium bicarbonate, 1.10; dihydrostreptomycin (as sulfate),[4] 0.66; and erythromycin (as glucoheptonate),[5] 0.095. The antibiotics concentrations are expressed as active bases.

The solution traveled upward to the top of the yolk-sac, displacing the heavier yolk down through the canal, and out around the needle. After displacement of most of the yolk, the embryos were returned to the incubator. On the following day (that is, after 4 days of incubation) this procedure was repeated to remove the small pool of yolk that had accumulated at the bottom of the yolk-sac. The egg was then transferred to a similar ring stand where, by means of a 17-gauge hypodermic needle, altered to dispense fluid horizontally at the tip, a final 40 ml of salts solution rapidly rinsed away remnants of the yolk.

Approximately two hours after this last step, the egg was carefully rotated to bring
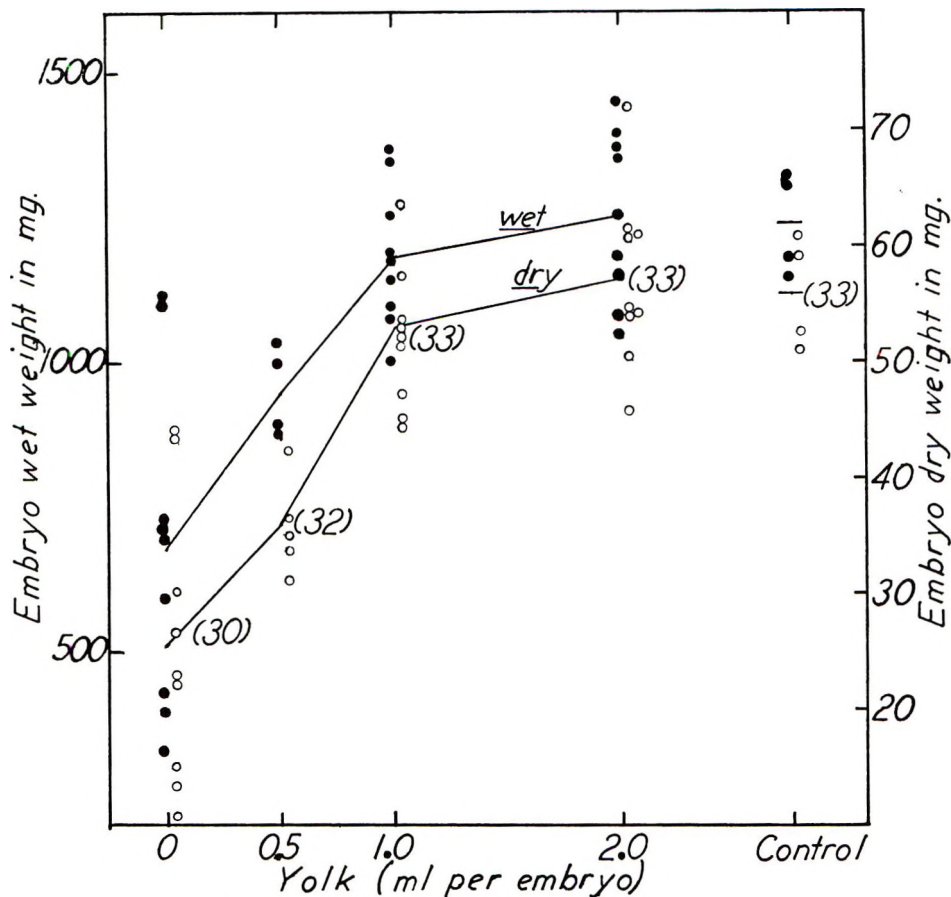
Fig. 1 Weights of 8-day chick embryos supplied with yolk from donor eggs at 4 days' incubation. Controls retained original yolk. Solid line connects mean weights. Closed circles are wet weights; open circles, dry weights. Developmental stages in parentheses.

the hole to the top, and donor yolk was injected through the canal. This yolk had been drawn by syringe from 4-day control embryos which had been prepared with canals, but which had not had their yolk displaced. The donor yolk was pooled in a sterile vessel and mixed thoroughly before the desired amount was injected. Between and after treatments, eggs were sealed with plastic tape[6] and maintained in an incubator at 37.5°C. Experiments were terminated at either 8 or 9 days' total incubation time (4 or 5 days after yolk replacement).

Samples of yolk-sac contents, allantoic fluid, and amniotic fluid were withdrawn and frozen, and the embryos were preserved in 10% formalin. Fluids analyzed for sodium and potassium were thawed, diluted to the proper range, and analyzed

in the Beckman 4100 flame photometer. Embryos were blotted with filter paper and weighed, then dried overnight in a vacuum oven at 100°C, cooled in a desiccator, and weighed again. In experiment 1, preserved embryos were examined under a dissecting microscope to determine the stage of development according to the criteria of Hamburger and Hamilton ('51).

RESULTS

In figure 1 is shown the direct relationship between yolk level and body weight of embryos sacrificed after 8 days total incubation. The mean dry weight of those that received only 1 ml of donor yolk/egg was twice that of the group receiving

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[6] Band Aid Plastic Tape, Johnson and Johnson, New Brunswick, New Jersey.

none, and was nearly equal to weights of the group receiving 2 ml, and of the positive control group, which was equipped with canals, but remained with original yolk intact. The mean developmental stage (parentheses, fig. 1) advanced from 30 (6.5 to 7 days' normal development) without yolk to 33, normal for this age.

Figure 2 shows weights of a similar experiment terminated at 9 days. At this age, 1 ml of yolk/egg was inadequate, but 2 ml permitted essentially normal growth.

Sodium-to-potassium ratios of amniotic fluid in experiment 1 (fig. 3) also increased as yolk increased, reaching normal range at 1 ml of yolk/egg. Allantoic fluid showed such fluctuations of Na/K ratios, however, that no pattern was detected.

No characteristic abnormalities were noted in embryos sacrificed at either 8 or 9 days, in contrast with effects resulting from vitamin or mineral deficiencies (Landauer, '61) or addition of antimetabolites (Karnofsky et al., '49; Ancel, '50; Landauer, '57). The parallels between wet-weight and dry-weight curves of figures 1 and 2 indicate that no edema ex-
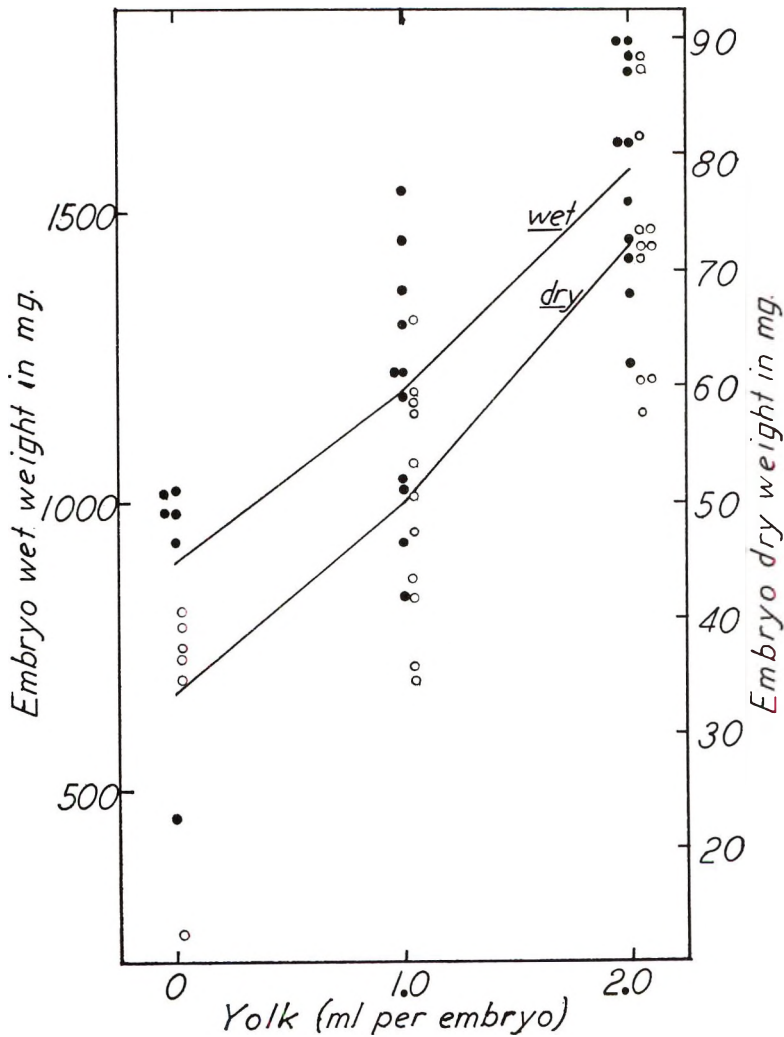


Fig. 2 Weights of 9-day chick embryos supplied with yolk from donor eggs at 4 days. Solid line connects mean weights. Closed circles are wet weights; open circles, dry weights.
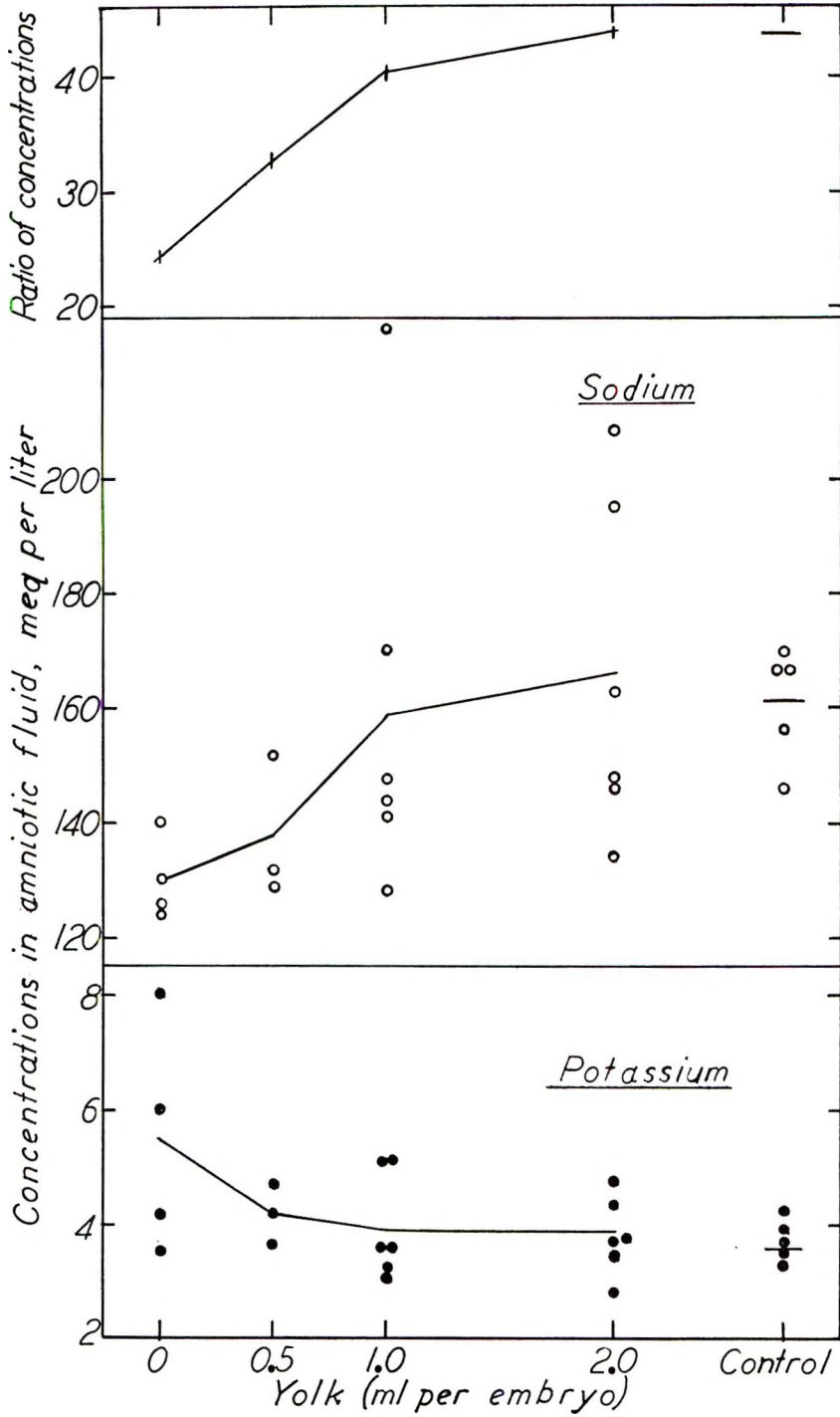
Fig. 3   Sodium and potassium in amniotic fluid of 8-day embryos supplied with donor yolk at 4 days. Lower panels: observed concentrations. Solid lines connect means. Top panel: Na/K ratios.

isted in any of the groups, nor did visual observation of embryos which had died earlier reveal edema.

## DISCUSSION

The direct response of embryos to various concentrations of yolk, starting at 4 days of age, indicates that they metabolize yolk to considerable degree in the first 9 days of incubation. This has been assumed by most investigators, but has never been shown experimentally. The increased need for yolk between 8 and 9 days, as compared with 4 to 8 days, reflects the rapid growth at these later stages of embryonic development. Embryos without sufficient yolk for normal development remain alive but weigh less than normal. From present observations, it is not possible to determine which parts of the yolk are exhausted first, or even whether there is selective utilization of certain fractions.

The mean sodium-to-potassium (Na/K) ratio in amniotic fluid of our normal 8-day controls was 44, compared with 40 in those embryos that had received 1 ml of yolk, and which had developed normally. This is in good agreement with a ratio of 41 found by Howard ('57) in amniotic fluid of 8-day embryos. Without supplemental yolk, however, 8-day embryos developed only to the stage of normal 6.5-day embryos, and had Na/K ratios averaging only 24, whereas Howard reported a ratio of 44 in normal 6-day embryos. Figure 3 shows that the low ratio found in embryos receiving no donor yolk is due to both lower sodium and higher potassium than in controls. As yolk is increased, these values approach normal at 1 ml/egg.

The same pattern was observed in several other experiments: when yolk was adequate to support normal growth, even when ionic balance in the replacement fluid was badly disturbed, the amniotic Na/K ratio remained normal in surviving embryos. Approaching exhaustion of yolk reduced the ratio before weight differences became evident.

The Na/K ratio of amniotic fluid may reflect that of the blood. Howard found similar levels of these cations in both amniotic fluid and plasma of chick embryos 12 days of age. She believed that blood, regulated by the mesonephros, or temporary functional kidney, corresponds closely to amniotic fluid in solute concentration. Since the mesonephros becomes functional on the fourth day of incubation (Hamilton, '52), there is no reason to suppose that this correspondence does not exist at that time.

Because the 91.2 mEq/liter of sodium and 22.8 mEq/liter of potassium present in Salts 25 are comparable to levels in subgerminal fluid (Howard, '57),[7] and thus differ considerably from levels noted in plasma and amniotic fluid, some regulatory mechanism appears to be still active. Addition of 1 ml of yolk at the expense of Salts 25 decreases total sodium per egg by only a negligible amount, 0.05 mEq (2%), and increases total potassium by about 0.05 mEq (9%). Since this results in an even greater difference between yolk and plasma potassium, it is likely that effects noted were due not to potassium, but to other metabolites present in the yolk.

The sodium and potassium levels of allantoic fluids were not affected in an orderly manner by yolk, but it was found that allantoic circulation was less well developed, and allantoic fluid volume was reduced, although the surface area of the membrane was normal, when only 0.5 ml of yolk was present. Fluid volume was normal with all other treatments, including that in which no supplemental yolk was supplied. Although we are unable to explain the significance of this observation, it appears that the extra-embryonic membranes and their fluids are sensitive detectors of metabolic state.

Although no abnormalities were noted in embryos that died before 9 days, or were sacrificed at 8 or 9 days, generalized edema occurred in skeletal musculature of similar embryos which received 2 ml of yolk at 4 days, and were incubated to 11 to 13 days total age. The viscera of these embryos appeared normal. A comparable group, given an additional 5 ml of yolk at 9 days of age, survived to 16 days, and these showed no edema at death. Apparently various metabolic processes are disturbed, depending upon the time of exhaustion of yolk or one of its components. Embryos receiving an initial injection of

[7] Unpublished data; S. M. Peters, of this laboratory, found 76.7 mEq of sodium and 36.7 mEq of potassium/liter of subgerminal fluid of 9 day embryos.

15 ml or more of yolk at 4 days reached the pipping stage, and appeared normal. Their ability to develop normally for 17 days after yolk replacement indicates that the rigorous steps of preparation were not harmful.

It is, of course, not unexpected that growth and development of the chick embryo are related to the amount of yolk available. The present experiments are, however, the first known to us which show this relationship clearly. Between 4 and 8 days of incubation, the embryo and its membranes require approximately 1 ml of yolk, which contains about 150 mg of protein and 320 mg of lipid, plus many other nutrients. We can speculate about the needs of the embryo for yolk nutrients, but no conclusions can be drawn from experiments using whole yolk; therefore, studies with yolk fractions are now under way. Development of techniques by which the nutritional environment can be controlled without altering normal physical relationships should make possible more precise inquiries into the nutrient requirements and metabolic processes of developing embryos.

## SUMMARY

When small amounts of yolk were supplied to chick embryos deprived of their own yolk after three days of incubation, survival, developmental stage, and body weight, measured at 8 or 9 days, were related directly to the amount of yolk provided. Embryos that were sacrificed, or that died of yolk exhaustion, show no characteristic abnormalities except for some edema among those of 11 to 13 days' incubation. Body weights of embryos sacrificed at 8 days were found to have increased as yolk increased to a maximum at 1 ml/embryo; for normal weight at 9 days, 2 ml of yolk were required. Yolk concentration affected allantoic development and the Na/K ratio in the amniotic fluid of 8-day embryos.

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# Serum Lipids and Diet: A Comparison between Three Population Groups with Low, Medium and High Fat Intake

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In a previous study (Roels et al., '59), some serum lipid levels of two groups of Africans with vastly different fat intakes were reported: one group (Banyaruanda from the Bufundu area in Ruanda) consume only 6.8% of their calories as fat, whereas the second group (Baniari from the Ituri district in the Congo) get 37.8% of their total caloric intake from lipids. Very marked differences were noted in the polyunsaturated fatty acid patterns in their sera, although there were no significant differences in their serum total fatty acids. The dietary fats of both groups came practically exclusively from vegetable sources.

It, therefore, seemed pertinent to compare their serum lipids with those of a group of American negroes who consume 48.3% of their calories as lipids, mainly of animal origin.

## GENERAL PROCEDURE AND METHODS

*Subjects, diet and clinical examination.* Details about the two African population groups were given previously (Roels et al., '59). The third group were American negroes attending the family clinic at Vanderbilt University Hospital, Nashville, Tennessee. In this group, 50 subjects were examined and bled. They belonged to 18 different families, and consisted of 16 men, 17 women, 9 boys and 8 girls. The boys and girls were from 8 to 16 years old and the men and women were between 17 and 60 years old, with the exception of one woman of 73 years.

The heads of all these families had steady work and earned from $150 to $350 a month.

The inventory or log book method (FAO, '49) was used for the dietary survey in Nashville. A complete inventory of all the foodstuffs present in the house on the day of examination was made; the housewife kept a record of all food purchased, number of meals eaten at home, number of visitors, the number of meals eaten away from home for one week. Exactly one week after the original inventory, the investigator returned to make a final inventory of all the food present in the house and to discuss the "log-book" kept by the housewife to try to ascertain whether anything had been forgotten.

Five per cent was allowed for wastage. Milk drunk by children in school was not accounted for in the calculation of the results and it is also possible that snacks eaten away from home, but not considered as a "meal out," were not recorded. This was done because of the uncertainty of the quantities and amounts exactly taken. However, this was not considered to affect the results appreciably. All the subjects who volunteered, except one, were healthy and were not under treatment for any disease at the time of the examination. None of these subjects was aware of any member of the previous generation (parents, aunts or uncles) suffering from or dying of heart disease. One of the subjects in the present study, a woman of 73, suffered from arteriosclerosis and had had thrombosis of the coronary artery at the age of 56.

To obtain 10 ml of fasting blood, the patients were visited in their homes before breakfast. The clinical examination consisted of weight, height, skin-fold thickness and blood pressure determinations. These were made before the blood was withdrawn.

*Biochemical methods.* The "fasting blood" was centrifuged after standing for about two hours at room temperature in the dark. The serum was pipetted off and kept at − 25°C in the dark in oxygen-free nitrogen. For the determination of the total and the polyethenoid fatty acids, the alkaline isomerization method of Pikaar and Nijhof ('58) was used. The alkaline isomerization procedure was checked by gas chromatography, using an ionization detector and chromosorb columns coated with adipate and succinate polyesters of ethylene glycol; the agreement between the two methods was excellent: the greatest difference between the alkaline isomerization and gas liquid chromatography was less than 2% of each individual fatty acid.

Total serum cholesterol was determined using the method of Mann ('61): 0.1 ml of serum was saponified and the saponification mixture was extracted with petroleum ether. An aliquot of the petroleum ether extract was evaporated and the residue taken up in glacial acetic acid. The ferric chloride/sulphuric acid reagent for color development was added and the transmission at 560 mμ was read exactly 30 minutes later in a Coleman 14 Spectrophotometer. A standard curve was established daily, using a highly purified, freshly recrystallized cholesterol standard.

## RESULTS

*Dietary survey.* The results of the dietary surveys conducted among the three population groups are summarized in figure 1.

Details about the diet of the two African population groups were given in a previous publication (Roels et al., '59). For the Nashville group, 16% of the lipid intake was of vegetable origin and 84% of animal origin. The animal fats they consumed came from lard, bacon, milk, bologna, ham, sausage, chicken, eggs and hamburger in that order of importance. Their vegetable fats were derived from margarine, bread and corn meal.

The data for the caloric intake given in figure 1 were calculated for inhabitant per day. These figures are comparable for the Nashville group and for the Banyaruanda from the Bufundu area, because the age distribution of the individuals was practically identical for both population samples studied in the course of the dietary surveys: for the Nashville sample, 24.0% were less than 4 years old and 29.8% were between 4 and 14 years of age. The corresponding figures for the Bufundu area were 23.1 and 29.7%. The Baniari on the other hand have a very low birth rate: only 32.8% of the individuals there were less than 17 years old. For this reason, the caloric intake per "man-value" a day was calculated for the three groups. For the Nashville negroes, the factors of Widdowson ('47) were used to obtain the number of "man-values" corresponding to the sample. For the Baniari and for the Banyaruanda from the Bufundu area, the factors established by Leurquin ('60) for Ruanda-Urundi were used. The caloric intake per "man-value" a day was thus found to be 3,175 for the Nashville sample, 3,124 for the Banyaruanda from the Bufundu area and 2,503 calories for the Baniari.

*Clinical observations.* The average weight of the adult male Banyaruanda was 57.5 kg, mean height 167.1 cm, and average blood pressure was 125/73 (Hiernaux, '52). No relative weight could be established for this group since their exact ages are unknown, even to the subjects themselves.

In the Nashville sample, the mean weight of the adult males was 72.4 kg, and mean height 171 cm. Mean relative weight was 100.8. The relative weight is the actual body weight expressed as a percentage of the standard weight for height and age, as given by the Association of Life Insurance Medical Directors and Actuarial Society of America ('12). The average blood pressure for the adult males was 117/79; this measurement was made early in the morning, before breakfast.

BUFUNDU          BANIARI          NASHVILLE
2,127 CALORIES    1,980 CALORIES   2,390 CALORIES

11.2  0.5  81.5  6.8      10.9  1.6  49.7  37.8      4.4  7.6  39.7  48.3

VEGETABLE PROTEINS

ANIMAL PROTEINS
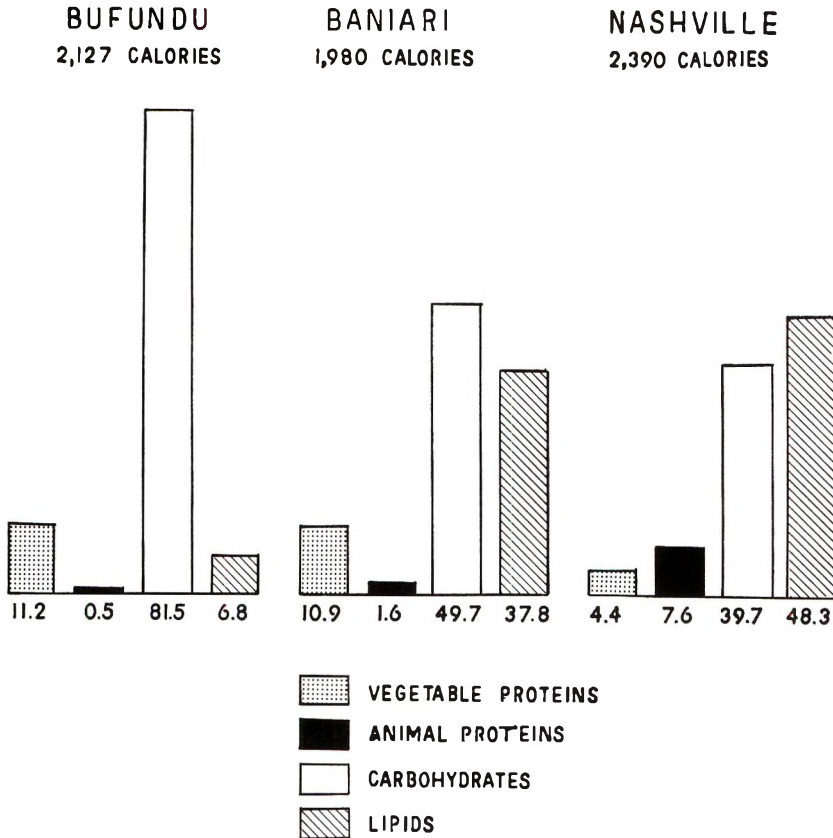
CARBOHYDRATES

LIPIDS

Fig. 1   Caloric percentage of major nutrients in the average daily dietary intake of three population groups studied.

Owing to recent political disturbances in the area, no comparative clinical observations could be made on the Baniari.

*Serum lipids.* The serum cholesterol levels, the total serum fatty acids, total polyunsaturated fatty acids and special groups of polyethenoid fatty acids of the three groups are given in table 1, listed by sex and age.

DISCUSSION

The differences in the serum cholesterol between the three groups classified by sex and age are shown in figure 2. The levels of significance of these differences are indicated in the same figure.

Within each sex/age group, the serum cholesterol level of the Nashville negroes was very much higher than that of the Baniari and that of the inhabitants of the Bufundu area of Ruanda-Urundi. No sig-

nificant differences were found between the serum cholesterol levels of the last two population groups except for the men, where the group with only 6.8% of lipids in their total caloric intake had a significantly higher ($P < 0.01$) serum cholesterol level (145 mg/100 ml) than those with 37.8% fat in their diet (124 mg/100 ml).

*Serum cholesterol and dietary cholesterol.* The Baniari (37.8% dietary fat) and the Banyaruanda (6.8% dietary fat) had practically no cholesterol in their diet, whereas the cholesterol content of the food of the Nashville negroes was much higher. This could have influenced their serum cholesterol levels somewhat, but probably not sufficiently so to be the only cause of the very great differences observed. Several authors have shown that there appears to be a homeostatic control of the endogenous cholesterol syn-

TABLE 1

Serum lipids of American Negroes compared with those of two groups of Africans

| Group | No. | Cholesterol | Total fatty acids | Dienoic | Trienoic | Tetraenoic | Pentaenoic | Hexaenoic | Total polyenoic |
|---|---|---|---|---|---|---|---|---|---|
| | | mg/100 ml | mg/100 ml | mg/100 ml | mg/100 ml | mg/100 ml | mg/100 ml | mg/100 ml | mg/100 ml |
| Men A[1] | 27 | 145.5±26.1[2] | 286.6±51.8 | 41.5±10.9 | 9.1±2.9 | 17.6± 3.5 | 8.8±2.4 | 6.1±1.2 | 83.0±15.4 |
| Men B | 25 | 124.2±26.8 | 277.9±83.2 | 61.9±16.4 | 3.7±1.3 | 22.2± 4.5 | 3.5±0.6 | 3.7±1.1 | 94.9±21.4 |
| Men N | 16 | 227.6±46.4 | 336.7±61.2 | 86.5±19.2 | 2.9±1.5 | 32.8± 9.6 | 4.3±1.9 | 5.2±2.7 | 131.6±28.1 |
| Women A | 24 | 156.1±30.0 | 292.7±64.7 | 41.6±11.4 | 9.6±2.7 | 16.4± 4.6 | 9.5±2.1 | 6.4±2.2 | 83.5±16.4 |
| Women B | 28 | 144.5±23.9 | 257.1±39.1 | 75.6±13.5 | 4.5±1.3 | 23.8± 4.4 | 4.1±0.6 | 4.1±1.5 | 112.1±17.3 |
| Women N | 17 | 209.0±49.4 | 332.0±58.7 | 87.2±14.7 | 2.6±1.8 | 30.3±10.1 | 4.8±1.5 | 4.6±2.9 | 129.4±22.3 |
| Boys A | 26 | 126.8±29.1 | 253.7±54.4 | 37.0±12.0 | 7.7±2.0 | 15.7± 3.1 | 8.9±1.6 | 6.9±1.3 | 76.2±16.4 |
| Boys B | 27 | 135.2±28.9 | 257.1±49.2 | 60.6±15.2 | 5.2±1.6 | 21.4± 5.0 | 4.4±1.1 | 5.6±2.0 | 97.2±21.4 |
| Boys N | 9 | 185.3±32.7 | 271.9±25.4 | 77.4±10.1 | 3.5±2.1 | 25.4± 5.7 | 3.2±1.0 | 4.4±2.8 | 113.9±14.7 |
| Girls A | 27 | 137.5±22.9 | 249.2±26.6 | 33.8± 7.7 | 7.5±2.3 | 15.4± 2.9 | 8.0±2.4 | 6.7±1.6 | 71.5±12.7 |
| Girls B | 24 | 137.2±15.0 | 265.7±32.3 | 56.8±13.7 | 5.5±1.6 | 21.9± 2.5 | 4.8±0.7 | 6.8±1.7 | 95.8±15.1 |
| Girls N | 8 | 190.4±29.5 | 319.0±33.7 | 83.7± 9.5 | 2.9±1.1 | 29.1± 3.6 | 4.0±1.4 | 5.9±1.7 | 124.7±11.3 |

[1] A indicates Banyaruanda, low fat intake; B, Baniari, high vegetable fat intake; N, Nashville negroes, high animal fat intake.
[2] Standard deviation.

thesis in man governed by the dietar cholesterol intake (Keys et al., '56). Thi was confirmed by Morris and co-worker ('57) in rats; they proved that wide var ations in dietary cholesterol had little o no effect on serum cholesterol levels bu greatly influenced the percentage of se rum cholesterol of endogenous syntheti origin.

*Serum cholesterol and dietary linolei acid.* About 6% of the total caloric in take of the Baniari (37.8% dietary fat came from linoleic acid contained in the peanut oil and palm oil in their diet. Fo the group on the low fat diet (6.8% of the dietary calories), less than 1% of thei total caloric intake came from linolei acid. The Nashville Group, with 48.3% dietary lipids, derived about 2% of their total caloric intake from linoleic acid and had by far the highest serum cholestero level. These figures for the linoleic acid intakes are approximate and are based upon the data of Hilditch ('56) and of the USDA ('59). It can be safely concluded that for the three groups studied, there is no direct proportional relationship between either the absolute amount of dietary lino leic acid and serum cholesterol or between serum cholesterol and the total amount of dietary fat.

When the dietary linoleic acid was ex pressed as a percentage of the lipid cal ories in the diet, it was found that this acid represents about 4% of the lipid cal ories for the Nashville negroes (48.3% dietary lipids), 15% for the Banyaruanda (6.8% fat in the diet) and 16% for the Baniari, who derive 37.8% of their calories from fat. These linoleic acid percentages are inversely related to the serum choles terol levels of the three groups under study. Hashim et al. ('59) however, using for mula feeding in 10 adult men, obtained results showing no direct relationship be tween the serum cholesterol-lowering ef fect of a dietary fat and its linoleic acid content. Funch et al ('60) observed this in rats: under certain circumstances, they found an increase of serum cholesterol in these animals when they changed their dietary lipids from a saturated fat to corn oil (rich in linoleic acid). Our study was not a controlled experiment but a com-

MEN          WOMEN          BOYS          GIRLS



228   145   124        209   156   144        185   127   135        190   137   137
p<001  p<01          p<001    N.S.          p<001    N.S.          p<001    N.S.

■  48.3% FAT INTAKE
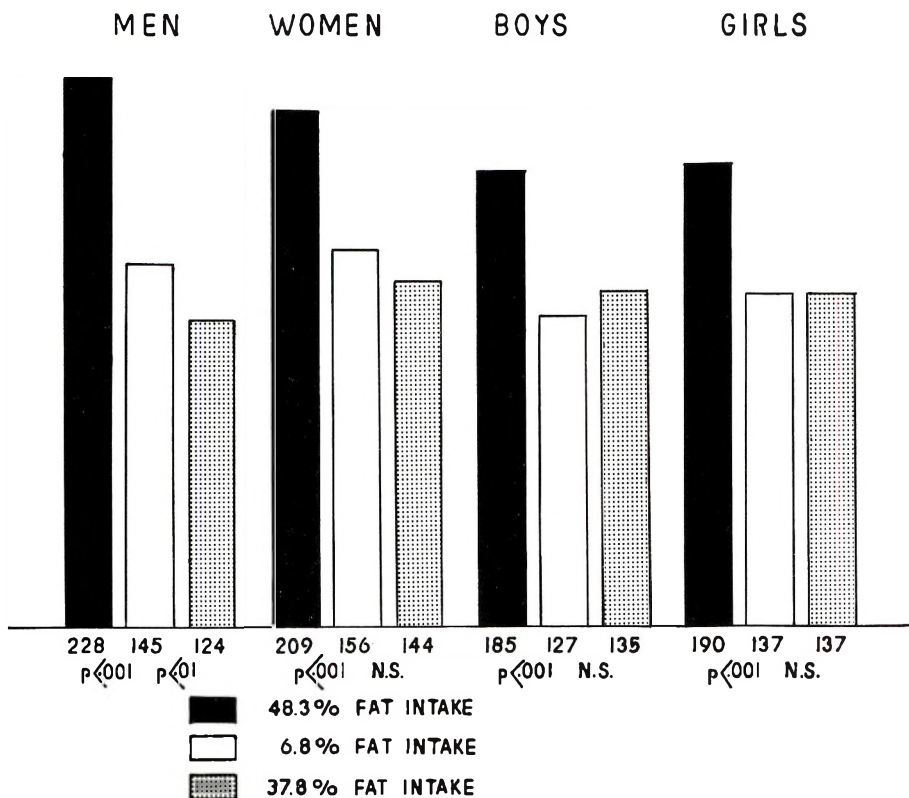
□  6.8% FAT INTAKE

▨  37.8% FAT INTAKE

Fig. 2   Serum cholesterol levels (mg/100 ml) of the three population groups studied.

parative survey of three population groups with very different eating habits. Many other direct and indirect dietary factors might have influenced their serum lipids in general and cholesterol in particular, such as the dietary proteins and carbohydrates which are vastly different in the three groups studied, and the endogenous lipogenesis which is undoubtedly influenced by the amount and the nature of the fats in the diet (Roels et al., '59).

*Serum cholesterol and serum fatty acids.* Except for the Nashville group, where the number of subjects examined was smaller, positive correlations were found between the serum cholesterol and the tetraenoic and total polyenoic serum fatty acid level within each sex/age group. Keys and coworkers ('59) observed a rapid increase in the serum cholesterol level of adult men when their diet was supplemented with arachidonic acid which is the principal tetraenoic acid in human serum.

The differences in serum total fatty acids and in the sums of the polyethenoid fatty acids as a percentage of the serum total fatty acids of the three population groups are shown in figure 3, together with the levels of significance between these differences.

Figure 4 shows the individual polyethenoid acids as a percentage of the total fatty acids and indicates the levels of significance of the differences between the three groups.

Vast differences in dietary fat intake appear to have little influence on the serum total fatty acids, although the Nashville group, with the highest fat intake (48.3%) had a higher serum total fatty acid level than the other two groups. The difference was, however, not always significant. There is very little difference between the serum total fatty acid level of the Baniari (37.8% dietary lipids) and that of the Banyaruanda (6.8% dietary
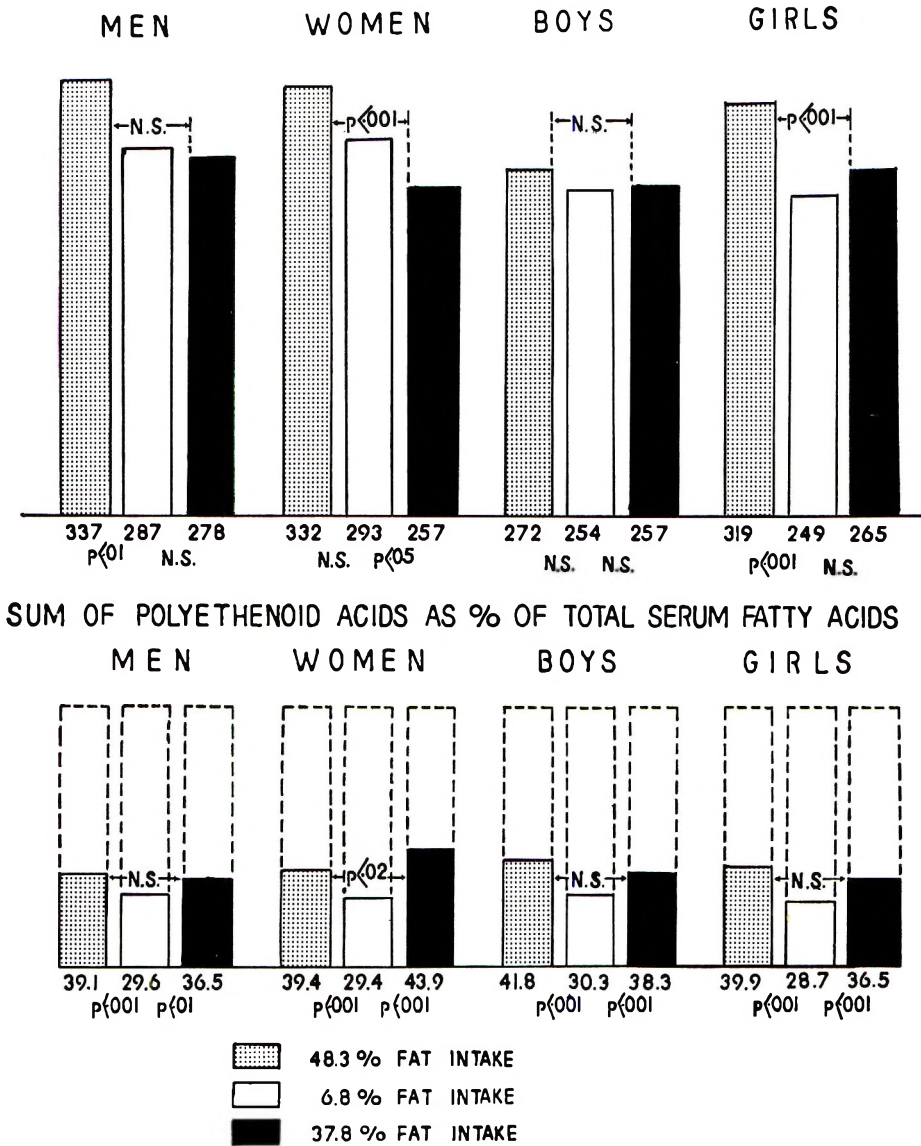
Fig. 3   Serum total fatty acids (mg/100 ml) of the three population groups studied.

lipids) despite the enormous difference in the fat in their food.

The differences in the dietary carbohydrate intakes of these three population groups are, of course, greater still (81, 50 and 40%, respectively) and they did not appear to influence their total serum fatty acid levels markedly. This is in agreement with the observation of Antonis and Bersohn ('61), who found that white and Bantu subjects, when subsisting for a long time on diets either low or high in fat-caloric content did not have significantly different fasting serum-triglyceride levels.

There is no difference in the sum of the serum polyethenoid fatty acids (as a percentage of total fatty acids) of the Nashville group and the Baniari, whereas the polyethenoid fatty acids of the Banyaruanda are significantly lower than these of the other two groups. This lowering of

MEN WOMEN BOYS GIRLS

DIENOIC

N.S. — p(05 — N.S. — p(05

25.7 14.9 23.9  26.6 14.8 29.6  28.4 14.6 23.9  26.4 13.6 21.6
p(001 p(001  p(001 p(001  p(001 p(001  p(001 p(001

TRIENOIC

p(001  p(001  p(001  p(001

0.9 3.2 1.4  0.8 3.3 1.8  1.3 3.1 2.0  0.9 3.0 2.1
p(001 p(001  p(001 p(001  p(001 p(001  p(001 p(001

TETRAENOIC

N.S. —  — N.S. —  N.S.

9.7 6.2 8.5  9.3 5.7 9.3  9.3 6.2 8.5  9.4 6.2 8.4
p(001 p(001  p(001 p(001  p(001 p(001  p(001 p(001

PENTAENOIC

N S  p(01  p(001

1.3 3.1 1.3  1.4 3.4 1.6  1.2 3.6 1.7  1.3 3.2 1.8
p(01 p(001  p(001 p(001  p(001 p(001  p(001 p(001

HEXAENOIC

N.S. —  N.S. —  N.S. —  N.S.

1.5 2.2 1.4  1.3 2.2 1.6  1.6 2.8 2.2  1.9 2.7 2.6
p(001 p(001  p(001 p(005  p(001 p(005  p(01 N.S.

■ 48.3 % FAT INTAKE
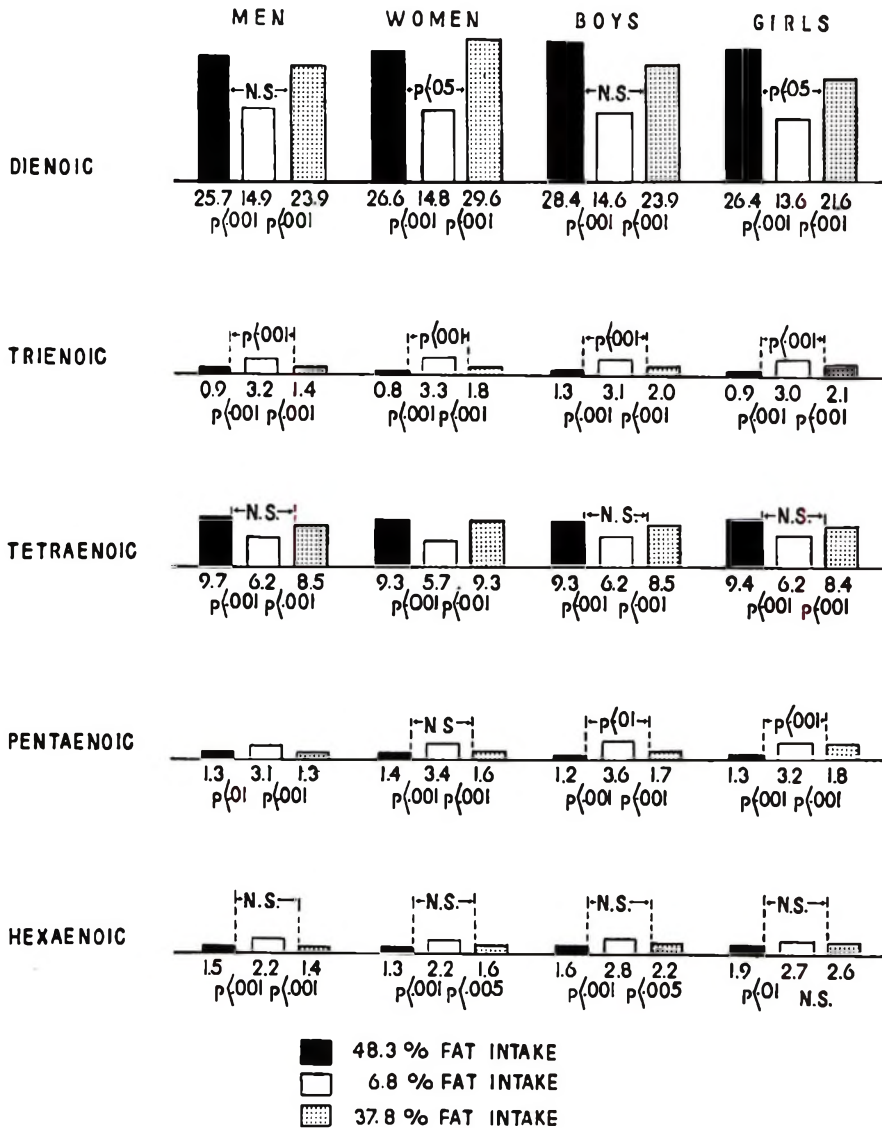□ 6.8 % FAT INTAKE
▨ 37.8 % FAT INTAKE

Fig. 4  Individual serum polyethenoid acids as percentage of total fatty acids.

the total polyunsaturated fatty acids is due to a significantly lower level of dienoic and tetraenoic fatty acids in the serum of the Banyaruanda, who have, however, significantly higher levels of trienoic, pentaenoic and hexaenoic acids than the other two groups.

It appears that when the dietary linoleic acid falls below a certain threshold, serum total polyunsaturated fatty acids are low owing to reduced dienoic and tetraenoic acids, whereas the trienoic, pentaenoic and hexaenoic serum fatty acids are then relatively high. Above this threshold, total serum polyenoic, dienoic and tetraenoic acids are higher and trienoic, pentaenoic and hexaenoic fatty acids tend to be lower in the serum. Once this minimal requirement for dietary linoleic acid has been satisfied, even fairly wide variation in the dietary intake of this acid (which, in the case of the Nashville group and of the Baniari, represents, respectively, 2 and 6% of the total caloric

intake) does not cause significant differences in the individual polyenoic fatty acid levels except for the trienoic acid which is significantly lower for the Nashville group. Holman ('60) found in a study in rats that when the ratio of trienoic-to-tetraenoic fatty acids in plasma is plotted against the dietary linoleic acid as a percentage of total calories, a hyperbola is obtained, in which the maximal rate of change of slope lies near 1% of calories. A similar pattern was observed in the present study for humans although our three groups had widely different total fat intakes.

In view of our observations and those of Holman ('60) in rats, it appears that when dietary linoleic acid falls below 1% of the total dietary calories in man, the percentage of saturated plus monoenoic fatty acids increases and endogenous lipogenesis produces higher levels of serum trienoic, pentaenoic and hexaenoic fatty acids. When the linoleic acid intake increases above this threshold, another pathway of endogenous synthesis of polyunsaturated fatty acids appears to cause considerably higher levels of serum dienoic and tetraenoic fatty acids, and to lower levels of trienoic, pentaenoic and hexaenoic acids, resulting in a higher level of serum total polyunsaturated fatty acids.

A suggestion for a possible mechanism of these pathways was given in a previous paper (Roels et al., '59).

## SUMMARY

Serum cholesterol, serum total and polyunsaturated fatty acids and diet of three populations were compared.

Of two groups of African negoes examined, one took 6.8 and the other 37.8% of its calories as lipids, almost exclusively of vegetable origin. The third group (negroes from Nashville) consumed 48.3% of its dietary calories as fats, mainly of animal origin. Their dietary linoleic acid represented, respectively, less than 1, 2 and 6% of their total caloric intake.

The Nashville group had the highest serum cholesterol level, but there was little difference in the serum cholesterol of the other two groups.

Serum total fatty acids of the three groups were not very different. From the observations of total and individual polyenoic fatty acids in these groups, it appears that there is a threshold value of dietary linoleic acid governing the pattern of serum fatty acids: below this threshold, serum total polyunsaturated fatty acids are low owing to a decline in dienoic and tetraenoic acids, despite relatively high trienoic, pentaenoic and hexaenoic acid levels. Above this threshold value, the opposite phenomenon was observed. In this range, a change from 2 to 6% dietary linoleic acid did not alter the serum polyenoic fatty acid levels appreciably, except perhaps for the trienoic acid.

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# Influence of the Dietary Protein Level on the Magnesium Requirement

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The first suggestion of a protein-magnesium relationship was made by Colby and Frye ('51) who showed that elevation of the protein content of a magnesium-deficient diet from 24 to 50% depressed weight gains and serum magnesium values. Unfortunately, weight gain was also depressed in those animals fed the magnesium-supplemented high protein diets, and diarrhea appeared in all the high protein-fed animals within 4 days.

Menaker ('54) presented additional evidence when he reported that protein-depleted rats gained significantly more weight with a low magnesium diet containing 7% protein than with one containing 14% protein. He also observed marked vasodilatation in the latter, but not the former. Similarly, Heinicke et al. ('56) demonstrated that high levels of potassium and magnesium were necessary to obtain satisfactory responses to amino acid supplements in the guinea pig.

The following study was designed to contribute further information both on the influence of dietary protein upon the magnesium requirement and on the possible mechanisms of any observed effects.

## EXPERIMENTAL

These studies involved two separate experiments. The first lasted 6 days and used 90 two-day-old male, Hy-line chicks divided into 6 groups. Three of these groups were fed a semipurified diet containing 100 ppm magnesium and either 12, 24, or 36% vitamin-free casein[1] as the sole source of protein. The remaining three groups were fed the same levels of protein and 600 ppm magnesium. The low mineral level is slightly less than one-half of the lowest reported magnesium requirement of the chick, whereas 600 ppm exceeds by 40 ppm the highest estimate for this mineral (O'Dell, '60). Magnesium was added as anhydrous magnesium sulfate and the changes in dietary protein and magnesium were made at the expense of glucose.[2] Other dietary components in gm/100 gm diet were corn oil, 5; salt mixture,[3] 5; powdered cellulose,[4] 4; glycine, 2; L-arginine·HCl, 2; L-cystine, 0.5; vitamin mixture,[5] 0.4; choline chloride, 0.2; and sufficient $NaH_2PO_4 \cdot H_2O$ to achieve a constant dietary phosphorus level of 0.57%.

For the second experiment, 48 weanling, male, Holtzman rats were divided into 6 lots and fed a similar diet that contained 0.6% calcium and either 12 or 36% protein at three levels of magnesium: 100, 200 or 600 ppm. Approximately 120 to 125 ppm magnesium are required for maximal weight gain in young (90- to 110-gm) Sprague-Dawley rats consuming a 12% protein, 0.6% calcium, semipurified ration (MacAleese and Forbes, '61). Magnesium was again added as anhydrous magnesium sulfate, but the variations in magnesium and casein were made at the expense of sucrose rather than glucose. In addition, the ration included in gm/100 gm diet, cottonseed oil,[6] 8; cellulose,[7] 5; salt mixture,[8] 4; vitamin mixture,[9] 0.4;

L-cystine, 0.4; and choline chloride, 0.2. The phosphorus level was kept constant at 0.36% by addition of the necessary amounts of phosphorus as $KH_2PO_4$. Total fecal and urine collections were made for 5 consecutive days during the second, fourth and sixth weeks of this trial. The rats were killed after 6 weeks and samples of blood serum, kidney and liver were taken for analysis. The excreta, kidneys and portions of each diet were dried to a constant weight, wet ashed (Reitz et al., '60) and analyzed for phosphorus by the Fiske-SubbaRow method (Hawk et al., '47) and for calcium and magnesium by a titrimetric method using EDTA and Eriochrome Black T (Robinson and Rathbun, '59). Magnesium alone was determined on the sera without a prior ashing, and on the livers following drying and wet ashing. Serum total protein was determined with the biuret method. The free amino acid content of the urine samples collected during the sixth week was determined with the Beckman amino acid analyzer.

## RESULTS

At a level of 600 ppm magnesium, no significant difference in weight gain was observed between the chicks fed 24 or 36% casein (table 1) although the weight of both groups considerably exceeded that of the 12% protein lot. Despite the weight difference, all animals appeared healthy and energetic. On the other hand, reduction of the magnesium level to 100 ppm resulted in severely restricted weight gain ($P < 0.01$) in the lots fed the higher levels of protein, but did not affect the 12% protein group. The chicks fed low protein

exhibited no symptoms of magnesium deficiency, whereas convulsions identical to those described by Bird ('49) were observed in the other two lots. Increase in the dietary protein at the lower intake of magnesium was accompanied by markedly greater mortality and decreased physical activity.

Similar results were obtained in the second experiment using weanling rats. At either 200 or 600 ppm magnesium, average weight gain (table 2) was significantly higher ($P < 0.01$) with the higher protein intake. At 100 ppm magnesium, however, the gain with the lower protein intake was far superior ($P < 0.01$) to that of the high protein lot, and the average weight of both groups was below that observed at the higher mineral intakes.

Vasodilatation resulting in a vivid red appearance of the ear is a characteristic feature of magnesium deficiency in the albino rat. Marked differences in the severity of hyperemia between the low and high protein lots were observed (table 2) at the two lower intakes of magnesium. Of particular interest was the occurrence of hyperemia in 6 of 8 rats receiving 36% protein and 200 ppm magnesium, a level of magnesium intake generally considered adequate for the rat (O'Dell, '60).

The results of the balance data (table 3) provide no evidence of a difference in apparent absorption of either magnesium or calcium between the diets used. Urinary loss of both was generally enhanced, however, by the higher protein intake. Urinary calcium levels were also elevated by increasing intakes of magnesium. The absorption and excretion of phosphorus was not significantly altered by the dietary

TABLE 1

*Effect of protein and magnesium on weight gain and mortality of the chick*

| Protein, % | 12 | 24 | 36 | 12 | 24 | 36 |
|---|---|---|---|---|---|---|
| Magnesium, ppm | 100 | 100 | 100 | 600 | 600 | 600 |
| No. of animals | 15 | 15 | 15 | 15 | 15 | 15 |
| Avg initial wt (gm ± SD) | 48.0 ± 4.6 | 48.5 ± 3.9 | 47.9 ± 5.2 | 48.2 ± 4.5 | 47.1 ± 3.5 | 48.1 ± 3.1 |
| Avg weight after 6 days (gm ± SD) | 69.9 ± 7.1 | 62.0 ± 5.5 | 59.7 ± 7.5 | 69.4 ± 8.9 | 85.4 ± 8.5 | 81.9 ± 8.6 |
| Number dead after 6 days | 0 | 4 | 8 | 0 | 0 | 0 |

TABLE 2

*Effect of protein and magnesium on weight gain and hyperemia in the rat*

| | | | | | | |
|---|---|---|---|---|---|---|
| Protein, % | 12 | 36 | 12 | 36 | 12 | 36 |
| Magnesium, ppm | 100 | 100 | 200 | 200 | 600 | 600 |
| No. of animals | 8 | 8 | 8 | 8 | 8 | 8 |
| Avg initial wt (gm ± SD) | 48.4 ± 3.3 | 47.9 ± 2.8 | 48.6 ± 3.9 | 48.8 ± 3.6 | 48.9 ± 3.2 | 48.8 ± 3.2 |
| Avg wt after 6 weeks (gm ± SD) | 230 ± 7 | 185 ± 14 | 244 ± 18 | 272 ± 14 | 250 ± 25 | 282 ± 6 |
| Avg wt after 8 days (gm ± SD) | 71.6 ± 4.9 | 71.9 ± 5.4 | 72.8 ± 4.6 | 89.0 ± 4.6 | 69.9 ± 4.1 | 93.1 ± 3.8 |
| No. of animals showing the following degrees of hyperemia after 8 days:   0 | 6 | 0 | 8 | 2 | 8 | 8 |
| 1+ | 2 | 0 | 0 | 6 | 0 | 0 |
| 2+ | 0 | 0 | 0 | 0 | 0 | 0 |
| 3+ | 0 | 8 | 0 | 0 | 0 | 0 |

variations of this study. The total retention of magnesium was considerably reduced by the high protein diet at the lowest level of dietary magnesium (100 ppm). Total retention was not greatly affected, however, by the differences in dietary protein at the higher magnesium intakes, although the greater weight gain of the rats fed 36% protein tended to reduce the accumulation of magnesium per gain in unit weight. Similarly, total retention of calcium was about equal in all 6 groups, but the greatly reduced weight gain with the lowest level of dietary magnesium resulted in an increased retention of calcium on the per unit gain basis.

The tissue analyses (table 4) revealed that, as in previous studies, the magnesium concentration of the kidney was not altered with changes in magnesium intake. A highly significant difference ($P < 0.01$) was observed, however, in the concentration of magnesium in the liver of rats fed the lowest as opposed to the highest mineral level whether dry weight or grams of nitrogen were used as the reference base. The values for the 200-ppm group fell between those observed at 100 and 600 ppm. Despite the constancy of the kidney magnesium values, the average kidney calcium concentration from both groups fed 100 ppm magnesium was higher than that at either 200 or 600 ppm.

Protein intake affected kidney calcium only at the highest magnesium intake. In the latter instance, practically no calcium was detectable in the kidneys of the low protein group, whereas the kidney calcium concentration of the high protein animals was statistically equivalent to that observed at 200 ppm magnesium. Serum magnesium varied as expected with changes in dietary magnesium, and was slightly depressed in the low magnesium, high protein group in agreement with the balance data.

The rats consuming the 100 ppm magnesium, 36% protein ration had an average total serum protein of $5.66 \pm 0.74$ (SD) gm/100 ml serum. This was significantly lower ($P < 0.05$) than the value of $6.51 \pm 0.43$ gm/100 ml recorded for the low magnesium, low protein group. The average values of the remaining lots ranged from 6.36 to 7.07 gm/100 ml, but the individual values showed a wider range and were not statistically significant.

The urinary excretion of free amino acids during the sixth week was approximately equal between the 36% protein groups. At the lower protein intake, however, the mean excretion of several amino acids (expressed as milligrams ± SD of amino acid per 5 days) was greatly increased in those rats fed the two lower mineral levels as opposed to the highest

TABLE 3

*Effect of protein and magnesium on calcium, phosphorus and magnesium balance in the rat*

| Protein, % | 12 | 36 | 12 | 36 | 12 | 36 |
|---|---|---|---|---|---|---|
| Magnesium, ppm | 100 | 100 | 200 | 200 | 500 | 600 |
| Number of rats | 6 | 6 | 6 | 6 | 6 | 6 |
| | Average cumulative totals[1] from three collection periods of 5 days each | | | | | |
| **Calcium** | | | | | | |
| Intake, mg | 865±16 | 781±21 | 950±17 | 1052±17 | 896±19 | 963±16 |
| Feces, mg | 216±27 | 222±42 | 371±79 | 306±32 | 249±32 | 278±21 |
| Feces, % | 25±4 | 28±4 | 39±8 | 29±2 | 28±3 | 29±1 |
| Urine, mg | 4.3±1.2 | 10.2±3.5 | 7.1±1.4 | 10.9±1.6 | 13.0±4.5 | 28.9±2.6 |
| Urine, % | 0.5±0.2 | 1.3±0.4 | 0.8±0.2 | 1.0±0.2 | 1.4±0.5 | 3.0±0.8 |
| Retained, mg | 645±69 | 548±43 | 572±75 | 735±36 | 634±49 | 657±26 |
| Retained, % | 74±4 | 70±4 | 60±8 | 70±2 | 71±3 | 68±1 |
| Retained, mg/100 gm gain | 1014±123 | 1123±80 | 832±155 | 928±48 | 926±51 | 787±39 |
| **Phosphorus** | | | | | | |
| Intake, mg | 664±39 | 502±43 | 633±36 | 587±30 | 654±44 | 684±35 |
| Feces, mg | 76±9 | 94±10 | 107±21 | 93±8 | 89±14 | 87±12 |
| Feces, % | 12±2 | 19±1 | 17±3 | 16±1 | 14±2 | 13±1 |
| Urine, mg | 126±17 | 82±11 | 135±16 | 84±11 | 91±13 | 81±11 |
| Urine, % | 19±2 | 16±1 | 22±3 | 15±2 | 14±1 | 12±2 |
| Retained, mg | 461±35 | 327±29 | 390±34 | 409±35 | 473±33 | 516±30 |
| Retained, % | 70±3 | 65±2 | 62±3 | 70±3 | 72±3 | 75±2 |
| Retained, mg/100 gm gain | 726±68 | 670±53 | 564±54 | 520±14 | 692±42 | 617±35 |
| **Magnesium** | | | | | | |
| Intake, mg | 20.1±1.2 | 14.6±1.2 | 36.9±2.1 | 34.1±1.7 | 100.1±6.8 | 105.1±5.4 |
| Feces, mg | 16.5±3.5 | 14.5±1.1 | 24.4±4.5 | 16.5±1.1 | 25.7±2.6 | 25.3±1.9 |
| Feces, % | 88±22 | 100±5 | 66±13 | 49±3 | 25±3 | 24±1 |
| Urine, mg | 2.1±0.3 | 2.2±0.3 | 4.3±0.8 | 5.6±0.6 | 18.2±3.2 | 30.1±5.5 |
| Urine, % | 8.4±4.6 | 15.1±2.2 | 11.7±2.7 | 16.6±1.9 | 18.2±2.6 | 30.1±4.1 |
| Retained, mg | 0.6±1.8 | − 2.1±0.8 | 8.2±5.3 | 11.9±1.5 | 56.8±6.3 | 48.1±4.4 |
| Retained, % | 1.9±2.3 | − 14.7±6.5 | 23.2±12.0 | 34.8±3.1 | 56.7±4.7 | 45.8±4.5 |
| Retained, mg/100 gm gain | 0.8±2.6 | − 4.4±1.9 | 12.1±8.0 | 15.0±1.2 | 83.1±8.3 | 57.6±5.6 |

[1] In either mg or % of total ±SD.

TABLE 4

*Effect of protein and magnesium on tissue mineral content*

| Protein, %<br>Magnesium, ppm | 12<br>100 | 36<br>100 | 12<br>200 | 36<br>200 | 12<br>600 | 36<br>600 |
|---|---|---|---|---|---|---|
| Serum Mg (avg mg/<br>100 ml ± SD) | 1.46 ± 0.10 | 1.04 ± 0.17 | 1.80 ± 0.22 | 1.82 ± 0.24 | 2.50 ± 0.20 | 2.36 ± 0.17 |
| Number of animals | 6 | 3 | 6 | 8 | 3 | 7 |
| Kidney Mg (avg mg/100<br>gm dry tissue ± SD) | 97.1 ± 14.3 | 96.1 ± 12.7 | 88.5 ± 8.8 | 89.9 ± 12.7 | 99.2 ± 15.9 | 85.6 ± 5.2 |
| Number of animals | 7 | 6 | 8 | 8 | 8 | 8 |
| Liver Mg (avg mg/100<br>gm dry tissue ± SD) | 54.8 ± 23.1 | 68.5 ± 11.1 | 61.1 ± 16.9 | 87.7 ± 19.6 | 106.9 ± 21.4 | 120.4 ± 34.7 |
| Number of animals | 7 | 6 | 8 | 8 | 8 | 8 |
| Kidney calcium (avg<br>mg/100 gm dry<br>tissue ± SD) | 152 ± 157 | 69 ± 19 | 25 ± 21 | 24 ± 20 | 4 ± 6 | 45 ± 10 |
| Number of animals | 7 | 6 | 8 | 8 | 8 | 7 |

magnesium intake. This was most pronounced with leucine (0.69 ± 0.74 and 0.69[10] ± 0.39 vs. 0.16[11] ± 0.03), isoleucine (0.22 ± 0.15 and 0.23[12] ± 0.10 vs. 0.05[13] ± 0.02), and valine (0.41 ± 0.22 and 0.60 ± 0.63 vs. 0.14 ± 0.06). Serine, methionine and phenylalanine showed only moderate differences, whereas none were observed for the remaining common amino acids.

## DISCUSSION

The growth, appearance and rate of mortality of the various groups in both the chick and the weanling rat experiments support the conclusion that the magnesium requirement of these species is directly related to the level of dietary protein. Menaker ('54) obtained very similar results using protein-depleted adult rats and came to the same conclusion. The balance data suggest that this increased requirement is due at least in part to an increased urinary loss of magnesium with the high protein diet. This is not sufficient, however, to explain the deficiency symptoms observed in the high protein lots at the higher magnesium intakes; namely, vasodilatation at 200 ppm and significant renal accumulation of calcium at 600 ppm, assuming that the latter was a function of magnesium nutrition in this case. The possibility exists that an in-

creased initial growth rate was an aggravating factor in the second experiment. On the other hand, the results of the first experiment tend to minimize the effect of the growth rate. In that case, 24 and 36% protein were found to yield equal growth with the 600 ppm magnesium intake, but the higher protein level greatly intensified the deficiency at 100 ppm magnesium as evidenced by increased mortality and diminished physical activity.

Most previous attempts to demonstrate changes in the magnesium content of soft tissues in magnesium-deficient animals have been unsuccessful. Significant but relatively small differences have been reported for muscle (MacIntyre and Davidsson, '58; McAleese and Forbes, '61), but no losses have been detected in kidney by the same authors. Liver magnesium was found unchanged in at least two studies with young rats (Tufts and Greenberg, '27; MacIntyre and Davidsson, '58). In both of these cases, however, the growth of the animals was severely restricted. Aikawa et al. ('62) reported a significant loss of magnesium from the lungs of mature rabbits fed a diet limiting in this mineral, but were unable to detect losses in the magnesium content of muscle, skin, kidney,

[10] Difference significant (P < 0.05).
[11] Difference significant (P < 0.05).
[12] Difference significant (P < 0.05).
[13] Difference significant (P < 0.05).

heart and liver in the same animals. In contrast, the lowest level of magnesium in the present study still permitted an appreciable weight gain, and a highly significant difference in liver magnesium was detected. It seems reasonable to conclude that magnesium is maintained tenaciously in the liver even in the acute deficiency state, but that levels of magnesium intake which will yield near normal growth in young rats are not sufficient to produce maximal concentrations of liver magnesium. The metabolic consequences of this reduced concentration, if any, are not known. The maintenance of normal concentrations of magnesium in the kidney under these conditions is unexpected. It is conceivable that in the deficient groups a portion of the magnesium is in irreversible association with crystalline deposits of apatite and, thus, is essentially nonfunctional, although we have no direct evidence in support of this speculation. Magnesium is often present, however, in crystalline kidney deposits in man.

Gershoff and Andrus ('61) have shown that although 400 ppm is a commonly used level of magnesium intake in the rat, one-quarter of the animals receiving this amount in a 15% casein diet developed minimal renal deposits of apatite. Elevation of the dietary magnesium to 4,000 ppm prevented this lesion. The results of the kidney calcium determinations in this study are in agreement with the suggestion of these workers that if the occurrence of calcium deposition in the kidney is the criterion of adequancy, then 400 ppm magnesium is a marginal intake for the rat fed a 12 to 15% protein diet.

A depression of serum proteins as a consequence of magnesium deficiency has recently been reported (Ko et al., '62). The observation that urinary excretion of various amino acids was diminished at the highest level of magnesium and the lower intake of protein is in agreement with the report by Sauberlich and Baumann ('49) that a moderate increase in urinary amino acids accompanied the consumption of a 10% protein, magnesium-deficient diet by mice.

The marked effect of dietary protein upon the magnesium requirement of the weanling rat and the chick suggests an important metabolic relationship between these two nutrients. This hypothesis is strengthened by the observation that the urinary excretion of certain amino acids increased and serum protein levels decreased in association with a reduction of magnesium intake.

## SUMMARY

The effect of the dietary protein intake upon the magnesium requirement of the chick and the rat was investigated. When magnesium was limiting, an increase in the dietary protein level retarded growth and increased mortality in the chick. Similarly, the higher level of protein restricted weight gain in the rat at a low level of magnesium intake. A classic symptom of magnesium deficiency, vasodilatation, was observed in the rats fed 36% protein and 200 ppm magnesium, a level of this mineral which is normally considered adequate.

A balance study in the rat failed to produce evidence of an effect of dietary protein on apparent absorption although urinary excretion of magnesium was increased with the higher protein diet. Urinary calcium was elevated following an increase in either dietary protein or magnesium.

Analyses of rat liver and kidneys showed that differences in the magnesium content of the former, but not the latter, occurred at different intakes of magnesium so long as appreciable growth was achieved. Kidney calcium was increased markedly by a decrease in dietary magnesium.

Changes in total serum protein and in the urinary excretion of leucine, isoleucine and valine were observed which were suggestive of an important role for magnesium in protein and amino acid metabolism.

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# Differences in Rat Strain Response to Three Diets of Different Composition

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An important factor in the conduct of nutritional experiments is the uniform quality of the animals used. Ancestry and disease contribute to the variation in response to diet, as is widely recognized in farm animal feeding. The development of different strains of mice with particular types of inborn characteristics has motivated many studies of their response to various types of diets and stimuli (Mayer, '53; Fenton et al., '51, '60; Bruell, '62).

From breeding a pair of unrelated rats, Morris et al. ('33) were able to establish by selection two strains, one of which was less efficient than the other in the utilization of food. Light and Cracas ('38) reported differences in the requirement for vitamin $B_1$ in three strains of white rats. Growth, organ weights and data on body composition of three strains of rats were reported in the review of Dunn et al. ('47). The data were collected from different laboratories, and the animals varied with respect to age and length of time fed the experimental diets which were of different or unknown composition. Widely different body weights were obtained which the authors attributed to strain differences, but they concluded that definite strain differences with respect to chemical composition had not been demonstrated unequivocally. Kohn ('50) noted differences in blood sugar and cholesterol levels in 4 strains of rats. Holmes ('52) observed striking differences in susceptibility to dental caries among three strains of rats maintained under identical environmental conditions. Differences in response of three strains of rats in the development of obesity were reported by Mickelsen et al. ('55), but the differences were not attributed to genetic variation. Heggeness ('60) reported differences in susceptibility of three strains of rats to the cataractogenic property of galactose and xylose. In a cooperative study of irradiated beef diets, differences in results from various laboratories were found to be due in part to the different strains of rats used (Mellette and Leone, '60).

Even though there are increasing numbers of reports in the recent literature of differences in rat strain response, there is a paucity of data on the nutritional response of different strains of rats to diet from studies conducted under otherwise identical conditions. The varied and often conflicting results recorded with rats fed the same diets in different laboratories may well be due to differences in the inherent metabolic characteristics of the rats used in the experiments. The availability in this laboratory of three strains of rats — Holtzman, Wistar and a mixed strain originally derived from two unrelated strains — made it possible to carry out a systematic study of the nutritional response of these three strains of rats to three different diets.

## EXPERIMENTAL

*Strains.* A stock colony of the laboratory mixed strain of rats, designated hereafter as BHE, was started in 1942 by breeding an albino Yale strain obtained from Columbia University with a black and white hooded strain from Pennsylvania State College. An assortment of albino, brown, gray, black, brown and white, gray and white or black and white rats resulted from the early breedings. Breeding was continued with animals having coat colors of black, black and white (piebald) and albino; animals of all other coat colors

were discarded. A closed-colony random-type breeding system was used through approximately 30 generations of animals. The BHE strain rats were fed a pelleted stock diet 1[1] for the last 10 generations.

Several litters from the breeding of mature male and female Holtzman rats (Holtzman Company, Madison, Wisconsin) were raised to 100 days of age with stock diet 2,[2] transferred to this laboratory and continued for three generations on stock diet 2. A stock colony of rats maintained since 1930 at the Bureau of Dairy Industry (now Dairy Cattle Research Branch) was originally purchased from the Wistar Institute and will be referred to hereafter as Wistar. For this investigation, Wistar rats were obtained as weanling males and females, raised in this laboratory to 100 days of age with stock diet 1 and transferred to stock diet 2 for three generations before their use in the study.

The animal quarters were air conditioned and were kept throughout the year, as closely as possible, at 77 to 82°F and 40 to 55% relative humidity. Lighting was regulated to give 12 hours of uniform illumination and 12 hours of darkness each day. Holtzman and Wistar rats, after a period of quarantine in separate rooms, were housed in the same quarters as the BHE strain and maintained thereafter under the same environmental conditions.

*Diets.* Littermates of male rats of all three strains were distributed as equally as possible among three diets. One of the diets was stock diet 1, which contained the following proximate composition: crude protein, not less than 25%; crude fat, not less than 4%; crude fiber, not more than 4%; ash, not more than 10% (designated hereafter as the stock diet). It was fed in pelleted form except during collection of urine and feces when it was ground to facilitate collection of scattered food. Food intake was measured for rats fed the stock diet only during the collection periods.

All three strains were also fed two semipurified diets: one of these, (SP) contained high nitrogen casein, 16%; lactalbumin, 8%; dried brewer's yeast, 10%; hydrogenated vegetable oil, 8%; salt mixture (modified Osborne and Mendel, '19), 4%; powdered cellulose, 2%; and sucrose, 52%.[3] The other semipurified diet

contained 75% of each of the ingredients of the semipurified diet (SP) and 25% reconstituted whole dried egg,[4] cooked, re-dried and ground before incorporation in the diet (SPE).

Approximately 10 gm of fresh kale were given to each animal twice weekly. One drop of vitamin A and D concentrate[5] was given orally twice weekly and 30 mg vitamin E in corn oil was given weekly to animals fed the SP and SPE diets. The SP and SPE diets were selected because of the basic information already available from studies of diet and longevity of the BHE strain of rats.[6]

*Procedures.* Over a period of two years, litters of male weanling rats, 21 to 23 days old, of all three strains, were housed individually and fed ad libitum until they were 330 to 335 days old. All animals were weighed weekly. For animals fed the SP and SPE diets, scattered food was carefully recovered and food intakes recorded weekly during the entire experimental period.

Urine and feces were collected quantitatively for 3 or 4 days from some of the rats of each strain fed all three diets when they were 155 and 325 days of age. Feces were collected for 6 days from some of the rats at 240 days of age. Feces were kept frozen until the end of the collection period and later dried, ground and analyzed for nitrogen and calorie content. Nitrogen was determined in the food, urine and feces by the macro-Kjeldahl method (AOAC, '50). Urine protein nitrogen was determined by the macro-Kjeldahl method after precipitation of the protein with trichloroacetic acid, and urinary protein calculated. The calorie value of samples of the food and of pooled fecal samples from each group of animals was determined in the Parr Bomb Adiabatic Calorimeter.

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[1] Animal Foundation Laboratory Diet, Standard Brands, Inc.

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

[3] Casein, Sheffield Chemical Company; lactalbumin, Whitson Division, Borden Company; yeast, Type 200 B, Standard Brands, Inc.; hydrogenated vegetable oil, Crisco; Cellu Flour, Chicago Dietetic Supply House, Inc.

[4] Whole spray dried egg, commercial pack, Henningsen, Inc.

[5] Percomorph Oil, Mead Johnson Company, containing 60,000 units vitamin A and 8,500 units vitamin D/gram.

[6] Unpublished data; manuscript in preparation by Dr. Mildred Adams. See report of Adams, ('62).

When the animals reached 330 to 335 days of age,[7] they were anesthetized with sodium amytal solution without previous fasting, the body cavity opened and blood removed by heart puncture. Any evidence of gross abnormalities was recorded. Livers were removed, carefully blotted to remove excess blood and weighed; a small portion was placed in formalin for histological examination and the remainder was frozen for analysis of nitrogen, fat and cholesterol content.

Kidneys were removed and carefully trimmed and weighed without removing the capsule. They were cut sagittally, examined for stones or other abnormalities and graded zero to 4 plus according to severity of nephrosis. Grading of occurrence and severity of nephrosis was in accordance with procedures developed in this laboratory for the evaluation of gross and histological findings.[8] Accordingly, grade one plus, a light mottling of the kidney surface occurred which was accompanied by a small increase in weight; grade two plus, surface mottling was more diffuse and accompanied by a larger increase in weight usually with the weight of one kidney in excess of 2 gm; grade three plus, the kidney surface was light brown in color and the weight per kidney over 3 gm; grade four plus, the kidney surface was pale tan in color and usually had a nodular surface.

The left adrenal and left thyroid (including the parathyroid) were removed, carefully trimmed and weighed. In addition to samples of livers, one-half of the right kidney, samples of lungs, pancreas, spleen, left testis, thyroids, adrenals and hearts were preserved in 10% formalin for histological examination (results to be published later). The remainder of the kidneys was added to the carcass. From some of the animals fed each diet, gastrointestinal contents were removed, the carcasses autoclaved at 15 pounds pressure for 15 minutes, ground and a weighed sample dried under infrared lamps.

Nitrogen was determined in the dried carcasses and fat content calculated from moisture and protein content assuming 3% ash and 2% residual moisture. Fat was determined in liver homogenates by the method of AOAC ('50) which includes acid hydrolysis. Cholesterol was determined in the liver homogenates by the method of Koval ('61). Cholesterol was determined in the blood serum by the method of Zlatkis et al. ('53). In some of the blood sera, protein and nonprotein nitrogen were determined and protein components were analyzed by moving boundary electrophoresis (results to be published later).

## RESULTS

For this study, 70 BHE, 43 Holtzman and 134 Wistar strain rats were started originally. Of these, complete data are included for 66 BHE, 19 Holtzman and 60 Wistar rats at 330 to 335 days of age. Thirty-seven Wistar rats were transferred to a study on longevity when they were 330 days old but their data for growth and food consumption are used in this report.

An increase in respiratory disease in rats has been usually encountered in long-term feeding studies. But in the present study, a difference in susceptibility to respiratory infection was noted in the three strains of rats. Holtzman and Wistar rats were particularly susceptible to a type of acute pneumonia after reaching about 200 days of age.

Only two of the 70 BHE rats used in this study developed symptoms of the disease. Thirty-seven of 97 Wistar and 24 of 43 Holtzman rats were killed at varying intervals of the study when symptoms appeared. Comparison of average growth and organ weight data of the smaller groups reported in this study with those of over 500, 300-day-old healthy BHE[9] and Wistar rats fed a stock diet in this laboratory during the past 5 years indicates a uniformity of response of these two strains unaffected by susceptibility to acute res-

[7] In order to have information on a larger number of Holtzman and Wistar rats, groups of 24 Holtzman and 32 Wistar rats were fed the three diets before the original groups were 330 days old. Because of the development of acute respiratory disease, data from only three Holtzman rats could be used. Thirty of the 32 Wistar rats were killed at 155 days of age when it was decided that a sufficient number of the original group of Wistar rats (60) had survived to 330 to 335 days. No fecal or urine collections were made for these animals and no carcasses were analyzed. They were anesthetized and killed, blood and organ weights obtained, livers analyzed for protein, fat and cholesterol, and serum cholesterol determined. For results see footnote to table 2.
[8] A manuscript, "Histological findings in rats as influenced by age and diet" is in preparation by Dr. Anna M. Allen.
[9] A study is in progress to determine by IBM analysis of records the genetic characteristics of BHE rats.

piratory infection. The response of these two strains to the other two diets studied is not affected by the disease. Therefore, it is highly unlikely that either chance differences of exposure or susceptibility to disease are responsible for the results of this study. The variety of responses of the same strain of rats to the different diets indicates that susceptibility alone is not the determining factor.

The data reported in figure 1 contain values for some of the Holtzman and Wistar rats that developed respiratory infection but data are omitted for the entire 11-week period during which the infection occurred. Average values were the same with or without the inclusion of these animals. In the values reported in all of the tables for liver analyses, other organ weights, serum cholesterol and carcass analyses, *only* animals are included that had not demonstrated signs of illness and appeared healthy at necropsy.

Although only a small number of Holtzman rats were available at 330 to 335 days of age, evaluation of the data from this strain is considered to be justifiable inasmuch as the standard errors of most of

the measurements are similar to those obtained for larger groups of BHE rats.

*Growth and food consumption.* In figure 1 are plotted the average body weights of BHE, Holtzman and Wistar rats at 11, 22, 33 and 44 weeks of the experiment. All strains of rats fed the stock diet grew at approximately the same rate and reached similar final body weights.[10] Weight gains of BHE and Holtzman rats fed the SP and SPE diets were 100 to 175 gm greater than those of Wistar rats fed the same diets. Total cumulative food intakes (fig. 1, upper curves) during 44 weeks of feeding were approximately the same for BHE and Holtzman rats fed the SP diet (5,309 and 5,315 gm), but about 15% more than intakes of Wistar rats (4,581 gm). Of the SPE diet, Holtzman rats ate approximately 5% more than the BHE rats, but both of these strains ate about

[10] Duncan's test ('55) for significance of differences between 9 means (3 strains × 3 diets) was applied to the various parameters after completion of the usual test for analysis of variance when the variance ratios were not significant for the 9 means compared. (See Cochran's test for homogeniety of variances, Dixon and Massey ['51] p. 147). When the variance ratios differed significantly, individual group comparisons were made by the *t* test and for these, only the significant differences are referred to in the text.
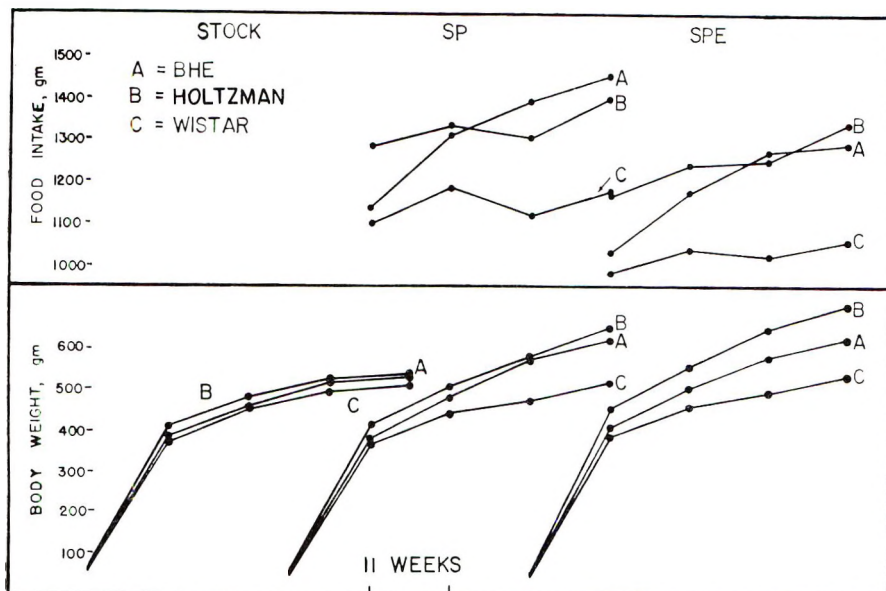


Fig. 1  Body weights and food intakes of three strains of rats fed three diets. Lower curves show body weights of BHE, Holtzman and Wistar rats on stock, SP and SPE diets at 11, 22, 33 and 44 weeks. Upper curves show food intakes of the three strains fed SP and SPE diets during the same intervals. Each point represents 19 to 24 BHE, 6 to 19 Holtzman and 25 to 38 Wistar rats per group.

10 to 15% more of this diet than did the Wistar rats. The average total gains per 100 gm food consumed after 44 weeks of feeding were similar for the BHE and Wistar strains but slightly higher for the Holtzman strain (SP diet, 10.8 (BHE), 10.1 (Wistar) and 11.2 (Holtzman); SPE diet, 11.9 (BHE), 11.7 (Wistar) and 12.8 (Holtzman)).

*Calorie and nitrogen data.* Gross calorie intakes[11] during 44 weeks of feeding of the SP and SPE diets were similar for any one strain.

In table 1 are the data showing the digestibility of the dietary energy and nitrogen supply, nitrogen balances and excretion of urine protein by the three strains of rats at 155 and 325 days of age. (Data collected at 240 days did not differ for any of the strains from the measurements at 325 days). The data substantiate an earlier report (Marshall et al., '59), that the energy and nitrogen supplied by the stock diet are not as digestible (% ADE and ADN)[12] as that of the semipurified diets. At 155 days, the percentage of ADE of the stock diet was slightly higher for Wistar rats than for BHE or Holtzman rats; but for all strains receiving the other two diets the values were quite similar. At 325 days, the percentage of ADE for the Holtzman rats fed the stock diet increased to that of the Wistar rats but the percentage of ADE for rats fed the SP and SPE diets was about the same for all of the strains. At all ages of measurement, the percentage of ADN was similar for all strains fed each diet.

Since the fat content was different for the two semipurified diets (9 vs. 17%), total gains per 100 digestible calories[13] were calculated and during 44 weeks of feeding were similar for the three strains (SP diet 2.5 (BHE), 2.6 (Holtzman), and 2.4 (Wistar); and for the SPE diet 2.5 (BHE), 2.7 (Holtzman) and 2.4 (Wistar)).

During the collection periods at 155 days of age, BHE rats ate less and stored significantly less nitrogen than either Holtzman or Wistar rats. There were no differences related to diet for any one strain except that Wistar rats fed the stock diet stored more nitrogen than Wistar rats fed the two semipurified diets; a similar trend was apparent for Holtzman

rats eating the stock vs. the SP diet. At 325 days of age each of the strains was storing nitrogen, and there were no significant differences related to strain. The group of Holtzman rats fed the SP diet stored less nitrogen than those fed the stock and SPE diets.

The BHE and Holtzman rats excreted significantly larger amounts of protein in the urine than did Wistar rats when fed the stock and SPE diets. In all cases the smallest excretion of urine protein was found for rats eating the SP diet. The BHE and Holtzman rats fed either the stock or SPE diet had significantly more urinary protein at 325 days of age than at 155 days but Wistar rats showed no difference between the diets at either age.

*Liver composition.* Size of liver, liver weight per 100 gm body weight or percentage of liver protein of Wistar rats were similar whether fed stock, SP or SPE diets, but when fed the SPE diet they had more liver fat than when fed stock or the SP diet (table 2). By contrast, BHE and Holtzman rats when fed the SPE diet had larger livers, larger livers per 100 gm body weight, a lower percentage of liver protein but more liver fat than when fed the SP diet. BHE and Holtzman rats receiving the SPE diet had larger livers, larger livers per 100 gm body weight, a lower percentage of liver protein and more liver fat than did Wistar rats.

Liver cholesterol was significantly higher, 2 to 10 times, in all rats fed the SPE diet (which contained 0.55% cholesterol), than in those receiving the stock or SP diet. The three strains fed the SPE diet differed significantly from each other in liver cholesterol levels.

*Other organs.* In each strain, the smallest amount of kidney damage observed was for those groups fed the SP diet and the largest was for those fed the SPE diet. Kidney stones, with or without hydronephrosis, were found in 9 of 22 (41%)

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[11] Gross calories per gm of diet, (average values for 9 different determinations each): SP diet, 4.61; SPE diet, 5.14.

[12] % ADE =
$$\frac{(\text{gross calorie intake} - \text{fecal calories})}{\text{gross calorie intake}} \times 100.$$
% ADN =
$$\frac{(\text{nitrogen intake} - \text{fecal nitrogen})}{\text{nitrogen intake}} \times 100.$$

[13] Average of values for % ADE for measurements at three age levels × gross calorie intakes.

## TABLE 1

Digestibility of dietary energy and nitrogen supply, nitrogen balance and urinary protein for BHE, Holtzman and Wistar strain rats at 155 and at 325 days of age

| Strain and diet[1] | No. rats | Body wt[2] | Intake Food | Intake Gross calories | Fecal calories | ADE[3] | Nitrogen Intake | Nitrogen In feces | Nitrogen In urine | Nitrogen Balance | ADN[3] | Urinary protein |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | gm | gm | Cal./day | Cal./day | % | mg/day | mg/day | mg/day | mg/day | % | mg/day |
| **At 155 days** | | | | | | | | | | | | |
| **Stock diet** | | | | | | | | | | | | |
| BHE | 15 | 438 | 16.5 | 65.5 | 11.9 | 81.6±0.5[4] | 774±25 | 178 | 594 | +2±21 | 76.8±0.6 | 119±15 |
| Holtzman | 10 | 502 | 19.1 | 74.9 | 14.2 | 81.0±0.2 | 956±16 | 242 | 612 | +102±9 | 74.7±0.5 | 217±44 |
| Wistar | 13 | 451 | 19.5 | 77.4 | 11.6 | 85.0±0.1 | 919±22 | 214 | 601 | +104±11 | 76.7±0.4 | 38±5 |
| **SP diet** | | | | | | | | | | | | |
| BHE | 14 | 454 | 13.6 | 63.9 | 4.9 | 92.3±0.2 | 525±18 | 48 | 467 | +12±10 | 90.8±0.2 | 63±6 |
| Holtzman | 10 | 501 | 15.6 | 70.5 | 4.8 | 93.3±0.2 | 652±18 | 52 | 535 | +65±10 | 92.1±0.3 | 58±7 |
| Wistar | 15 | 455 | 16.1 | 73.9 | 5.0 | 93.3±0.1 | 644±19 | 60 | 521 | +64±5 | 90.7±0.2 | 23±2 |
| **SPE diet** | | | | | | | | | | | | |
| BHE | 15 | 501 | 11.5 | 59.5 | 4.0 | 93.2±0.2 | 547±18 | 45 | 515 | −13±5 | 91.8±0.2 | 164±28[5] (14)[6] |
| Holtzman | 10 | 572 | 15.3 | 77.3 | 5.7 | 92.6±0.1 | 758±25 | 70 | 607 | +79±12 | 90.7±0.2 | 161±20 |
| Wistar | 15 | 469 | 14.2 | 72.8 | 4.3 | 94.0±0.1 | 691±25 | 57 | 581 | +52±7 | 91.7±0.2 | 35±6 |
| **At 325 days** | | | | | | | | | | | | |
| **Stock diet** | | | | | | | | | | | | |
| BHE | 12 | 531 | 20.7 | 86.1 | 15.9 | 81.5±0.1 | 1039±30 | 247 | 723 | +69±8 | 76.2±0.4 | 308±44 |
| Holtzman | 5 | 557 | 18.2 | 72.4 | 10.6 | 85.3±0.2 | 841±58 | 181 | 594 | +66±20 | 78.4±0.8 | 406±79 |
| Wistar | 12 | 540 | 19.2 | 74.9 | 10.9 | 85.5±0.4 | 924±31 | 207 | 666 | +50±13 | 77.6±0.7 | 84±19 (11) |
| **SP diet** | | | | | | | | | | | | |
| BHE | 12 | 613 | 17.8 | 84.4 | 5.3 | 93.7±0.1 | 735±23 | 53 | 651 | +34±9 | 92.7±0.1 | 93±17 |
| Holtzman | 8 | 655 | 17.3 | 79.5 | 5.6 | 93.0±0.2 | 677±25 | 61 | 605 | +11±8 | 91.0±0.3 | 108±23 |
| Wistar | 13 | 506 | 15.8 | 72.3 | 4.7 | 93.4±0.1 | 637±23 | 60 | 543 | +34±10 | 90.6±0.2 | 25±4 (12) |
| **SPE diet** | | | | | | | | | | | | |
| BHE | 12 | 632 | 15.7 | 83.6 | 4.9 | 94.1±0.1 | 791±34 | 61 | 679 | +50±7 | 92.3±0.2 | 292±72[7] (11) |
| Holtzman | 5 | 724 | 16.9 | 86.2 | 5.3 | 93.9±0.2 | 822±23 | 69 | 688 | +64±6 | 91.5±0.4 | 373±36 |
| Wistar | 15 | 518 | 13.9 | 72.1 | 4.6 | 93.6±0.1 | 694±27 | 59 | 599 | +37±8 | 91.5±0.3 | 35±10 (14) |

[1] BHE = laboratory mixed strain started in 1942 from a Yale strain obtained from Columbia University and a hooded strain obtained from Pennsylvania State College; stock diet = Animal Foundation Laboratory diet; SP = semipurified diet; SPE = semipurified diet with egg.
[2] Average body weight of groups of rats at beginning of the collection period.
[3] ADE = apparent digestible energy; ADN = apparent digestible nitrogen.
[4] Standard error.
[5] Excludes one extreme value of 939 mg; average for 15 rats = 215 ± 58 mg.
[6] Numbers in parentheses are number of samples analyzed.
[7] Excludes one extreme value of 1785 mg; average for 12 rats = 416 ± 141 mg.

TABLE 2

Liver composition, serum cholesterol and other organ weights of 330 to 335-day-old BHE, Holtzman and Wistar strain rats[1]

| Strain and diet[2] | No. rats | Body wt | Liver[3] | | | | | Serum cholesterol | Kidney wt[4] | Adrenal wt[5] | Thyroid wt[6] |
| | | | Weight | Weight/Body wt | Protein | Fat | Cholesterol | | | | |
| | | gm | gm | % | % | % | mg/gm | mg% | gm | mg | mg |
| **Stock diet** | | | | | | | | | | | |
| BHE | 22 | 539±13[7] | 20.9±1.0 | 3.9±0.1 | 21.0±0.1 | 4.9±0.1 | 3.3±0.1 | 272±41 | 4.6±0.4 | 23.2±1.1 | 22.3±0.9 |
| Holtzman | 5 | 553±10 | 22.5±0.6 | 4.1±0.1 | 19.7±0.2 | 3.9±0.03 | 2.7±0.2 | 230±25 | 4.4±0.3 | 32.6±2.3 | 18.4±1.7 |
| Wistar | 20 | 515±11 | 17.6±0.6 | 3.4±0.1 | 20.6±0.1 | 3.9±0.1 | 2.7±0.1 | 140±10 | 3.5±0.1 | 18.8±1.0 | 15.5±0.7 |
| **SP diet** | | | | | | | | | | | |
| BHE | 23 | 636±13 | 20.0±0.7 | 3.1±0.1 | 19.9±0.2 | 7.9±0.5 | 3.4±0.1 | 171±11 | 3.8±0.1 | 21.8±0.8 | 12.9±0.4 |
| Holtzman | 8 | 659±18 | 19.0±0.5 | 2.9±0.1 | 19.3±0.4 | 4.4±0.2 | 2.6±0.1 | 164±11 | 3.4±0.1 | 28.0±1.9 | 11.5±0.7 |
| Wistar | 17 | 516±15 | 15.9±0.4 | 3.1±0.1 | 20.2±0.2 | 4.3±0.1 | 2.6±0.1 | 119±6 (16)[8] | 3.2±0.1 | 15.3±1.4 | 11.3±0.8 |
| **SPE diet** | | | | | | | | | | | |
| BHE | 21 | 623±14 | 30.5±1.5 | 4.9±0.2 | 17.5±0.3 | 19.4±1.2 | 41.0±3.1 | 358±43[9](20) | 5.2±0.6[10](20) | 28.3±1.9 | 18.2±0.9 |
| Holtzman | 6 | 714±19 | 31.2±1.4 | 4.4±0.1 | 17.5±0.4 | 12.8±1.5 | 23.6±4.7 | 334±14 | 5.2±0.5 | 39.2±2.0 | 15.2±1.4 |
| Wistar | 21 | 523±11 | 18.1±0.7 | 3.4±0.1 | 20.3±0.2 | 7.0±0.4 | 9.6±1.0 | 193±10 (20) | 3.3±0.1 | 17.5±1.5 | 14.0±0.9 |

[1] Body weights of 155-day-old Wistar rats fed stock or SP diet were significantly smaller than those of 330 to 335-day-old rats, but by 155 days, rats fed the SPE diet were as large as the older Wistar rats. Liver weight per 100 gm of body weight for 155-day-old rats fed all three diets was significantly higher than for the older rats. With the SPE diet, liver fat and liver cholesterol (mg/gm) were significantly higher and serum cholesterol lower at 155 days than at 330–335 days.

[2] See footnote 1, table 1.

[3] All calculations based on wet weight.

[4] Weight of both kidneys.

[5] Weight of left adrenal.

[6] Weight of left half of thyroid.

[7] Standard error.

[8] Numbers in parentheses are number of samples analyzed.

[9] Excludes one extreme value of 1204 mg %; average of 21 values = 398 ± 58 mg/100 ml.

[10] Excludes one extreme value of 17.72 gm; average of 21 values = 5.79 ± 0.8 gm.

BHE rats fed the stock diet and in 2 of 23 (9%) fed the SP diet. Eighteen of 21 (86%) BHE rats fed the SPE diet and 14 of 22 (64%) fed the stock diet had nephrosis varying from one plus to four plus. Four of 20 (20%) Wistar rats fed the stock diet had kidney stones and 4 had mild nephrosis; one rat fed the SPE diet had nephrosis (++) but in the remaining 5, other forms of kidney damage were mild. Nephrosis (+ to ++++) occurred in all Holtzman rats fed the SPE diet and in 4 of 5 (80%) rats fed the stock diet. It was of doubtful significance in 2 of 8 (25%) rats fed the SP diet.

Kidney, adrenal and thyroid weights are shown in table 2. The kidney weights reflected to some extent kidney damage and for the SPE diet, larger kidney weights were associated with increased liver size. The BHE and Holtzman rats receiving the stock or SPE diets had larger kidneys than did the Wistar rats.

Holtzman rats fed all diets had larger adrenals, both total and per unit of body weight, than either BHE or Wistar rats. The BHE and Holtzman rats had larger adrenals when fed the SPE diet than when fed either the stock or SP diets, but Wistar rats showed no significant difference in adrenal size related to diet.

The smallest thyroid weights for all strains were obtained for rats fed the SP diet. The BHE and Holtzman rats fed the stock diet had larger thyroids than Wistar rats. The BHE rats fed the SPE diet had larger thyroids than either Holtzman or Wistar rats.

*Serum cholesterol.* Wistar rats had significantly lower serum cholesterol levels (table 2) than either BHE or Holtzman rats fed all three diets. Wistar rats showed some elevation when they received the SPE diet, but significantly less elevation than did the BHE and Holtzman rats fed this diet. Differences in distribution of serum cholesterol values for BHE and Wistar rats are shown in figure 2 (values for Holtzman rats are not included here because of the small numbers of these rats). This figure is included to point out the skewed distribution of the values of the BHE rats, particularly for the SPE diet, indicating the presence of animals within the BHE strain which show a different response to the same diet.

*Carcass composition.* In table 3 are the data showing the carcass composition of groups of each strain of rats. When fed the stock diet, there were no significant strain differences in carcass weight, fat-free carcass weight, percentage of protein or percentage of fat. When fed the SP and SPE diets, BHE rats had the smallest percentage of carcass protein. For any one strain, there were no differences in per-
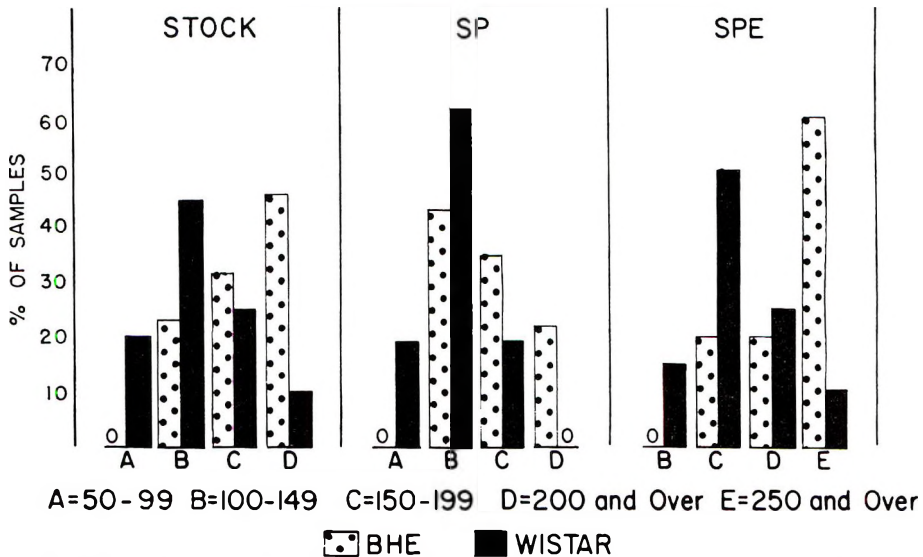


Fig. 2   Range of serum cholesterol values for BHE and Wistar strain rats (mg/100 ml).

TABLE 3

*Carcass composition of 330 to 335-day-old BHE, Holtzman and Wistar strain rats*

| Strain and diet[1] | No. rats | Carcass[2] | | | | | Protein in fat-free carcass | Moisture in fat-free carcass |
|---|---|---|---|---|---|---|---|---|
| | | Weight[3] | Moisture | Protein | Fat[4] | Fat-free wt[5] | | |
| | | *gm* | % | % | % | *gm* | % | % |
| Stock diet | | | | | | | | |
| BHE | 12 | 482 ± 18[6] | 54.9 | 19.6 ± 0.4 | 22.5 ± 1.5 | 373 ± 13 | 25.2 | 71.0 |
| Holtzman | 5 | 495 ± 9 | 57.3 | 20.6 ± 0.2 | 19.1 ± 1.1 | 400 ± 7 | 25.4 | 70.9 |
| Wistar | 11 | 484 ± 17 | 55.6 | 20.2 ± 0.4 | 21.2 ± 1.4 | 380 ± 10 | 25.6 | 70.8 |
| SP diet | | | | | | | | |
| BHE | 12 | 574 ± 17 | 47.0 | 16.4 ± 0.3 | 33.5 ± 1.1 | 381 ± 11 | 24.7 | 70.8 |
| Holtzman | 8 | 614 ± 18 | 48.2 | 17.9 ± 0.3 | 30.9 ± 0.9 | 425 ± 15 | 25.9 | 69.6 |
| Wistar | 12 | 476 ± 16 | 52.0 | 18.9 ± 0.3 | 26.1 ± 1.6 | 349 ± 8 | 25.5 | 70.9 |
| SPE diet | | | | | | | | |
| BHE | 12 | 577 ± 21 | 50.8 | 17.4 ± 0.3 | 28.7 ± 1.1 | 411 ± 15 | 24.4 | 71.4 |
| Holtzman | 6 | 657 ± 19 | 49.1 | 18.5 ± 0.2 | 29.4 ± 1.2 | 463 ± 8 | 26.2 | 69.7 |
| Wistar | 14 | 472 ± 11 | 53.6 | 19.6 ± 0.3 | 23.7 ± 1.1 | 359 ± 8 | 25.7 | 70.5 |

[1] See footnote 1, table 1.
[2] All calculations based on wet weight.
[3] Weight as analyzed, does not include liver.
[4] Estimated from analysis of moisture and protein.
[5] Carcass weight minus carcass fat.
[6] Standard error.

centage of carcass protein for rats fed the SP and SPE diets. The BHE and Holtzman groups fed the stock diet had a significantly higher percentage of carcass protein than rats fed the SPE diet but this was not true for the Wistar rats. In all cases rats fed the SP diet had a greater percentage of body fat than those fed the stock diet. Only the BHE rats showed a difference in body fat when fed the SP compared with the SPE diet. The BHE and Holtzman rats fed both the SP and SPE diets had a significantly higher body fat content than did the Wistar rats; when fed these two diets, BHE rats had 3.5 to 3.8 gm of carcass fat for each 100 gm of food consumed, as compared with 2.8 gm for Wistar rats.

In all strains fed all three diets the composition of the fat-free body was similar. Many workers (Moulton, '23; Pace and Rathbun, '45; Babineau and Pagé, '55) have found a constancy in the fat-free body of rats, regardless of dietary treatment. But Holtzman rats fed the SP and SPE diets had larger fat-free body weights and more carcass protein than BHE or Wistar rats, which perhaps indicates a better utilization of the SP and SPE diets for protein storage by Holtzman than by BHE or Wistar rats.

## DISCUSSION

The interpretation of the results of this experiment would be greatly simplified if differences in response of the three strains of rats could be attributed to differences in food intake. Although food intake of rats fed the stock diet was measured only during collection periods, Wistar rats were consuming as much or more than the other two strains. Wistar rats fed the stock diet had livers 19% smaller than those of BHE and Holtzman rats. The BHE rats ate 15% more of the SP and SPE diets than did the Wistar rats, but their livers were 25 (SP) and 70% (SPE) larger and had 45 (SP) and 65% (SPE) more fat. The BHE rats ate 5% less of the SPE diet than did the Holtzman rats; livers of the two strains were of comparable weight but BHE rats had 35% more liver fat. In addition, the relatively innocuous increase in liver and serum cholesterol and the absence of severe kidney damage in Wistar rats after feeding increased amounts of fat and cholesterol (SPE diet) supports the theory of a different mechanism for fat synthesis in these animals when compared with the other two strains. The apparent difference in fat metabolism among the

strains may be influenced by food intake. The high urinary protein excretion of both BHE and Holtzman rats may be associated with the increased food intakes of which the excess calories after protein replacement would be deposited as fat.

Inborn defects of glucose metabolism in the Yale strain of rats have been reported (Cole and Harned, '38; Orten and Sayers, '42; Sayers et al., '44; Beach et al., '56) which were not discernible in the Wistar strain. Since the BHE rats were derived in part from the Yale strain of rats, it is conceivable that a defect in glucose metabolism may be present in some of the BHE rats.[14] No information is available on the characteristics of the original hooded-strain ancestry of the BHE rats. The work of Sayers et al. ('44) led Mayer ('55) to characterize the Yale strain as hereditary obese animals. Zucker and Zucker ('62) recently reported the development of a hereditary obese group of rats which appeared spontaneously (25% /litter) in one strain in their laboratory. The response of this group in comparison with that of normal littermates fed diets high or low in fat indicated pathological lesions in the kidney, genetic hyperlipemia, hypercholesterolemia and increased body fat. They pointed out that the response of the "new fatty mutation" differed from that of Yale rats in that abnormal amounts of body fat were produced when the mutant rats were fed a low-fat stock diet. However, if differences in susceptibility to different types of physiological stress are characteristic of the "biochemical constitution" of various strains of rats, a knowledge of the response of these rats to diet seems pertinent in view of the increasing awareness of and difficulty in the evaluation and interpretation of data obtained in experiments with human subjects having known or unknown hereditary defects.

The wide variation in results obtained for the BHE rats for some of the measurements, particularly the urinary protein, kidney weights, serum and liver cholesterol values, indicates that, within this strain, hybridization has resulted in the production of groups of rats with different genetic characteristics and therefore different responses to diet. The wide variation obtained for certain measurements on these rats reduces the significance of the difference for some comparisons and makes it necessary to use large groups for nutritional experiments.

Fewer strain differences in the measurements were found when the stock diet was fed than when the animals received the two semipurified diets. Since all three strains fed the three diets showed similar digestibilities of the nitrogen and of the energy supply of the diets, similar gains per 100 gm of food consumed, and similar gains per 100 Cal. ingested, differences in response must be due to inherent metabolic characteristics of the rats.

## SUMMARY

Male weanling rats were housed individually and fed ad libitum one of three diets until they were killed, without fasting, when 330 to 335 days old. A mixed strain (BHE), Holtzman and a strain of Wistar rats were studied. The three diets fed to all strains included a pelleted stock diet, a semipurified diet (SP) and a semipurified diet containing 75% of the SP diet and 25% reconstituted, cooked and re-dried whole egg (SPE).

Weight gain for rats fed the stock diet was similar for rats of all strains. But when fed the SP and SPE diets BHE and Holtzman rats ate more and were heavier than Wistar rats. Gross calorie intakes were similar for the SP and SPE diets for any one strain. Nitrogen balances at 155 days of age indicated that BHE rats stored significantly less nitrogen than Holtzman or Wistar rats, but by 325 days all three strains were storing nitrogen and there were no significant differences related to strain. The BHE and Holtzman rats fed stock or SPE diets excreted larger amounts of protein in the urine than did Wistar rats. When fed the SPE diet, BHE and Holtzman rats had larger livers, larger livers per 100 gm of body weight, a smaller percentage of liver protein and more liver fat and cholesterol, more kidney damage, larger kidneys, larger adrenals, a smaller percentage of carcass protein, more carcass fat and higher serum cholesterol than did Wistar rats.

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[14] Some families of BHE rats with history of kidney damage have been inbred (brother-sister matings) for 9 generations in this laboratory. An increase in incidence and severity of nephrosis is now apparent and a few hooded rats were born in the 7th generation.

It was concluded that certain measurements such as body growth, food intake, urinary protein, liver, kidney and adrenal weights, carcass protein, carcass fat and serum cholesterol are influenced by strain of rat as well as by diet. The similarity for all three strains in digestibility of the dietary nitrogen and energy supply, and of gains per 100 gm of food and gains per 100 Cal. consumed in contrast with the differences in liver and body composition indicates that differences in response among strains must be related to differences in inherent metabolic characteristics of the rats.

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# Dye Binding by Soybean and Fish Meal as an Index of Quality[1]

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Soybean meal must be heat processed for maximal nutritional value. However, either underheating or overheating will produce a product of inferior nutritional value. Apparently underheating does not completely destroy or inactivate toxic substances (Alumot and Nitsan, '61; Liener, '53) or the proteins are not denatured to a point where they are completely digested (Fisher and Johnson, '58). Overheating, on the other hand, apparently results in reducing availability of lysine so that a deficiency of this amino acid is produced (McGinnis and Evans, '47). The degree of lysine inactivation depends upon time and temperature under appropriate moisture conditions. The mechanism responsible for the inactivation of lysine is thought to be primarily the browning or Maillard reaction in which the epsilon amino acid group reacts with free reducing sugar, such as glucose, under appropriate conditions or temperature and moisture to form a complex which is unavailable to the animal or from which the lysine is released too late to be utilized with the other amino acids that are released more rapidly (Lea, '50; Block et al., '46).

An analysis that would indicate the degree of lysine availability would in effect indicate the degree of heating of the soybean meals. Frölich ('54) first used dye binding as a method of indicating soybean protein quality after processing. This method was based on the increased capacity of heated protein to bind Cresol Red under acidic conditions. Olomucki and Bornstein ('60) later modified Frölich's method with respect to classification of quality with dye binding.

Udy ('56) used Orange G (1-phenylazo-2-naphthol-6, 8-disulfonic acid sodium salt) in the determination of protein content of wheat. Orange G, under acidic conditions, binds specifically to either free amino groups, the imidazole group of histidine, or the guanidyl group of arginine provided they are in a free or dissociated state (Fraenkel-Conrat and Cooper, '44). If the lysine or soybean meal is inactivated by complexing at the epsilon amino group during processing, then differences should be expected in its capacity to bind Orange G.

It was the purpose of the studies reported in this paper to examine the Orange G binding properties of both soybean and fish meals subjected to different processing treatments and to determine whether a relationship could be demonstrated between dye binding capacity, protein quality, and heat treatment.

## EXPERIMENTAL

*Heat treatment and assay of the soybean meal.* Whole ground soybean meal was spread approximately one inch thick (2.5 cm) on stainless steel pans and heated at 120° for various allotted times (table 1) in a Scanlan Morris steam-operated laboratory autoclave. Samples from each treatment were then reground in a small laboratory hammermill to a consistency of 24 mesh; 900- to 1,000-mg portions were weighed out to the nearest milligram and transferred to 250-ml Erlenmeyer flasks. The dye solution (one mg of Orange G/ml in distilled water buffered[2] to pH 2.2) was delivered to the flask with a calibrated 50-ml automatic buret at the rate of 1 ml/10

TABLE 1

*Effect of autoclaving soybean meal on dye binding*

| Auto-claving | Dye bound[1] | Ratio[2] | Protein[3] | Protein[4] |
|---|---|---|---|---|
| *min at 120°* | *mg/gm meal* | | % | Δ% |
| 0 | 70.8(a)[5] | 1.00 | 43.8 | — |
| 15 | 68.5(b) | 0.97 | 41.6 | −2.2 |
| 30 | 68.4(b) | 0.97 | 41.5 | −2.3 |
| 45 | 63.5(b) | 0.97 | 41.6 | −2.2 |
| 60 | 63.8(c) | 0.90 | 37.2 | −6.6 |
| 90 | 63.2(c) | 0.89 | 36.6 | −7.2 |
| 120 | 61.2(d) | 0.86 | 34.7 | −9.1 |

[1] An average of three determinations on a moisture-free basis.
[2] Ratio of milligrams of dye bound per gram of the respective treatments over that of the original raw meal.
[3] As determined by the dye binding technique.
[4] The change in protein content of the respective treatment in comparison with the original raw meal as determined by the dye binding technique.
[5] Letters in parentheses refer to significance at the 1% level: treatments with a common letter are not statistically different from each other, whereas those treatments without a common letter are.

TABLE 2

*Basal diet[1]*

| | % |
|---|---|
| Whole ground soybean meal[2] | 35.0 |
| Ground corn | 57.7 |
| Dehydrated alfafa (17% protein) | 3.0 |
| Limestone | 2.0 |
| Dicalcium phosphate | 1.0 |
| Methionine hydroxy analogue | 0.5 |
| NaCl | 0.3 |
| Choline chloride (25% mix) | 0.2 |
| Supplement[3] | to 100.0 |

[1] Contains 18.9% of protein (N × 6.25).
[2] All samples regardless of heat treatment assayed 43.5% of protein (N × 6.25) on a moisture-free basis.
[3] Supplemented with the following per pound (454 gm): vitamin A, 1,500 IU; vitamin $D_3$, 200 ICU; riboflavin, 1 mg; calcium pantothenate, 2 mg; niacin, 5 mg; vitamin E, 2 IU; ethoxyquin, 200 mg.

mg of the soybean meal (on a dry-weight basis) to the nearest tenth of a milliliter. The mixture was agitated by hand approximately every 5 minutes for one hour. The mixture was then filtered through Whatman no. 1 filter paper and a 1-ml aliquot of the filtrate was diluted 1:100 in a volumetric flask.

Optical density of the diluted filtrate was obtained at a wave length of 470 mμ in a Beckman DU spectrophotometer. The amount of unbound dye in solution was then calculated from a predetermined regression [$Y = 0.019 + 2.216X$, where Y is the concentration of Orange G in mg/ml and X is the optical density at 470 mμ ($r = 0.994$)]. This regression equation was obtained by appropriately diluting the original dye solution with distilled water over a range of 0.1 to 1.0 mg/ml at 0.1-mg intervals and determining the optical density of each. A second regression equation [$Y = 0.945X − 23.1$ ($r = 0.961$), where Y is the percentage of protein (N × 6.25) and X is the milligrams of Orange G bound per gram of meal] was obtained after plotting the amount of dye bound by different amounts of protein from raw soybean meal after reacting with a constant amount of the dye solution. The quantity of raw meal was varied to represent meals ranging from 35 to 45% of protein (N × 6.25). By reference to this equation, the amount of protein as determined by the dye binding method for an unknown was calculated. The difference in protein content as determined by this procedure and the Kjeldahl method (N × 6.25) reflects the destruction or inactivation of three basic amino acids (including lysine).

*Diets and design.* To study dye binding as an index of protein quality, chick growth trials were conducted on the same heat treated soybean meals, both with and without supplementary lysine. The basal diet (table 2) was formulated to contain a suboptimal amount of protein (18.9% of protein, N × 6.25). Thus, any amino acid deficiencies, as created by heat treatment, would be readily reflected in the growth trials.

Five male and five female chicks (New Hampshire × Delaware) were randomly allotted to pens of electrically heated and temperature controlled multitiered battery brooders with raised wire floors. Three pens of birds were fed each diet for a period of 4 weeks. Individual body weights and group feed consumption were recorded at weekly intervals. Feed and water were supplied ad libitum.

The 4-week chick results and dye binding data were analyzed statistically in compliance with the completely randomized design (Federer, '55), followed with Duncan's multiple range test (Duncan, '55) to find lot differences.

*Assay of fish meal.* Various samples of fish meals[3] which had been biologically

[3] The samples of fish meal that had been growth-rated and analyzed for available methionine and lysine were supplied by Dr. L. E. Ousterhout, Bureau of Commercial Fisheries, Department of the Interior, College Park, Maryland.

tested as the sole source of protein for chicks and analyzed for available methionine and lysine were obtained and analyzed for dye binding capacity. The samples were extracted with diethyl ether in a soxhlet apparatus before proceeding as with the soybean meals. Because of the wide range of protein content (N × 6.25), samples for dye binding were weighed out to contain 70 mg of nitrogen. To this was added a constant volume of dye solution (100 ml). Thus, a ratio of protein to dye was maintained so that dye binding capacity would be the only variable.

## RESULTS

*Dye binding results of soybean meal.* The capacity of the soybean meal to bind Orange G was decreased by heat treatment (table 1). The shortest heat treatment significantly reduced the amount of dye bound ($P < 0.01$). However, there was no further significant reduction until the meal had been heated for 60 minutes. The meals autoclaved for 60 and 90 minutes gave similar results, but were significantly different from the meal heated for 120 minutes ($P < 0.01$).

By using a ratio of the dye binding capacity of a processed meal to that of the raw meal, or a change in the percentage of protein as determined by the regression equation, an index of quality was established. The quality, as measured by these indices, did not change significantly until the soybean meal was autoclaved for more than 45 minutes. A further significant de-

crease in quality was observed in the meal autoclaved for more than 90 minutes.

Protein quality, as estimated by chick growth (table 3) correlated well with dye binding and the suggested indices with the exception of the raw meal. Although the raw meal gave optimal dye binding values, it supported poor growth which is attributed primarily to factors other than lysine availability. The meals autoclaved for 15, 30, 45 and 60 minutes were not statistically different among themselves, but significantly different from the 90-minute autoclaved meal ($P < 0.01$). The meal treated for 120 minutes gave a significantly lower gain than all other meals. Both the chick growth and dye binding indices demonstrate no significant differences between the effects of heat treatment for 15, 30, and 45 minutes. This suggests a rather extensive period of time in which little damage to the protein occurs, under conditions used in this test.

Lysine supplementation of all meals increased chick growth except for the meal heated 45 minutes. This then indicates that lysine was the most limiting amino acid in all treatments. When the growth data of the lysine supplemented diets were statistically analyzed, only the raw meal and that heat-treated for 45 minutes were significantly different from the others ($P < 0.01$). The difference obtained with the raw meal was expected, but not with the 45-minute heat treatment. The latter is not understood, but it may have been caused by factors other than the dietary variable studied.

TABLE 3

*Effect of autoclaving soybean meal on chick growth and feed utilization*

| Autoclaving treatment | No supplementation | | 0.5% Lysine supplementation[2] | |
|---|---|---|---|---|
| | 4-Week wt[1] | Gain/feed consumed | 4-Week wt | Gain/feed consumed |
| *min at 120°* | *gm* | | *gm* | |
| 0 | 229 ± 7[3](a)[4] | 0.43 | 240 ± 9(a) | 0.45 |
| 15 | 312 ± 8 (b) | 0.54 | 328 ± 8(c) | 0.55 |
| 30 | 315 ± 8 (b) | 0.51 | 347 ± 8(c) | 0.55 |
| 45 | 309 ± 6 (b) | 0.52 | 276 ± 8(b) | 0.48 |
| 60 | 293 ± 7 (b) | 0.50 | 339 ± 6(c) | 0.54 |
| 90 | 265 ± 7 (c) | 0.47 | 313 ± 7(c) | 0.52 |
| 120 | 185 ± 7 (d) | 0.36 | 315 ± 8(c) | 0.52 |

[1] Each average represents three pens of 10 chicks per pen.
[2] Feed grade lysine, containing 109 gm L-lysine per pound (454 gm), was replacing an equivalent amount of corn in the basal ration.
[3] Mean ± SE of the mean.
[4] See footnote 5 of table 2.

TABLE 4

*Fishmeal quality and dye binding[1]*

| Meal | Species | Type of drier | Growth rating | Protein[2] | Available[3] sulfur amino acid | Available[4] lysine | Dye binding |
|------|---------|---------------|---------------|------------|------------------------------|---------------------|-------------|
|      |         |               |               | %          |                              |                     | mg bound/ 70 mg N |
| GH1-1D70 | Herring | vacuum | superior | 57.8 | high | 7.96 | 77.3 ± 1.5[5] |
| GM1-1A70 | Menhaden | steam | superior | 62.9 | high | — | 70.0 ± 1.0 |
| GR1-1C79 | Redfish | flame | superior | 54.8 | high | 7.8 | 66.0 ± 0.0 |
| GG1-1B79 | Menhaden | steam | excellent | 62.9 | high | 7.7 | 72.0 ± 1.4 |
| GM1-4G60 | Menhaden | flame | excellent | 56.9 | high | — | 69.5 ± 0.7 |
| GM2-2B60 | Menhaden | flame | good | 60.8 | almost high | 7.43 | 72.7 ± 0.6 |
| GG1-6C79 | Menhaden | steam | fair | 59.6 | low | 7.0 | 66.0 ± 0.0 |
| GM1-7B80 | Menhaden | steam | fair | 57.4 | fair | — | 66.0 ± 0.0 |
| GG1-6F79 | Menhaden | flame | fair | 59.4 | fair | 6.6 | 60.7 ± 1.5 |
| GM1-4B120 | Menhaden | steam | poor | 60.4 | low | — | 64.3 ± 0.6 |
| GG1-365 | Menhaden | flame | poor | 62.0 | low | 5.9 | 41.3 ± 1.5 |
| GE-8 | Tuna | flame | poor | 48.6 | low | 5.0 | 54.0 ± 3.0 |

[1] All data in this table, other than the dye binding results, were supplied by Dr. L. E. Ousterhout, Bureau of Commercial Fisheries, Department of the Interior, College Park, Maryland.
[2] Protein (N × 6.25) on an "as is" basis.
[3] As determined by the method of Ousterhout et al. ('59) and Ousterhout, L. E., and D. G. Snyder 1960 The nutritional evaluation of fishmeals. Poultry Sci., 39: 1281 (abstract).
[4] On an "as is" basis by the method of Carpenter ('60) as percentage of protein.
[5] Standard deviation of three determinations.

*Dye binding results of fish meal.* A very definite tendency toward a decreasing ability to bind dye with decreasing quality was observed (table 4). However, there were samples for which dye binding values would indicate good quality in contrast to a lower quality rating based on chick feeding trials. These meals which would have a high nutritional rating based on dye binding were low in sulfur amino acid content, and in some instances, they were prepared from different species of fish or through different processing methods. Dye binding values do not give an indication of sulfur amino acid content.

### DISCUSSION

*Soybean studies.* The use of Orange G in the determination of soybean meal quality was selected because of its specificity, under acidic conditions, to bind either free amino groups, the imidazole group of histidine, or the guanidyl group of arginine provided they are in a free and dissociated state (Fraenkel-Conrat and Cooper, '44). The decreased Orange G binding by soybean meal, observed with increased autoclaving, is probably caused by the binding of the epsilon amino group of lysine with reducing sugars. Del Cueto et al. ('60) found that autoclaving the chick pea at 121° for 60 minutes destroyed about 10% of the lysine whereas the arginine

and histidine content were unaffected. The fact that supplementing overheated meals with lysine completely overcame the chick growth depression suggests that lysine was the principal, if not the only, amino acid involved in the changes in dye binding of soybean meal by autoclaving.

In contrast with the results of Frölich ('54) and Olomucki and Bornstein ('60) where binding of Cresol Red increased with heat treatment, Orange G binding decreased. The increased capacity to bind Cresol Red dye (nonspecific) is probably due to an increased availability of amphoteric groups resulting from increased protein denaturation.

With the use of either method, dye binding and the quality of the protein are well correlated with bioassay results. However, the Orange G method is considered to be more specifically related to nutritional value as it reacts with amino acids of critical importance in nutrition.

*Fish meal studies.* Because the quality of fish meal varies considerably after processing, it appeared possible to apply dye binding to this product also. However, it was apparent that this one simple test could not be used to indicate quality alone. Samples of fish meal that had low nutritional value because of low sulfur amino acid content still showed high dye binding properties. Dye binding can be

used to estimate the lysine content, but some other method would have to be used to estimate the available sulfur amino acids. Even then, dye binding may not be a reliable index to quality of fish meal as it appears to be for soybean meal either because of the fluctuations in histidine and arginine content among species, or parts of fish used in fish meal production, or both. These fluctuations may indirectly be observed in the discrepancies between Carpenter's method for the detection of available lysine and the dye binding values (table 4).

Some factors that must be considered when using the Orange G binding method are hydrogen ion concentration, particle size of sample, reaction time and fat content. The pH of 2.2 was used because Fraenkel-Conrat and Cooper ('44) indicated that complete dissociation of basic protein groups was approached at this point in the presence of dyes. Assuming that 2.2 is the optimal pH for groups associated with Orange G binding, the deviations from this could adversely affect the accuracy of this method.

Particle size of the meal is of great importance because the finer the meal the faster dye binding occurs. This is critical in this analysis because of the relatively short reaction time. Thus, the meals should be ground as finely and uniformly as possible, both within and between series of analyses.

Because dye binding is a process of equilibration, time is an important factor. Fraenkel-Conrat and Cooper ('44) showed that equilibrium could be attained in 20 hours with most proteins. Udy ('56) used a three-minute equilibration time with wheat; however, he used flour and the mixture was pulverized in a special mill.[4] It is felt that the present reaction time of one hour used in this analysis is sufficient to obtain the relative dye binding capacity accurately.

Since the fat content of samples may interfere with the ability of the water-soluble dye to bind to the protein, the amount present should be constant or it should be extracted. The fat content of the soybean meal was constant in all treatments and, therefore, was not removed. The fishmeals, in contrast, varied considerably in this respect and extraction was necessary prior to dye binding tests.

## SUMMARY

A chemical method for the determination of soybean meal quality after processing is proposed. The method is based on the reaction of the dye, Orange G, with the basic amino acids in the meal. The amount bound depends upon the availability of either free amino, imidazole, or guanidyl groups. Differences in the binding capacity of soybean meal are caused primarily by differences in the availability of the epsilon amino group of lysine.

The dye binding capacity of soybean meals heated for varying periods of time was closely correlated with growth of chicks fed the meals.

This method was also applied to fish meal, but because of variations in methionine (which was not detected by this method), and differences in arginine and histidine content between various meals, the correlation with chick growth was not satisfactory.

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# Prevention of "Meat Anemia" in Mice by Copper and Calcium[1]

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It has been shown previously (Adler, '58; Ilan et al., '60; Ilan and Guggenheim, '60, '62) that young mice maintained with a diet composed entirely of meat develop severe anemia which is mainly macrocytic and slightly hyperchromic. The bone marrow was found to be hyperplastic with megaloblast-like cells. The anemia could neither be cured nor prevented by any one of the hematopoietic factors, such as iron, pyridoxine, folic acid or vitamin $B_{12}$ or combinations of these. Beef liver, on the other hand, proved to be very effective in preventing and curing the anemia. It was assumed that a hematopoietic factor other than pyridoxine, folic acid, vitamin $B_{12}$ or iron is present in beef liver in a higher concentration than in beef muscle. Since addition of copper to the meat diet in the quantities present in the liver added was able to prevent the anemia, it was assumed that the "meat anemia" of mice is a copper deficiency anemia.[2] Copper deficiency has recently been reported (Moore, '62) in rats fed a meat diet. Further studies on the effect of copper on prevention of the "meat anemia" and on the correlation between copper content of the diet, hemoglobin and copper concentration in livers of mice are presented in this paper.

Growth of mice subsisting on the meat diet lagged considerably behind controls. This was assumed to be due to a deficiency of calcium, which is present in meat in very small amounts. Calcium carbonate was, therefore, added to the diet of mice rendered anemic by the meat diet. This treatment improved growth, and also cured the anemia. Calcium supplements have recently been reported to alleviate the anemia developing in rats fed meat (Moore, '62). The effect of calcium on the prevention of the "meat anemia" and the utilization of copper by mice was, therefore, studied.

## METHODS

Male Swiss mice, three weeks old, were used, if not otherwise stated. Before they were taken for experiment they and their mothers were fed a stock diet, as described by Ilan et al. ('60). The experimental diet consisted for the most part of raw beef muscle. Copper sulfate ($CuSO_4 \cdot 5H_2O$) or the different compounds listed in tables 1 and 2 were incorporated into the minced meat in varying amounts. All these compounds were analytically pure.

The milk diet used was composed of dried fat-free milk, 50; glucose, 45; and vegetable oil, 5. It was enriched with 0.125% of $FeSO_4$. Hemoglobin was examined in tail blood by the cyanmethemoglobin method (Crosby et al., '54). Copper was determined by the method of Eden and Green ('40). The yellow color formed in the presence of copper with sodium diethyldithiocarbamate was extracted with amyl alcohol, and the absorption at 500 m$\mu$ was measured in a Klett apparatus.

## RESULTS

*Effect of supplementation of the meat diet with copper.* Seven groups of young mice and 5 groups of adult mice were used in these experiments. The young mice were three weeks old and fed the meat diet for 6 weeks, whereas the adult, 6-week-old mice were observed for 8 weeks. Various amounts of copper (as

TABLE 1

*Effect of calcium salts on growth and hemoglobin of mice fed a meat diet for 6 weeks[1]*

| Compound | No. of mice | Calcium added to food | Increase of weight | Change of hemoglobin |
|---|---|---|---|---|
| | | gm/kg | gm | gm/100 ml |
| Control, no calcium added | 29 | — | 3.9 ± 0.61 | − 12.2 ± 0.62 |
| Carbonate | 19 | 0.15 | 8.9 ± 0.92 | − 5.6 ± 0.94 |
| | 16 | 0.75 | 5.9 ± 0.82 | − 2.0 ± 0.82 |
| | 16 | 2.25 | 10.1 ± 0.51 | − 0.9 ± 0.56 |
| Chloride | 7 | 0.15 | 3.0 ± 1.13 | − 9.4 ± 1.23 |
| | 9 | 0.75 | 6.7 ± 0.89 | − 6.0 ± 1.40 |
| | 7 | 2.25 | 7.0 ± 1.96 | − 0.7 ± 0.89 |
| Phosphate | 17 | 0.15 | 7.6 ± 0.77 | − 4.8 ± 0.87 |
| | 11 | 0.75 | 9.1 ± 0.88 | − 2.4 ± 0.92 |
| | 18 | 2.25 | 8.7 ± 0.69 | + 1.5 ± 0.40 |
| Lactate | 9 | 0.15 | 5.8 ± 1.71 | − 9.5 ± 1.42 |
| | 15 | 0.75 | 6.2 ± 0.70 | − 5.8 ± 0.80 |
| | 12 | 2.25 | 8.3 ± 1.08 | − 2.5 ± 0.74 |
| Gluconate | 27 | 0.15 | 4.1 ± 0.61 | − 10.8 ± 0.58 |
| | 20 | 0.75 | 7.9 ± 0.78 | − 5.1 ± 0.81 |
| | 15 | 2.25 | 12.8 ± 0.60 | − 1.6 ± 0.64 |
| All compounds | 79 | 0.15 | 6.1 | − 8.0 |
| | 71 | 0.75 | 7.1 | − 4.3 |
| | 68 | 2.25 | 9.7 | − 0.5 |

[1] For initial weight of mice and hemoglobin content of their blood see footnote of table 3. Values are expressed as means and their standard errors.

TABLE 2

*Effect of strontium and magnesium chlorides on growth and hemoglobin of mice fed a meat diet for 6 weeks[1]*

| Compound | No. of mice | Metal added to food | Increase of weight | Change of hemoglobin |
|---|---|---|---|---|
| | | gm/kg | gm | gm/100 ml |
| Control | 29 | — | 3.9 ± 0.61 | − 12.2 ± 0.62 |
| Strontium chloride | 5 | 0.75 | 3.8 ± 0.42 | − 11.7 ± 0.69 |
| Strontium chloride | 16 | 1.50 | 3.0 ± 0.54 | − 8.3 ± 0.73 |
| Strontium chloride | 12 | 2.25 | 7.1 ± 0.85 | − 2.3 ± 0.91 |
| Magnesium chloride | 12 | 2.25 | 4.8 ± 0.97 | − 7.3 ± 0.85 |
| Calcium chloride | 7 | 2.25 | 7.0 ± 1.96 | − 0.7 ± 0.90 |

[1] For initial weight of mice and hemoglobin content of their blood see footnote of table 3. Values are expressed as means and their standard errors.

CuSO₄) were incorporated into the meat as indicated in table 3. The table presents changes of weight and hemoglobin as well as of concentrations of copper in the livers of these mice.

Data in table 3 confirm previous findings that severe anemia develops in mice fed a meat diet. Adding increasing amounts of copper gradually prevents the anemia and simultaneously increases the copper concentration of the liver. With additions of 1.0 to 2.0 mg of copper/kg of meat in young mice and with 0.5 to 1.0 mg in adult mice, anemia can largely be prevented. These supplements induce levels of 4 to 6 mg of copper/kg of liver. Larger amounts of copper were needed in young mice than in adults to obtain a similar degree of prevention of anemia. This may be explained on the basis of the amounts of copper present in the liver at the start of the experiment. Livers of 20 young,

TABLE 3

*Effect of supplementation of a meat diet with copper on weight, hemoglobin and copper content of livers of young and adult mice[1]*

| No. of mice | Age | Supple-mentation of copper | Change of weight | Change of hemoglobin | Copper content of liver |
|---|---|---|---|---|---|
| | *weeks* | *mg/kg* | *gm* | *gm/100 ml* | *mg/kg* |
| 18 | 3 | — | $+6.1 \pm 0.97$ | $-9.4 \pm 0.98$ | $2.28 \pm 0.40$ |
| 16 | 3 | 0.1 | $+5.2 \pm 0.82$ | $-8.9 \pm 0.77$ | $3.21 \pm 0.25$ |
| 15 | 3 | 0.25 | $+5.3 \pm 0.52$ | $-7.9 \pm 0.70$ | $4.46 \pm 0.20$ |
| 12 | 3 | 0.5 | $+6.5 \pm 0.84$ | $-5.2 \pm 1.17$ | $4.23 \pm 0.12$ |
| 30 | 3 | 1.0 | $+9.1 \pm 0.66$ | $-1.4 \pm 0.78$ | $5.50 \pm 0.15$ |
| 17 | 3 | 2.0 | $+6.9 \pm 0.95$ | $+0.7 \pm 0.56$ | $7.14 \pm 0.13$ |
| 14 | 3 | 4.0 | $+6.5 \pm 0.73$ | $+1.5 \pm 0.19$ | $8.75 \pm 0.14$ |
| 18 | 6 | — | $-0.9 \pm 1.39$ | $-8.2 \pm 0.79$ | $2.87 \pm 0.14$ |
| 15 | 6 | 0.1 | $+2.9 \pm 0.84$ | $-4.2 \pm 1.01$ | $3.18 \pm 0.16$ |
| 10 | 6 | 0.25 | $-3.1 \pm 1.46$ | $-4.3 \pm 0.89$ | $4.05 \pm 0.20$ |
| 12 | 6 | 0.5 | $-2.6 \pm 0.39$ | $-1.2 \pm 0.30$ | $4.25 \pm 0.17$ |
| 13 | 6 | 1.0 | $+3.5 \pm 0.67$ | $-0.7 \pm 0.67$ | $5.82 \pm 0.42$ |

[1] Copper was added as $CuSO_4$. The young and the adult mice were 3 and 6 weeks old, respectively, at the beginning of the experiments which lasted 6 and 8 weeks, respectively. The initial weight of the young mice was 11.0 to 14.0 gm and that of the adult mice, 21.0 to 28.0 gm. Their blood contained 14.0 to 17.0 gm of hemoglobin/100 ml. The figures indicate means and standard errors.

three-week-old mice were found to contain $4.40 \pm 0.22$ mg of copper/kg as against $5.57$ ($\pm 0.11$) mg/kg present in 10 livers of adult, 6-week-old mice.

These experiments demonstrated that the "meat anemia" can be prevented by dietary copper and that a certain level of copper in the liver appears to be necessary to maintain normal hematopoiesis. To study further the possible role of copper in the prevention of "meat anemia," a milk diet was fed to young mice. The results are presented in table 4.

The milk diet provided similar amounts of copper per gram as the meat. The mice fed the milk diet gained more weight and their hemoglobin decreased much less than that of the mice offered meat. The copper content of their livers, however, was only insignificantly higher than that found in the livers of mice fed the unsupplemented meat diet. Average meat consumption amounted to 7 gm per mouse per day, and this quantity provided 9.1 µg of copper. The mice subsisting on the milk diet ate about 5 gm of food/day which contained 7.5 µg of copper. It appears, therefore, that the copper content of the liver is related to the dietary intake, but that hematopoiesis is not necessarily dependent on the copper content of the liver. When the diet consists of meat, higher amounts of copper are needed to prevent anemia. This result does not support the assumption that the "meat anemia" is a dietary copper deficiency anemia.

*Effect of supplementation of the meat diet with calcium, strontium and magnesium.* In the first series of experiments, 5 calcium salts were studied: three inorganic (carbonate, chloride, phosphate) and two organic (lactate, gluconate) salts. Different groups of mice received the meat diet which was supplemented with 0.15,

TABLE 4

*Weight increase, hemoglobin and copper content of livers of mice fed milk and meat diets for 6 weeks[1]*

| No. of mice | Diet | Copper content of diet | Increase of weight | Change of hemoglobin | Copper content of liver |
|---|---|---|---|---|---|
| | | *mg/kg* | *gm* | *gm/100 ml* | *mg/kg* |
| 22 | Milk | 1.5 | $12.6 \pm 0.76$ | $-1.5 \pm 0.32$ | $3.18 \pm 0.25$ |
| 18 | Meat | 1.3 | $6.1 \pm 0.97$ | $-9.4 \pm 0.98$ | $2.28 \pm 0.40$ |

[1] For initial weight of mice and hemoglobin content of their blood see footnote of table 3. Values are expressed as means and their standard errors.

0.75 and 2.25 gm, of calcium/kg, respectively. The results obtained after 6 weeks are presented in table 1. A supplement of 2.25 gm of calcium/kg of meat prevented anemia, whereas smaller amounts were less effective or ineffective. It appears that among the salts tested phosphate was the most efficient in preventing anemia, followed by carbonate and chloride, whereas the two organic salts, lactate and gluconate, exhibited the weakest potency.

As expected, addition of calcium salts to the meat diet, which is very poor in calcium, improved growth of the mice (table 1). Large supplements induced a weight increase of 7 to 13 gm, whereas the weight of mice receiving the smaller supplements of calcium increased by 3 to 7 gm only.

Next, the effects of magnesium and strontium were studied. Both these elements closely resemble calcium with respect to their physical and chemical properties and physiological activities. The effects of supplementation of the meat diet with $MgCl_2$ and $SrCl_2$ are shown in table 2. Even supplements as large as 2.25 gm of magnesium/kg of meat did not prevent the anemia and their effect was inferior to that of corresponding quantities of calcium salts. Furthermore, magnesium did not induce normal growth and differed in this respect, too, from the calcium salts tested. Strontium, on the other hand, had an anemia-preventing effect. It appears, however, to be less able to prevent anemia than calcium.

*Effect of calcium on the utilization of copper.* The question arose whether the beneficial effect of calcium on the prevention of "meat anemia" can be explained by its action on the utilization of copper. Contamination with copper of the calcium compounds used could be excluded; only traces of copper which were much too small to prevent anemia could be detected. On the other hand, the phosphate content of meat is very high and its calcium content low. The possibility exists, therefore, that such a low calcium-to-phosphorus ratio may depress the absorption of the small amounts of copper present in meat, and that supplementation with calcium counteracts the high intake of phosphorus. The effect of an addition of calcium on the utilization of copper present in or added to meat was studied in 6 groups of mice. They were fed the meat diet supplemented with either calcium or copper or with both minerals, as indicated in table 5. After supplying these diets for 6 weeks the copper content of the livers was examined. It is well known that the liver is the main storage organ in the body for copper and provides a reasonably reliable index of the copper status of the animal (Underwood, '56).

The data of table 5 show that calcium, as expected, improved growth and prevented anemia. It had, however, a slight effect only on copper concentration of the liver, the differences between the corresponding groups being statistically insignificant. Thus, calcium does not appear to improve the nutritional availability of copper. Moreover, it prevented anemia even when the copper content of the liver was much lower than the minimal concentration permiting normal hematopoiesis of mice fed the meat diet.

TABLE 5

*Copper concentrations of livers of young mice fed for 6 weeks a meat diet supplemented with calcium and with different amounts of copper[1]*

| No. of mice | Supplementation | | Increase of weight | Change of hemoglobin | Copper content of liver |
|---|---|---|---|---|---|
| | Calcium | Copper | | | |
| | *mg/kg* | *mg/kg* | *gm* | *gm/100 ml* | *mg/kg* |
| 16 | — | — | 6.1 ± 0.97 | − 9.4 ± 0.98 | 2.28 ± 0.40 |
| 25 | 2.25 | — | 8.4 ± 0.78 | − 0.3 ± 0.39 | 3.04 ± 0.08 |
| 15 | — | 0.25 | 5.3 ± 0.52 | − 7.9 ± 0.70 | 4.46 ± 0.20 |
| 12 | 2.25 | 0.25 | 14.3 ± 0.53 | − 0.4 ± 0.44 | 4.88 ± 0.12 |
| 12 | — | 0.50 | 6.5 ± 0.84 | − 5.2 ± 1.17 | 4.23 ± 0.12 |
| 13 | 2.25 | 0.50 | 12.7 ± 0.91 | + 0.6 ± 0.44 | 5.01 ± 0.43 |

[1] For initial weight of mice and hemoglobin content of their blood see footnote of table 3. Calcium was added to meat as $CaCO_3$ and copper as $CuSO_4$. Figures indicate means and their standard errors.

## DISCUSSION

Our studies show that certain minerals, such as copper and calcium, when added to a meat diet prevent the anemia which invariably develops in mice, whereas other minerals, such as magnesium and iron (Ilan et al., '60) have no effect. Strontium had a slight anemia-preventing effect only.

It has previously been suggested[3] that the "meat anemia" is a copper deficiency anemia, and the following arguments have been brought forward in support of this suggestion. (1) Skeletal muscle is a poor source of copper (1.3 mg/kg). Supplementation of muscle meat with beef liver which contains 22.1 mg of copper/kg has been shown to prevent the anemia (Ilan et al., '60; Ilan and Guggenheim, '62). (2) Livers of mice that had subsisted on a meat diet and were probably depleted of copper had no therapeutic effect on the anemia (Ilan and Guggenheim, '60). (3) Feeding heart muscle in place of skeletal muscle (Ilan and Guggenheim, '62) did not result in anemia as the copper content of this tissue is higher (Cunningham, '25). (4) Supplementation of the meat diet with copper sulfate prevents the "meat anemia."

The data presented in this paper cast some doubt on whether the "meat anemia" is a dietary copper deficiency anemia. (1) A milk diet which does not supply more copper than meat, does not induce anemia in 6 weeks. It would probably have caused anemia (Hart et al., '28) if the observation period had been extended. But the fact that feeding meat causes severe anemia in such a short time as 6 weeks points to another etiology than dietary copper deficiency. (2) Although the addition of copper sulfate to meat increases the copper content of the liver, anemia is prevented only when the copper level of the liver is much higher than that found in non-anemic mice maintained with a milk diet. (3) As has been pointed out,[4] rats, in contrast with mice, are quite resistant to "meat anemia," and their hemoglobin level does not decrease even after consuming a meat diet for 12 weeks. This was thought to be because rats acquire a much higher copper reserve during their suckling period than mice. Whereas milk is a rela-

tively poor source of copper, rat milk has been reported to be an exception, containing 10 times more copper than the milk of other species (Cox and Mueller, '37). The peculiar sensitivity of mice to meat is, however, apparently not the result of low liver storage of copper. The livers of 20 weanling mice of our stock colony contained $4.40 \pm 0.22$[5] mg of copper/kg as against $4.68 \pm 0.18$ mg present in the livers of 10 weanling rats.[6] (4) The possibility that the copper present in meat cannot be efficiently absorbed owing to its high phosphate content as proposed by Moore ('62) is not supported by our observations. Addition of calcium to meat prevents the anemia without significantly increasing the copper content of the liver. This would be expected, if calcium improves absorption of copper. And among the 5 calcium compounds tested, phosphate was the most efficient in preventing anemia. Thus, calcium does not act by increasing absorption of copper. On the other hand, the anemia is not caused by deficiency of dietary calcium, since cow's liver, which contains no more calcium than muscle (Watt and Merrill, '50), when incorporated into a meat diet, prevents and cures the anemia (Ilan et al., '60; Ilan and Guggenheim, '62).

Schultze and associates ('34) found that copper was not utilized by rats when given as its hematoporphyrin. It may be speculated that the copper is present in meat as hematoporphyrin. On the other hand, our data on liver concentration of copper indicate that the copper in meat is not much less available for absorption than that in milk. The main question appears to be not the possible depresson of absorption but the apparently diminished availability of copper present in the liver for hematopoiesis, when mice subsist on a meat diet. Schultze et al. ('34) mention the possibility that copper is absorbed in the intestine but cannot take effect on account of its chemical complexity. If we adopt this suggestion, we have also to assume that rats, in contrast with mice, are able to liberate the copper of the complex.

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[3] See footnote 2.
[4] See footnote 2.
[5] SE.
[6] Guggenheim, K., and E. Tal. unpublished results.

Another possibility is the presence in meat of a toxic substance which is overcome by copper or calcium. Copper is known to counteract excessive amounts of molbydenum. Moreover, the co-existence of symptoms of copper deficiency with normal concentrations of copper in blood and tissues has been postulated, at high intakes of molybdenum (Underwood, '56). Also different species exhibit different degrees of susceptibility to molybdenum poisoning; cattle and sheep are most susceptible, whereas pigs and horses are most tolerant.

Finally, it may be possible that dietary calcium is needed for efficient utilization of copper in hematopoiesis. These possibilities are now under investigation.

## SUMMARY

1. Young mice, when fed for 6 weeks a diet of muscle meat, developed severe anemia.

2. The anemia could be prevented by supplementing the meat with 1 mg of copper (as $CuSO_4$) per kg. Smaller amounts were less effective.

3. Only when the copper content of the liver reached 4 to 6 mg/kg was anemia prevented in mice fed a meat diet. On the other hand, mice maintained with a milk diet that provides amounts of copper similar to those in meat did not develop anemia in 6 weeks, and after that period their livers contained less than 4 mg of copper/kg.

4. Various calcium salts (carbonate, chloride, phosphate, lactate, gluconate) were incorporated into the meat diet. Supplements of 2.25 gm of calcium/kg of meat prevented the anemia. Calcium phosphate was the most efficient followed by carbonate and chloride, whereas lactate and gluconate were less effective. Strontium chloride exhibited also a marked potency in preventing the anemia, whereas magnesium chloride was almost ineffective.

5. When the meat diet was supplemented with calcium carbonate and with different amounts of copper, insignificantly more copper was found in the livers of mice in comparison with that in the livers of mice maintained with a similar diet without the addition of calcium. It appears that calcium does not act by improving absorption of the small amounts of copper present in meat or of the larger amounts added to meat.

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# Observations on a Magnesium-fluoride Interrelationship in Chicks[1]

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Gardiner et al. ('61) showed that the addition of 0.08% fluoride and 0.25% magnesium in chick diets caused a greater depression of growth than did 0.08% fluoride alone. In addition, the birds fed the high magnesium-high fluoride diet developed a characteristic leg weakness and exhibited reduced bone ash which was not noted in the presence of either supplemental magnesium or fluoride alone. The leg weakness, first noted at 5 or 6 days of age, increased in severity until the birds were 12 to 14 days old. At this age a gradual improvement began and continued until most of the birds had recovered at the end of the 4-week experimental period. It was suggested that the depressed growth rate with the use of high levels of magnesium and fluoride may have been caused by reduced calcification of the bone and the resultant impairment of mobility of the bird, or from inefficient utilization of carbohydrate due to the inhibition of enolase.

The classical *in vitro* studies by Warburg and Christian ('42) demonstrated that magnesium participates in the inhibition of enolase by fluoride. Similarly, Goldenberg and Sobel ('51) observed that *in vitro* calcification was inhibited in the presence of both magnesium and fluoride but not in the presence of fluoride alone. *In vivo* results reported by Schuck ('38) indicated that dietary magnesium has no effect on fluoride toxicity in rats. On the other hand, Dibak and Ginter ('59) reported inhibition of fluoride toxicity when magnesium was added to rat diets containing fluoride.

The experiments reported here were designed to investigate further the magnesium-fluoride interrelationship in growing chicks.

## EXPERIMENTAL

Four experiments were conducted using White Cross, male chicks. The birds were randomly assigned to replicates, banded, weighed, and placed in electrically heated, wire-floor starting batteries. Feed and water were provided ad libitum and the birds were weighed weekly. Four replicates of 10 birds each were started on each of the various treatments. The replicates were assigned so that no replicate of a treatment could be in the same deck level or the same battery as another replicate of that treatment. Thus, replicates were actually deck levels and were considered fixed in analyzing the data. When tissue and blood samples were taken at the end of the experiments, all birds in the 4 replicates were considered in the statistical analysis of growth. However, when birds were killed prior to termination of the experiment, the birds were all taken from one replicate or deck level and that replicate was not used in evaluating treatment effects on growth. Statistical analysis of the data was made by the analysis of variance (Snedecor, '60) and the treatment means were compared by the method of Newman ('39) as modified by Keuls ('52). The basal diets were supplemented with reagent grade magnesium carbonate and sodium fluoride to obtain the desired levels of magnesium and fluorine.

Experiment 1 was conducted to determine the effect of calcium on the magnesium-fluoride interrelationship. The basal diet, composed chiefly of ground yellow corn, dehulled soybean oil meal, soybean

TABLE 1

*Influence of calcium on the magnesium-fluoride interrelationship in young chicks*

| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Supplemental F, % | — | 0.08 | — | 0.08 | — | 0.08 | — | 0.08 |
| Supplemental Mg, % | — | — | 0.25 | 0.25 | — | — | 0.25 | 0.25 |
| Total Ca, % | 1.0 | 1.0 | 1.0 | 1.0 | 1.50 | 1.50 | 1.50 | 1.50 |
| Avg wt, gm 2-week | 236 | 204 | 229 | 131 | 219 | 212 | 209 | 152 |
| Avg wt, gm 4-week | 613 | 522 | 620 | 354 | 564 | 547 | 571 | 442 |
| Alkaline phosphatase[1] 2-week | 165 | 270 | 159 | 250 | | | | |
| Alkaline phosphatase[1] 4-week | 46 | 135 | 51 | 119 | 60 | | | 98 |
| Bone ash,[2] % 2-week | 45.4 | 43.6 | 43.9 | 34.7 | 45.7 | | | 43.6 |
| Bone ash,[2] % 4-week | 43.7 | 44.0 | 43.8 | 38.0 | 45.5 | | | 43.0 |
| Bone F,[2] ppm 2-week | 83.3 | 9058 | 77.9 | 5089 | 83.3 | | | 4477 |
| Bone F,[2] ppm 4-week | 65.9 | 9632 | 85.6 | 5302 | 80.4 | | | 4715 |

[1] Expressed as millimole units as defined by Bessey ('46).
[2] Based on dry, fat-free bone.

oil, and condensed fish solubles, was the same as that used by Gardiner et al. ('61) except ground yellow corn, ground limestone,[2] and dicalcium phosphate[3] were used at the rate of 46.4, 1.10 and 1.80%, respectively. Glucose was used to adjust all diets to 100%. This basal diet was supplemented with two levels of calcium, magnesium and fluoride as indicated in table 1. The birds were placed on treatment at one day of age and after two weeks one replicate from treatments 1 to 4 was killed. Blood samples were taken by cardiac puncture with heparin as an anticoagulant and the right femur was removed for bone analyses. After centrifugation of the blood, the plasma and bone samples were frozen immediately on dry ice and stored at − 20°C until analyzed. Alkaline phosphatase activity of the blood plasma was determined using the procedure of Bessey ('46). The ash content of the individual bone samples was determined as described by Gardiner et al. ('61). Bone ash from the various treatments was composited and analyzed for fluoride content using the procedure of Schall and Williamson ('55). A second replicate was killed after 4 weeks and samples were obtained and analyzed as described above.

The effect of age on the magnesium-fluoride interrelationship was studied in experiment 2. One group of birds was placed on treatment at one day of age; a second group was maintained with the basal diet for two weeks and then placed on treatment. The basal diet, the same as that used in experiment 1, was supplemented with two levels of magnesium and two levels of fluoride (table 2). One replicate of the birds started on experiment at one day of age was killed after two weeks on treatment. One replicate of the birds started on treatment at two weeks of age was killed after two weeks and another replicate was killed after 4 weeks. Blood plasma and bone analysis were the same as in experiment 1. In addition, samples of gastrocnemius muscle were collected and analyzed for the conversion of 3-phosphoglycerate to phosphoenolpyruvate by the method of Kun ('60).

The effects of a semipurified diet and a greater range of supplemental magnesium and fluoride were studied in experiment 3. The percentage composition of the basal diet was: isolated soy protein, 30; corn oil, 5; cellulose, 3; DL-methionine, 0.50; glycine, 0.30; butylated hydroxytoluene (25%), 0.05; vitamin premix, 1.26; mineral mix, 4.41; glucose, as necessary to total 100%. The vitamin premix supplied

[2] Commercial grade.
[3] See footnote 2.

**TABLE 2**

*Influence of age on the development of the magnesium-fluoride interrelationship*

| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age at start, weeks | 0 | 0 | 0 | 0 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Age at sacrifice, weeks | 2 | 2 | 2 | 2 | 4 | 4 | 4 | 4 | 6 | 6 | 6 | 6 |
| Supplemental F, % | — | 0.08 | — | 0.08 | — | 0.08 | — | 0.08 | — | 0.08 | — | 0.08 |
| Supplemental Mg, % | — | — | 0.25 | 0.25 | — | — | 0.25 | 0.25 | — | — | 0.25 | 0.25 |
| Avg weight, gm | 229 | 214 | 236 | 175 | 605 | 596 | 624 | 598 | 1,063 | 1,018 | 1,086 | 1,030 |
| Alkaline phosphatase[1] | 122 | 257 | 155 | 405 | 48 | 156 | 55 | 123 | 33 | 93 | 30 | 57 |
| Bone ash,[2] % | 42.3 | 41.6 | 40.9 | 38.9 | 42.7 | 44.0 | 42.8 | 42.4 | 42.1 | 42.5 | 41.7 | 42.4 |
| Bone F,[2] ppm | 906 | 10,268 | 377 | 6,757 | 1,339 | 7,798 | 248 | 4,754 | 143 | 8,823 | 976 | 6,622 |
| Enolase activity[3] | 18.2 | 17.2 | 17.3 | 17.0 | 17.7 | 16.9 | 17.3 | 17.4 | — | — | — | — |

[1] Expressed as millimole units as defined by Bessey ('46).

[2] Based on dry, fat-free bone.

[3] The activity measured depends on the action of both phosphoglyceromutase and enolase. Activity expressed in terms of micrograms of iodine labile phosphorus released per 15 minutes per gram of tissue.

**TABLE 3**

*Effect of supplementary magnesium and fluoride in a semipurified diet*

| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Supplemental F, % | — | 0.05 | 0.08 | 0.11 | — | 0.05 | 0.08 | 0.11 |
| Supplemental Mg, % | — | — | — | — | 0.25 | 0.25 | 0.25 | 0.25 |
| Avg weight, gm | 250 | 226 | 162 | 131 | 250 | 205 | 172 | 141 |
| Alkaline phosphatase[1] | 65 | 136 | 142 | 139 | 66 | 161 | 148 | 176 |
| Bone ash,[2] % | 41.6 | 40.3 | 41.2 | 41.3 | 41.3 | 36.1 | 38.3 | 30.1 |
| Leg weakness,[3] %, 1-week | 2.6 | — | — | — | — | 43.6 | 61.5 | 55.0 |
| Leg weakness, %, 2-week | — | 2.5 | — | — | — | 10.3 | 10.5 | 24.3 |
| Enolase activity[4] | 17.5 | 16.1 | 17.0 | 15.0 | 17.9 | 16.4 | 15.4 | 15.2 |

[1] Expressed as millimole units as defined by Bessey ('46).

[2] Based on dry, fat-free bone.

[3] Percentage of birds showing severe leg weakness. Only those birds which were unable to stand longer than a few seconds were counted as having leg weakness.

[4] See table 2.

TABLE 4

*Effect of high levels of magnesium on the magnesium-fluoride interaction*

| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Supplemental F, % | — | — | — | — | 0.08 | 0.08 | 0.08 | 0.08 |
| Supplemental Mg, % | — | 0.10 | 0.50 | 0.90 | — | 0.10 | 0.50 | 0.90 |
| Avg wt, gm, 1-week | 113 | 106 | 107 | 84 | 89 | 88 | 67 | 67 |
| Avg wt, gm, 2-week | 238 | 235 | 221 | 165 | 161 | 177 | 120 | 94 |
| Bone ash,[1] %, 1-week | 37.9 | 39.1 | 36.0 | 29.8 | 38.6 | 37.0 | 27.5 | 20.1 |
| Bone ash, %, 2-week | 40.3 | 41.9 | 38.8 | 29.3 | 40.8 | 40.2 | 30.9 | 19.9 |
| Leg weakness,[2] 1-week | — | — | — | 10 | — | — | 65 | 92 |
| Leg weakness, 2-week | — | — | — | 10 | — | — | 36 | 95 |

[1] Based on dry, fat-free bone.
[2] See table 3.

the following minimal amounts per 100 gm of diet: choline chloride, 200 mg; vitamin A, 1,000 IU; vitamin $B_{12}$, 0.66 µg; procaine penicillin, 0.44 mg; riboflavin, 0.89 mg; Ca pantothenate, 1.77 mg; niacin, 3.96 mg; vitamin $D_3$, 150 ICU; vitamin E acetate, 0.56 IU, menadione sodium bisulfite, 0.07 mg; inositol, 24 mg; pyridoxine·HCl, 1.34 mg; thiamine·HCl, 0.79; folic acid, 0.37 mg; biotin, 0.026 mg. The mineral mix contributed the following levels of minerals to the diet (mg/100 gm): $Ca_3(PO_4)_2$, 2,100; $K_2HPO_4$, 850; $CaCO_3$, 650; NaCl, 500; $FeSO_4·7H_2O$, 80; $MnSO_4·H_2O$, 50; $ZnCO_3$, 15; $(MgCO_3)_4$ Mg $(OH)_2·4H_2O$, 160; $CuSO_4·5H_2O$, 2; $Na_2MoO_4·2H_2O$, 0.25; NaI, 0.12; Co $Cl_2·H_2O$, 0.10.

The basal diet was supplemented with three levels of magnesium and 4 levels of fluoride as indicated in table 3. Treatment began at one day of age and 5 birds from one replicate of each treatment were killed two weeks later. Plasma and bone samples were collected and analyzed as described earlier. The incidence of leg weakness was noted, counting only those birds that were severely affected.

Experiment 4 was conducted to study the effect of varying the magnesium content of a semipurified diet over a greater range than had been studied previously. The basal diet was the same as in experiment 3 with the following exceptions: the mineral mix supplied 250 mg of NaCl/100 gm of diet and constituted 4.17% of the diet; sodium carbonate was used to maintain a constant sodium level in all diets. Sodium fluoride and magnesium carbonate were added to the basal diet as indicated in table 4. The treatments were started at one day of age and 5 birds from one repli-

cate of each treatment were killed to obtain plasma and bone samples at one week of age. Five birds from another replicate of each treatment were killed at two weeks of age.

## RESULTS AND DISCUSSION

The results of experiment 1 are summarized in table 1. Analysis of variance indicated a significant ($P < 0.01$) depression in growth as the result of increased dietary fluoride. Although there was an effect due to magnesium, the addition of magnesium depressed growth significantly only in the presence of fluoride, which was reflected in a significant magnesium × fluoride interaction ($P < 0.01$). The main effect of calcium was not significant, but a significant calcium × fluoride interaction ($P < 0.01$) was obtained. A comparison of means showed that the higher level of calcium reduced growth in the absence of supplemental fluoride, and increased growth in the presence of supplemental fluoride. The magnesium × calcium and magnesium × fluoride × calcium interactions were not significant.

Alkaline phosphatase activity of blood plasma was increased ($P < 0.01$) by the addition of fluoride to the diet and decreased with age ($P < 0.01$) but did not reflect the magnesium × fluoride interaction that was observed in growth. The ash content of the bones was markedly reduced when the diets were supplemented with both magnesium and fluoride, but when added alone neither element significantly affected bone ash. A significant interaction ($P < 0.01$) of magnesium × fluoride on bone ash was observed. It also appears that added magnesium depressed the deposition of fluoride in the bone tissue

but these data could not be treated statistically because the individual bone samples from the various treatments were composited for fluoride analysis.

The results of experiment 2 (table 2) showed that the addition of excess fluoride alone reduced growth in chicks placed on the experimental diets at either one day of age or two weeks of age. However, the additional reduction in growth, the reduction in bone ash, and the leg weakness syndrome characteristic of high levels of dietary magnesium and fluoride were observed only when one-day-old birds were given the experimental diets; the magnesium × fluoride interaction could not be demonstrated in chicks between two and six weeks of age. Hence, this effect is age dependent and occurs during the period of rapid bone calcification.

As in experiment 1, alkaline phosphatase activity increased with the addition of fluoride to the diet and decreased with age. Activity was also increased on both levels of fluoride by the addition of magnesium to the diets of birds that were placed on treatment at one day of age ($P < 0.05$). Although the magnesium × fluoride interaction was nonsignificant, the increased phosphatase activity due to supplemental magnesium was greater with the high fluoride diet than with the low fluoride diet. This effect of magnesium on plasma alkaline phosphatase activity was not observed in birds that were placed on treatment at two weeks of age.

The only effect on bone ash in the second experiment was a reduction by the addition of both magnesium and fluoride to the diets of the birds that were killed at two weeks. The bone fluoride values followed the same pattern as in experiment 1, again suggesting that magnesium in the diet somehow decreases the deposition of fluoride in bone tissue. The conversion of 3-phosphoglycerate to phosphoenolpyruvate by muscle homogenates was not affected by the various treatments. This indicates that the harmful effect of excess magnesium and fluoride is not mediated by the inhibition of phosphoglyceric mutase or enolase, the enzymes which catalyze this conversion.

The weight data from experiment 3 (table 3), with a semipurified diet, closely followed the pattern established in experiments 1 and 2 showing a significant magnesium × fluoride interaction ($P < 0.01$). The addition of either magnesium or fluoride caused an increase in alkaline phosphatase activity ($P < 0.01$), but the magnesium × fluoride interaction was not significant. However the increased phosphatase activity caused by dietary magnesium appeared to be limited to the highest level of supplemental magnesium (treatments 9–12) and the greatest phosphatase activity was obtained with the highest level of magnesium with supplemental fluoride. The addition of fluoride to the diet increased alkaline phosphatase activity at the lowest level (0.05%) and higher levels of supplemental fluoride caused no further changes.

The bone ash data were in agreement with those in experiment 1, in that the addition of both magnesium and fluoride to the diet reduced bone ash. Magnesium alone at the highest level (0.50%) decreased bone ash slightly whereas fluoride alone had no effect.

The characteristic leg weakness, which occurred almost exclusively in the treatments supplemented with both magnesium and fluoride, followed the same pattern as described by Gardiner et al. ('61). A further observation was the occurrence of watery droppings by the birds on fluoride supplemented diets. This symptom, which became more severe with increased dietary fluoride, was attenuated noticeably by increasing the magnesium level of the diet. The conversion of 3-phosphoglycerate to phosphoenolpyruvate by muscle homogenates was not significantly affected by the various treatments.

The results of experiment 4 are presented in table 4. The one-week and two-week weight as well as the bone ash data confirm the results of previous experiments by showing a significant magnesium × fluoride interaction ($P < 0.01$) — becoming more pronounced as dietary magnesium approaches the toxic level. The depression in growth and bone ash with the highest level of magnesium (0.90%) without supplemental fluoride was apparently due to magnesium toxicity. As in experiment 3, there was a slight reduction in bone ash on the 0.50% level of

magnesium without fluoride (treatment 3), whereas fluoride alone did not influence bone ash. The incidence of leg weakness, as in experiment 3, was largely limited to the high magnesium-high fluoride diets.

Attempts at this station to demonstrate a magnesium × fluoride interaction in weanling rats under conditions similar to those described above were unsuccessful. The results with rats agree with those reported by Schuck ('38) and also agree with the data on chicks fed experimental diets at two weeks of age. Since weanling rats were three weeks of age when placed on experiment, the inability to show the interaction with rats may not be due to a species difference, but may be a reflection of the age relationship in the production of the syndrome. The influence of age on the magnesium × fluoride interaction was also demonstrated in the decreased incidence or severity of the leg weakness of birds with age although no changes were made in the diets. As mentioned previously, this magnesium × fluoride relationship apparently is manifested during the period of rapid bone calcification, which suggests that the effect on growth might be mediated entirely through the influence on calcification; however in experiment 4, bone ash on the highest level of magnesium alone (treatment 4) was lower than on the next lower level of magnesium plus fluoride (treatment 7), yet growth and leg weakness were more severely affected with the combination of elements. From these observations, it appears that the syndrome observed in the chicks maintained with high levels of dietary magnesium and fluoride is not solely a reflection of reduced bone calcification; other factors such as muscular weakness brought about by impaired energy metabolism may be involved.

## SUMMARY

The addition of high levels of both magnesium and fluoride to either practical or semipurified diets complete in all known nutrients produced the following results in young chicks: (1) greater reduction in growth than with either magnesium or fluoride alone; (2) the development of a characteristic leg weakness; (3) decreased calcification of the bone. Increasing the calcium level in the diets from 1.0 to 1.5% reduced but did not prevent the magnesium-fluoride relationship. The growth reduction, leg weakness and reduced bone ash caused by high dietary magnesium and fluoride were obtained only when one-day-old chicks were fed the experimental diets; these results were not observed when chicks at two weeks of age were fed the same diets, nor could the interaction be shown in weanling rats. Plasma alkaline phosphatase activity in chicks was elevated about twofold by added fluoride regardless of the dietary magnesium or calcium levels. The level of alkaline phosphatase in the plasma decreased with age in all groups at approximately the same rate. Dietary magnesium or fluoride levels did not influence the conversion of 3-phosphoglycerate to phosphoenolpyruvate by muscle homogenates.

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# Utilization of a Delayed Lysine Supplement by Young Rats[1]

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Although some vitamins administered once a day separate from the diet of young rats appear to be utilized as well as those incorporated in the diet (Sarett and Morrison, '60), little is known of the utilization of the amino acids given apart from the diet. Geiger ('47) showed that the amino acids, administered apart from the deficient diets, were not efficiently utilized by the young rats. Recently, Yang et al. ('61) found that a lysine supplement administered once a day to young rats apart from a lysine-deficient diet appeared to be as well utilized as when incorporated in the diet, but the experimental procedures were different from those used by Geiger. The lysine supplement was administered by stomach tube in single daily doses (Yang et al., '61), whereas in Geiger's studies, the supplemental amino acid was incorporated in an otherwise adequate but protein-free basal diet, alternating with the deficient diet at 12-hour intervals.

Therefore, experiments were conducted to compare the two methods of administering lysine supplement to determine whether a true difference exists in the results obtained, and also to elucidate the causes for the dissimilar results obtained by Geiger ('47) and Yang et al. ('61).

## EXPERIMENTAL PROCEDURES

Male weanling rats of the Sprague-Dawley strain were individually housed in suspended wide-mesh cages in an air-conditioned room maintained at 24°C and 40% of relative humidity. During a 4-week experimental period, deionized water was given ad libitum to all the animals, and the food allowances among groups were varied.

Three experiments comprised this investigation. Experiment A was conducted to repeat some of Geiger's studies on the utilization of the delayed amino acid supplement incorporated in an otherwise adequate but protein-free basal diet (Geiger, '47). Groups of 6 rats were fed ad libitum the following diets: group 1, diet 1 containing zein supplemented with both L-lysine monohydrochloride (LMH) and L-tryptophan; group 2, diet 2 containing zein without any amino acid supplement; and group 3, diet 3 containing zein supplemented with L-tryptophan and diet 4 consisting of the protein-free basal diet supplemented with LMH, alternating at 12-hour intervals. The composition of diets 1, 2, 3 and 4 (table 1) was patterned, respec-

TABLE 1

*Composition of the diets used in experiment A*

|  | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|
|  | % | % | % | % |
| Basal diet[1] | 81.3 | 70 | 68 | 94 |
| Zein | 17.0 | 30 | 30 | — |
| L-Tryptophan | 0.5 | — | 2 | — |
| L-Lysine·HCl | 1.2 | — | — | 6 |

[1] With the following percentage composition: cornstarch, 87.47; cottonseed oil, 5.13; salt mixture, USP XIV, 5.13; cod liver oil (E. R. Squibb and Sons, New York, each gram contained 1,700 and 170 USP units of vitamins A and D, respectively), 1.27; and cornstarch-vitamin mixture (similar to that used by Yang et al., '59, except that oleum percomorphum was omitted), 1.00.

tively, after that of diets 4, 5, 8 and 7 used by Geiger, except that the rice bran concentrate which was no longer available was replaced by a mixture of vitamins.

Experiment B was conducted to compare the utilization of the lysine supplement given by stomach tube in single daily doses

with that incorporated in an otherwise adequate but protein-free basal diet. Groups of 6 rats were fed for 12 hours daily (10 PM to 10 AM) the following diets: groups 1, 3 and 4, diet 6 (tables 2 and 3) containing wheat gluten and a mixture of amino acids at levels similar to those used by Gupta et al. ('58); and group 2, diet 7 consisting of diet 6 supplemented with 0.5% of LMH, which supported satisfactory growth of young rats (Calhoun et al., '60). For the next 12 hours (10 AM to 10 PM), they were fed the following diets: groups 1 and 2, diet 5 consisting of the protein-free basal diet only; group 3, diet 8 consisting of diet 5 supplemented with LMH at the 5% level;[4] and group 4, diet 5 and a single daily dose of LMH given by stomach tube at 4 PM.

The food allowances among groups were equalized. The amount of food given to groups 2–4 was restricted to the average amount of food consumed on the previous day by group 1.

The amount of LMH solution given by stomach tube to the animals in group 4 was adjusted daily so that it was equivalent to the amount of the supplemental lysine consumed by the animals in group 2 receiving the lysine supplement incorporated in diet 7. The animals receiving no LMH solution (groups 1, 2 and 3) were given an equivalent amount of deionized water by stomach tube to equalize the effect of handling.

Experiment C was conducted to determine the explanation for the difference between the authors' findings and those of Geiger. Groups of 5 rats were fed for 12 hours daily the following diets: groups 1, 3, and 4, diet 9 (tables 2 and 4) containing zein and a mixture of amino acids at the levels used by Benton et al. ('55), and group 2, diet 10 consisting of diet 9 supplemented with 0.8% of LMH. For the next 12 hours, they were fed the following diets: groups 1 and 2, diet 5 consisting of the protein-free basal diet only; group 3, diet 11 consisting of diet 5 supplemented with 8% of LMH; and group 4, diet 5 and a single daily dose of LMH given by stomach tube at 4 PM in an amount equivalent to the supplemental lysine in diet 10 that was consumed by group 2. The amount of food given to groups 1, 2 and 4 was restricted to the average amount of the

TABLE 2

*Composition of the protein-free basal diet (diet 5) used in experiments B and C*

|  | % |
|---|---|
| Cornstarch[1] | 88 |
| Cottonseed oil | 5 |
| Salt mixture, USP XIV | 5 |
| Cod liver oil[2] | 1 |
| Cornstarch-vitamin mixture[2] | 1 |

[1] Dietary proteins and amino acid supplements were added at the expense of cornstarch.
[2] See footnote 1, table 1.

[4] High levels of LMH were added to the protein-free basal diet used in experiments B and C (diets 8 and 11) to simulate the high levels of amino acid supplement used by Geiger ('47).

TABLE 3

*Effects of feeding rats for 4 weeks a diet containing wheat gluten and an amino acid mixture with delayed administration of lysine supplement (experiment B)*

| Group no.[1] | Lysine supplement | Food intake | | Weight gain[2] | Protein efficiency[2,3] | Biological value[2,4] |
|---|---|---|---|---|---|---|
| | | 10 PM to 10 AM | 10 AM to 10 PM | | | |
| | | gm | gm | gm | | % |
| 1 | None | 113(6)[5] | 77(5) | 8 | 0.32 | 21.7 |
| 2 | Mixed in gluten diet | 114(7) | 84(5) | 27 | 1.23 | 43.9 |
| 3 | Mixed in basal diet | 114(6) | 83(8) | 33 | 1.31 | 42.9 |
| 4 | By stomach tube at 4 PM | 113(6) | 79(5) | 21 | 0.99 | 38.2 |

[1] Six rats/group.
[2] Combination of two diets.
[3] Weight gain in grams/gram of protein eaten.
[4] (Nitrogen in food — nitrogen in urine — nitrogen in feces) × 100

$$\frac{\text{(Nitrogen in food — nitrogen in urine — nitrogen in feces)} \times 100}{\text{Nitrogen in food — nitrogen in feces}}$$

[5] Figures within parentheses indicate the numbers of the following diets: diet 5 consisting of the protein-free basal diet only (table 2); diet 6 containing 20% wheat gluten, 0.2% L-histidine·HCl, 0.2% DL-methionine, 0.2% DL-threonine, and 0.05% L-tryptophan, which provided 18% protein (N × 6.25) and 0.27% L-lysine; diet 7 consisting of diet 6 supplemented with 0.5% L-lysine monohydrochloride (LMH); and diet 8 consisting of the protein-free basal diet (diet 5) supplemented with 5% LMH.

TABLE 4

*Effects of feeding rats for 4 weeks a diet containing zein and an amino acid mixture with delayed administration of lysine supplement (experiment C)*

| Group no.[1] | Lysine supplement | Food intake | | Weight gain[2] | Protein efficiency[2,3] | Biological value[2,4] |
|---|---|---|---|---|---|---|
| | | 10 PM to 10 AM | 10 AM to 10 PM | | | |
| | | gm | gm | gm | | % |
| 1 | None | 49(9)[5] | 93(5) | −13 | −0.88 | 53.6 |
| 2 | Mixed in zein diet | 138(10) | 106(5) | 42 | 1.04 | 55.0 |
| 3 | Mixed in basal diet | 136(9) | 101(11) | 37 | 0.90 | 46.6 |
| 4 | By stomach tube at 4 PM | 136(9) | 85(5) | 27 | 0.66 | 48.0 |

[1] Five rats/group.
[2] Combination of two diets.
[3] Weight gain in grams/gram of protein eaten.
[4] See footnote 4, table 3.
[5] Figures within parentheses indicate the numbers of the following diets: diet 5 consisting of the protein-free basal diet only (table 2); diet 9 containing 24% zein, 0.2% L-tryptophan, 0.4% DL-methionine, 0.4% L-histidine·HCl, 1.2% DL-threonine, 1.6% DL-valine, 1.0% DL-isoleucine and 0.2% L-arginine·HCl, which provide 27.5% protein (N × 6.25) and 0.029% L-lysine; diet 10 consisting of diet 9 supplemented with 0.8% L-lysine monohydrochloride (LMH); and diet 11 consisting of the protein-free basal diet (diet 5) supplemented with 8% LMH.

food consumed on the previous day by group 3.

Food intakes and body weights were recorded for all rats. Measurement of the biological value and nitrogen and lysine content was conducted according to the procedures previously described (Yang et al., '59, '61). Data obtained were subjected to analysis of variance (Snedecor, '56) and Duncan's multiple range test (Duncan, '55), using 1% ($P < 0.01$) as the significant level.

RESULTS AND DISCUSSION

As shown in table 5, the animals in group 1 fed diet 1 containing zein supplemented with both LMH and L-tryptophan grew suboptimally; the animals (group 2) given diet 2 containing zein without any amino acid supplement lost weight. The animals in group 3, fed diet 3 containing zein supplemented with L-tryptophan and diet 4 consisting of the protein-free basal diet supplemented with LMH at 12-hour

intervals, also lost weight. These results are in excellent agreement with the observations of Geiger ('47), although the rice bran concentrate used by Geiger was replaced by a mixture of vitamins.

The animals in group 3 consumed only 13 gm of diet 3 during the 4-week feeding period, whereas those in group 1 consumed 213 gm of diet 1, and in group 2, 132 gm of diet 2. The low intake of diet 3 may be owing, in part, to the amino acid imbalance induced by the excessive amount of L-tryptophan (2%). Since the animals in group 3 consumed significantly less protein ($P < 0.01$) than the animals in groups 1 or 2, it appears that the weight loss of group 3 was due to the feeding of an imbalanced diet rather than the delayed lysine supplement. Data obtained in experiment C (table 4) appear to support this hypothesis.

Geiger ('47) had already pointed out that a normal rate of growth of young rats cannot be obtained with a zein diet supple-

TABLE 5

*Effects of feeding rats for 4 weeks a diet containing zein with delayed administration of lysine supplement (experiment A)*

| Group no.[1] | | Weight gain | Food intake | Protein intake |
|---|---|---|---|---|
| | | gm | gm | gm |
| 1 | Basal + zein + lysine + tryptophan | 7 | 213(1)[2] | 36.0 |
| 2 | Basal + zein | −15 | 132(2) | 36.8 |
| 3 | Basal + zein + tryptophan, (10 AM to 10 PM) | −16 | 13(3) | 9.0 |
| | Basal + lysine, (10 PM to 10 AM) | | 102(4) | 3.8 |

[1] Six rats/group.
[2] Figures within parentheses indicate the diet numbers shown in table 1.

mented with both lysine and tryptophan, and the reason for such failure had not been determined at that time. Further studies (Geiger et al., '52), however, showed that the threonine and valine in zein were not readily available to the rats. Benton et al. ('55) also found that the young rats receiving the zein diet had an abnormally high requirement for isoleucine and the young rats grew normally when fed a diet containing 24% of zein supplemented with a mixture of 8 essential amino acids.

Food intakes and growth data of experiment B, comparing the utilization of the lysine supplement given by stomach tube with that incorporated in a protein-free basal diet, are summarized in table 3. The mean value of weight gains, protein efficiencies and biological values for the rats in group 1 receiving the lysine-deficient gluten diet (diet 6) were significantly lower ($P < 0.01$) than the comparable values obtained in groups 2–4 receiving lysine supplements administered by various methods. The mean weight gain and protein efficiency of the rats in group 3 receiving the lysine supplement incorporated in the protein-free basal diet (diet 8) were not significantly different ($P > 0.05$) from the values obtained in group 2 receiving the lysine supplement incorporated in the diet containing wheat gluten (diet 7), but were greater ($P < 0.01$) than the values of the animals in group 4 receiving the supplemental lysine given by stomach tube. There were no significant differences among the biological values of the rats in groups 2–4. Somewhat similar results were also obtained when the young rats were fed a diet containing 10% of wheat flour proteins, the same diet supplemented with 0.25% of LMH, or the same flour diet with the supplemental lysine either given by stomach tube or incorporated in the protein-free basal diet (2.5% LMH).[5]

Data obtained in experiment B showed that the delayed supplementation of lysine, that is, the lysine supplement incorporated in a protein-free basal diet or that given by stomach tube, was utilized by the young rats. These results are not in agreement with those of Geiger ('47), who found that the delayed supplementation of lysine was ineffective; it did not promote the growth of the rats when fed with a lysine-deficient diet at 12 hour intervals. But, in Geiger's experiment (table 5), zein was used as the dietary protein, whereas in the present studies, wheat gluten was used. Before drawing further conclusions on the utilization of the delayed lysine supplement, it seemed necessary to determine whether the poor growth obtained in Geiger's studies was due to the feeding of an imbalanced diet (experiment C).

As shown in table 4, when the amount of food given to groups 1, 2 and 4 during the 4-week feeding period was adjusted to that consumed by group 3, the animals in group 1 consumed only 49 gm of diet 9 containing zein and a mixture of 7 amino acids. This may be owing, in part, to the amino acid imbalance. The weight gain and protein efficiency obtained by group 1 resulting from the feeding of diet 9 were significantly lower ($P < 0.01$) than the values obtained by any other dietary group tested. The weight gain and protein efficiency obtained by group 2 receiving the lysine supplement incorporated in the diet containing zein (diet 10) were not significantly different ($P > 0.05$) from the values obtained by group 3 receiving the lysine supplement incorporated in the protein-free basal diet (diet 11), but were greater ($P < 0.01$) than the values obtained by group 4 receiving the lysine supplement given by stomach tube. No significant differences were found in the biological values obtained by group 1–4.

Data obtained in this investigation appear to indicate that young rats can synthesize protein when fed an otherwise adequate diet containing proteins deficient in lysine for 12 hours and then, in the next 12 hours, fed an otherwise adequate but protein-free basal diet and a single daily dose of lysine given by stomach tube, or the same basal diet supplemented with lysine. Although the present observations are not in agreement with Geiger's conclusions on the utilization of the delayed lysine supplement, they do not contradict the concept that for protein synthesis all the essential components have to be pres-

[5] S. P. Yang, unpublished data.

ent simultaneously in the cell. The utilization by young rats of the delayed lysine supplement may be related to the slow turnover rate of lysine in the body or the ability of the animals to re-use some of the lysine obtained from protein breakdown, or both (Bender, '60).

The superiority of the lysine supplement incorporated in the basal diet over that given by stomach tube as shown in tables 3 and 4 may be due to a more efficient utilization by absorption over a longer period of time. Also note that the animals receiving the lysine supplement incorporated in the basal diet consumed more lysine than the animals receiving the supplement by stomach tube.

## SUMMARY

Experiments were conducted to determine the utilization by young rats of the delayed lysine supplement administered by stomach tube or incorporated in an otherwise adequate but protein-free basal diet.

Data obtained indicate that young rats can synthesize protein when fed an otherwise adequate diet containing proteins deficient in lysine, for 12 hours, and then, in the next 12 hours, fed a protein-free basal diet and a single daily dose of lysine given by stomach tube, or the same basal diet supplemented with lysine.

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# ERRATUM

Snyderman, S. E., L. E. Holt, Jr., J. Dancis, E. Roitman, A. Boyer and M. E. Balis 1962 "Unessential" nitrogen: A limiting factor for human growth. J. Nutrition, 78: 57.

An omission occurred in the title of table 6, page 69. The title should have appeared as follows:

TABLE 6

*Plasma amino acid levels (mg/100 ml)*