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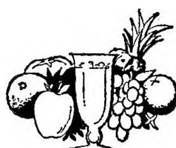

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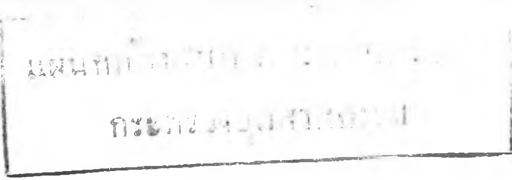

# The Influence of Vitamin $B_{12}$ on the Content, Distribution and in Vivo Synthesis of Thiamine Pyrophosphate, Flavin Adenine Dinucleotide and Pyridine Nucleotides in Rat Liver[1]

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Apart from the well-known interrelationships among the B vitamins, there is reason to believe that folic acid and vitamin $B_{12}$ may influence the functioning of other vitamins as cofactors. Thus, dietary folic acid has been known to determine rat liver stores of coenzyme A (CoA) and adenotriphosphate (ATP) (Popp and Totter, '52; Totter, '53); a decrease in liver DPN is also caused by aminopterin[2] (Strength et al., '54). The *in vivo* incorporation of nitcotinamide into pyridinonucleotides in rat liver is affected in a deficiency of vitamin $B_{12}$ (Nadkarni et al., '57). Low blood level of citrovorum factor in the hyperthyroid, vitamin $B_{12}$-deficient rat is corrected by administration of vitamin $B_{12}$ (Pfander et al., '52). In liver homogenates from vitamin $B_{12}$-deficient hens, the synthesis of citrovorum factor from added folic acid is less than in those from animals injected with the vitamin (Doctor et al., '54). The potentiating effect of vitamin $B_{12}$ in the mobilization of folic acid has also been reported from this laboratory (Sreenivasan, '51; Fatterpaker et al., '55a). The general influence of vitamin $B_{12}$ on carbohydrate and lipid metabolism has been linked to a primary relation to sulphydryl biosynthesis (Ling and Chow, '54; Register, '54; Kasbekar et al., '56, '59a). Distinguished from these apparently collateral findings is the reported elevation of CoA in livers of vitamin $B_{12}$-deficient rats and mice (Boxer et al., '53, '55; Wong and Schweigert, '56).

The present work relates to a study of the influence of vitamin $B_{12}$ on the intracellular distribution of thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD) and pyridine nucleotides (PN), and to their *in vivo* synthesis from the corresponding administered vitamins, in the rat liver. Data on the distribution of these cofactors in liver cells of the normal rat are available in the works of Goethart ('52) and Dianzani and Dianzani Mor ('57) on TPP, of Carruthers and Suntzeff ('54) and Dianzani ('55) on pyridine nucleotides (PN) and of Schneider and Hogeboom (Schneider, '56) on FAD.

## EXPERIMENTAL

Young, male Wistar rats weighing approximately 100 gm each were used. The animals, housed individually in raised mesh-bottom cages, were initially depleted of their vitamin $B_{12}$ reserves by maintenance on a purified, iodo-casein ration. This consisted of the following percentage composition: hot, alcohol-extracted casein, 18; iodinated casein,[3] 0.15; arachis oil, 6; shark liver oil, 2; sucrose, 9.85; maize starch 60; and salt mixture (U.S.P. XIV), 4; with vitamins to provide in milligrams per kilogram of diet: thiamine·HCl, 6; riboflavin, 10; nicotinic acid, 30; calcium pantothenate, 20; pyridoxine·HCl 6; biotin, 1; folic acid, 5; p-aminobenzoic acid, 100; choline·Cl, 500; inositol, 500; 2-methyl-1, 4-naphthoquinone, 10; and α-tocopherol,

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[1] This work was supported by a research grant from the Williams-Waterman Fund, Research Corporation, New York.

[2] Strength, D. R., and N. I. Mondy 1953 Choline dehydrogenase activity and DPN content of rat livers following aminopterin injection. Federation Proc., *12:* 276 (abstract).

[3] Protomone, obtained from Cerophyl Laboratories, Kansas City, Mo.

50. The vitamin additions provided in this basal diet were considered adequate for the hyperthyroid condition. At the end of 4 weeks, animals were divided into two groups, one of which continued to receive the basal diet modified by the omission of iodinated casein and *p*-aminobenzoic acid and substitution by 2% of succinyl sulphathiazole. The latter addition was compensated for by adjusting the percentage of starch. The second, control group, received the modified diet with a supplement of vitamin $B_{12}$ (150 μg/kg of diet). After a further 5-week period, the animals in both groups were divided into 4 sub-groups of 7 to 8 rats each, one of which was killed immediately to establish the liver distribution pattern of the cofactors in the vitamin $B_{12}$-deficient and replete states. The remaining three sub-groups of each group were used to study the synthesis of each of the three cofactors from the respective, administered vitamins. The rats were injected, intraperitoneally, on 5 successive days, with 5 mg of thiamine·HCl, 5 mg of riboflavin, or 1 mg of nicotinamide per rat per day. All animals were killed on the 5th day, 8 hours following the final injection of the test-vitamin.

The normal intracellular distribution pattern of the cofactors was secured on liver of 10 adult male rats weighing approximately 200 gm, maintained on the laboratory stock diet consisting of (gm per 100 gm of diet): whole wheat flour, 75; whole milk powder, 2; casein, 12; dried yeast, 2; arachis oil, 3; shark liver oil, 2; sodium chloride, 2; and calcium carbonate, 2.

Animals were exsanguinated and livers were perfused with ice-cold 0.25 M sucrose, promptly excised, blotted and made into 10% homogenates with 0.25 M sucrose in a Potter-Elvehjem homogenizer. The homogenates were separated by the differential centrifugation procedure of Schneider and Hogeboom ('50) into nuclear, mitochondrial and microsomal plus supernatant fractions using an International (PR-2) refrigerated centrifuge. Total and free thiamine in portions of whole homogenates and fractions were determined by a modification of the fluorometric method of Hennessy ('41) before and after hydrolysis of TPP complexes with taka-diastase for 18 hours at 37°C, the TPP content being calculated by difference. The total and non-FAD (flavin mono nucleotide, FMN plus free riboflavin) riboflavin were determined by the fluorometric procedure of Bessey et al. ('49) and the FAD content derived from these values with use of proper conversion factor, as indicated by these authors. The determination of total PN in homogenates and in fractions, as well as the differential determination of their oxidized and reduced forms in whole homogenates, was carried out fluorometrically by the procedure outlined by Dianzani ('55).

For determinations of blood erythrocyte count and hemoglobin content and of plasma vitamin $B_{12}$ concentration, the animals were bled from tail veins and adequate samples collected in heparinated vials. The erythrocyte count was made by the standard method. Hemoglobin was determined by acid-hematin method in a Klett-Summerson photoelectric colorimeter (Kolmer et al., '51). Plasma vitamin $B_{12}$ was determined by the method of Ross ('52) using *Euglena gracilis* as the test organism.

Portions of liver homogenates were incubated at 37°C for 12 hours under toluene with papain (25 mg/gm of fresh liver) to liberate the bound vitamin $B_{12}$, which was assayed using *E. gracilis* according to the method of Hoff-Jorgenson ('54).

The results were analyzed for statistical significance by calculating the t value of Fisher (Fisher and Yates, '53). Only the differences with a t value corresponding to a probability $P < 0.05$ were accepted as significant.

## RESULTS

*Content and distribution of cofactors and free vitamins in normal liver cytoplasm.* The data obtained with liver homogenates of normal, stock diet-fed animals are reported in table 1. About 30% of the TPP of the homogenate was found contained in mitochondria, while the rest was present almost exclusively in the supernatant. The distribution pattern compares with data reported by others (Goethart, '52; Dianzani and Dianzani Mor, '57) under similar conditions. Free thiamine, also maximally localized in the supernatant

TABLE 1

*Content and distribution of cofactors and free vitamins in cytoplasm fractions of normal rat liver. Mean values from 10 independent determinations*

| Cytoplasm fraction | TPP | | Free thiamine | | FAD | | FMN + free riboflavin | | Total PN | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $\mu g/gm$[1] | % | $\mu g/gm$ | % | $\mu g/gm$ | % | $\mu g/gm$ | % | $\mu g/gm$ | % |
| Homogenate | 17.20 ± 1.31[2] | | 4.91 ± 0.34 | | 24.80 ± 1.40 | | 8.56 ± 0.61 | | 845 ± 25 | |
| Nuclear | 0.69 ± 0.13 | 4.3 ± 0.9 | 1.01 ± 0.09 | 22.9 ± 2.1 | 4.26 ± 0.81 | 17.7 ± 2.5 | 1.80 ± 0.21 | 21.9 ± 2.3 | 184 ± 16 | 24.5 ± 2.1 |
| Mitochondrial | 5.01 ± 0.92 | 31.4 ± 4.9 | 0.51 ± 0.06 | 11.6 ± 1.4 | 13.94 ± 1.13 | 58.2 ± 4.7 | 1.80 ± 0.19 | 21.9 ± 2.3 | 121 ± 11 | 16.2 ± 1.2 |
| Supernatant | 10.26 ± 1.11 | 64.3 ± 5.6 | 2.89 ± 0.13 | 65.5 ± 4.3 | 5.75 ± 0.61 | 24.0 ± 3.1 | 4.63 ± 0.88 | 56.2 ± 4.3 | 442 ± 18 | 59.2 ± 2.9 |
| Recovery, % | | 92.8 ± 2.6 | | 89.8 ± 1.8 | | 96.6 ± 3.4 | | 96.3 ± 3.1 | | 88.4 ± 3.4 |

[1] Gram weight of fresh tissue.
[2] Standard error of mean.

TABLE 2

*Growth rate and blood and liver values in vitamin $B_{12}$ deficiency*[1]

| Group | Body weight[2] | | Erythrocytes | Hemoglobin | Vitamin $B_{12}$ | |
|---|---|---|---|---|---|---|
| | Initial | Final | | | Plasma | Liver |
| | *gm* | *gm* | $\times 10^6/mm^3$ | *gm/100 ml* | $\mu\mu g/ml$ | $m\mu g/gm$ |
| Vitamin $B_{12}$-deficient | 147 ± 4[3] | 188 ± 4 | 4.0 ± 0.3 | 11.7 ± 0.5 | 87 ± 41 | 29.3 ± 9.2 |
| Vitamin $B_{12}$-fed | 146 ± 3 | 256 ± 5 | 6.9 ± 0.3 | 14.8 ± 0.7 | 834 ± 34 | 102.5 ± 11.3 |
| Normal[4] | — | — | 7.8 ± 0.4 | 15.1 ± 0.4 | 688 ± 29 | 86.4 ± 6.5 |

[1] Results are average values obtained from at least 7 independent determinations in each series.
[2] Initial weight refers to weight at time of grouping after 4 weeks of iodo-casein feeding; final weight is 5 weeks after grouping and maintenance on succinyl sulphathiazole-containing diets.
[3] Mean value ± standard error of mean.
[4] Adult rats of approximately 200 gm maintained on laboratory stock diet.

**TABLE 3**

*Effect of vitamin $B_{12}$ on the content and distribution of TPP and free thiamine in liver cytoplasm and on the incorporation of thiamine administered into these components*[1]

| Cytoplasm fraction | Vitamin $B_{12}$-fed (8 rats) | | | | Vitamin $B_{12}$-deficient (8 rats) | | | | Thiamine-injected | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Vitamin $B_{12}$-fed (7 rats) | | Vitamin $B_{12}$-deficient (9 rats) | |
| | TPP | | Free thiamine | | TPP | | Free thiamine | | TPP | Free thiamine | TPP | Free thiamine |
| | | | | | | | | | Increase over uninjected control | | | |
| | μg/gm | % | μg/gm | % | μg/gm | % | μg/gm | % | % | % | % | % |
| Homogenate | 13.20 ± 1.01[2] | | 4.28 ± 0.13 | | 7.23 ± 0.83 | | 3.97 ± 0.30 | | 78.7 ± 4.1 | 90.8 ± 3.8 | 48.2 ± 2.1 | 94.2 ± 3.9 |
| Nuclear | 0.62 ± 0.02 | 5.2 ± 0.4 | 0.76 ± 0.07 | 19.2 ± 1.6 | 0.56 ± 0.04 | 8.8 ± 0.6 | 0.63 ± 0.02 | 17.9 ± 1.0 | 124.2 ± 5.3 | 64.5 ± 3.0 | 12.5 ± 0.8 | 106.3 ± 8.8 |
| Mitochondrial | 3.49 ± 0.11 | 29.3 ± 2.1 | 0.34 ± 0.01 | 8.6 ± 0.6 | 0.86 ± 0.03 | 13.4 ± 1.2 | 0.33 ± 0.03 | 9.3 ± 0.8 | 100.6 ± 2.9 | 70.6 ± 3.1 | 62.8 ± 2.1 | 112.1 ± 7.3 |
| Supernatant | 7.81 ± 0.81 | 65.5 ± 6.2 | 2.86 ± 0.03 | 72.1 ± 1.8 | 4.99 ± 0.29 | 77.8 ± 5.3 | 2.57 ± 0.11 | 72.8 ± 4.3 | 74.4 ± 1.8 | 109.8 ± 4.3 | 51.4 ± 3.3 | 86.8 ± 2.3 |
| Recovery, % | | 90.3 ± 3.3 | | 92.6 ± 2.7 | | 88.7 ± 3.1 | | 88.9 ± 2.0 | 93.4 ± 2.9 | 95.8 ± 1.1 | 89.6 ± 2.0 | 88.2 ± 3.0 |

[1] Animals injected intraperitoneally with 5 mg of thiamine·HCl/rat/day for 5 consecutive days and killed 8 hours following final injection on the 5th day.

[2] Standard error of mean.

TABLE 4

*Effect of vitamin $B_{12}$ on the content and distribution of FAD and FMN + free riboflavin in liver cytoplasm and on the incorporation of riboflavin[1] administered into these components*

| Cytoplasm fraction | Vitamin $B_{12}$-fed (8 rats) | | | | Vitamin $B_{12}$-deficient (8 rats) | | | | Riboflavin injected | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Vitamin $B_{12}$-fed (7 rats) | | Vitamin $B_{12}$-deficient (9 rats) | |
| | FAD | | FMN + free riboflavin | | FAD | | FMN + free riboflavin | | FAD | FMN + free riboflavin | FAD | FMN + free riboflavin |
| | | | | | | | | | *Increase over uninjected control* | | | |
| | *µg/gm* | % | *µg/gm* | % | *µg/gm* | % | *µg/gm* | % | % | % | % | % |
| Homogenate | 24.50 ± 1.61[2] | | 10.13 ± 0.42 | | 12.79 ± 1.83 | | 12.48 ± 1.32 | | 67.6 ± 2.6 | 75.1 ± 3.8 | 41.4 ± 2.9 | 68.9 ± 3.1 |
| Nuclear | 4.36 ± 0.33 | 19.5 ± 3.6 | 1.65 ± 0.01 | 16.9 ± 0.9 | 3.37 ± 0.03 | 29.4 ± 1.2 | 2.55 ± 0.04 | 23.3 ± 1.2 | 92.2 ± 3.7 | 38.8 ± 1.6 | 54.3 ± 2.2 | 27.8 ± 1.5 |
| Mitochondrial | 13.69 ± 0.58 | 61.3 ± 2.4 | 2.43 ± 0.02 | 24.8 ± 1.2 | 4.63 ± 0.05 | 40.4 ± 1.8 | 1.74 ± 0.04 | 15.9 ± 1.3 | 54.9 ± 1.4 | 53.1 ± 2.7 | 20.7 ± 2.1 | 70.7 ± 4.6 |
| Supernatant | 4.29 ± 0.17 | 19.2 ± 2.9 | 5.70 ± 0.32 | 58.3 ± 2.4 | 3.45 ± 0.05 | 30.1 ± 1.6 | 6.65 ± 0.21 | 60.8 ± 1.9 | 82.3 ± 2.9 | 75.6 ± 3.7 | 52.5 ± 3.9 | 84.2 ± 2.1 |
| Recovery, % | | 91.2 ± 2.7 | | 96.5 ± 1.3 | | 89.5 ± 3.4 | | 87.6 ± 3.1 | 91.1 ± 2.1 | 90.3 ± 3.9 | 88.8 ± 3.8 | 87.6 ± 3.9 |

[1] As for thiamine (table 3), with injections of 1 mg of riboflavin/rat/day.
[2] Standard error of mean.

TABLE 5

*Effect of vitamin $B_{12}$ on the content and distribution of total PN in liver cytoplasm and on its synthesis from administered nicotinamide[1]*

| Cytoplasm fraction | Vitamin $B_{12}$-fed (8 rats) | | Vitamin $B_{12}$-deficient (8 rats) | | Nicotinamide-injected | |
|---|---|---|---|---|---|---|
| | | | | | Vitamin $B_{12}$-fed (7 rats) | Vitamin $B_{12}$-deficient (9 rats) |
| | | | | | *increase over uninjected control* | |
| | *µg/gm* | % | *µg/gm* | % | % | % |
| Homogenate | 641 ± 23[2] | | 392 ± 22 | | 65.2 ± 3.9 | 43.8 ± 3.1 |
| Nuclear | 115 ± 11 | 20.1 ± 1.8 | 89 ± 5 | 26.2 ± 1.9 | 101.7 ± 4.2 | 13.5 ± 1.2 |
| Mitochondrial | 100 ± 8 | 17.4 ± 1.8 | 25 ± 4 | 7.6 ± 0.5 | 50.0 ± 3.8 | 60.0 ± 2.9 |
| Supernatant | 357 ± 11 | 62.5 ± 4.0 | 224 ± 13 | 66.2 ± 2.7 | 57.1 ± 3.1 | 56.2 ± 1.9 |
| Recovery, % | | 89.2 ± 3.3 | | 86.3 ± 1.8 | 89.0 ± 2.3 | 87.1 ± 2.1 |

[1] As for thiamine (table 3), with injections of 5 mg of nicotinamide/rat/day.
[2] Standard error of mean.

fraction was, however, present in appreciable amounts in the nuclear fraction, its concentration in this fraction even exceeding that of TPP ($P < 0.05$).

The distribution pattern of FAD differed from that of TPP, in its greater association with the mitochondria than with the supernatant fraction. The proportion of FAD found in association with the mitochondria was similar to that reported (about 65%) by Schneider and Hogeboom (Schneider, '56). Although about 60% of the non-FAD riboflavin was contained in the supernatant, the proportion associated with mitochondria was small as compared with the FAD content of this fraction.

A major fraction of the total liver PN was localized in the supernatant, in confirmation of earlier observations (Carruthers and Suntzeff, '54; Dianzani, '55). A significant proportion was, however, contained in the nuclear fraction as well.

*Alterations in vitamin $B_{12}$ deficiency.* The data on blood erythrocyte count and hemoglobin concentration and especially on plasma and liver content of vitamin $B_{12}$ (table 2), apart from observations on growth noted below, connote the severity of the deficiency attained. During the 5-week period for which the animals were maintained on the succinyl sulphathiazole- containing diets, the vitamin $B_{12}$-deficient ration promoted an average gain in body weight of 8 gm/week compared with an average of 22 gm using the control ration with vitamin $B_{12}$ (table 2). Data on blood erythrocyte count and hemoglobin concentration and especially on plasma and liver content of vitamin $B_{12}$ (table 2) also connote the severity of the deficiency attained. Table 6 records the values obtained for oxidized and reduced forms of PN. The effects of vitamin $B_{12}$ deficiency are summarized as follows: (a) there was a marked reduction in the liver content of the cofactors studied, being about 44% for TPP, 48% for FAD and 64% for total PN; (b) the mitochondria exhibited the largest depletion of the cytoplasm fractions. The reductions were 75, 66 and 75%, respectively, for TPP, FAD and PN. Significant reductions in the concentrations of these cofactors were also apparent in the other two fractions, especially in the supernatant; (c) the content and distribution of free thiamine remained essentially unaltered. With non-FAD riboflavin, there was small but significant elevation in both nuclear ($P < 0.005$) and supernatant ($P < 0.05$) fractions, the total being not significantly altered; and (d) the observed decrease in the proportion of oxidized to reduced forms of pyridine nucleotides (PN/PNH ratio) in livers of vitamin $B_{12}$-deficient animals (table 6) is in accordance with the findings of Nadkarni et al., '57). It is of interest to note that the changes in PNH content were much less pronounced in vitamin $B_{12}$ deficiency.

*Effect of vitamin $B_{12}$ on incorporation of administered vitamins into liver cofactors.* Tables 3 to 5 also include the results of experiments on the conversion by liver enzymes *in vivo* of the administered vitamins into their respective cofactors. Incorporation of thiamine, riboflavin and nicotinamide into their coenzyme forms is considerably less in the vitamin $B_{12}$-defi-

TABLE 6

*Effect of vitamin $B_{12}$ on oxidized and reduced PN in liver homogenates*

| Group | No. of rats | Oxidized pyridine nucleotides (PN) | Reduced pyridine nucleotides (PNH) | PN/PNH |
|---|---|---|---|---|
| | | $\mu g/gm$ | $\mu g/gm$ | |
| Normal | 10 | 639 ± 22[1] | 206 ± 9 | 3.10 ± 0.09 |
| Vitamin $B_{12}$-deficient | 8 | 237 ± 18 | 155 ± 5 | 1.53 ± 0.11 |
| Vitamin $B_{12}$-fed | 8 | 476 ± 23 | 165 ± 6 | 2.88 ± 0.13 |
| Vitamin $B_{12}$-deficient— nicotinamide injected | 7 | 343 ± 26 | 221 ± 7 | 1.55 ± 0.14 |
| Vitamin $B_{12}$-fed nicontinamide injected | 8 | 809 ± 19 | 250 ± 11 | 3.24 ± 0.19 |

[1] Standard error of mean.

cient animals, although the extent of incorporation is appreciable even in this group. A relatively greater proportion of free thiamine and of non-FAD riboflavin was observed in the deficient group. The PN/PNH ratio was not significantly altered as a result of nicotinamide administration, the difference between the deficient and control groups being maintained (table 6).

The decreased ability in vitamin $B_{12}$ deficiency of the liver enzymes for conversion of the administered vitamins into their respective cofactors is reflected in all of the cytoplasmic fractions and is largely seen in the nuclear fraction for TPP and PN and in the mitochondrial fraction for FAD. The deficient animals showed higher gains of free thiamine in the nuclear and mitochondrial fractions, whereas the differences with respect to non-FAD riboflavin were confined to the supernatant fraction.

## DISCUSSION

The present observations demonstrate an impairment in the retention of TPP, FAD and PN in the vitamin $B_{12}$-deficient rat liver, as well as in their biosynthesis from the corresponding vitamins administered. In general, the observed effects point to greater depletion of cofactors from mitochondria than from other fractions. If these changes are not always reflected in liver levels of the free vitamins, it may be because of some losses through excretion; this may also imply a decreased use of the free vitamins for synthesis of coenzymes. Dianzani ('55) and Dianzani and Dianzani Mor ('57) have reported similar modifications in the distribution of TPP and PN in mitochondria from fatty livers caused by choline deficiency or $CCl_4$ poisoning. As discussed by these authors, such changes could result from decreased rates of synthesis of the cofactors or their increased degradation or from both causes. Thus it is possible that the synthesis of TPP through phosphorylation of thiamine and of DPN through the Kornberg reaction is diminished *in vivo* as a consequence of the reduced concentration of ATP in fatty livers; increased decomposition of TPP and DPN may be favored by increased acid phosphatase activity and through pyrophosphorolytic cleavage, respectively. Increased degradation of PN may also occur through partial displacement from the mitochondrial into the supernatant fraction, where DPNase is very active. Since both vitamin $B_{12}$ and choline could exert similar lipotropic effects, it is probable that the same types of causes operate in either deficiency.

A rapid depletion of rat liver and its mitochondrial vitamin $B_{12}$ could result from hyperthyroidism (Kasbekar et al., '59a) and acute carbon tetrachloride poisoning (Kasbekar et al., '59b). These conditions are known to cause morphological damage to mitochondrial structure with consequent displacement of intramitochondrial constituents into the surrounding medium (Dianzani, '54, '55; Dianzani and Dianzani Mor, '57; Maley and Lardy, '55; Kasbekar and Sreenivasan, '56). The protection afforded by prior administration of vitamin $B_{12}$ under these conditions of stress (Fatterpaker et al., '55b; Kasbekar et al., '59a, '59b) could arise from its general lipotropic effect and from its known influence on sulphydryl conservation which, in turn, is essential for maintenance of mitochondrial integrity (Tapley, '56; Hunter et al., '56). It has also been suggested that vitamin $B_{12}$ may be necessary for the synthesis of porphyrin-containing proteins of the cell (O'Dell et al., '55) which, apart from their function as respiratory carriers, are apparently also of importance in determining mitochondrial morphology (Gamble, '57). The observed alterations in mitochondrial cofactors in vitamin $B_{12}$ deficiency may thus have an important though indirect, bearing on this function of the vitamin in the maintenance of mitochondrial organization in so far as the nucleotidation and phosphorylation reactions involved in the synthesis of cofactors are ATP-dependent and any structural damage to mitochondria renders the synthesis of ATP inoperative (Kielley and Kielley, '51; Dianzani, '54).

The effect of vitamin $B_{12}$ also has to be assessed in terms of its known relationship to nucleotide biosynthesis. Although the nature of this relationship is obscure, evidence exists to suggest its involvement in the biosynthesis of both ribose[4] and de-

[4] Ling, C. T., and B. F. Chow 1954 Effect of vitamin $B_{12}$ on ribose formation in erythrocytes. Federation Proc., *13:* 253 (abstract).

oxyribose (Downing and Schweigert, '56; Wong and Schweigert, '57) moieties of nucleic acids.

It is interesting to note that the injection of a vitamin could enhance the liver content of the corresponding cofactor despite dietary adequacy of the vitamin concerned. Kaplan and coworkers ('56) had observed a 10-fold increase in liver PN following administration of nicotinamide into normal mice.

According to Hogeboom and Schneider ('52), the DPN synthesis is localized in the liver cell nucleus. In the vitamin $B_{12}$-deficient rats, the extent of impairment in PN synthesis from administered nicontinamide is more pronounced in the nuclear fraction (table 5). A similar impairment in TPP synthesis from administered thiamine is again better reflected in the nuclear fraction than in the mitochondria and least in the supernatant fraction (table 3); this nuclear impairment is accompanied by an appreciable rise in free thiamine in this fraction, suggesting that TPP, like DPN, may be synthesized in the liver nucleus from thiamine, a process susceptible to vitamin $B_{12}$-deficiency.

On the other hand, the impairment in FAD synthesis from riboflavin administered to the vitamin $B_{12}$-deficient rat (table 4) is better reflected in the mitochondrial fraction. The increase in the proportion of non-FAD riboflavin in the vitamin $B_{12}$-deficient animals, with or without riboflavin administration, is, however, confined mainly to the supernatant fraction. The synthesis of FAD from FMN and ATP is also localized in the supernatant fraction (Schneider, '56).

An increase in the proportion of reduced PN in fatty livers (Dianzani, '55) and in those from vitamin $B_{12}$-deficient rats (Nadkarni et al., '57) has been reported. Such a condition may provoke predomination of fatty acid synthesis as compared with breakdown (Lynen, '54). The decreased content (table 6) of oxidized PN in vitamin $B_{12}$ deficiency (Nadkarni et al., '57) may point to its selective destruction by the DPNase (McIlwain and Rodnight, '49; Zatman et al., '53) contained in mitochondrial and supernatant fractions, as well as to decreased synthesis.

SUMMARY

Rats depleted of their vitamin $B_{12}$ reserves were used to study the effects of dietary vitamin $B_{12}$ on (1) the content and distribution in liver cytoplasm of thiamine pyrophosphate (TPP) and free thiamine, flavin adenine dinucleotide (FAD) and non-FAD riboflavin (FMN + free riboflavin) and total pyridine nucleotides (PN), and (2) the *in vivo* synthesis of these cofactors from the respective vitamins.

In the normal, stock diet-fed animals, about 30 % of total liver TPP was localized in the mitochondria while the rest was found almost exclusively in the supernatant fraction (including microsomes). Free thiamine was distributed between the supernatant (65% ) and the nuclear fraction (23% ). About 60% of total liver FAD content was associated with mitochondria while about 55% of the total non-FAD content was contained in the supernatant fraction, the balance in either case being almost equally distributed between the remaining two fractions. A major portion (60% ) of the total liver PN was localized in the supernatant; the nuclear fraction also contained appreciable amounts (25% ).

The liver content of the cofactors was markedly affected in vitamin $B_{12}$ deficiency with average reductions of 45, 48 and 64% , respectively, in TPP, FAD and PN. The effects were largely confined to the mitochondrial fraction and were not accompanied by corresponding changes in the content of the free vitamins. A decrease in oxidized pyridine nucleotides in liver homogenates with proportional lowering of the ratio of oxidized to reduced pyridine nucleotides (PN/PNH) was observed.

The incorporation of injected thiamine, riboflavin and nicotinamide into the liver coenzymes was impaired in vitamin $B_{12}$ deficiency. The impairment in TPP and PN synthesis was reflected to a greater extent in the nuclear fraction than in other fractions, while that in FAD synthesis was seen to an almost equal extent in all fractions.

In the vitamin $B_{12}$-deficient animal there was also appreciable incorporation of administered vitamins into their cofactors;

the concentration of the free forms of the vitamins was, however, relatively greater.

The results, suggesting an impairment in the biosynthesis of these cofactors in vitamin $B_{12}$ deficiency, are discussed. Conditions arising out of possible damage to mitochondrial integrity, as well as the effects of the vitamin in relation to the mode and site of synthesis of the cofactors, are discussed.

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# Effects of Nicotinic Acid and Related Compounds on Sterol Metabolism in the Chick and Rat[1]

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Biochemical and physiological effects of nicotinic acid (NAc) have been measured periodically ever since its recognition as a vitamin, but relatively little is known of the alterations induced in the body by very high doses. Currently 3 to 6 gm doses of NAc are being fed daily as a therapeutic agent to lower the level of cholesterol in human blood[4] (Parsons and Flinn, '59) NAc appears to be one of the most potent agents known for this purpose (Altschul et al., '55; Parsons et al., '56). Apparently something other than vitamin activity is involved, since comparable doses of nicotinamide (NAm) fail to lower blood cholesterol appreciably in man (Miller et al., '58; Parsons and Flinn, '57).

In the present study high doses of NAc, NAm and certain related compounds were fed to rats and chicks. The measurements made include weight, blood cholesterol; liver fat, cholesterol, and pyridine nucleotides; and fecal excretion of fatty acids, sterols and bile acids.

## METHODS

*Rat experiments.* Male albino rats of the Holtzman strain weighing approximately 100 gm were fed diets containing from 0.1 to 1.0% of NAc, NAm, isonicotinic acid (INA), 1% of benzoic acid or 2% of DL-tryptophan.

Basal diet 1 consisted of the following in per cent: casein, 18; sucrose, 67; lard 10; salts, 4; (Wesson, '32), vitamin mixture,[5] 0.1; choline chloride, 0.1; vitamin and halibut liver oil, 0.09. Consumption of E solution (50 mg α-tocopherol/ml), 0.09; the diet was ad libitum and was recorded during periods of the experiment. Animals were decapitated after a period of from one to 28 days on the experimental diets.

*Chick experiments.* One per cent of NAc, NAm, INA or benzoic acid was added to basal diet 2 containing in per cent: sucrose, 56; alcohol extracted casein, 23; gelatin, 10; Salts V (Briggs et al., '43), 6; soybean oil, 4; feeding oil (300 I.U. of vitamin A, 60 I.U. of vitamin $D_3$, 0.3 mg of vitamin E/100 gm of diet), 1; L-cystine, 0.3; and water-soluble vitamins.[6]

*Blood cholesterol.* Whole blood cholesterol values were obtained for both the chick and the rat by a modified Sperry-Webb procedure (Wells and Baumann, '54). Satisfactory recoveries of sterol from

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[4] Goldsmith, G. A., J. G. Hamilton and O. N. Miller 1959 Excretion of sterols, bile acids and niacin derivatives in man in relation to serum lipids, dietary fats and nicotinic acid administration. Fed. Proc., *18:* 526 (abstract).

[5] The vitamin mixture consisted of the following in milligrams: inositol, 100; calcium pantothenate, 20; niacin, 10; menadione, 10; riboflavin, 2; thiamine, 2; pyridoxine, 2.5; biotin, 0.1; folic acid, 0.2; vitamin $B_{12}$, 0.02; and glucose to 1 gm.

[6] The following vitamins were added in milligrams per 100 gm of diet: thiamine, 0.6; riboflavin, 0.9; calcium panothenate, 2.0; niacin, 5.0; pyridoxine, 0.8; biotin, 0.03; inositol, 100; choline chloride, 200; folic acid, 0.2; vitamin $B_{12}$, 0.002; and menadione 0.02.



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precipitated blood were obtained by this procedure.[7]

*Liver cholesterol and fat.* Dried, powdered livers were extracted for 24 hours in a Soxhlet extractor with diethyl ether. Liver fat values were based on the weight of lipid extracted. Cholesterol assays were performed on aliquots of the extracts.

*Sterols, fatty acids and bile acids in feces.* Dried feces were exhaustively extracted with Skellysolve B in a Soxhlet extractor. The residue remaining after evaporation was saponified and the sterols extracted into Skellysolve A. Assays of $\Delta^7$-cholestenol, cholesterol, methostenol and coprostanol were made by the method of Coleman et al. ('56). The extracted residue was acidified to pH 4 with 10% aqueous sulfuric acid and the fatty and bile acids extracted with diethyl ether. After evaporation of the extract to dryness, the fatty acids were taken up in hot Skellysolve A (Siperstein et al., '52), the bile acids remaining as a residue. The fatty acid solution was evaporated to dryness, ethanol was added and the acidity determined by titration with 0.02 N NaOH to the thymolphthalein end point. The acidity of the bile acid fraction was determined similarly after solution in aqueous ethanol. Fatty acids were expressed as stearic acid equivalents, and bile acids as cholic acid equivalents.

## EXPERIMENTAL

*Rats fed a hypercholesterolemia-inducing diet.* Weanling male rats were made hypercholesterolemic by the addition of 1% of cholesterol (purified by the dibromide) and 0.5% of cholic acid to basal diet 1 at the expense of sucrose. The diet was fed for two weeks to three groups of 12 each. During this period the cholesterol content of the blood increased from an initial value of 100 mg/100 ml of whole blood to an average of 203 mg%. The average weight was 111 gm.

Thereafter, one group was continued on the diet without supplementation, and the other groups received additions of 0.2 or 1% of nicotinic acid, the latter level supplying a NAc/calorie ratio roughly equivalent to that consumed by human subjects receiving therapeutic doses of 3.0 to 6.0 gm of NAc daily (Parsons et al., '56). Blood was drawn by heart puncture from 6 rats of each group at bi-weekly intervals and the cholesterol content determined on individual samples of whole blood. During the next weeks the cholesterol level of the blood continued to rise both in the presence and absence of nicotinic acid (table 1). However the rate of increase during the first three weeks was somewhat less in rats receiving 1.0% of NAc than in those receiving no supplement (table 1, lines 2, 3 and 4). Peak values for blood cholesterol were reached at 4 weeks (table 1, line 5) and the moderate lowering effect of NAc also disappeared at this time. The averages for all values during the 7 weeks of supplementation were 297, 306 and 292 mg% for the three groups. Thus, in contrast to results with rabbits or men (Altschul et al., '55; Altschul, '56; Merrill and Lemley-Stone, '57; Parsons et al., '56 and Miller et al., '58) nicotinic acid failed to lower blood cholesterol to any substantial degree in these hypercholesterolemic rats. No adverse effects were noted upon growth owing to the high levels of NAc administered.

*Rats fed a normal diet.* The drastic dietary conditions necessary for hypercholesterolemia in the rat (1% of cholesterol and 0.5% of cholic acid) differ markedly from those under which nicotinic acid is normally effective in man. Accordingly, NAc and related substances were fed to rats receiving basal diet 1 in which 10% of lard furnished the only source of dietary cholesterol. The supplements included 0.1 to 1% of NAc, 0.1 to 1% of NAm, 0.1 to 1% of INA, 2% of tryptophan, or 1% of benzoic acid.

[7] Kraupp et al., ('58) and Kraupp and Schnetz, ('59) have suggested that the low values for cholesterol in NAc-fed men might be an artifact due to altered adherence of sterol to the proteinaceous coagulum formed on contact with serum and alcohol-acetone (Zak procedure). In our hands exhaustive extraction of such precipitates from human serum with hot chloroform revealed the residual sterol to range from 7.3 to 15.2% of the total sterol in the sample, whereas coagula of rat whole blood samples contained only 1%; samples from rats fed NAc were indistinguishable from others in this respect. Analysis of coagula from 4 samples of human serum (courtesy of Dr. W. B. Parsons, Jr.) failed to reveal any substantial dependence upon NAc intake.

After 4 weeks of ad libitum consumption, the three levels of NAc fed caused substantial increases in the total cholesterol content of the blood (table 2, lines 2, 3 and 4) and even more significant increases in free cholesterol, exceeding those in total cholesterol. Free cholesterol was also increased when 2% of tryptophan was fed. Nicotinamide or benzoic acid failed to alter the blood cholesterol level whereas INA tended to depress it (table 2). The observed elevation of blood cholesterol by NAc in rats with an initial normal level of blood cholesterol differs from the definite lowering effect due to NAC observed by Altschul and Hoffer in normal young adult men ('58) and by Altschul in rabbits with a normal serum cholesterol level ('56); rather, our results with rats parallel those of O'Reilly et al. ('57) who showed NAc to increase blood cholesterol in man when the pretreatment level of cholesterol was low.

*Chicks fed normal or hypercholesterolemia-inducing diets.* Barred Plymouth Rock chicks of mixed sexes initially weighing 400 to 500 gm were fed basal diet 2 ad libitum. In certain experiments 10%

TABLE 1

*Effect of nicotinic acid on whole blood cholesterol in rats fed diets containing cholesterol and cholic acid*

| Weeks on diet | Blood cholesterol | | |
|---|---|---|---|
| | Control | 0.2% NAc | 1.0% NAc |
| | *mg%* | *mg%* | *mg%* |
| 0 | 203 ± 8.73[1] | 203 ± 8.73 | 203 ± 8.73 |
| 1 | 232 ± 20.0 | 302 ± 28.8 | 238 ± 17.0 |
| 2 | 329 ± 18.1 | 284 ± 13.6 | 255 ± 14.5 |
| 3 | 291 ± 13.5 | 271 ± 14.4 | 253 ± 19.3 |
| 4 | 397 ± 35.8 | 340 ± 71.3 | 450 ± 42.8 |
| 5 | 319 ± 40.0 | 326 ± 18.7 | 308 ± 38.0 |
| 6 | 282 ± 13.8 | 313 ± 12.7 | 278 ± 17.3 |
| 7 | 231 ± 34.3 | 309 ± 35.0 | 261 ± 21.6 |
| Av. (1–7 weeks) | 297 | 306 | 292 |
| | Body weight | | |
| | *gm* | *gm* | *gm* |
| Initial | 111 | 111 | 111 |
| Final | 260 | 256 | 251 |

[1] Standard error of the mean.

TABLE 2

*Cholesterol in whole blood of rats fed nicotinic acid or related compounds*

| Supplement[1] | No. of rats | Whole blood cholesterol | |
|---|---|---|---|
| | | Free | Total |
| | | *mg/100 ml* | *mg/100 ml* |
| None | 10 | 58.6 ± 2.31[2] | 80.3 ± 3.42 |
| 0.1% Nicotinic acid | 6 | 79.1 ± 4.66[3] | 92.3 ± 4.50[4] |
| 0.5% Nicotinic acid | 4 | 70.8 ± 3.34[3] | 87.1 ± 4.32 |
| 1.0% Nicotinic acid | 10 | 71.4 ± 3.06[3] | 91.7 ± 4.28[4] |
| 0.1% Nicotinamide | 4 | 53.3 ± 4.67 | 80.1 ± 5.00 |
| 0.5% Nicotinamide | 2 | 60.2 ± 5.00 | 78.7 ± 2.50 |
| 1.0% Nicotinamide | 10 | 58.1 ± 0.79 | 75.9 ± 0.92 |
| 1.0% Benzoic acid | 5 | 57.1 ± 0.84 | 75.2 ± 0.61 |
| 0.1% Isonicotinic acid | 4 | 60.9 ± 4.02 | 67.8 ± 2.84[4] |
| 1.0% Isonicotinic acid | 4 | 47.1 ± 4.70[4] | 67.1 ± 5.95 |
| 2.0% Tryptophan | 4 | 65.6 ± 1.71[4] | 84.9 ± 2.06 |

[1] Basal diet 1 containing 10% of lard but no other source of cholesterol.
[2] Standard error of the mean.
[3] Highly statistically significant ($P < 0.01$).
[4] Statistically significant ($P < 0.05$).

of lard or 10% of lard plus 0.5% of purified cholesterol were added at the expense of the soybean oil and some of the sucrose. One per cent of NAc or related compounds was added to the diets at the expense of sucrose. After two and 4 weeks on the diets, blood was drawn from the brachial vein for cholesterol analysis.

The chicks fed cholesterol showed an increase of approximately 100% in the total cholesterol content of the whole blood (table 3, line 1 vs. line 4), most of the increase being in the esterified fraction. After 2 weeks, 1% of NAc significantly depressed the total blood cholesterol level of chicks fed 0.5% of cholesterol (table 3, line 2, vs. line 1) and the depression persisted but was less severe at 4 weeks. NAc also appeared to lower blood cholesterol in chicks fed lard with no added cholesterol (line 5 vs. line 4), although the differences were not significant. One per cent of NAm appeared to lower blood cholesterol slightly after the first two weeks, but this effect had disappeared by 4 weeks.

Chicks fed the basal diet containing 4% of soybean oil showed a constant total blood cholesterol level of 137 mg/100 ml of blood. One per cent of NAc or of INA depressed this level significantly, while 1% of NAm produced a non-significant depression and 1% of benzoic was without effect. Thus chicks appeared to resemble man, in that dietary nicotinic acid lowered blood cholesterol whereas nicotinamide was much less effective.

*Pyridine nucleotide contents of livers from rats and chicks fed NAc or analogues.* Livers were removed from decapitated rats or chicks and frozen in an acetone-dry ice mixture within 30 seconds after decapitation. Weighed samples were taken from the pulverized, frozen tissue and homogenized for two minutes in a trichloracetic acid solution. The concentration of oxidized pyridine nucleotides in this extract was then determined by the method of Feigelson et al. ('50). Both NAc and NAm increased the levels of the pyridine nucleotides in the liver (table 4, lines 1–4). The increase due to 1% of NAc was about 100% in the rat and somewhat less in the chick. In both species the increases due to NAm substantially exceeded those due to NAc. Values at 4 weeks for the higher level of NAc or NAm were essentially similar to those after two weeks of feeding. Neither INA nor benzoic acid altered the levels of the pyridine nucleotides significantly (table 4, lines 1, 6–8).

Preiss and Handler ('58a and '58b) reported NAc to be a better precursor of pyridine nucleotides both *in vivo* and *in vitro* than NAm. Kaplan et al. ('56) reported that an injection of NAm into mice produced greater increases in the pyridine nucleotide content of normal livers than

TABLE 3

*Effect of nicotinic acid and related compounds on blood cholesterol in chicks*

| Diet | 2 weeks | | 4 weeks | |
|---|---|---|---|---|
| | Free | Total | Free | Total |
| | *mg%* | *mg%* | *mg%* | *mg%* |
| 10% Lard + 0.5% cholesterol[1] | 119 ± 5.99 | 235 ± 14.6 | 122 ± 4.91 | 285 ± 7.60 |
| 10% Lard + 1% NAc[1] | 95 ± 4.42[4] | 191 ± 4.97[4] | 116 ± 8.13 | 253 ± 9.61[4] |
| 10% Lard + 1% NAm[1] | 107 ± 5.43 | 198 ± 5.31[4] | 127 ± 4.41 | 274 ± 9.99 |
| 10% Lard[1] | 70.4 ± 1.00 | 116 ± 7.51 | 71.7 ± 2.40 | 137 ± 6.00 |
| 10% Lard + 1% NAc[1] | 69.0 ± 0.745 | 102 ± 4.69 | 61.4 ± 1.92[3] | 118 ± 9.43 |
| 10% Lard + 1% NAm[1] | 70.0 ± 2.47 | 106 ± 3.24 | 71.4 ± 2.10 | 134 ± 9.50 |
| 4% Soybean oil[2] | 89.5 ± 3.09 | 137 ± 3.04 | 80.0 ± 3.60 | 137 ± 5.40 |
| 4% Soybean oil + 1% NAc[2] | 68.8 ± 1.70[3] | 105 ± 2.00[3] | 60.5 ± 2.86[3] | 103 ± 2.45[3] |
| 4% Soybean oil + 1% NAm[2] | 86.7 ± 0.736 | 123 ± 7.56 | 73.0 ± 3.36 | 126 ± 3.98 |
| 4% Soybean oil + 1% INA[2] | 69.2 ± 2.08[3] | 114 ± 4.33[3] | 62.1 ± 2.33[3] | 118 ± 4.52[4] |
| 4% Soybean oil + 1% benzoic acid[2] | 87.0 ± 7.93 | 143 ± 16.0 | 71.2 ± 4.78 | 129 ± 8.53 |

[1] Five chicks per group.
[2] Six chicks per group.
[3] Highly significant ($P < 0.01$).
[4] Statistically significant ($P < 0.05$).

NAc, but they have recently shown NAm to be converted to NAc prior to its incorporation into pyridine nucleotides (Langan et al., '59). Our results suggest that the greater effectiveness of NAc over NAm on blood cholesterol in the chick is not due to any greater accumulation of pyridine nucleotides by NAc.

*Liver fat and cholesterol.* For studies of the liver lipids rats were fed basal diet 1 plus 1% of NAc, NAm, isonicotinic acid, or benzoic acid, or the basal diet supplemented with 1% of cholesterol and 0.5% of cholic acid. Chicks were fed either basal diet 2 containing 10% of lard or an additional 0.5% of cholesterol plus the nicotinic acid analogues. The diets were fed for 4 weeks, with 4 to 8 animals per group (table 5).

As expected, the feeding of cholesterol markedly increased the cholesterol content of the liver: from a control value of 3.8 mg/gm dry weight to 21 to 25 mg/gm for rats fed cholesterol, and from chick con-

TABLE 4

*Pyridine nucleotides in livers from rats and chicks fed nicotinic acid or analogues, determined by wet weight*

| | Rat | | Chick |
|---|---|---|---|
| | 2 weeks | 4 weeks | 4 weeks |
| | μg/gm | μg/gm | μg/gm |
| Basal[1] | 570 ± 11.8[2] | 505 ± 23.1 | 584 ± 41 |
| Basal + 0.1% nicotinic acid | 965 ± 265 | 707 ± 137 | |
| Basal + 1.0% nicotinic acid | 1030 ± 260 | 1045 ± 170 | 982 ± 63 |
| Basal + 0.1% nicotinamide | 849 ± 15.0 | 665 ± 51.4 | |
| Basal + 1.0% nicotinamide | 1277 ± 231 | 1296 ± 111 | 1380 ± 186 |
| Basal + 0.1% isonicotinic acid | 706 ± 174 | 670 ± 5.0 | |
| Basal + 1.0% isonicotinic acid | 697 ± 93 | 556 ± 10.0 | 563 ± 70 |
| Basal + 1.0% benzoic acid | | 513 ± 8.38 | 551 ± 25 |

[1] 10% of lard for rats; 4% of soybean oil for chicks.
[2] Standard error of the mean.

TABLE 5

*Liver fat and cholesterol levels of rats and chicks, determined by dry weight*

| Line no. | | Fat | | Cholesterol | |
|---|---|---|---|---|---|
| | | Rat | Chick | Rat | Chick |
| | | mg/gm | mg/gm | mg/gm | mg/gm |
| 1 | Basal[1] | 203 | 121 | 3.81 | 8.6 |
| 2 | Basal + 1% NAc | 221 | 123 | 3.50 | 7.8 |
| 3 | Basal + 1% NAm | 230 | 161 | 3.94 | 8.1 |
| 4 | Basal + 1% INA | 182 | 96 | 3.63 | 8.0 |
| 5 | Basal + 1% benzoic acid | 227 | 125 | 4.24 | 8.3 |
| 6 | Chick (10% lard) | — | 77 | — | 5.2 |
| 7 | Chick (10% lard + 1% NAc | — | 98 | — | 8.2 |
| 8 | Chick (10% lard) + 1% NAm | — | 82 | — | 7.5 |
| 9 | Chick (10% lard) + ½% cholesterol | — | 141 | — | 26.4 |
| 10 | Chick (10% lard) + ½% cholesterol + 1% NAc | — | 209 | — | 40.9 |
| 11 | Chick (10% lard) + ½% cholesterol + 1% NAm | — | 182 | — | 38.7 |
| 12 | Rat (10% lard) | — | — | 3.81 | — |
| 13 | Rat (10% lard) + 1% cholesterol and ½% cholic acid | — | — | 21.8 | — |
| 14 | Rat (10% lard + 1% cholesterol and ½% cholic acid + 0.2% NAc | — | — | 24.4 | — |
| 15 | Rat (10% lard) + 1% cholesterol and ½% cholic acid + 1% NAc | — | — | 25.6 | — |

[1] Rat, 10% lard; chick, 4% soybean oil as fat.

trol values of 5 to 8 mg/gm to 26 to 40 mg/gm for those fed cholesterol. Neither NAc nor NAm lowered liver cholesterol under these conditions, and in the chick both produced substantial elevations (table 5, lines 9—11, 13—15).

In the absence of dietary cholesterol, NAc, NAm and benzoic acid appeared to increase liver fat in the rat. INA lowered the level of liver fat both in the rat and chick (table 5, line 4).

In chicks fed cholesterol, NAc raised liver cholesterol from 26.4 to 40.9 mg/gm, while NAm raised it to 38.7. In the rat, the respective values were 21.8, 24.4 and 25.6 mg/gm. Thus our results differ from those of Schön ('58) who reported NAc to lower liver cholesterol in rats on a "cholesterol-free hypolipotropic diet" (composition not stated) in which the control liver fat was 25% of the fresh weight and cholesterol was 1181 mg% . In our studies the values for liver cholesterol were relatively uniform in both rats and chicks on the basal diets, with no significant effect due to any of the nicotinic acid analogues (table 5, lines 1–5); when 10% of lard was fed to chicks, NAc and NAm increased liver cholesterol somewhat (lines 6–9). The percentages of liver fat tended to reflect the variations in liver cholesterol when cholesterol was fed, NAc raising liver fat values somewhat more than NAm.

*Growth rates in rats and chicks fed NAc and analogues.* The small but significant elevations in liver fat in rats fed 1% of NAm was accompanied by a depressed growth rate similar to that previously observed by Handler and Dann ('42). One per cent of NAm or 2% of tryptophan limited growth to about 40% of the control rate for rats weighing about 100 gm, whereas nicotinic acid or INA at this level did not depress growth. These depressions in growth rate were paralleled by decreases in food consumption.

Chicks fed 1% of nicotinic acid grew slightly less than control animals (8 to 17%) and nicotinamide or benzoic acid depressed growth somewhat more; INA was without effect.

*Lipid excretion by the rat.* Since bile acids represent a major pathway by which cholesterol can be removed from the body, the amounts of bile acids as well as fatty acids were measured in feces from rats fed diets containing NAc, NAm, or INA at the 1% levels. Pooled fecal samples of 6 rat-days collected after three weeks on the experimental diet were extracted as described under "Methods" and the fatty and bile acids isolated for titration.

There appeared to be no significant alteration in the fatty acids excreted when NAc was fed (table 6). High levels of NAm may have elevated the fatty acids excreted, as these values paralleled an enhanced bile acid excretion. One per cent of INA resulted in an elevated bile acid excretion without change in fatty acid excretion. Since INA depressed blood cholesterol (table 2) and enhanced bile acid excretion, the overall effect of this compound was similar to that reported by Goldsmith et al. ('59) for humans receiving NAc in massive doses. By way of contrast, our NAm-fed rats showed a similar excre-

TABLE 6
*Excretion of fatty acids, bile acids and sterols by the rat*

| Diet | Fatty acids | Bile acids | Total sterols[1] |
|---|---|---|---|
| | *mg/day* | *mg/day* | *mg/day* |
| Control | 47.3[2] | 7.7 | 10.7 |
| Control + 0.1% NAc | 57.3 | 9.7 | 9.9 |
| Control + 1.0% NAc | 47.0 | 7.2 | 11.1 |
| Control + 0.1% NAm | 43.3 | 9.4 | 10.8 |
| Control + 1.0% NAm | 65.8 | 13.6 | 10.6 |
| Control + 0.1% INA | 30.3 | 9.7 | 9.1 |
| Control + 1.0% INA | 43.0 | 15.1 | 9.9 |

[1] Sums of the various sterols determined separately.
[2] All of the values represent the pooled feces collected from 6 rat-days.

tion pattern but no depressed blood cholesterol levels.

The total sterols excreted by the rat were unaltered by NAc, and the amounts of the individual sterols, $\Delta^7$cholestenol, cholesterol, methostenol and coprostanol, varied only slightly. Furthermore, no difference was found in the conversion of cholesterol to coprostanol during an 8-day anaerobic fermentation (Coleman and Baumann, '57) by bacteria isolated from the intestines of rats fed either the basal diet or basal diet plus NAc or its analogues.

## DISCUSSION

The hypocholesterolemic action of NAc observed in our chicks appears to be analogous to that repeatedly observed in man. This phenomenon has also been studied in the dog, rabbit, guinea pig and rat. The blood cholesterol level of the dog does not appear to respond to NAc (Narcia et al., '59), the response in the rabbit has been best observed only when the blood level of cholesterol had been elevated abnormally (Altschul, '56; Merrill and Lemley-Stone, '57), whereas the response was marginal or in an opposite direction in the rat (Duncan and Best, '58) and the guinea pig.[8]

Although our results have not revealed the means by which NAc lowers blood cholesterol, a comparison of the effects of NAc and its analogues on blood cholesterol, and on liver pyridine nucleotide and cholesterol permits a tentative evaluation of possible mechanisms.

Since NAc elevated the liver pyridine nucleotide concentrations in both the rat or the chick and the blood cholesterol-lowering effect was observed only in the chick, the amount of pyridine nucleotide present does not appear to determine the cholesterol-lowering effect. Furthermore, NAm, which increased the level of pyridine nucleotides in the chick to a greater extent than NAc, did not produce a significant depression in the blood cholesterol level. Isonicotinic acid, a compound structurally similar to NAc but unable to alter the liver pyridine nucleotide levels of either the chick or the rat, exerted a significant hypocholesterolemic action in both of these species.

Miller et al. ('58) have observed a large increase in the excretion of nicotinuric acid by humans administered therapeutic doses of NAc, while no comparable elevation was observed in individuals receiving NAm. The principal excretion product of nicotinic acid in the rat has been shown to be nicotinuric acid, while it is only a minor product of nicotinamide metabolism (Lin and Johnson, '53). Compounds like NAc are detoxified by activation with coenzyme A followed by conjugation with glycine in the rat or with ornithine in the chick[9] (Henderson, '56). Hence, an explanation for the cholesterol-lowering effects of NAc and INA might be sought among these pre-excretory reactions. The absence of a hypocholesterolemic action of benzoic acid in either the rat or chick indicated that neither the activation nor conjugation appeared to be responsible for the effects observed.

O'Reilly et al., ('57) have suggested that NAc exerts a homeostatic effect on blood cholesterol since the drug depressed blood cholesterol in hypercholesterolemic patients and elevated it in normal subjects. The diverse effects of NAc in the chick and the rat appear to be in agreement, at least superficially, with this hypothesis: NAc raised the blood cholesterol level in the rat, in which it is normally low, and lowered it in the chick which normally has a higher level.

Since the therapeutic level of NAc is more than 100 times that required for its vitamin function, it was noteworthy that the toxic effects of NAc or its analogues on growth or liver fat were so small. Of the substances tested, INA appeared to produce the least alteration in growth, liver pyridine nucleotide levels, or liver cholesterol concentrations, while yielding the most consistent lowering of blood cholesterol in both the rat and the chick.

## SUMMARY

1. High levels of nicotinic acid (NAc), comparable to those used in human therapy, were fed to rats and chicks, and the effects compared with those resulting from

[8] Gaylor, J., unpublished data.

[9] Sarkar, N., M. Fuld and D. E. Green 1951 Studies on the synthesis of hippuric acid. Fed. Proc., *10:* 242 (abstract).

nicotinamide (NAm), isonicotinic acid (INA) or benzoic acid.

2. Nicotinic acid did not alter the blood cholesterol level of rats fed a hypercholesterolemia-inducing diet (basal plus 0.5% of cholic acid and 1% of cholesterol). The blood cholesterol level of chicks fed a cholesterol-containing diet (0.5% ) was depressed by NAc and altered only slightly by NAm.

3. Nicotinic acid and INA significantly depressed blood cholesterol levels of chicks fed a low cholesterol diet while NAm produced less significant depressions and benzoic acid was without effect. Blood cholesterol of rats fed the basal diet was depressed only by INA, while NAc significantly elevated the level.

4. Liver pyridine nucleotide levels were markedly elevated by both NAc and NAm while INA and benzoic acid produced no alteration in either the chick or the rat.

5. The liver fat levels of chicks were elevated by NAm and depressed by INA; INA also depressed the liver fat levels of rats. No marked changes were observed in the liver sterol levels.

6. Nicotinic acid did not alter total sterol, bile acid or fatty acid excretion by the rat. Bile acid excretion was increased somewhat by high dietary levels of NAm or INA.

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# The Effect of Environment on Chick Growth[1]

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The work of Coates et al. ('51), Hill et al. ('53) and Lillie et al. ('53) has demonstrated clearly that response to an antibiotic disappears or decreases when chicks are raised in new quarters. A number of workers have also shown that the requirement for certain nutrients may be lower when an antibiotic is included in the feed (Jukes, '55). Coates ('53) reported that after depopulating, thoroughly cleaning and disinfecting chick quarters, the antibiotic growth effect in chicks disappeared; however, after a few successive lots of chicks were grown, the growth response to penicillin reappeared.

In our nutrition laboratory, fumigation was neglected because of overlapping experiments during the past year. As a result of not having a routine for fumigation of this laboratory, a number of interesting observations have been made on data collected during the past 18 months. These observations indicate strongly that the cleanliness of quarters may have a pronounced effect on both rate of growth and quantitative requirements for certain specific nutrients by young growing chickens.

## EXPERIMENTAL

Nutrition research trials are conducted in two rooms, each approximately 32′ by 20′, with a connecting wash room approximately 30′ by 15′. Our policy has been to clear the laboratory periodically of all research work, clean the laboratory thoroughly with water and a disinfecting soap and fumigate with formalin and potassium permanganate (3500 gm of potassium permanganate and 6300 ml of formalin, divided into 7 vessels spaced throughout the three rooms). White Plymouth Rock cockerels obtained from a local commercial hatchery are used for most of our nutritional research. When breeding work is involved, Athens-Canadian random-bred chicks, hatched in our incubators, are used. All birds are wing-banded and placed on experimental diets when approximately one day old.

The experimental procedure in the present study involves comparison between experiments initiated immediately after the brooder room was fumigated and those started when older birds were in the brooder room with the quarters not cleared or fumigated.

In the magnesium and methionine studies, chicks were housed in electrically-heated battery brooders having wire mesh floors, for 21 days. In the zinc studies, the birds were housed in plastic cloth and wooden cages with pine-shaving litter on floors. The chicks were brooded with infra-red lamps for the experimental period, 14 days.

Composition of rations used in these studies is shown in table 1. Rations A and B were used in the magnesium studies. Ration A was calculated to contain 25% of protein and 1050 Cal. of productive energy per pound, while ration B contained 33.33% of protein and 1400 Cal. of productive energy per pound. The purified, isolated soybean-protein diet used in the zinc studies was similar to that used in a previous study by Edwards et al. ('58) except that corn oil replaced hydrogenated vegetable fat; also tricalcium phosphate and calcium carbonate were used to obtain the desired calcium and phosphorus levels (1.2 and 0.6%, respectively). The practical-type ration used in the zinc studies was the corn-soybean oil meal ration previously used in this laboratory by Edwards et al. ('59). Ration C as shown in table 1 was used in the methionine studies.

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TABLE 1

*Composition of basal rations*

| | Diet | | |
|---|---|---|---|
| | A | B | C |
| | *gm* | *gm* | *gm* |
| Dextrose | 60.95 | 28.41 | 70.84 |
| Isolated soybean protein | 27.80 | 37.00 | 16.67 |
| Cellulose[1] | 3.329 | 0.047 | 3.00 |
| Corn oil | 0.50 | 25.00 | 3.00 |
| DL-Methionine | 0.69 | 0.918 | |
| Glycine | 0.34 | 0.452 | 0.34 |
| Microcel[2] | 1.00 | 1.00 | |
| $CaH(PO_4)_2$ | 2.38 | 3.165 | |
| $CaCO_3$ | 0.713 | 0.948 | |
| Vitamin A concentrate (20,000 U.S.P. units/gm) | 0.05 | 0.067 | 0.05 |
| NaCl | 0.75 | 0.998 | 0.75 |
| KCl | 0.60 | 0.798 | 0.60 |
| D-α Tocopheryl acetate concentrate (20,000 I.U./pound) | 0.154 | 0.205 | 0.154 |
| Choline Cl concentrate (70.7%) | 0.282 | 0.375 | 0.282 |
| $ZnSO_4 \cdot 7H_2O$ | 0.088 | 0.117 | 0.088 |
| $MgSO_4$ | | | 0.255 |
| Defluorinated phosphate | | | 3.529 |
| $MnSO_4 \cdot H_2O$ | 26.4 | 35.11 | 26.4 |
| $FeSO_4 \cdot 7H_2O$ | 11.0 | 14.63 | 11.0 |
| $CuSO_4 \cdot 5H_2O$ | 1.1 | 1.46 | 1.1 |
| $CoCl_2 \cdot 6H_2O$ | 1.1 | 1.46 | 1.1 |
| KI | 1.1 | 1.46 | 1.1 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.11 | 0.146 | 0.11 |
| Inositol | 110.0 | 146.3 | 110.0 |
| *p*-Aminobenzoic acid | 22.0 | 29.26 | 22.0 |
| Calcium pantothenate | 2.2 | 2.926 | 2.2 |
| Niacin | 2.64 | 3.511 | 2.64 |
| Thiamine·HCl | 1.32 | 1.755 | 1.32 |
| Riboflavin | 1.32 | 1.755 | 1.32 |
| Pyridoxine·HCl | 0.66 | 0.878 | 0.66 |
| Folic acid | 0.44 | 0.585 | 0.44 |
| Menadione | 0.22 | 0.2931 | 0.22 |
| Biotin | 0.044 | 0.0591 | 0.044 |
| Vitamin $B_{12}$ | 0.022 | 0.029 | 0.022 |
| Vitamin $D_3$ concentrate (15,000 I.U./gm) | 13.332 | 17.731 | 13.332 |

[1] Solka Floc BNB-4C, The Brown Co., Berlin, New Hampshire.

[2] Microcel E, a synthetic hydrated calcium silicate, Johns-Manville, New York.

### *Magnesium studies*

The results of two experiments in which graded levels of magnesium were added to diets A and B (table 1) are shown in table 2. When diet A was fed under contaminated conditions (laboratory not washed or fumigated and with older birds present), no plateau cf response was obtained and the growth rate increased through the highest magnesium level used. Mortality decreased as the level of magnesium in the diet increased, but it was still 13% with 200 ppm of supplemental magnesium in the diet. In the experiment conducted under the fumigated conditions (laboratory washed and fumigated, no older birds present), not over 100 ppm of magnesium was needed to obtain maximum growth rate and to reduce mortality to zero when the low energy diet, or diet A, was fed.

When the high energy diet, or diet B, was fed, no plateau of response was obtained under contaminated conditions. Growth rate was poor and mortality very high at the lower levels of magnesium in the diet. Even with 200 ppm of magnesium in the diet, mortality was 17%. When the high energy diet was fed under fumigated conditions, a plateau of the growth response curve was obtained and the requirement for growth appeared to be

TABLE 2

*Studies of the magnesium requirement of the chick*

| Supplemental Magnesium | Low energy diet | | | | High energy diet | | | |
|---|---|---|---|---|---|---|---|---|
| | Contaminated | | Fumigated | | Contaminated | | Fumigated | |
| | Av. weight 3 weeks[1] | Mortality | Av. weight 3 weeks[1] | Mortality | Av. weight 3 weeks[1] | Mortality | Av. weight 3 weeks[1] | Mortality |
| *ppm* | *gm* | % | *gm* | % | *gm* | % | *gm* | % |
| 0 | 222 | 57 | 282 | 43 | 235 | 93 | 282 | 83 |
| 25 | 260 | 43 | | | 218 | 77 | | |
| 50 | 269 | 40 | | | 248 | 63 | | |
| 100 | 280 | 20 | 332 | 0 | 285 | 47 | 343 | 27 |
| 150 | | | 328 | 3 | | | 366 | 17 |
| 200 | 325 | 13 | 326 | 0 | 308 | 17 | 346 | 7 |
| 250 | | | 328 | 3 | | | 357 | 7 |
| 300 | | | 326 | 0 | | | 347 | 0 |

[1] Average of three groups of 10 White Rock cockerels.

in the range 100 to 150 ppm. However, some mortality continued to occur at the higher levels of magnesium and was prevented only with the use of 300 ppm of magnesium in the diet.

The effect of contamination of environment on the magnesium requirement of the young chick may help to explain some of the differences in requirements reported by other workers. Almquist ('42) noted a requirement for growth of approximately 400 ppm of magnesium; whereas, Scott, et al., ('56) reported that 100 ppm of magnesium in rations supported maximum growth.

The data presented in table 2 indicate that both the calorie and protein content of the ration, as well as the environment, exert major effects on the chick's requirement for magnesium when expressed as a percentage of the diet. The calorie and protein influence is exerted through the overall efficiency of feed utilization, whereas the environment appears to exert an influence that may be much more specific, and which could influence one nutrient and not another.

*Zinc studies*

Data are presented in table 3 which show the effect of environment on the growth rate and zinc requirement of chicks fed a purified dextrose,[2] isolated soybean-protein diet. Under contaminated conditions, apparently the growth rate of the chicks is slower and the zinc requirement higher than under the fumigated conditions. Chicks raised under fumigated conditions did not show an additional growth response when more than 20 ppm of supplemental zinc was used.

TABLE 3

*Zinc studies using the purified diet*

| Supplemental zinc | Contaminated Av. weight, 2 weeks[1] | Fumigated Av. weight, 2 weeks[1] |
|---|---|---|
| *ppm* | *gm* | *gm* |
| 0 | 81 | 84 |
| 10 | 114 | 138 |
| 20 | 136 | 162 |
| 40 | 143 | 162 |

[1] Average of three groups of 10 White Rock cockerels.

The results of experiments using practical-type rations are presented in table 4.

TABLE 4

*Zinc studies using the practical-type diet*

| Supplemental zinc | Contaminated Av. weight, 2 weeks[1] | Fumigated Av. weight, 2 weeks[1] |
|---|---|---|
| *ppm* | *gm* | *gm* |
| 0 | 129 | 179 |
| 10 | 140 | 172 |
| 20 | 140 | 183 |

[1] Average of 4 groups of 10 White Rock cockerels.

Under contaminated conditions, poor growth was observed in chicks receiving the basal ration and a growth response was noted with the use of supplemental zinc. Under fumigated conditions, rapid growth was observed using the basal

[2] Cerelose.

ration, whereas no response was obtained with the use of supplemental zinc. This can be interpreted to mean that the requirement for zinc was higher under the contaminated conditions. It should be noted that an antibiotic (oxytetracycline) was present in the basal rations in these experiments.

### *Methionine studies*

Two experiments were conducted using chicks from the same single-male matings of Athens-Canadian random-bred stock for each test. The results are shown in table 5. Chicks raised under contaminated conditions did not grow as fast as chicks raised under the fumigated conditions. Complete response curves for the two experiments are not available and it is, therefore, difficult to determine accurately the methionine requirement under the various conditions. However, when the data are plotted on semi-log paper, the requirement for methionine under the two conditions appears to be the same. Thus, apparently a deficiency of some nutrient other than methionine may have caused the poor growth of chickens receiving this ration under the contaminated condition.

TABLE 5
*Methionine requirement studies using individually pedigreed chicks*

| Supplemental methionine | Contaminated Av. weight, 3 weeks[1] | Fumigated Av. weight, 3 weeks[1] |
|---|---|---|
| % | *gm* | *gm* |
| 0.0 | 73 | 85 |
| 0.05 | | 107 |
| 0.10 | | 127 |
| 0.20 | 115 | 137 |
| 0.40 | 120 | 140 |
| 0.60 | 113 | |
| 0.80 | 107 | |

[1] Average of three groups of 12 chicks each selected from Athens-Canadian random-bred stock.

## DISCUSSION

Results observed indicate that more rapid chick growth occurs in fumigated than in contaminated chick quarters. This effect may be similar to the failure to get an antibiotic response in new quarters reported by Coates, et al. ('51). However, since an antibiotic was present in one of the rations in which this effect was observed, it can be concluded that the presence of an antibiotic will not cause the effect of fumigation to disappear. However, another antibiotic or a mixture of antibiotics could conceivably have improved the growth of the chicks reared in contaminated quarters to a greater extent than the antibiotic used. Also a response might possibly be obtained from an unidentified growth-factor supplement, under contaminated conditions, and the phenomenon apparent in our work be the same as that reported by Barnett and Bird ('56) wherein a response was obtained from unidentified factor supplements when unsterilized poultry feces at a low level were included in the diet. The data presented suggest that many of the nutrients known to be required are necessary in greater amounts for chicks grown in a contaminated environment than for chicks grown after the environment has been decontaminated. This observation points up further complications for studies aimed to establish a definite nutrient requirement. Furthermore, special consideration must be given to studying requirements in contaminated environments in respect to the level of other nutrients in the diet, in order to avoid compounding deficiencies. Since all nutrients may not be present in adequate amounts, the growth pattern of the nutrient under study may be influenced by a deficiency of a nutrient not under consideration.

While numerous factors may have affected the results obtained, the authors do not believe that source or type of chick was a factor in these experiments. Further work, under more controlled conditions, is being undertaken to study such factors as environmental temperature, specific and non-specific disease and overall diet composition.

## SUMMARY

Chickens grown in an experimental laboratory immediately after the laboratory had been cleaned and fumigated with formalin and potassium permanganate grew at a greater rate than chicks grown in the same laboratory with older chicks present from the start of the experiment. The data presented also suggest that the

requirement of the chick for certain nutrients may be much greater when chicks are grown in contaminated quarters as compared with chicks grown in fumigated quarters. The significance of the findings is discussed.

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# Comparative Protein Requirement of the Rat and Mouse for Growth, Reproduction and Lactation Using Casein Diets

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Relatively little is known of the dietary protein requirement of the mouse. With highly purified diets, containing 22 to 30 % of casein, growth of mice was excellent but reproduction and lactation were poor (Cerecedo and Vinson, '44; Murphy and Dunn, '50). Bosshardt, Ydse, Ayres and Barnes, ('46) observed that the dietary level of casein at which maximum utilization for growth was obtained was 9 to 12% and that although the absolute maximal protein efficiency ratios are not necessarily the same for both rats and mice, proteins bear the same relative nutritional value to each other when assayed by rats or by mice. They indicated the necessity for standardizing conditions, such as pretreatment of test animals, duration of test period, level of test protein, caloric intake, adequacy of other nutrients and strain of animal. Fenton and Carr ('51) noted a wide range of protein efficiency ratios when mice of 4 highly inbred strains were studied under identical conditions.

The minimal protein concentration of a rice-beans-casein diet, which supported growth, reproduction and lactation in several generations of rats, such as that obtained with an adequate diet, was 16.7% (Goettsch, '46; '48; '49). From determined values for "true" digestibility and biological value of the dietary protein, the net protein concentration was 10.4%. The diet contained 4.9 mg net of nitrogen per calorie.

With the purpose of determining whether protein from another source would support adequate growth, reproduction and lactation in the rat at a similar minimal concentration of net nitrogen per calorie, casein diets were investigated. The diets were fed to both rats and mice and their protein requirements compared under the given conditions.

## EXPERIMENTAL

*Diets.* Crude casein, containing 12.78% of total nitrogen was the source of protein. The "true" digestibility and biological value were determined by Goyco and Asenjo ('47) with rats and observed to be 99.2 and 71.7 respectively. The casein was held at refrigerator temperature until used. The composition and calculated nutritive values of the casein diets are given in table 1. Proximate analyses were made occasionally and good agreement was noted between the calculated and observed values. Protein was apparently the limiting factor of the diet. The diets contained approximately 3.7 Cal./gm.

*Procedure.* Detailed procedures for obtaining data on growth, reproduction and lactation have been published for rats (Goettsch, '48; '49) and for mice (Goettsch, '42). The experiments were carried out under the conditions of a tropical marine climate with acclimatized Wistar rats and Swiss mice, known as STM strains.

The average food consumption of three rats in a cage was determined. On account of food wastage, it was necessary to house the mice separately. They were given daily a weighed portion of diet, moistened with water to form a paste. The following day any uneaten food was collected, dried at 100°C weighed and the value used to correct the original weight. Food consumption was recorded during

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TABLE 1

*Composition and calculated nutritive values of the casein diets*

| | Percentage dietary protein (T.N. × 6.38) | | | |
|---|---|---|---|---|
| | 16.3 | 13.6 | 11.4 | 9.4 |
| Constituents[1] | | | | |
| Casein (technical) | 20.0 | 16.7 | 14.0 | 11.6 |
| Cornstarch (raw) | 68.0 | 71.3 | 74.0 | 76.4 |
| Vegetable oil[2] | 7.0 | 7.0 | 7.0 | 7.0 |
| Cod liver oil | 1.0 | 1.0 | 1.0 | 1.0 |
| Salt mixture[3] | 4.0 | 4.0 | 4.0 | 4.0 |
| Nutritive values | | | | |
| Total nitrogen, % | 2.56 | 2.17 | 1.79 | 1.48 |
| Protein calories, % | 17.6 | 14.7 | 12.3 | 10.0 |
| Total nitrogen/Cal., *mg* | 7.0 | 5.9 | 4.8 | 4.0 |
| Net nitrogen (T.N. × 99.7 × 71.7)/Cal., *mg* | 5.0 | 4.2 | 3.4 | 2.9 |

[1] Synthetic vitamins added in milligrams as supplement to each 100 gm of diet: thiamine·HCl, 0.5; pyridoxine·HCl, 0.5; riboflavin, 1.0; niacin, 2.0; vitamin K, 0.5; *p*-aminobenzoic acid, 1.0; Ca pantothenate, 5.0; inositol, 40; choline Cl, 50; folic acid, 0.4; and biotin, 0.04. Each animal received orally each week, one drop of sesame oil containing 2 mg $\alpha$-tocopherol acetate. Vitamins supplied through the courtesy of Hoffman-La Roche, Inc.

[2] Argol.

[3] Modified Hawk-Oser ('31) salt mixture, with $CaCO_3$ substituted for Ca citrate.

early growth: for rats, a 28-day period beginning at the time when the rat weighed 60 gm; for mice, a 14-day period beginning at the time when the mouse weighed 9 gm. During gestation the food consumption was measured for the first 21 days in the rat and 18 days in the mouse. During lactation food consumption was measured for the first 17 days in both the rat and mouse.

## DISCUSSION

*Growth.* As will be seen in table 2, the gain in body weight of rats fed the casein diets, containing 16.3 and 13.6% of protein, compares favorably with that observed with the minimal protein rice-beans-casein diet (Goettsch, '48). Rats fed the 11.4 and 9.4% protein diets grew less rapidly. With the 9.4% protein diet males and females grew at approximately the same rate.

In mice, less difference was observed between the sexes in the gain in body weight. The rate of growth decreased gradually with restriction of protein.

*Protein efficiency.* The maximum protein efficiency under the given conditions occurred in rats with the 13.6, 11.4 and 9.4% protein diets, being 2.8 for males and 2.6 for females. Assuming a direct relationship between the maximal protein efficiency and the net utilization of protein, the maximal protein efficiency of an ideal protein may be calculated as $2.8/99.2 \times 71.7 = 3.9$, which is in agreement, essentially, with the value calculated from the rice-beans-casein data (Goettsch, '48) and with that obtained experimentally by Barnes, Maack, Knights and Burr ('45) using whole egg.

In mice, the maximal protein efficiency did not differ greatly between sexes and occurred with the 11.4 and 9.4% protein diets—approximately 1.7. The results with mice are in accord with those of Bosshardt, Ydse, Ayres and Barnes ('46).

*Caloric intake.* In male rats, the maximal caloric consumption was observed with the 13.6% protein diet, which also induced the maximal protein efficiency ratio (Barnes and Bosshardt, '46).

All the the mice, except males fed the 16.3% protein diet, ate 10.4 Cal./day, regardless of the protein level, sex or rate of growth (table 2).

*Minimal protein concentration for growth.* Assuming that the essential amino acids are utilized most economically when the supply is restricted (Osborne, Mendel and Ferry, '19; Mitchell, '23–'24) apparently the diets containing 13.6 to

TABLE 2

*Growth, food intake and protein efficiency in the rat and mouse fed with casein diets*[1]

| Dietary protein | Animals | Mean gain in body weight | Mean food intake | Mean protein intake | Mean gain per gm protein | Mean daily food intake | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Amount | Cal. | Protein |
| *%* | *no.* | *gm* | *gm* | *gm* | *gm* | *gm* | *no.* | *gm* |
| | | | | Rat | | | | |
| *Male* | | | | | | | | |
| 16.7[2] | 12 | 120 | 343 | 57 | 2.1 | 12.2 | 42 | 2.0 |
| 16.3 | 12 | 134 | 321 | 52 | 2.6 | 11.5 | 43 | 1.9 |
| 13.6 | 8 | 132 | 345 | 47 | 2.8 | 12.3 | 46 | 1.7 |
| 11.4 | 9 | 111 | 336 | 38 | 2.9 | 12.0 | 44 | 1.4 |
| 9.4 | 20 | 56 | 221 | 21 | 2.7 | 8.0 | 30 | 0.8 |
| *Female* | | | | | | | | |
| 16.7[2] | 12 | 96 | 318 | 53 | 1.8 | 11.4 | 39 | 1.9 |
| 16.3 | 18 | 103 | 293 | 48 | 2.2 | 10.5 | 39 | 1.7 |
| 13.6 | 15 | 91 | 254 | 35 | 2.6 | 9.1 | 34 | 1.3 |
| 11.4 | 21 | 72 | 238 | 27 | 2.7 | 8.5 | 31 | 1.0 |
| 9.4 | 32 | 56 | 236 | 22 | 2.6 | 8.4 | 31 | 0.8 |
| | | | | Mouse | | | | |
| *Male* | | | | | | | | |
| 16.3 | 9 | 10.3 | 42.1 | 6.9 | 1.5 | 3.0 | 11.1 | 0.49 |
| 13.6 | 12 | 8.2 | 38.7 | 5.3 | 1.5 | 2.8 | 10.4 | 0.38 |
| 11.4 | 12 | 7.2 | 39.5 | 4.5 | 1.6 | 2.8 | 10.4 | 0.32 |
| 9.4 | 12 | 6.2 | 39.9 | 3.7 | 1.7 | 2.8 | 10.4 | 0.26 |
| *Female* | | | | | | | | |
| 16.3 | 9 | 8.2 | 39.2 | 6.4 | 1.3 | 2.8 | 10.4 | 0.46 |
| 13.6 | 12 | 8.0 | 38.7 | 5.3 | 1.5 | 2.8 | 10.4 | 0.38 |
| 11.4 | 12 | 7.9 | 38.9 | 4.4 | 1.8 | 2.8 | 10.4 | 0.31 |
| 9.4 | 24 | 6.2 | 39.4 | 3.7 | 1.7 | 2.8 | 10.4 | 0.26 |

[1] Collection of data: rat, 28 days from the time when the rat weighed 60 gm; mouse, 14 days from the time that the mouse weighed 9 gm.

[2] Data obtained with the minimal protein rice-beans-casein diet (Goettsch '48).

9.4% of protein did not meet the growth requirement of rats. The minimal protein concentration which supported growth in rats, which was equal to that of several generations with an adequate diet, was 16.3%. This diet supplies 17.6% of protein calories or 7.0 mg of total nitrogen/Cal.

The protein requirement for growth in the mouse apparently is less than that in the rat. Since the maximal protein efficiency occurred with the 11.4 and 9.4% protein diets, apparently the 13.6% protein diet contained the minimal protein concentration for adequate growth in the mouse. This diet supplies 14.7% of protein calories or 5.9 mg of total nitrogen/Cal.

*Reproduction in the rat.* The mean age body weight at the time of first estrus and the length of the estrus cycles were within the normal limits with the 16.3 to 11.4% protein diets. With the 9.4% protein diet, a delay was observed in the onset of sexual maturity, with longer intervals between periods of estrus.

Observations of fertility of the male and female rats were not included in table 3 since they were essentially the same as those for the rice-beans-casein diets (Goettsch, '49).

Gestation data using the 16.3 and 13.6% protein diets compared favorably with observations noted when using the minimal protein rice-beans-casein diet, in size of litter, birth weight of young and gain in body weight of female. With restricted protein, the litters contained fewer young, although the birth weight was within normal limits and the female gained less body weight during gestation.

Lactation performance was better with the 16.3% diet than with any of the lower protein diets or with the minimal protein

rice-beans-casein diet. The body weight of young rats 21 days old, and the gain in body weight of female and young during lactation decreased as the protein level decreased. Using the casein diets, regardless of protein level, 98% of the young survived the period of lactation. These results are not in agreement with those observed with the rice-beans-casein diets (Goettsch, '49), in which protein restriction resulted in a reduction in number of survivors.

As with the rice-beans-casein diets (Goettsch, '49) apparently the minimal protein diet which supports gestation and lactation, as when feeding an adequate diet, is also the minimal protein diet for growth.

*Protein intake.* With feeding the 16.3% protein diet, daily protein intake during gestation was 2.3 gm and during lactation 4.1 gm. Compared with the protein intake (1.7 gm) of females during the early growth period, these values represent increases of 30 and 140%, respectively. It will be seen in table 3 that the food consumption during lactation was greater with the casein diets than with the rice-beans casein diet (Goettsch, '49).

*Reproduction in the mouse.* A delay in the onset of sexual maturity was observed in mice fed the 11.4 and 9.4% protein diets, but the length of the estrus cycles was within normal limits regardless of the concentration of protein. As in the case of rats, the mean body weight at the time of first estrus tended to be more constant than the mean age.

The mice of all groups were fertile (greater than 95%).

Gestation was similar with the 16.3 and 13.6% protein diets. At lower levels of protein, although the size of the litter was unchanged, a slight reduction in birth weight occurred as well as reduction in body weight gain of the female.

TABLE 3

*Reproduction, lactation and food intake in the rats fed casein diets*

| | Percentage dietary protein | | | | |
|---|---|---|---|---|---|
| | 16.7[1] | 16.3 | 13.6 | 11.4 | 9.4[2] |
| Estrus | | | | | |
| Mean age at first estrus, *days* | 38.5 | 36.9 | 37.8 | 43.1 | 50.4 |
| Mean weight at first estrus, *gm* | 102.9 | 89 | 89 | 90 | 100 |
| Mean length of cycle, *days* | 5.6 | 5.3 | 5.5 | 5.3 | 7.4 |
| Gestation | | | | | |
| Mean size of litter, *no.* | 8.2 | 9.5 | 9.0 | 8.0 | 6.2 |
| Mean birth weight, *gm* | | | | | |
| Male | 6.0 | 5.4 | 5.5 | 5.4 | 5.6 |
| Female | 5.9 | 5.3 | 5.2 | 5.1 | 5.4 |
| Gain in weight of female, *gm* | 84 | 96 | 91 | 73 | 69 |
| Lactation | | | | | |
| Mean weight at 21 days, *gm* | | | | | |
| Male | 33.7 | 38.8 | 29.3 | 23.6 | 18.4 |
| Female | 33.9 | 36.0 | 31.0 | 24.7 | 17.8 |
| Gain in weight of female and young, *gm* | 106 | 172 | 137 | 59 | 2 |
| Mean daily food intake | | | | | |
| Gestation, 21 days | | | | | |
| Food intake, *gm* | 14.0 | 14.4 | 13.9 | 12.8 | 12.4 |
| Calories, *no.* | 48 | 53 | 51 | 47 | 46 |
| Protein, *gm* | 2.3 | 2.3 | 1.9 | 1.5 | 1.2 |
| Lactation, 17 days | | | | | |
| Food intake, *gm* | 20.7 | 25.2 | 22.2 | 18.2 | — |
| Calories, *no.* | 70 | 93 | 82 | 67 | |
| Protein, *gm* | 3.4 | 4.1 | 3.0 | 2.1 | |

[1] Data obtained with the minimal protein rice-beans-casein diet (Goettsch, '48).

[2] There was great variation in lactation performance, especially in food consumption.

TABLE 4

*Reproduction, lactation and food intake in the mouse fed casein diets*

| | Percentage dietary protein | | | |
|---|---|---|---|---|
| | 16.3 | 13.6 | 11.4 | 9.4 |
| Estrus | | | | |
| Mean age at time of first estrus, *days* | 31.7 | 30.5 | 36.7 | 38.2 |
| Mean weight at time of first estrus, *gm* | 14.8 | 13.6 | 15.3 | 14.4 |
| Mean length of cycle, *days* | 5.6 | 4.9 | 5.2 | 5.5 |
| Gestation | | | | |
| Mean size of litter, *no.* | 7.1 | 7.5 | 7.4 | 7.1 |
| Mean birth weight, *gm* | | | | |
| Male | 1.4 | 1.4 | 1.3 | 1.2 |
| Female | 1.3 | 1.3 | 1.2 | 1.2 |
| Gain in weight of female, *gm* | 15.2 | 15.4 | 13.9 | 11.7 |
| Lactation | | | | |
| Mean weight at 21 days, *gm* | | | | |
| Male | 8.4 | 6.3 | 6.0 | 4.4 |
| Female | 8.2 | 6.5 | 5.6 | 4.4 |
| Gain in weight of female and young, *gm* | 34.7 | 24.3 | 19.0 | 9.0 |
| Mean daily food consumption | | | | |
| Gestation, first 18 days | | | | |
| Food intake, *gm* | 4.2 | 4.0 | 3.8 | 3.6 |
| Calories, *no.* | 15.5 | 14.8 | 14.0 | 13.2 |
| Protein, *gm* | 0.68 | 0.54 | 0.43 | 0.34 |
| Lactation, first 17 days | | | | |
| Food intake, *gm* | 7.1 | 7.6 | 7.2 | 5.2 |
| Calories, *no.* | 26.0 | 28.1 | 26.6 | 19.2 |
| Protein, *gm* | 1.16 | 1.03 | 0.82 | 0.49 |

Lactation performance was best with the highest protein diet, but the young mice appeared obese. With each decrease in protein concentration, a corresponding reduction in the body weight of the young at 21 days of age was observed as well as in body weight gain of females and young.

As judged from the appearance of the mice, the diet containing 13.6% of protein met the minimal protein requirement for reproduction and lactation. It was also the minimal protein diet for growth.

*Protein intake.* With feeding the 13.6% protein diet the daily protein intake during gestation was 0.54 gm and during lactation, 1.03 gm. Compared with the protein intake (0.38 gm) of females during the early growth period, these values represent increases of 40 and 170% respectively.

*Minimal net protein requirement for growth in the rat.* Goettsch ('48) has suggested that the protein requirement for growth be expressed as net protein, a characterization which takes into account the "true" digestibility and biological value of the protein and which would permit the prediction of the minimal requirement of any protein.

The protein requirement for growth in the rat is apparently met by the casein diet containing 16.3% of protein. This diet supplies 17.6% of protein calories or 7.0 mg of total nitrogen/Cal. It will be seen in table 1 that this diet contains 5.0 mg of net nitrogen/Cal., a value confirming that obtained when feeding the rice-beans-casein diets (Goettsch, '48).

In view of the biological variation among rats in growth rate and food intake, the protein requirement may be expressed preferably as ranges rather than as precise values (Mitchell, '44). Since growth of the rats was apparently within the normal limits with the 16.3 and 13.6% protein diets, and since the maximal protein efficiency ratio was induced by the latter, a casein diet containing 15.0% of protein was investigated. This diet was similar to the 16.3% protein diet, except that a dif-

ferent sample of casein was used and 1.6% of the casein was replaced by an equal amount of cornstarch. It was fed to rats with the following results: the average gain in body weight of 12 males was 125 gm, the protein intake, 45.9 and the maximal protein efficiency ratio was 2.7; for 12 females, the respective values were 85, 38 and 2.2 gm. These values lie closer to those obtained with the 16.3% diet (table 2).

It is tentatively suggested that the protein requirement for growth in the rat is met by a diet containing 4.6 to 5.0 mg of net nitrogen/Cal.


SUMMARY

Rats (159) and mice (102) were observed during the periods of growth, reproduction and lactation when fed diets ranging from 16.3 to 9.4% of protein, supplied by crude casein. The animals were fed ad libitum, and food consumption measured.

1. The minimum protein concentration which supports growth, reproduction and lactation, such as that obtained with several generations of rats fed an adequate diet, was 16.3%. This diet supplied 17.6% of protein calories or 7.0 mg of total nitrogen/Cal.

2. The minimum protein concentration which supports adequate growth, reproduction and lactation in the mouse was 13.6%. This diet supplied 14.7% of protein calories or 5.9 mg of total nitrogen/Cal.

3. Since the "true" digestibility and biological value of the casein were 99.2 and 71.7 respectively, the 16.3% protein diet contained 5.0 mg of net nitrogen/Cal.

4. Reasons are given for suggesting tentatively that the protein requirement for growth in the rat is 4.5 to 5.0 mg of net nitrogen/Cal.


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# An Unidentified Water-Soluble Factor in Alfalfa Which Improves Utilization of Vitamin A[1]

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Available data indicate that alfalfa contains a factor (or factors) apparently distinct from any of the known nutrients which accentuated symptoms of hypervitaminosis A in immature rats fed massive but relatively non-toxic doses of this vitamin (Ershoff et al., '57). Both the dried alfalfa juice and the water-washed pulp remaining after the extraction of the juice were active in this respect. Subsequent findings indicated that the water-soluble extract of alfalfa meal had similar activity.[2] The suggestion was made that alfalfa contains an unidentified factor (or factors) which was effective in promoting an increased absorption or utilization (or both) of vitamin A. The above studies were conducted with rats fed excessive amounts of this vitamin. The following investigation was undertaken to determine the effects of a water-soluble extract of alfalfa on rats fed suboptimal amounts of vitamin A.

## PROCEDURE AND RESULTS

The alfalfa extract used in the present experiment was prepared as follows:[3] alfalfa leaf meal was introduced into a pressure vessel along with water in a ratio of one gallon per pound of meal. The slurry was autoclaved at 15 pounds of steam pressure for two hours. The liquid was drained off and a rinse of the residue was made by adding one-fourth the volume of water used in the autoclaving step. After the rinse liquid was collected and combined with the first fraction, the total extract was concentrated to 8.5% of its volume The syrup was then dried to a solid on a drum dryer and passed through a hammer-mill. The resulting material had the following percentage composition: moisture, 7.16; crude fat (ether extract), 0.55; crude fiber, 0.36; crude protein (3.55% N $\times$ 6.25), 22.18; ash (mineral matter), 22.35; and nitrogen-free extract (by difference), 47.4. The carotene content of this material (measured as β-carotene) was 0.59 ppm.[4] Since one international unit (I.U.) of provitamin A is equivalent to 0.6 μg of β-carotene, the alfalfa extract used in the present experiment (assuming that all carotenoid present was β-carotene) contained approximately 1 I.U. of provitamin A/gm. Alfalfa, however, contains carotenoids other than β-carotene and these are less active than β-carotene as sources of provitamin A. The actual provitamin A potency of the alfalfa extract, therefore, in terms of its carotenoid content was less than 1 I.U./gm. In the present report observations are presented on (1) the effects of the alfalfa extract indicated above on the response of vitamin A-depleted rats fed suboptimal amounts of vitamin A; (2) the effects of the alfalfa extract described above on time required for depletion, the maximum weight attained and the length of survival of immature rats placed at weaning on a highly purified diet deficient in vitamin A;

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[2] Ershoff, B. H., unpublished data.

[3] The alfalfa extract was kindly provided by the Research and Development Division of Nutrilite Products Inc., Buena Park, California.

[4] Carotene was determined by the procedure outlined in Official Methods of Analysis of the Association of Official Agricultural Chemists, ed. 8, 1955, p. 816.

(3) the comparative effects of a water-soluble extract of alfalfa and graded levels of β-carotene and vitamin A palmitate on the growth and survival of immature male rats; and (4) the comparative effects of a water-soluble alfalfa extract and supplements of the known nutrients on the weight increment and length of survival of immature rats placed at weaning on a highly purified diet deficient in vitamin A.

*Experiment 1. Effects of a water-soluble extract of alfalfa on the response of vitamin A-depleted rats fed sub-optimal amounts of vitamin A*

Thirty female rats of the Holtzman strain, which had been raised from weaning on a natural food stock ration, were selected at three to 4 months of age and bred to normal males. Litters were reduced to 7 at 5 days of age, and on the 10th day mothers and litters were placed on a vitamin A-free basal diet having the following percentage composition: sucrose, 66; casein[5], 24; salt mixture[6], 5; hydrogenated cottonseed oil, 3; and cottonseed oil, 2. To each kilogram of the above diet the following vitamins were added: thiamine-HCl, 20 mg; riboflavin, 20 mg; pyridoxine-HCl, 20 mg; Ca pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin $B_{12}$, 150 μg; 2-methyl, 1, 4 naphthoquinone, 5 mg; choline chloride, 2 gm; vitamin $D_2$, 500 U.S.P. units; and 250 mg of mixed tocopherols[7] equivalent to 186.25 I.U. of vitamin E. The vitamins were added in place of an equal amount of sucrose. The young rats were weaned when 21 days old and the males continued on the above diet until they were depleted of vitamin A. The end of the depletion period was set as the 5th day on which rats on the vitamin A-free diet showed stationary or decreasing weight. The average time of depletion was 24 days post-weaning and average body weight at depletion was 108 gm. At this time 60 male rats ranging from 88 to 120 gm in body weight were divided into 6 comparable groups of 10 animals each, with all animals in each group derived from a different litter. Three of the groups were fed the basal vitamin A-free diet indicated above supplemented with 1% alfalfa extract and zero, 250 or 500 U.S.P. units of vitamin A/kg of diet. The group designations $A_1$, $A_2$ and $A_3$ were given to these three groups, respectively The alfalfa extract was used in the above diets in place of an equal amount of sucrose. The remaining three groups were fed the basal vitamin A-free diet plus 6 μg of β-carotene/kg of diet (the equivalent of 10 I.U. of provitamin A) and zero, 250 or 500 U.S.P. units of vitamin A/kg of ration. The group designations $B_1$, $B_2$ and $B_3$ were given to these groups, respectively. The 6 μg of β-carotene which was added per kilogram of diet is the same amount of carotenoids as supplied by the alfalfa extract when fed at a 1% level. The vitamin A in the above diets was supplied in the form of synthetic vitamin A palmitate in corn oil[8] which was thoroughly mixed in appropriate amounts with the cottonseed oil of the diet at the time rations were prepared. Beta-carotene was supplied by mixing the crystalline material with casein at the time diets were prepared. Diets were made up weekly and stored under refrigeration. Animals were placed in metal cages with raised screen bottoms (2 rats per cage) and fed daily ad libitum the diets indicated above. All food not consumed 24 hours after feeding was discarded. These measures were used to minimize oxidative changes in the diet. Animals that died during the first week on experimental diets were not included in the tabulation of data but were replaced by depleted animals of comparable weight. Feeding was continued for 28 days or until death whichever occurred earlier. Results are summarized in table 1.

[5] Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[6] Hubbell, Mendel and Wakeman Salt Mixutre, General Biochemicals, Inc., Chagrin Falls, Ohio.

[7] Mixed Tocopherols Concentrate N.F., Type 4–50, Distillation Products Industries, Rochester, New York.

[8] Synthetic vitamin A palmitate in corn oil (1 million U.S.P. units/gm) obtained from Hoffman-La Roche, Inc., Nutley, New Jersey. The vitamin A content of this material was determined chemically prior to mixing in the diet to assure that no loss of potency had occurred.

TABLE 1

*Effects of a water-soluble extract of alfalfa on the weight increment of vitamin A-depleted male rats fed suboptimal amounts of vitamin A (10 animals per group)*

| Dietary group | Vitamin A supplement per kg/diet | Survival[1] | Av. weight increase after 28 days of feeding[2] |
|---|---|---|---|
| | *U.S.P. units* | *%* | *gm* |
| Vitamin A-free + 1% alfalfa extract | 0 | 60 | 5.2 ± 7.8 |
| Vitamin A-free + 10 I.U. provitamin A/kg/diet[3] | 0 | 0 | |
| Vitamin A-free + 1% alfalfa extract | 250 | 100 | 114.0 ± 8.6 |
| Vitamin A-free + 10 I.U. provitamin A/kg/diet[3] | 250 | 100 | 70.1 ± 7.9 |
| Vitamin A-free + 1% alfalfa extract | 500 | 100 | 122.4 ± 7.2 |
| Vitamin A-free + 10 I.U. provitamin A/kg/diet[3] | 500 | 100 | 123.1 ± 4.4 |

[1] Experimental period, 28 days.

[2] Including standard error of the mean calculated as follows:

$$\sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}{n-1}} / \sqrt{n}$$

where "X" equals the weight gained and "n" is the number of observations.

[3] Beta-carotene was added at a level of 6 µg (which is the equivalent of 10 I.U. provitamin A)/kg of diet. This is the amount of carotenoids supplied by the alfalfa extract when fed at a 1% level in the diet.

Findings indicate that the water-soluble alfalfa extract when incorporated at a 1% level in the basal vitamin A-free diet significantly increased the average survival time of rats depleted of vitamin A. Whereas none of the rats fed diet $B_1$ (the basal vitamin A-free diet plus 6 µg of β-carotene/kg of diet) survived for 28 days, 60% of the rats fed diet $A_1$ (the basal vitamin A-free diet plus 1% of water-soluble alfalfa extract) were still alive after 28 days of feeding when the test was discontinued. The two groups indicated above also differed in weight response. All the rats administered diet $B_1$ lost weight from the first day of feeding. Of the 10 rats fed diet $A_1$, however, 8 gained weight during the experimental period. The average maximum weight increase was 20.2 gm (range 9 to 28 gm). Six of the 8 rats indicated above which gained weight exhibited their maximum weight increase during the third week of feeding after which they rapidly lost weight; the remaining two animals in this group were still gaining at the time feeding was discontinued.

The water-soluble extract of alfalfa when incorporated at a 1% level in the diet also increased significantly the average weight increment of vitamin A-depleted rats fed a diet containing a suboptimal amount of vitamin A. Thus, the average weight increment of rats fed diet $A_2$ (which contained 1% of water-soluble alfalfa extract and 250 U.S.P. units of vitamin A/kg of diet) was 114.0 ± 8.6 gm in contrast to an average weight increment of 70.1 ± 7.9 gm for rats fed a comparable diet with the alfalfa extract omitted (diet $B_2$). At a higher level of vitamin A supplementation (namely, 500 U.S.P. units/kg of diet) no significant difference was observed between the weight increment of rats fed diets with or without the alfalfa extract.

*Experiment 2. Effects of a water-soluble extract of alfalfa on time required for depletion, the maximal weight attained and the length of survival of immature rats placed at weaning on a highly purified diet deficient in vitamin A*

Twenty-four female rats of the Holtzman strain which had been raised from weaning on a natural food stock ration were bred to normal males when approximately 4 months old. Litters were reduced to 7 at 5 days of age and on the 10th day mothers and litters were placed on the basal vitamin A-free diet used in experiment 1. The young were weaned when 21 days old and divided into 5 comparable groups consisting of 10 male and 6 female rats per group with all animals in each group derived from a different litter. Animals were placed in metal cages with raised screen bottoms (2 rats per cage) and fed the basal vitamin A-free diet plus the following supplements: group 1, 6 µg of β-carotene (10 I.U. of provitamin A)/kg of diet; group 2, 15 µg of β-carotene (25 I.U. of provitamin A) /kg of diet; group 3, 50 µg of β-carotene (83.3 I.U. of provitamin A) /kg of diet; group 4, 1% of water-soluble alfalfa extract; and group 5, 2.5% of water-soluble alfalfa extract. The 6 µg and 15 µg of β-carotene added per kilogram of diet in groups 1 and 2 are the same amounts of carotenoids as supplied by the alfalfa extract when fed at a 1% and 2.5% level, (groups 4 and 5, respectively). The diet fed group 3 contained over three times as much carotene as that provided by the highest dose of alfalfa extract (group 5). Crystalline β-carotene and the alfalfa extract were used in the above diets in place of an equal amount of sucrose. Animals were fed these diets ad libitum and observations made as to time of depletion (namely, the 5th day on which rats showed stationary or decreasing body weight), the maximal weight increase attained and length of survival. Feeding was continued for 300

TABLE 2

*Effects of a water-soluble extract of alfalfa on the weight increment, depletion time and length of survival of immature rats fed a vitamin A-free diet*

| Group | Supplements/kg diet | Number of animals | Time on diet until depletion | Maximum weight increase | Average survival time[1] |
|---|---|---|---|---|---|
| | | | *days* | *gm* | *days* |
| | | Male rats | | | |
| 1 | 6 µg β-carotene (10 I.U. provitamin A) | 10 | 31.3 | 110.2 | 47.6 ± 2.4 |
| 2 | 15 µg β-carotene (25 I.U. provitamin A) | 10 | 31.3 | 115.9 | 52.6 ± 2.6 |
| 3 | 50 µg β-carotene (83.3 I.U. provitamin A) | 10 | 29.6 | 114.2 | 51.3 ± 1.9 |
| 4 | 10 gm alfalfa extract containing 5.9 µg carotene | 10 | 50.9 | 168.3 | 76.3 ± 7.2 |
| 5 | 25 gm alfalfa extract containing 14.8 µg carotene | 10 | 71.5 | 236.5 | 116.3 ± 9.3 |
| | | Female rats | | | |
| 1 | 6 µg β-carotene (10 I.U. provitamin A) | 6 | 34.7 | 98.5 | 50.7 ± 2.2 |
| 2 | 15 µg β-carotene (25 I.U. provitamin A) | 6 | 35.8 | 90.3 | 50.3 ± 2.8 |
| 3 | 50 µg β-carotene (83.3 I.U. provitamin A) | 6 | 34.9 | 93.1 | 53.9 ± 2.1 |
| 4 | 10 gm alfalfa extract containing 5.9 µg carotene | 6 | 50.0 | 121.3 | 81.4 ± 6.0 |
| 5 | 25 gm alfalfa extract containing 14.8 µg carotene | 6 | 96.2 | 189.5 | 210.7 ± 20.5[2] |

[1] Including standard error of the mean. See footnote 2, table 1.

[2] One rat in this group was still alive after 300 days on diet.

days or until death, whichever occurred earlier. Results are summarized in table 2.

Findings indicate that the water-soluble alfalfa extract when fed at levels of one or 2.5% of the diet significantly increased the length of time required for depletion, the maximum weight increment attained and the length of survival of both male and female rats fed a highly purified diet deficient in vitamin A. Results were more pronounced at the 2.5 than the 1% level of supplementation. The effects of the water-soluble alfalfa extract were not due to its carotenoid content, inasmuch as β-carotene, when fed at levels corresponding to the amounts present in the 1% and 2.5% alfalfa concentrate supplements was far less active than the alfalfa extract in this regard. That the activity of the water-soluble alfalfa extract was not due to its carotenoid content is indicated further by the fact that a supplement of this material when fed at a 1% level in the diet was significantly more active than β-carotene even when the latter was fed at a level of 50 μg/kg of diet which was over 8 times the carotenoid content of the 1% alfalfa extract supplement. Similar results were obtained with both male and female rats.

*Experiment 3. Comparative effects of a water-soluble extract of alfalfa and graded levels of β-carotene and vitamin A palmitate on the growth and survival of immature male rats*

Ninety-one partially depleted male rats of the Holtzman strain similar to those used in experiment 2 were weaned when 21 days old and divided into 13 comparable groups of 7 animals each. One group was fed the basal vitamin A-free diet; the remaining groups were fed the basal vitamin A-free diet plus the supplements indicated in table 3. The supplements were added in place of an equal amount of sucrose. Animals were placed in metal cages with raised screen bottoms (2 rats per cage) and fed the various rations ad libitum for 134 days or until death, whichever occurred earlier. Observations of weight increment and length of survival of the various groups are summarized in table 3.

In agreement with experiment 2, the results indicate that the water-soluble alfalfa extract when fed at levels of 1% or 2.5% of the diet gave a significant increase in weight and length of survival of immature male rats fed the basal vitamin A-free ration. Crystalline β-carotene, however, when fed at levels of 30 μg (50 I.U. of provitamin A) or 60 μg (100 I.U. of provitamin A) /kg of diet was without significant activity in this respect. Since the alfalfa extract, when fed in the above amounts, provided only 5.9 and 14.8 μg of carotene, respectively, per kilogram of diet, its protective effect was apparently caused by some factor other than its carotenoid content. The possibility that the negative results obtained with β-carotene at the above dose levels resulted from an impaired utilization of this material in the absence of the alfalfa extract appears to be ruled out by the observation that vitamin A palmitate when fed at comparable levels (i.e., 50 or 100 U.S.P. units of vitamin A/kg of diet) gave results similar to those obtained with the crystalline β-carotene material. At higher levels of β-carotene and vitamin A palmitate administration, effects were obtained which equaled or surpassed that obtained with the alfalfa extract. A supplement of 120 μg of β-carotene (200 I.U. of provitamin A) or 200 U.S.P. units of vitamin A/kg of diet gave results comparable to those obtained with the alfalfa extract at a 2.5% level of feeding. Higher levels of β-carotene (namely, 400 and 800 I.U. provitamin A/kg of diet) and vitamin A palmitate (400 and 800 U.S.P. units vitamin A/kg of diet) resulted in increased body weight and an average survival time significantly greater than that obtained with the alfalfa extract

*Experiment 4. Comparative effects of a water-soluble alfalfa extract and supplements of the known nutrients on the weight increment and length of survival of rats placed at weaning on a diet deficient in vitamin A*

The experimental procedure was similar to that used in experiment 2. Male rats, the mothers of which were fed the basal

TABLE 3

*Comparative effects of a water-soluble extract of alfalfa and graded levels of β-carotene and vitamin A palmitate on the growth and survival of immature male rats (7 rats per group)*[1]

| Supplements fed with basal vitamin A-free diet | Gain in body weight after following days of feeding | | | | | Average survival time[2,3] | Survival |
|---|---|---|---|---|---|---|---|
| | 28 | 56 | 84 | 112 | 134 | | |
| | *gm* | *gm* | *gm* | *gm* | *gm* | *days* | % |
| None | 119 | | | | | 48.4 ± 1.7 | 0 |
| 10 gm alfalfa extract containing 5.9 μg carotene | 126 | 151 | 177 (2)[4] | 156 (1) | | 72.5 ± 4.8 | 0 |
| 25 gm alfalfa extract containing 14.8 μg carotene | 146 | 213 | 222 (6) | 229 (4) | 201 (3) | 110.8 ± 6.6 | 42.9 |
| 50 I.U. provitamin A (30 μg β-carotene) | 123 | 79 (2) | | | | 51.7 ± 3.1 | 0 |
| 50 units vitamin A[5] | 99 | 83 (4) | | | | 48.8 ± 2.1 | 0 |
| 100 I.U. provitamin A (60 μg β-carotene | 84 | 89 (4) | | | | 60.4 ± 2.8 | 0 |
| 100 units vitamin A | 123 | 105 (2) | | | | 52.3 ± 3.4 | 0 |
| 200 I.U. provitamin A (120 μg β-carotene) | 151 | 173 | 262 | 265 (6) | 225 (1) | 125.1 ± 3.1 | 14.3 |
| 200 units vitamin A | 127 | 161 | 174 (4) | 174 (1) | 0 | 94.8 ± 7.0 | 0 |
| 400 I.U. provitamin A (240 μg β-carotene) | 156 | 269 | 315 | 316 | 325 | 134 ± 0 | 100 |
| 400 units vitamin A | 145 | 240 | 293 | 285 | 362 | 134 ± 0 | 100 |
| 800 I.U. provitamin A (480 μg β-carotene) | 159 | 262 | 320 | 335 | 352 | 134 ± 0 | 100 |
| 800 units vitamin A | 197 | 297 | 356 | 376 | 405 | 134 ± 0 | 100 |

[1] Average initial body weight of rats in the various groups ranged between 44.0 and 47.6 gm.

[2] Data were calculated on the basis of an average survival time of 134 days for animals alive at termination of experiment.

[3] Including standard error of the mean. See footnote 2, table 1.

[4] The values within parentheses indicate the number of animals alive at the time and on which averages are based, and represent only those groups where the number per group differs from the number started.

[5] Synthetic vitamin A palmitate in corn oil (1 million U.S.P. units/gm), obtained from Hoffman-La Roche, Inc., Nutley, New Jersey.

vitamin A-free diet from the 10th day of lactation, were weaned when 21 days old and divided into 4 comparable groups of 8 animals each, with all animals in each group derived from a different litter. Group A was fed the basal vitamin A-free diet supplemented with 15 μg of β-carotene/kg of ration; group B received the basal vitamin A free diet plus 2.5% of the water-soluble alfalfa extract; group C received the basal vitamin A-free diet plus 15 μg of β-carotene/kg of diet plus the following supplements per kilogram of ration: casein[5], 12 gm; salt mixture[6], 2.5 gm; cottonseed oil, 2.5 gm; thiamine-HCl, 1 mg; riboflavin, 1 mg; pyridoxine-HCl, 1 mg; Ca pantothenate, 3 mg; nicotinic acid, 5 mg; ascorbic acid, 10 mg; biotin. 200 μg; folic acid, 500 μg; *p*-aminobenzoic acid, 20 mg; inositol, 40 mg; vitamin $B_{12}$ 7.5 μg; 2-methyl-1, 4 naphthoquinone, 250 μg; choline chloride, 100 mg; vitamin $D_2$, 25 U.S.P. units; and mixed tocopherals[7], 12.5 mg; and group D received the basal vitamin A-free diet plus 5000 U.S.P. units of vitamin A[8] in the form of vitamin A palmitate per kilogram of diet. The supplements in group C were added in amounts equal to or exceeding the quantity of such nutrients supplied by the alfalfa extract when fed at a 2.5% level in the diet. All supplements were fed in place of an equal amount of sucrose. Feeding was continued ad libitum for 60 days or until death, whichever occurred earlier. All rats in groups A and C were depleted within 30 days, as judged by stationary or decreasing body weight for a 5-day period and the onset of ocular symptoms characteristic of vitamn A deficiency. The average survival time of these rats was 44 and 42 days, respectively, for groups A and C. Subsequent findings indicated that a supplement of 1% of lemon bioflavonoid complex also had no significant effect on rate of depletion or length of survival under comparable experimental conditions. In contrast, all rats in groups B and D survived the experimental period of 60 days during which time they showed a continuous increase in body weight and appeared grossly normal in all respects. These observations indicated that the active factor in the water-soluble alfalfa extract is distinct from any of the known nutrients.

## DISCUSSION

Present findings indicate that alfalfa contains a water-soluble factor apparently distinct from any of the known nutrients which significantly improved vitamin A utilization in the rat. This is indicated by demonstration that the oral administration of a water-soluble extract of alfalfa prolonged the survival time of rats depleted of vitamin A and increased significantly the average weight increment of rats depleted of vitamin A and subsequently fed a suboptimal amount of this vitamin. In addition, findings indicate that the water-soluble alfalfa extract increased the length of time required for depletion, the maximum weight increment attained and the length of survival of rats fed a purified diet deficient in vitamin A. The activity of the alfalfa extract was not due to its carotenoid content and was not duplicated by supplements of the known nutrients when fed in amounts equal to or exceeding the quantity of such nutrients provided by the alfalfa extract. The latter, however, could not completely replace carotene or vitamin A in the diet as evidenced by the fact that rats fed the basal vitamin A-free diet supplemented with the alfalfa extract eventually plateaued in body weight and developed symptoms of vitamin A deficiency.

That unidentified factors exist in natural foodstuffs which promote the utilization of administered vitamin A has been reported by a number of investigators. Palm kernel meal, coconut cake, acetone-extracted herring roe (Tainsh and Wilkinson, '39; Gridgeman et al., '40), soybean lecithin (Slanetz and Scharf, '43, '45), yeast (Patrick and Morgan, '43), fish solubles (Harmes et al., '56a, b), alfalfa and other succulent plants (Ershoff et al., '57), liver (Ershoff et al., '57) and other substances including the tocopherols (Moore, '40; Davies and Moore,

[5] See footnote 5, page 314.
[6] See footnote 6, page 314.
[7] See footnote 7, page 314.
[8] See footnote 8, page 314.

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'41; Hickman et al., '44), xanthophyll[9], aureomycin (Murray and Campbell, '55a, b), *N*, $N^1$-diphenyl-*p*-phenylenediamine (DPPD) (Matterson et al., '55; Cox et al., '57) and bioflavonoids (Ershoff, '57) have all been shown to improve the utilization of administered vitamin A as judged by various indices in experimental animals. Observations noted in the present experiment indicate that alfalfa contains a water-soluble factor which improved utilization of *endogenous* vitamin A as well. It is becoming increasingly apparent that dietary factors exist which are not themselves essential nutrients but which promote an increased utilization of such nutrients by the body. Present findings indicate that alfalfa contains such a factor in respect to vitamin A. Further studies are indicated to determine the value of this factor, not only in vitamin A-deficiency states resulting from the ingestion of diets deficient in this vitamin, but also in those caused by an impaired absorption or utilization of this nutrient as well.

## SUMMARY

Supplements of a water-soluble extract from alfalfa increased the length of time required for depletion, the maximum weight increment attained and the length of survival of rats fed a purified diet deficient in vitamin A. The alfalfa extract also prolonged the survival time of rats depleted of vitamin A and caused weight increase in such rats following administration of a suboptimal amount of vitamin A. It could not, however, replace completely the carotene or vitamin A in the diet as evidenced by the fact that the weight of rats fed the basal vitamin A-free diet supplemented with the alfalfa extract eventually plateaued and the rats developed symptoms of vitamin A deficiency. The active factor (or factors) in alfalfa is distinct from any of the known nutrients.

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# The Effect of Scurvy on Thyroid Activity in the Guinea Pig[1]

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In studies of scurvy in the guinea pig, many diverse symptoms have been noted. Among the early observations was the effect of scurvy on metabolic rate. Fidlar and coworkers ('39), Mosonyi and Rigo ('33), Hamne ('41) and other groups reported that oxygen consumption was increased in the ascorbic acid depleted guinea pig. Other workers were unable to detect any change in oxygen consumption and several reported decreased values (Söderström and Törnblom, '33; Scoz et al., '37; Baucke, '39). While the observations of McHenry and others suggested increased thyroid activity, histological examinations of the thyroid glands gave no support to the hypothesis (MacLean, Sheppard and McHenry, '39); these findings were similar to those of Löwy ('23). Other groups have reported various types of enlargement of the thyroid gland in scurvy (McCarrison, '19; Bessesen, '22; Harris and Smith, '28; Schulze and Linneman, '38).

It is likely that one of the causes of the conflicting results in the early studies was the diversity of crude diets employed. Undoubtedly some of these diets produced multiple deficiencies rather than simple ascorbic acid depletion. Recently, purified semi-synthetic diets have been designed for the feeding of guinea pigs. It seemed worthwhile to reinvestigate the problem of thyroid activity in scurvy, using these newer diets.

## METHODS

The diet employed was basically that of Reid and Briggs ('53). It consisted in percentage composition of casein (vitamin-free), 26%; vitamin powder (in casein),[2] 4%; sucrose, 10.3%; glucose, 7.8%; corn starch, 20%; α-cellulose,[3] 15.0%; salts mixture,[1] 9.0%; corn oil, 7.3%; choline chloride, 0.2%; inositol, 0.2%; and the following amounts of the fat soluble vitamins per kilogram of diet: vitamin D, 1600 I. U. vitamin A, 17000 I.U. vitamin E, 20 I.U.; menadione, 2.0 mg. This diet supplemented with 5 mg of ascorbic acid per day, produced continuous weight gains for at least 6 months when fed to young guinea pigs. These animals appeared comparable in health to chow-fed animals and did not show intestinal upset. If vitamin C was omitted from the diet, signs of scurvy began to appear within 10 to 15 days and the animals usually died within 20 to 25 days. The signs of scurvy were similar to those reported in the literature, including diarrhea, loss of appetite, stiffening of the hind quarters and at autopsy, enlargement of the costochondral junction, hemorrhages at the "knee" joint and local subcutaneous sites and enlarged adrenal glands. By giving the animals a daily dose of 0.2 mg of ascorbic acid, it was possible to produce the early symptoms of scurvy within two to three weeks, but to keep the animals alive, in a "chronic deficiency" state for two to three months.

In the experiments to be reported, young, in-bred guinea pigs of both sexes from the Connaught Medical Research Laboratories' colony were used. The animals were housed in individual screen-bottom cages and provided with water ad libitum. Pair-feeding of the control

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[1] Supported by a grant from the National Research Council of the Government of Canada.

[2] The vitamin powder and salts mixture were as described by Reid and Briggs ('53), except that p-aminobenzoic acid was incorporated at a level of 2 gm/800 gm of vitamin powder.

[3] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

groups was instituted in some of the experiments, as noted, to avoid discrepancies owing to possible metabolic effects of inanition and to avoid gross weight differences in oxygen uptake studies. All groups received the ascorbic acid-free diet. Control groups were given orally 5 mg of ascorbic acid in water per day. Depleted groups were given either 0.2 to 0.4 mg of ascorbic acid orally (chronic studies) or no ascorbic acid (acute studies). The animals were fed daily and weighed at least once a week in chronic studies and twice a week in acute studies. The diet was kept refrigerated prior to feeding.

Oxygen consumption was determined in an apparatus similar to that described by Schabe and Griffeth ('38), which provided a continuous recording of oxygen consumption. Carbon dioxide was absorbed on soda lime placed in the animal chamber; mercury was substituted for barium hydroxide in the pump to provide circulation of air within the chamber. Determinations were carried out over a 25 to 30-minute period following a 10-minute equilibration period; if the graphic recording was not linear, the determination was repeated. The metabolic rate was calculated as cubic centimeters per square meter per hour, using the following formula for surface area: $A = 12.54\ W^{0.60}$, where $A$ = surface area in square centimeters, and $W$ = body weight in grams. Results were expressed also as cubic centimeters of oxygen consumed per hour, and per kilogram of body weight per hour.

Urinary tyrosine excretion was determined by the method of Medes ('32), using "urine blanks" without added sodium nitrite, to correct for cloudiness, instead of recentrifugation as suggested by Medes. Tubes were read 2 to 3 minutes after the addition of the sodium nitrite. The guinea pigs were given an oral dose of 100 mg of *l*-tyrosine in 2 ml of water and were then placed in metabolism cages, without food for 18 hours. The urine was collected, in the presence of sulphuric acid, under mineral oil. Before the urine was assayed for tyrosyl compounds, it was diluted and clarified with Lloyd's reagent.

In one study, carrier-free radioiodine was injected intraperitoneally and thyroid radioactivity was observed by placing the animal on a stage with the thyroid region above the opening of a vertically mounted, collimated scintillation counter. An estimation of the radioactivity was obtained from an attached count-rate meter. Measurements of radioactivity in the abdomen and thigh were negligible two days after the injection of iodine$^{131}$; the neck counts reflected the thyroid radioactivity rather than that of the blood or tissues. In the third of the three radioiodine studies, a standard solution of the radioactive isotope was prepared so that daily comparison of the observed count-rate of the neck and of the standard solution could be made. The standard was so constructed that the results of this study could be expressed as "percentage uptake." In this experiment, 0.6 μc of radioiodine were injected. The standard consisted of 0.3 μc of radioiodine in 5 ml of saline contained in a 1.5 cm internal diameter vial mounted 0.5 cm above the platform of the scintillation counter; the count-rate of the standard was multiplied by two before the observed values were compared to it.

In a series of 6 separate experiments, the metabolic effects of both chronic and acute scurvy were studied in the guinea pig. The results of three experiments are presented to illustrate the main findings.

*Experiment 1.* Young guinea pigs were divided into a depleted and a control group of 7 animals each. The groups consisted of males and females and were balanced with respect to an initial average body weight of 156 gm. Both groups were fed ad libitum, but the depleted group received no ascorbic acid. The average body weights of the depleted and control groups were as follows: at two weeks, 204 and 270 gm, respectively; at three weeks, 186 and 306 gm respectively. After three weeks of deprivation the experiment was terminated owing to the scorbutic condition and probable imminent death of the ascorbic acid-depleted animals.

*Experiment 2.* In experiments 2 and 3, the effects of chronic scurvy were studied. In experiment 2, the groups, comprised of 5 male and 5 female guinea pigs each, had an initial average body weight of 255 gm. The weight gains of the depleted, pair-fed control and ad libitum-fed control groups are shown in figure 1. All

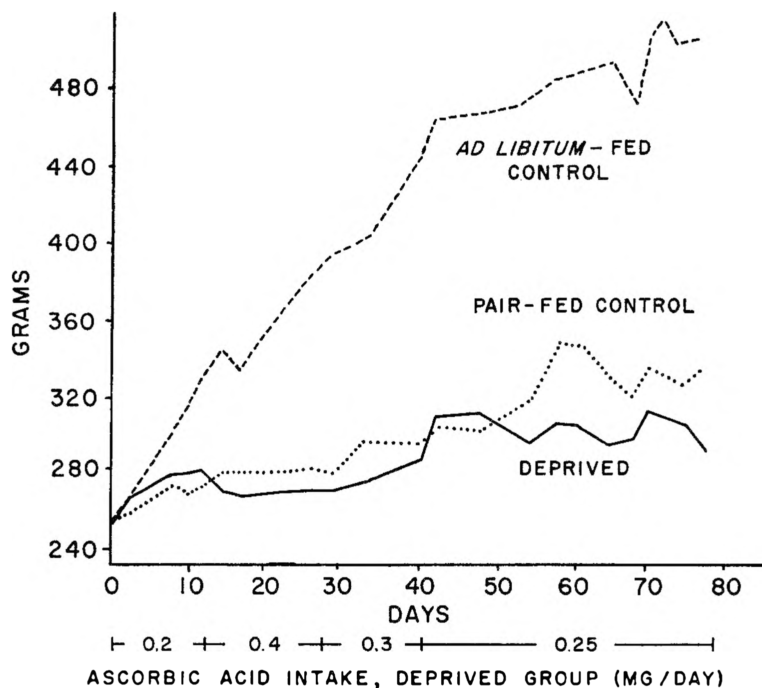


**Fig. 1** Body weight changes in chronic scurvy (exp. 2). All control animals received 5 mg of ascorbic acid/day; the deprived group received the amounts shown.

of the control animals received 5 mg of ascorbic acid per day. The ascorbic acid intake of the depleted animals was varied between 0.2 and 0.4 mg/day in an attempt to maintain a chronic scorbutic condition as judged by weight gains; the actual amounts given are shown in figure 1.

*Experiment* 3. In the third experiment, all of the animals were deprived of dietary iodine for a period of two and a half months before the deprivation of ascorbic acid began; during this period, all of the animals were fed the synthetic diet without added potassium iodide, supplemented with 5 mg of ascorbic acid per day. The purpose of this deprivation was to avoid saturation of the thyroid gland with dietary iodine and the subsequent low uptake of injected radioiodine. No attempt was made to restrict the iodine obtained in the tap water used for drinking or as a contaminant of other dietary constituents, nor was the intake from these sources measured. No signs of iodine deficiency were apparent nor was any effect upon metabolic rate noted; apparently sufficient iodine was ingested to prevent undue elevation of the iodine uptake figures as subsequently determined. The omission of dietary iodine was continued through the experiment until 4 days after the last injection of radioiodine had been given. The initial weight of the animals at the beginning of the ascorbic acid depletion was 575 gm; the groups consisted of 7 males and 7 females. The ascorbic acid depleted animals received 0.2 mg of ascorbic acid, whereas the pair-fed controls received 5.0 mg of the vitamin.

## RESULTS

### *Oxygen consumption*

The results of the oxygen uptake studies carried out in experiments 1, 2 and 3 are shown in table 1. Until the 6th week of experiment 2, the animals were not fasted before the oxygen uptake measurements were made. After that time, all animals were fasted at least 18 hours. Apparently both acute and chronic ascorbic acid deprivation will increase oxygen consumption. The results in the acute experiments were more variable than those in the

TABLE 1

*Oxygen consumption in scurvy*[1]

| Time on diet | Ascorbic acid[2] intake of depleted group | $O_2$/hour | | | $O_2$/kg/hour | | | $O_2/m^2$/hour | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Depleted | Pair-fed | Ad libitum-fed | Depleted | Pair-fed | Ad libitum-fed | Depleted | Pair-fed | Ad libitum-fed |
| *weeks* | *mg/day* | | | | | | | | | |
| | | Experiment 1, acute scurvy | | | | | | | | |
| 2 | 0 | 255 ± 10 | — | 262 ± 28 | 1250 ± 72 | — | 970 ± 67[3] | 117 ± 5 | — | 107 ± 9 |
| 3 | 0 | 210 ± 16 | — | 276 ± 30 | 1130 ± 65 | — | 900 ± 78[4] | 102 ± 7 | — | 100 ± 9 |
| | | Experiment 2, chronic scurvy | | | | | | | | |
| 2 | 0.4 | 355 ± 13 | 320 ± 23 | 360 ± 21 | 1250 ± 48 | 1120 ± 84 | 1130 ± 67 | 135 ± 6 | 120 ± 10 | 129 ± 8 |
| 3 | 0.4 | 380 ± 25 | 325 ± 16 | 365 ± 19 | 1350 ± 57 | 1156 ± 52[3] | 1030 ± 43[4] | 148 ± 4 | 125 ± 6[3] | 124 ± 6[4] |
| 4 | 0.4 | 330 ± 24 | 270 ± 15[3] | 375 ± 10 | 1185 ± 50 | 935 ± 55[4] | 1015 ± 25[4] | 120 ± 7 | 103 ± 6[3] | 125 ± 3 |
| 5 | 0.3 | 325 ± 17 | 290 ± 13 | 400 ± 16[4] | 1205 ± 93 | 1005 ± 58 | 992 ± 36[5] | 129 ± 7 | 111 ± 6 | 127 ± 4 |
| 6 | 0.25 | 310 ± 10 | 280 ± 9 | 365 ± 10[4] | 1175 ± 57 | 945 ± 29[4] | 890 ± 30[4] | 125 ± 3 | 105 ± 3[4] | 114 ± 4 |
| 8 | 0.25 | 300 ± 16 | 285 ± 11 | 370 ± 22[4] | 1020 ± 39 | 930 ± 36 | 810 ± 25[4] | 113 ± 3 | 105 ± 4 | 107 ± 4 |
| 9 | 0.25 | 345 ± 22 | 300 ± 12 | 370 ± 7 | 1130 ± 40 | 900 ± 36[4] | 790 ± 14[4] | 127 ± 4 | 105 ± 4[4] | 107 ± 2[4] |
| 12 | 0.25 | 265 ± 15 | 250 ± 9 | 375 ± 10[4] | 900 ± 33 | 760 ± 58 | 740 ± 27[4] | 100 ± 3 | 89 ± 3[5] | 95 ± 5 |
| | | Experiment 3, chronic scurvy, iodine depleted | | | | | | | | |
| 2 | 0.2 | 470 ± 14 | 420 ± 10 | — | 805 ± 28 | 810 ± 30 | — | 114 ± 4 | 112 ± 3 | — |
| 3 | 0.2 | 405 ± 15 | 365 ± 10[5] | — | 720 ± 29 | 685 ± 20 | — | 100 ± 3 | 95 ± 3 | — |
| 4 | 0.2 | 415 ± 15 | 345 ± 11[4] | — | 725 ± 23 | 665 ± 24[4] | — | 102 ± 3 | 91 ± 3[3] | — |
| 5 | 0.2 | 400 ± 15 | 345 ± 14[3] | — | 731 ± 33 | 670 ± 23[5] | — | 101 ± 4 | 91 ± 3 | — |
| 6 | 0.2 | 410 ± 11 | 340 ± 6[4] | — | 780 ± 34 | 702 ± 16[5] | — | 105 ± 3 | 93 ± 2[4] | — |
| 8 | 0.2 | 350 ± 16 | 280 ± 9[4] | — | 695 ± 43 | 615 ± 17 | — | 93 ± 14 | 79 ± 6 | — |

[1] Mean ± S.E.

[2] All control animals received 5.0 mg ascorbic acid/day.

[3] $P \leq 0.02$, statistical comparison with depleted group.

[4] $P \leq 0.01$, statistical comparison with depleted group.

[5] $P \leq 0.05$, statistical comparison with depleted group.

chronic studies, perhaps owing to greater individual variation in the rate of depletion of body stores of the vitamin in the acute group. Total oxygen consumption per animal would not appear to be a valid method of comparing groups owing to the large differences in body weight between depleted and ad libitum-fed control groups. Nevertheless when pair-fed and depleted groups of similar weight (experiment 3) are compared, increased total oxygen consumption is evident in the depleted group. When oxygen consumption is corrected for body weight by expressing it in terms of body weight or of surface area, then increased oxygen consumption in the depleted group becomes even more apparent. These results are in agreement with those of Fidlar et al. ('39).

No consistent difference was noted between male and female guinea pigs of the same group with respect to oxygen consumption. In the chronic studies, however, female depleted animals showed increases in oxygen consumption earlier than the males. A similar difference was seen with respect to the onset of scorbutic symptoms. Since both sexes received the same intake of the vitamin it may be that the requirement of the female is greater or that the initial body store was smaller; thus a critical level of depletion was reached earlier in the female than in the male.

*Tyrosine excretion*

The results of tyrosine load tests carried out in experiments 1, 2 and 3 are shown in table 2. In spite of a high degree of individual variation in the depleted groups, it was possible to demonstrate a significant increase in the tyrosyl compound excretion in scorbutic animals. These observations are in agreement with those reported in the literature. In preliminary experiments, the excretion of tyrosyl compounds in control animals was found to be almost the same after the administration of water or of tyrosine; No detectable difference was noted between the amount excreted by control and scorbutic animals unless tyrosine was given. Apparently, then, the scorbutic guinea pig is unable to metabolize loads of tyrosine as efficiently as can the normal animal.

*Radioiodine studies*

During the course of experiment 3, radioactive iodine$^{131}$ was administered on three successive occasions in a study of thyroid iodine uptake. The interval between injections was of sufficient duration to permit the thyroid radioactivity to fall to negligible levels. Before the as-

TABLE 2

*Urinary tyrosine excretion following an oral load*[1]

| Time on diet | Ascorbic acid intake of depleted group[2] | Depleted | Pair-fed control | Ad libitum-fed control |
|---|---|---|---|---|
| *weeks* | *mg/day* | *mg* | *mg* | *mg* |
| | | Experiment 1, acute scurvy | | |
| 3 | 0 | 63 ± 15 | — | 5 ± 4[3] |
| | | Experiment 2, chronic scurvy | | |
| 3 | 0.2 | 33 ± 7 | 8 ± 0.6[3] | 8 ± 0.6[3] |
| 4 | 0.4 | 29 ± 8 | 8 ± 0.8[3] | 6 ± 1[3] |
| 5 | 0.4 | 43 ± 14 | 8 ± 0.7[4] | 7 ± 0.8[3] |
| 7 | 0.25 | 19 ± 5 | 12 ± 2 | 10 ± 1 |
| 10 | 0.25 | 25 ± 9 | 10 ± 2 | 9 ± 2 |
| | | Experiment 3, chronic scurvy, iodine depleted | | |
| 9 | 0.2 | 47 ± 9 | 22 ± 2[4] | — |

[1] Mean ± S.E.
[2] All control animals received 5 mg of ascorbic acid per day.
[3] $P \leq 0.01$, statistical comparison to depleted group.
[4] $P \leq 0.02$, statistical comparison to depleted group.

corbic acid deprivation was initiated, all of the animals were fed the synthetic diet without added iodine for two and a half months to avoid the anomalous effects on iodine uptake of a high thyroid $I^{127}$ content. No symptoms of iodine deficiency were seen. After 11 days of ascorbic acid deprivation a single dose of 0.2 μc of $I^{131}$ was administered to each animal. The resultant thyroid radioactivity did not exceed 450 net counts per minute. On the 25th day of experimental feeding, 0.5 μc of radioiodine was administered. The thyrod radioactivity on the day after injection was (net counts per minute): female deprived, 1210 ± 105; female control, 853 ± 90; male deprived, 925 ± 80; male control, 885 ± 90. On the 49th day of deprivation, 0.6 μc of $I^{131}$ was injected. The radioactivity on the first day after injection was (net counts per minute): female deprived, 1785 ± 146; female control, 1045 ± 95; male deprived, 1435 ± 87; male control, 955 ± 37.

The data collected after the second injection was compared with an $I^{131}$ solution and by the application of dilution factors, an estimate of the percentage uptake of the injected $I^{131}$ into the thyroid was obtained. At the time of the third injection, a phantom standard was prepared and the thyroid radioactivity was compared directly with this estimated percentage uptake. The results of these studies are shown in figure 2.

The female ascorbic acid-deprived guinea pigs exhibited increased thyroid $I^{131}$ uptake by the 25th day of deprivation whereas no change was evident in the males at this time. By the 49th day of deprivation, deprived animals of both sexes showed significantly increased radioiodine

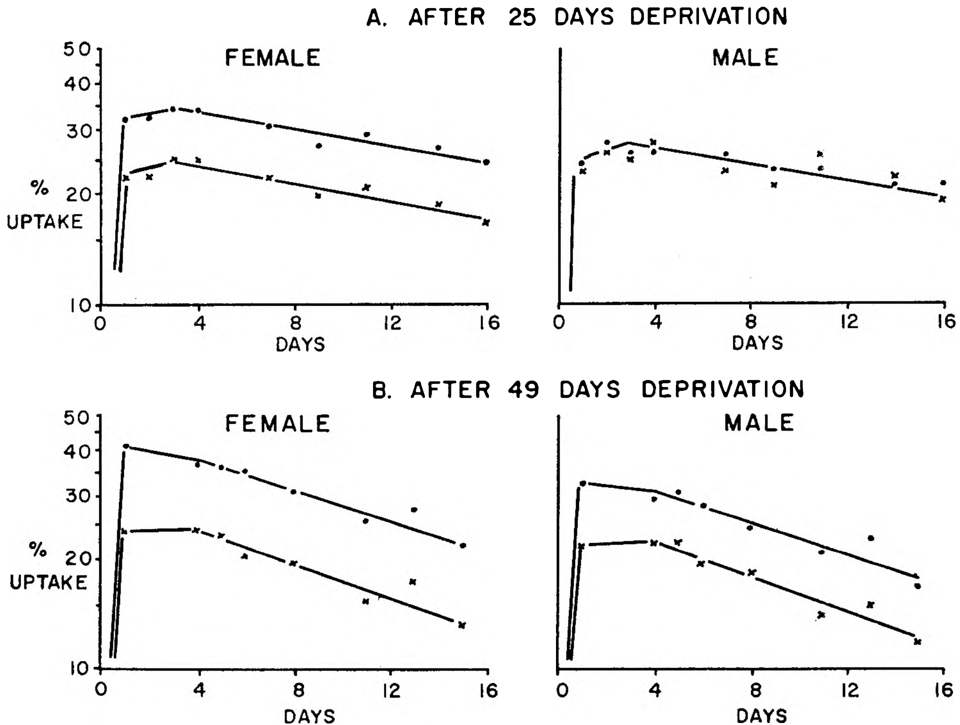


Fig. 2 Radioiodine uptake in ascorbic-acid-deprived (●) and pair-fed control (×) guinea pigs. Following the administration of 0.5 μc of $I^{131}$ on the 25th day of deprivation, the radioiodine uptake was significantly increased in females ($P < 0.05$ on days 1, 2, 4; $P < 0.02$ on days 7, 9, 11, 16) but was not altered in males. Following the administration of 0.6 μc of $I^{131}$ on the 49th day of deprivation, the radioiodine uptake was significantly increased in females ($P < 0.01$ on days 1 to 13; $P < 0.02$ on day 15) and in males ($P < 0.01$ on days one to 11; $P < 0.02$ on days 13, 15.).

uptakes. On the 25th day of deprivation, female guinea pigs were showing early signs of scurvy and losing body weight whereas the males showed no symptoms of scurvy and were maintaining body weight. By the 49th day, both sexes exhibited signs of scurvy.

The removal of radioiodine was increased in all animals, after the last injection of $I^{131}$, by the addition of extra iodine to the diet. Iodine was added on the 4th day after injection in an amount to supply approximately 30 µg/animal per day. Whether added dietary iodine was present (third study) or absent (second study) during the decay period, very little difference was noted in the rates of decay between scorbutic and control animals.

In view of the prolonged control of dietary variables, the control of food intake and the control of dietary iodine, at a low but adequate level of intake, it would appear that the increased uptake of $I^{131}$ must be related to ascorbic acid intake and no doubt reflects an increased thyroid activity.

## DISCUSSION

The results presented indicate cleary that oxygen consumption is increased in scorbutic guinea pigs. This observation suggests an increased thyroid activity. While no histological examination of the thyroid gland was carried out, the hypothesis of increased activity is supported by the increased iodine uptake in scurvy. Further support for this hypothesis comes from the earlier work of Mosonyi and Kezdi ('41). They demonstrated that thyroidectomized guinea pigs, rendered scorbutic, did not show any appreciable increase in oxygen uptake. Intact guinea pigs rendered scorbutic on the same diet showed marked increases in oxygen uptake. The results of Mosonyi and Kezdi demonstrated that the thyroid gland was essential for the effect of scurvy on the metabolic rate.

While the present evidence is sufficient to show increased thyroid activity in the scorbutic guinea pig, it does not reveal the mechanism of this effect of scurvy. A possible connection between scurvy, the thyroid gland, and oxygen uptake lies in the metabolism of tyrosine. It has been postulated that tyrosine is a precursor of the thyroid hormones. In the present studies, as in the studies of other workers, it has been possible to demonstrate that scorbutic animals excrete tyrosine and "tyrosyl" derivatives in the urine following oral administration of the amino acid. Scorbutic animals are unable to catabolize tyrosine normally. It may be that an interference with tyrosine catabolism leads to its accumuation in the thyroid gland and thus promotes the production of excess thyroid hormone.

Regardless of the increased thyroid activity, scorbutic animals lose little more weight than pair-fed controls. It would seem from the results shown in figure 1, that the effect of ascorbic acid on body weight is largely one of food intake rather than of a specific effect on the growth processes. After 28 days of absolute deprivation, scorbutic animals in experiment 1 were eating an average of about 7 gm of diet per day; ad libitum-fed control animals in this experiment ate about 23 gm/day. When ascorbic acid was again given to the scorbutic pigs, a prompt increase in food intake occurred and a subsequent increase in body weight.

In both chronic and acute studies, the deprivation of ascorbic acid has very little effect on food intake or body weight for 12 to 16 days and then both begin to decrease. It is likely that this time represents the depletion of body stores of the vitamin to a critical level. In female animals this change is seen earlier than in males, especially in the chronic studies. The early symptoms of scurvy also appear in the females earlier than in the males. In experiment 3, it was demonstrated that iodine uptake was increased in female deprived animals before males. Apparently the female guinea pig is more susceptible to scurvy than is the male. It may be that the female has a higher requirement for vitamin C than the male, or that the female has a smaller initial store of the vitamin.

## SUMMARY

Using a purified diet of known composition, it has been possible to substantiate earlier reports of an increased oxygen uptake by scorbutic guinea pigs. It was also

demonstrated that these animals showed increased thyroidal uptake of $I^{131}$. The evidence indicates that thyroid activity is increased in scurvy in the guinea pig. This increased activity may be linked to the abnormal metabolism of tyrosine, a thyroid hormone precursor, in scurvy. Female guinea pigs are more susceptible to scurvy than are males, perhaps owing to a higher requirement of a lower initial store of the vitamin.

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# Nutritional Studies with the Guinea Pig

## VI. TRYPTOPHAN (WITH AMPLE DIETARY NIACIN)

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The quantitative requirement for tryptophan of the rat, chick, dog, swine, and man has been determined, but has not been established for the guinea pig. Cannon et al. ('46) studied the inadequacy of certain natural diets for guinea pigs. On a corn-soybean oil meal-alfalfa ration supplemented with adequate amounts of vitamins A, D, E and C plus a mixture of water-soluble vitamins (including niacin), growth was improved by the addition of linseed oil meal, soybean oil meal or casein. It was pointed out that the effectiveness of these additions was proportional to their tryptophan content and that they could be replaced in part by a supplement of 0.3% of DL-tryptophan. Heinicke et al. ('55) reported that tryptophan became a limiting factor for guinea pigs started on a 20% casein-sucrose diet when 8 weeks old. Their results indicated that animals fed a 25% casein-sucrose diet did not need supplementary tryptophan. In contrast, work in this laboratory[1] showed no definite increase in growth with the addition of tryptophan to a 20% casein diet. The lack of agreement in the results cited is probably attributable to some difference in the diets other than tryptophan. The diet of Heinicke et al. contained 78% of sucrose or dextrin as the sole source of carbohydrate whereas the diet used in this laboratory contained a mixture of carbohydrates, including 20% of starch. Another important difference in the diets was in their mineral content; the Heinicke diet contained 4.0% of salts (approximately 30% of which was $CaCO_3$) as compared with 6% of salts in our diet.

The present studies were initiated to determine the tryptophan requirement of the guinea pig for growth and for maintaining a healthy condition of the eyes in the presence of ample dietary niacin and essential amino acids other than tryptophan.

### METHODS

Male guinea pigs of the Hartley strain, 3 to 5 days old, weighing from 95 to 115 gm, were placed on experimental diets. In most of the experiments 5 animals were used in each group. During the first two weeks the animals were housed in stainless steel cages of ¼ inch mesh, and later were moved to cages with ½ inch mesh. A few extra animals were placed on the deficient diets in each experiment for use as substitutes for animals which failed to adjust to the experimental regimen. No substitutions were made after the third day. Procedures used in the care of the animals were similar to those described previously (Reid and Briggs, '53). Minor modifications in technique have been used in the present studies. No greens were fed, and the only water available was that in the drinking bottles. Two animals were placed in a cage during the first three days so that they might learn from each other to use the food and water supply. The need of such very young animals for companionship may have contributed to the success of the procedure. The diets were prepared in amounts to last no longer than two weeks and were kept refrigerated until used.

The basal diet used was similar to a purified diet for guinea pigs described previously (Reid and Briggs, '53) except for variations in the amount and kind of pro-

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[1] Reid, M. E. 1956 Protein and amino acid studies with the guinea pig. Federation Proc., 15: 570 (abstract).

tein and in the incorporation of an amino acid mixture. The ration consisted of the following in percentage amounts: purified soybean protein,[2] 10; gelatin, 10; corn oil, 7.3; sucrose, 10.2; cellophane spangles,[3] 15; cornstarch, 20; glucose,[4] 13.9; potassium acetate, 2.5; magnesium oxide, 0.5; salts (Briggs et al., '52), 6; choline chloride, 0.2; ascorbic acid, 0.2; inositol, 0.2; liberal amounts of the known vitamins, and an amino acid mixture, 4.0. The amino acid mixture had the following composition (gm/100 gm of diet):

| | | | |
|---|---|---|---|
| L-cystine | 0.04 | DL-methionine | 0.43 |
| L-glutamic acid | 0.43 | DL-phenylalanine | 0.26 |
| L-histidine·HCl | 0.13 | DL-threonine | 0.43 |
| DL-isoleucine | 0.86 | L-tyrosine | 0.26 |
| L-leucine | 0.34 | DL-valine | 0.60 |
| L-lysine·HCl | 0.22 | Total | 4.0 |

With the exception of tryptophan and methionine, the addition of this amino acid mixture to the diet resulted in a total level of the essential amino acids approximately equal to that furnished by a 20% level of purified soybean protein. Since the amount of methionine in a 20% level of this protein was found to be inadequate for maximum growth (Reid, '56) and since the amount of this amino acid in gelatin is even less, additional methionine was incorporated into the amino acid mixture to make the total dietary allowance adequate (approximately 0.4% of L-methionine). The basal diet contained 0.108% of L-tryptophan furnished by the soybean protein. L-Tryptophan was added in different tests in amounts ranging from none to 0.2%. In another series of tests to determine the comparative value of L- and D-tryptophan and mixtures of the two, the amounts added varied from none to 0.12 and 0.24%. All of the tests were conducted with an ample supply[5] of dietary niacin (20 mg %).

Slit-lamp examination of the eyes at ×20 magnification was made at weekly intervals and at the end of the experiment both eyes were enucleated, embedded in celloidin and stained with hemotoxylin and eosin. The major portion of a series of 160 animals was killed at intervals of one to 14 weeks after being placed on the diets.

## RESULTS

As shown in the data summarized in table 1, growth of the animals without a tryptophan supplement was slightly retarded and one death occurred in this group as a result of the deficiency. Alopecia was extreme in a few of the animals (fig. 1). Surprisingly, these animals, although unable to retain the first coat of fur, were soon able to grow a new coat. Some of the animals, on the other hand, showed little loss of fur. No definite evidence was noted of a lowering of hemoglobin values (determined after 6 weeks on the diets) and no discoloration of the teeth. On autopsy, no deposition of fat was found around the kidneys or other internal organs. A tendency to soft feces and/or

[2] Drackett Assay Protein C-1.

[3] Cellophane Spangles, obtained from Rayon Processing Co., Pawtucket, Rhode Island.

[4] Cerelose.

[5] Results of studies of niacin/tryptophan relations in the guinea pig will be reported in publication VII of this series (Reid, '60).

TABLE 1

*Tryptophan requirement with a diet containing ample niacin and 20% of protein (containing 1.08% tryptophan) supplemented with adequate amounts of the essential amino acids other than tryptophan*

| L-Tryptophan supplement | No. of experiments | No. of animals | No. of survivors | Average weights, Weeks 2 | 4 | 6 |
|---|---|---|---|---|---|---|
| % | | | | gm | gm | gm |
| None | 4 | 20 | 19 | 144 ± 14[1] | 222 ± 20 | 286 ± 34 |
| 0.02 | 3 | 12 | 12 | 157 ± 17 | 239 ± 35 | 319 ± 31 |
| 0.03 | 3 | 15 | 15 | 160 ± 15 | 249 ± 24 | 327 ± 27 |
| 0.04 | 2 | 9 | 9 | 151 ± 18 | 244 ± 27 | 321 ± 37 |
| 0.05 | 2 | 10 | 10 | 158 ± 20 | 245 ± 44 | 326 ± 48 |
| 0.10 | 3 | 10 | 10 | 160 ± 26 | 260 ± 20 | 339 ± 21 |
| 0.20 | 1 | 5 | 5 | 154 ± 18 | 249 ± 24 | 343 ± 18 |

[1] Standard deviation.

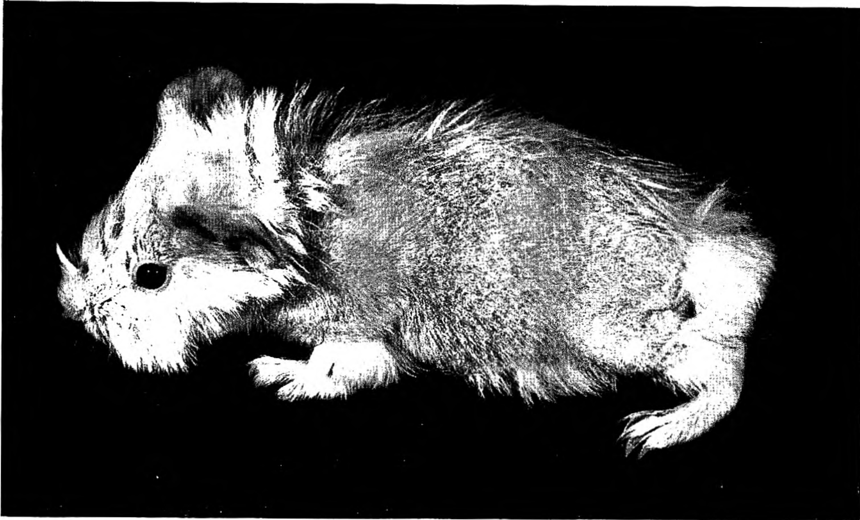

Fig. 1 Tryptophan-deficient animal losing fur over most of its body and at the same time a fairly thick growth of new hair is starting.

diarrhea and to swelling of the abdomen due to distention of the cecum and large intestine were characteristic of this group. The distended condition of the abdomen partially accounts for the fact that only a slight difference in weight was found between the deficient and control animals.

Next to the fur condition, the most pronounced effect of the deficiency occurred in the lens of the eye. The eyes of all animals on the diet with no added tryptophan showed lens changes which eventually progressed to advanced cataract. The first changes were observed in 7 of 21 animals one week after they were placed on the experimental diet. At the two-week interval, 20 out of 24 showed incipient lesions. All animals on this diet eventually developed cataract.

The first histologic lesions consisted of hydropic swelling at the ends of the lens fibers, particularly at the sutures. Later, the histopathology involved the deep cortex and spread from there along the suture toward the surface. All components of the lens equator preserved their morphologic integrity even in advanced cataract. The epithelial cells of the lens did not undergo degeneration but in some cases multiplied around the sutures. Corneal vascularization as a result of tryptophan deficiency was not observed in the slit-lamp examinations of the eyes, but it could be seen in a great number of the histological preparations. Except for the corneal vascularization, no abnormal findings were noted in parts of the eye other than the lens. A full report of the observations of the eyes appears in another publication (Von Sallmann et al., '59).

With the addition of only 0.02% of L-tryptophan to the basal diet (table 1) there was an improvement in growth and in general well-being of the animals, together with a very noticeable diminution of cataractous changes in the eyes. The addition of 0.03% produced approximately maximal growth. Each increase in added L-tryptophan decreased the incidence and extent of lenticular changes but not until an added level of 0.1% was reached was there complete protection from cataract. Studies with added levels of tryptophan between 0.06 and 0.1% were not made. For semiquantitation of the effects of tryptophan deficiency on the lens, the severity of the lesions was graded arbitrarily on the basis of the biomicroscopic and histologic appearance as : suggestive (+), incipient (++), extensive (+++), and far advanced (++++). Each designation represents an average of both eyes. In some animals slight differences between the two eyes were observed in the pattern of cataractous change but not in the degree of change. In table 2, the dependency of the

TABLE 2

*Effect of different supplementary levels of L-tryptophan on degree[1] of cataractous change*

| Time after start of diet | Tryptophan supplement (%) | | | |
|---|---|---|---|---|
| | None | 0.02 | 0.03 | 0.06–0.1 |
| *weeks* | | | | |
| 4½ | ++++ (1)[1,2]<br>+++ (1)<br>++ (2) | ++ (1)<br>+ (4) | | 0 (5) |
| 6 | ++++ (6)<br>+++ (3) | +++ (1)<br>+ (4) | ++ (3)<br>+ (1)<br>0 (1) | 0 (9) |
| 8 | ++++ (7)<br>+++ (1) | +++ (2)<br>++ (1)<br>+ (1)<br>0 (1) | + (4)<br>0 (5) | 0 (4) |

[1] Degree of cataractous change is graded as (+) suggestive, (++) incipient, (+++) extensive, (++++) far advanced.

[2] Numbers within parentheses indicate number of animals showing indicated changes.

cataractous changes on the quantities of the supplement is shown. Only the experiments in which 0.02, 0.03 and 0.1% of tryptophan were added to the basal diet were used in the table because these groups comprised a sufficient number of animals. The effect on weight owing to increased levels of tryptophan was less than the effect on the lens. This relation is shown clearly in figure 2.

In another series of experiments involving a total number of 85 animals the comparative values of L- and D-tryptophan were studied. In 20 animals, reared without addition of tryptophan to the diet, three deaths occurred (table 3). With a 0.03% level of added L-tryptophan, approximately maximal growth was obtained. This is in agreement with the results of the previous experiments. At this level of D-tryptophan, three of the 10 animals died and growth of the survivors appeared to be inferior, though possibly not significantly so, to that of the unsupplemented group. No definite protective effect was observed on the condition of the lenses as a result of the lower added levels of the D-supplement (table 4). The relative inactivity of the D-isomer to affect growth was apparent even at the 0.03% level as shown by the similarity in weight gains of the animals receiving 0.06% of DL-tryptophan compared with that using 0.03% of the L-isomer.

With 0.06% of added D-tryptophan, some of the animals showed almost maximal growth, whereas others appeared definitely unthrifty. However, the eyes of this group showed little improvement, if any, over the unsupplemented group as viewed by examination with the slit lamp. With 0.12% of the DL-compound, maximal growth was obtained and the eyes were almost free of cataractous change. With 0.12% of the D-isomer the eyes of three of the 5 animals showed slight or incipient

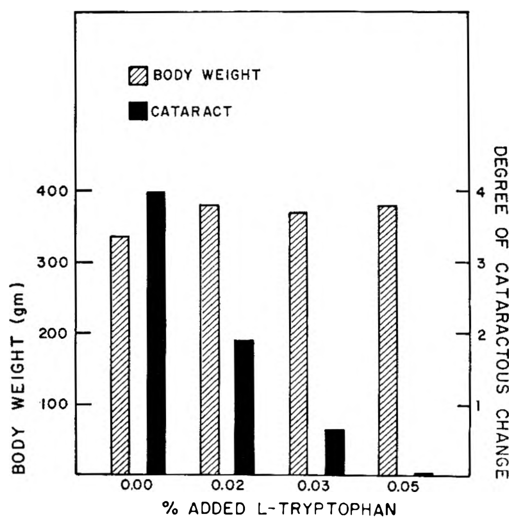


Fig. 2 Degree of cataractous changes (after 8 weeks) and body weight (gm) in relation to added tryptophan.

TABLE 3

*Comparative value of* L- *and* D-*tryptophan in a diet containing ample niacin and 20% of protein supplemented with amino acids other than tryptophan*

| Tryptophan supplement | No. of Experiments | No. of Animals | No. of Survivors | Average weights: Weeks 2 | 4 | 6 |
|---|---|---|---|---|---|---|
| % | | | | gm | gm | gm |
| None | 4 | 20 | 17 | 148 ± 13 | 228 ± 27 | 301 ± 36 |
| 0.03 L-[1] | 2 | 10 | 10 | 162 ± 17 | 257 ± 23 | 333 ± 28 |
| 0.03 D-[2] | 2 | 10 | 7 | 145 ± 16 | 216 ± 22 | 256 ± 36 |
| 0.06 DL-[3] | 2 | 10 | 10 | 154 ± 15 | 230 ± 29 | 303 ± 36 |
| 0.05 L- | 1 | 5 | 5 | 167 ± 9 | 261 ± 19 | 339 ± 36 |
| 0.05 D- | 1 | 5 | 4 | 157 ± 14 | 249 ± 18 | 333 ± 30 |
| 0.10 DL- | 1 | 5 | 5 | 165 ± 26 | 266 ± 29 | 351 ± 27 |
| 0.06 L- | 1 | 5 | 5 | 162 ± 10 | 255 ± 13 | 342 ± 14 |
| 0.06 D- | 1 | 5 | 5 | 145 ± 14 | 237 ± 23 | 318 ± 35 |
| 0.12 DL- | 1 | 5 | 5 | 160 ± 9 | 248 ± 27 | 339 ± 30 |
| 0.12 L- | 1 | 5 | 5 | 156 ± 4 | 266 ± 6 | 352 ± 13 |
| 0.12 D- | 1 | 5 | 5 | 147 ± 7 | 235 ± 8 | 328 ± 6 |
| 0.24 DL- | 1 | 5 | 5 | 161 ± 3 | 266 ± 15 | 352 ± 28 |

[1] L-Tryptophan: optical rotation $[\alpha]_D^{20}$ – 0.5% – 29.2°; nitrogen (%) calculated 13.72; found 13.52.

[2] D-Tryptophan: optical rotation $[\alpha]_D^{20}$ – 0.5% + 31.5; nitrogen (%) calculated 13.72; found 14.07.

[3] DL-Tryptophan: optical rotation $[\alpha]_D^{20}$ – 0.25% + 0.20; nitrogen (%) calculated 13.72; found 13.76.

The tryptophan preparations were made by Nutritional Biochemicals Corporation, Cleveland.

TABLE 4

*Comparison of cataractous change with different levels of* L-, D,- *or* DL-*tryptophan supplements*

| Time after start of diet | Tryptophan supplement (%): None | Form | 0.03 | 0.06 | 0.12 | 0.24 |
|---|---|---|---|---|---|---|
| weeks | | | | | | |
| 6 | ++++ (3)[2]<br>+++ (7)<br>++ (3) | L- | +++ (1)<br>++ (1)<br>+ (2)<br>0 (1) | + (3)<br>0 (1) | + (1)<br>0 (4) | |
| | | D- | ++++ (1)<br>+++ (1)<br>++ (2) | ++++ (1)<br>+++ (3)<br>++ (1) | ++ (1)<br>+ (2)<br>0 (2) | |
| | | DL- | | ++ (1)<br>+ (3)<br>0 (1) | + (1)<br>0 (4) | |
| 8 | ++++ (4) | L- | + (4) | | | |
| | | D- | ++++ (2)<br>++ (1) | | | |
| | | DL- | | +++ (1)<br>++ (1)<br>+ (3) | | 0 (5) |

[1] Severity of cataractous changes was graded as (+) suggestive; (++) incipient; (+++) extensive; (++++) far advanced.

[2] Numbers in parentheses represent the number of animals showing the indicated changes.

changes. The eyes of the group on 0.24% of the DL-compound appeared to be fully protected.

## DISCUSSION

With a diet containing a total level of amino acids approximately equal, (with the exception of tryptophan and methionine) to that in a 20% level of purified soybean protein, the requirement for L-tryptophan for maximum growth appears to be approximately 0.13%. This is slightly less than the requirement for maximum

growth in the rat (0.17%, Salmon, '54; approximately 0.2%, Oesterling and Rose, '52), slightly less than that for the chick (0.15%, Fisher et al., '55; Morrison et al., '56) but greater than that of swine (0.115%, Becker et al., '55). The amount of L-tryptophan required to maintain the eyes of the guinea pig in a healthy condition is more than 0.16% but possibly somewhat less than 0.2%.

Under the dietary conditions used in this study, the young guinea pig, unlike the rat (Berg and Potgieter, '32; du Vigneaud, Sealock and Van Etten, '32), appeared to show only a slight ability to utilize D-tryptophan. The results suggest that it may have a growth-promoting value of from one-fourth to one-third that of the L-form. In most of the experiments no beneficial effect was observed during the first two to three weeks, but later, a slow improvement was observed when the D-form was fed at the higher levels. Also, a marked variability was noted in the response of different individuals to this isomer. Some conversion to the L-form may occur, such as that found in the rat by Oesterling and Rose ('52). Anderson et al. ('50) observed that the D-isomer was of value in the chick when the diet contained starch. The improvement, using starch, as compared with glucose as the source of carbohydrate was attributed to a change in the bacterial flora of the digestive tract. When sulfasuxadine was fed, the benefit derived from D-tryptophan was reduced. The starch content of the diet in the present experiments may have facilitated the animals' adaptation to utilize the D-isomer. The beneficial results with the D-isomer were more obvious in improved growth than in increased protection of the eyes.

The most outstanding finding in these studies with the guinea pig is that the requirement for tryptophan of a specific organ, the eye, is greater than that for maximum growth of the whole animal. It is probably the first observation of a higher requirement for an amino acid by an individual organ in relation to the body as a whole. It parallels the vitamin C situation in the guinea pig with respect to the teeth, namely, that the requirement to maintain the teeth and the surrounding tissue in a healthy condition is greater than that for normal body growth.

The cataractous changes described here are the first to be reported for the guinea pig. This animal was found to be especially suitable for the eye studies since the cataractous changes developed early and the animal could be maintained for a long time on a tryptophan-deficient diet. Also, the size of the lens as compared with that of the rat facilitated the analysis of the slit-lamp picture and microscopic examination. The observation of dietary-induced lens changes without other systemic signs of the deficiency-state might have application for certain types of juvenile cataract. It is possible that some perinuclear lens opacities, not induced by hyperparathyroidism, are related to amino acid deficiencies, including that of tryptophan.

Under the conditions of the present experiments, the deficiency effects which developed were the result of a shortage of tryptophan as such. There was no possibility of complications developing owing to a shortage of niacin (Pike, '51) or of an inadequacy at any time of any of the essential amino acids other than tryptophan. One of the most interesting observations made was that cataractous changes occurred while growth, and presumably protein synthesis, was continuing. In the studies reported with rats, cataracts were produced after the animals had stopped growing and were suffering severely from the deficiency—even losing weight (Totter and Day, '42; Buschke, '43; Von Sallmann et al., '59). This has been interpreted as being a result of a general halt in protein synthesis from which the lens suffers along with other tissues (Schaeffer and Geiger, '47; Schaeffer and Murray, '50). The tryptophan content (as percentage of protein) of normal and cataractous lenses in the rat was found to be the same (Schaeffer and Murray, '50).

Results of this study with the guinea pig suggest that protein synthesis, as indicated by growth, and lens changes in tryptophan deficiency can occur simultaneously. The following possibilities, either acting separately or in combination, are suggested to explain the phenomena: (1) the percentage content of tryptophan in the lenses of deficient and normal animals

may be the same as that shown for the rat but the total amount of lens protein formed is less in the deprived animal; (2) the tryptophan content of one or more of the lens proteins is higher than that of the body as a whole, this protein thus being expected to suffer more in the deprived state, and (3) with an inadequacy of dietary tryptophan some degenerative (physical) change occurs in the lens protein.

## SUMMARY

The requirement of the guinea pig for tryptophan was studied using a diet containing protein and which was adequate in all essential amino acids other than tryptophan.

Feeding the basal diet containing 0.108% of tryptophan, growth was slightly retarded and some fatalities occurred; after 6 to 8 weeks on the diet the eyes of all of the animals showed advanced cataractous changes and some corneal vascularization which could be seen on histological examination. Most of the animals showed alopecia, all had swollen abdomens and excreted soft, unformed feces. No discoloration of the teeth and no definite lowering of hemoglobin values were observed.

Maximum growth was obtained by the addition of 0.03% of L-tryptophan to the diet. At this level a definite improvement in the condition of the eyes occurred. Protection was not complete with the addition of 0.06% but it was with 0.1%. The requirement for complete eye protection and for good growth, therefore, is more than 0.16% but possibly somewhat less than 0.2%.

The D-isomer appeared to have from one-fourth to one-third the growth-promoting activity of the L-form. An addition of 0.06% to the diet was necessary to obtain 100% survival. Whether the D-form was at all protective to the eyes is doubtful. The presence of the D-isomer did not appear to suppress the protective action of the L-form on the eyes and at the higher supplemental levels it may possibly have had a slight additional effect.

The most outstanding finding in these studies is that the requirement for tryptophan by one specific organ, the eye, is greater than that for maximum growth. It is probably the first observation of a higher amino acid requirement of a specific organ in comparison to the body as a whole.

## ACKNOWLEDGMENTS

The authors wish to express appreciation to Dr. Olaf Mickelsen for suggesting this cooperative study, to Patricia Grimes for assistance in the experiments and assembling of the data for publication, to Dr. W. C. Alford and coworkers for determination of the nitrogen content and optical rotation of the tryptophan employed, to Howard Bakerman for a microbiological determination of the tryptophan content of the casein and purified soybean protein, to Drs. Z. M. Tolgay and W. C. Alford for the nitrogen and moisture determination of the soybean protein, to Helen Hood and Esther Hurley for preparation of the diets, and to Mary Stull and Woodrow Duvall for the care of the animals.

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# The Availability of Lysine in Wheat, Flour, Bread and Gluten[1]

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It is recognized that the nutrient content of a food as determined by analysis does not necessarily establish the amount of that nutrient available when that food is consumed by man or laboratory animals. Few studies have been reported on the availability of lysine in wheat and wheat products. Kuiken and Lyman ('48) found 92.8% of the lysine in wheat to be available to the rat as determined by fecal excretion. Guthneck et al. ('53) reported the lysine of wheat germ to be 65% available as measured by gains in body weight of protein-depleted adult male rats. Gupta et al. ('58) reported the lysine of wheat flour to be 70% available as measured by the growth of young rats. These authors reviewed the recent literature on the availability of lysine from different products.

In view of the paucity of information on the availability of amino acids from wheat and wheat products, this laboratory has initiated a program to study methods suitable for such determinations on these products. This paper presents the lysine studies. It is hoped that when results obtained in these studies are compared with those obtained from human feeding studies, a better understanding of both the usefulness and limitations of animal feeding tests for determining availabilities will eventuate.

## EXPERIMENTAL

*Sample description.* A white bread flour (95% patent representing approximately 68% of the cleaned wheat) together with the cleaned wheat blend from which it was milled was obtained from a commercial flour mill. The wheat blend was comprised of hard red spring and hard red winter wheat. Prior to incorporation into the diets, the wheat was ground in a hammer mill using a 0.024-inch screen.

Bread was made of the flour using a formula and procedure simulating commercial practice (Hepburn et al., '57). The formula included 4 parts of nonfat dry milk and 2.5 parts of compressed yeast per 100 parts of flour. The loaves were baked, cooled, wrapped and allowed to stand overnight at room temperature. They were then sliced and dried in a slow stream of unheated air. After drying, the bread was ground in a hammer mill to pass a 0.024-inch screen.

Nitrogen was determined in the samples by the Kjeldahl-Gunning procedure. Wheat, flour, bread and gluten contained 2.46, 2.31, 2.42 and 12.0% of nitrogen, respectively, on the "as fed" basis.

Lysine was determined microbiologically by the method of Hepburn et al. ('57). The values were 0.494, 0.352 and 1.61% of lysine (as hydrochloride) for wheat, flour and gluten, respectively. The bread prepared for experiment 1 was found to contain 0.434%, and that for experiment 2, 0.448% of lysine.

*Basal diets.* Two separate feeding experiments were performed. The basal diet fed in the first experiment (basal A, table 1) was patterned after that of Gupta et al. ('58) and included 20% of gluten having a nitrogen content of 13.5%. The basal diet in the second experiment (basal B, table 1) contained a mixture of 17 amino acids (omitting lysine) in the amounts of the active forms calculated to be present in the gluten of experiment 1 (Home Economics Research Report no. 4) plus the amounts of histidine, methionine, threonine and tryptophan used as supplements in basal A. The quantities of DL-isoleucine, DL-threonine and DL-valine were doubled to

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TABLE 1
*Composition of basal diets*

| Basal diet A | | Basal diet B | |
|---|---|---|---|
| Ingredient | | Ingredient | |
| | % | | % |
| Gluten[1] | 20.00 | DL-Alanine | 0.346 |
| L-Histidine HCl·$H_2O$ | 0.20 | L-Arginine HCl | 0.816 |
| DL-Methionine | 0.20 | DL-Aspartic acid | 0.558 |
| DL-Threonine | 0.20 | L-Cystine | 0.334 |
| DL-Tryptophan | 0.05 | L-Glutamic acid | 5.664 |
| Salts 4[2] | 4.00 | Glycine | 0.566 |
| Corn oil[3] | 5.00 | L-Histidine HCl·$H_2O$ | 0.676 |
| Vitamins[4] | + | DL-Isoleucine | 1.420 |
| Starch[5] | 70.14 | L-Leucine | 1.158 |
| Total | 100.00 | DL-Methionine | 0.468 |
| | | DL-Phenylalanine | 0.840 |
| | | L-Proline | 1.968 |
| | | DL-Serine | 0.732 |
| | | DL-Threonine | 1.020 |
| | | DL-Tryptophan | 0.216 |
| | | L-Tyrosine | 0.502 |
| | | DL-Valine | 1.464 |
| | | Urea[6] | 1.196 |
| | | Salts 4[2] | 4.00 |
| | | Corn oil[3] | 5.00 |
| | | Vitamins[7] | + |
| | | Starch[5] | 70.35 |
| | | Total | 100.00 |

[1] Contained 13.51% of nitrogen.
[2] Hegsted et al. ('41).
[3] Mazola.
[4] Vitamins were supplied in a portion of the gluten in mg/100 gm of diet as follows: thiamine·HCl, 1; riboflavin, 1; pyridoxine·HCl, 1; nicotinic acid, 10; *i*-inositol, 20; *p*-aminobenzoic acid, 20; folic acid, 0.1; biotin, 0.1; menadione, 2.0; Ca pantothenate, 4; choline chloride, 150; vitamin $B_{12}$, 0.004. Each rat received weekly 2 mg α-tocopherol acetate dissolved in 2 drops of corn oil. Vitamins A and D were supplied by a drop of halibut liver oil given to each rat weekly.
[5] Aytex P, General Mills.
[6] Added to make basal diet B isonitrogenous with basal diet A.
[7] See footnote 4, this table. Starch served as the carrier.

compensate for the inacivity of the D form. The D isomers of methionine, phenylalanine and tryptophan were assumed to be completely active. The use of wheat starch and wheat gluten or an amino acid mixture patterned after gluten in the basal diets provided for minimal change in the pattern of composition of the diet (except for lysine) upon the addition of the samples (wheat, flour, bread or gluten).

*Sample diets.* The wheat, flour and bread samples were incorporated into both basal diets at 70% of the diet at the expense of starch. In the second experiment, gluten was also fed as a sample at the level of 20% of the diet.

A standard growth curve was established in experiment 1 by adding zero, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.8% of L-lysine hydrochloride to basal diet A, and in experiment 2 by adding zero, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8 and 1.0% of L-lysine hydrochloride to basal diet B. All additions to the basal diets were made at the expense cf starch. The prepared diets were stored at −20°F.

*Experimental procedure 1.* Groups of 5 weanling male rats (Sprague-Dawley strain) were closely matched in weight. One group was sacrificed as the zero time control. The remaining groups were assigned at random to the various basal A diets. Average initial weight was 44.2 gm. The animals were housed individually in suspended wire-bottom cages in an environmental temperature maintained at approximately 77°F. The diets, replenished daily, and distilled water were fed ad libitum for three weeks. Food intakes and

body weights were recorded weekly. Feces were collected during the 15th, 16th and 17th day of the experiment for subsequent determination of lysine. Two per cent of ferric oxide was added to the diets at the beginning and at the end of the 3-day period to serve as markers.

After three weeks, the animals were weighed and sacrificed in random order using chloroform, the intestinal contents removed and empty body weights taken. The carcasses were frozen at −20°F, chopped into small pieces and ground in a Hobart grinder with the aid of dry ice snow. The ground carcasses were stored at −20°F until analyzed. Nitrogen was determined in duplicate on each carcass by the Kjeldahl-Gunning procedure.

*Experimental procedure* 2. Because some animals refused the amino acid diet on the first day, it was deemed necessary to permit gradual adjustment to this type of diet. This was accomplished by feeding a grain-based stock diet on the second day; on subsequent days increasing proportions of basal diet B plus 0.3% of added lysine hydrochloride were incorporated into the diet so that 100% amino acid diet was attained on the 6th day. On the 7th day the animals were divided into groups of 5 and assigned to the basal B diets at random, with one group sacrificed as the zero time control. The experimental procedure during the three-week feeding period was as described for experiment 1.

Upon sacrificing, the livers were removed for determination of moisture, fat and nitrogen. Moisture was determined as the loss of weight after drying the whole livers in an air oven. Fat was determined by extracting the dried, ground entire livers with ether for 4 hours in the Goldfisch extraction apparatus. Nitrogen was determined by the Kjeldahl-Gunning procedure on duplicate samples of the ether-extracted residue of each liver.

## RESULTS AND DISCUSSION

*Growth rates.* Good gains in weight were obtained with both the basal A diet (37.4 gm/week) and the basal B diet (40.9 gm/week) when adequate lysine supplementations were made (tables 2, 3). This indicates that the basal diets were satisfactory in meeting all nutritional requirements for growth, other than lysine. The maximum growth rate with basal B diet was considerably greater than the 28.7 gm/week reported by Ramasarma et al. ('49) on their best amino acid diet or the 28.8 gm/week reported by Womack et al. ('57) on their amino acid control diet. Perhaps the amino acid pattern used in the present experiments was better balanced, especially with respect to the non-essential amino acids. Possibly, for maximum growth rate, some of the "non-essentials" must be included in the diet. Ramasarma et al. ('49) have shown that of the non-essential amino acids, glutamic acid proved to be the most effective in increasing growth rate. Womack and Rose ('47) found that the inclusion of proline and especially glutamic acid increased the

TABLE 2

*Average food intake, weight gain, empty weight gain and carcass nitrogen gain at the end of the three-week period in the first experiment*

| Group | Supplement to basal diet | Food intake | Weight gain | | Empty weight gain | | Carcass nitrogen gain | |
|---|---|---|---|---|---|---|---|---|
| | | *gm* | *gm* | *gm/100 gm food* | *gm* | *gm/100 gm food* | *gm* | *gm/100 gm food* |
| 1 | None | 150.1 | 23.0 | 15.3 | 21.2 | 14.1 | 0.576 | 0.384 |
| 2 | 0.1% LMHCl[1] | 173.4 | 40.4 | 23.3 | 38.0 | 21.9 | 1.061 | 0.612 |
| 3 | 0.2% LMHCl | 197.6 | 62.0 | 31.4 | 56.8 | 28.7 | 1.534 | 0.776 |
| 4 | 0.3% LMHCl | 229.6 | 86.4 | 37.6 | 80.2 | 34.9 | 2.199 | 0.958 |
| 5 | 0.4% LMHCl | 248.1 | 98.2 | 39.6 | 90.6 | 36.5 | 2.614 | 1.054 |
| 6 | 0.5% LMHCl | 249.0 | 107.2 | 43.0 | 101.0 | 40.6 | 3.014 | 1.210 |
| 7 | 0.8% LMHCl | 240.8 | 112.2 | 46.6 | 105.6 | 43.8 | 3.340 | 1.387 |
| 8 | 70% wheat | 196.7 | 66.8 | 34.0 | 57.4 | 29.2 | 1.665 | 0.846 |
| 9 | 70% flour | 183.1 | 54.2 | 29.6 | 47.8 | 26.1 | 1.319 | 0.720 |
| 10 | 70% bread | 197.0 | 65.0 | 33.0 | 57.0 | 28.9 | 1.583 | 0.804 |

[1] Lysine monohydrochloride.

TABLE 3

*Average food intake, weight gain, empty weight gain and carcass nitrogen gain at the end of the three-week period in the second experiment*

| Group | Supplement to basal diet | Food intake | Weight gain | | Empty weight gain | | Carcass nitrogen gain | |
|---|---|---|---|---|---|---|---|---|
| | | *gm* | *gm* | *gm/100 gm food* | *gm* | *gm/100 gm food* | *gm* | *gm/100 gm food* |
| 1 | None | 108.1 | −10.2 | −9.4 | −8.4 | −7.8 | −0.113 | −0.104 |
| 2 | 0.1% LMHCl[1] | 117.9 | −2.8 | −2.4 | −1.4 | −1.2 | 0.081 | 0.069 |
| 3 | 0.2% LMHCl | 180.4 | 10.2 | 5.6 | 11.6 | 6.4 | 0.348 | 0.193 |
| 4 | 0.3% LMHCl | 193.7 | 28.2 | 14.6 | 28.6 | 14.8 | 0.807 | 0.417 |
| 5 | 0.4% LMHCl | 225.8 | 48.4 | 21.4 | 44.8 | 19.8 | 1.259 | 0.558 |
| 6 | 0.5% LMHCl | 252.5 | 71.0 | 28.1 | 67.0 | 26.5 | 1.882 | 0.745 |
| 7 | 0.8% LMHCl | 276.7 | 109.4 | 39.5 | 103.0 | 37.2 | 3.247 | 1.173 |
| 8 | 1.0% LMHCl | 293.5 | 122.8 | 41.8 | 114.0 | 38.8 | 3.579 | 1.219 |
| 9 | 70% wheat | 169.9 | 22.4 | 13.2 | 18.0 | 10.6 | 0.581 | 0.342 |
| 10 | 70% flour | 157.4 | 11.0 | 7.0 | 11.2 | 7.1 | 0.380 | 0.241 |
| 11 | 70% bread | 172.4 | 19.0 | 11.0 | 19.0 | 11.0 | 0.588 | 0.341 |
| 12 | 20% gluten | 175.2 | 20.0 | 11.4 | 20.0 | 11.4 | 0.583 | 0.333 |

[1]Lysine monohydrochloride.

rate of gain when young rats were fed an amino acid diet otherwise complete in the remaining 16 commonly occurring amino acids. The amino acid mixture used in the present experiment contained approximately 35% of glutamic acid and 10% of proline. The use of wheat starch as the carbohydrate may also have contributed to the good growth of the animals fed the amino acid diet. Spivey et al. ('58) and Harper and Spivey ('58) have commented upon the physiological significance of different carbohydrates in the diet of rats.

*Liver data.* Liver moisture, nitrogen and fat values obtained from animals from the second experiment are given in table 4. Moisture and nitrogen values appear to be quite similar on all diets. Liver fat was lowered from about 16% (diets 1–4) to about 8.5% by adequate supplements of L-lysine hydrochloride (diets 7, 8). This agrees with the findings of Gillespie et al. ('45) who showed that adequate supplementation with lysine lowered the percentage of liver fat otherwise obtained by feeding a lysine-deficient diet. No elevated liver fats were obtained with diets containing samples under test to indicate an adverse imbalance of amino acids. In fact, the feeding of wheat (diet 9) and the feed-

TABLE 4

*Average percentage of moisture, nitrogen and fat in the livers of animals in the second experiment*

| Group | Supplement to basal diet | Moisture | Nitrogen | Fat |
|---|---|---|---|---|
| | | % | %, *dry* | %, *dry* |
| 1 | None | 74.2 | 12.3 | 12.1 |
| 2 | 0.1% LMHCl[1] | 72.7 | 11.9 | 16.3 |
| 3 | 0.2% LMHCl | 72.5 | 11.2 | 18.1 |
| 4 | 0.3% LMHCl | 71.9 | 11.4 | 16.3 |
| 5 | 0.4% LMHCl | 72.7 | 12.0 | 14.0 |
| 6 | 0.5% LMHCl | 72.8 | 11.4 | 12.7 |
| 7 | 0.8% LMHCl | 73.3 | 11.8 | 8.7 |
| 8 | 1.0% LMHCl | 73.3 | 12.0 | 8.5 |
| 9 | 70% wheat | 73.1 | 12.2 | 9.1 |
| 10 | 70% flour | 73.0 | 11.9 | 11.6 |
| 11 | 70% bread | 72.8 | 11.7 | 11.0 |
| 12 | 20% gluten | 72.3 | 11.6 | 8.6 |
| 13 | Zero time controls | 75.1 | 13.8 | 16.0 |

[1] Lysine monohydrochloride.

TABLE 5

*Percentage of lysine from wheat, flour, bread and gluten excreted in the feces during the 15, 16 and 17th day of feeding*

| | Basal diet A | | | Basal diet B | | | |
|---|---|---|---|---|---|---|---|
| | Wheat | Flour | Bread | Wheat | Flour | Bread | Gluten |
| Total lysine in feces, *mg* | 210 | 167 | 270 | 147 | 57 | 103 | 40 |
| Lysine not from sample,[1] *mg* | 84 | 74 | 186 | 38 | 34 | 37 | 37 |
| Lysine from sample, *mg* | 126 | 93 | 84 | 109 | 23 | 66 | 3 |
| Sample lysine fed, *mg* | 481 | 303 | 937 | 444 | 278 | 388 | 399 |
| Sample lysine excreted, % | 26 | 31 | 9 | 24 | 8 | 17 | 1 |

[1] $\frac{\text{Lysine excreted by group 1}}{\text{Food intake of group 1}} \times$ food intake of experimental group.

ing of gluten (diet 12) resulted in lower percentages of liver fat than could be ascribed to their content of lysine. Flour and bread affected liver fat less than did wheat and gluten. Harper et al. ('55) have also shown that wheat lowered liver fat when fed in combination with a lysine-deficient diet.

*Fecal excretion of lysine.* Assuming that the amount of lysine excreted from the basal diet was proportional to the grams of diet consumed, this amount of lysine was substracted from the total amount of fecal lysine excreted over the 3-day collection period. The difference was ascribed to the ingestion of sample and the percentage of sample lysine appearing in the feces was calculated (table 5).

Although fecal excretion has been employed as a means of measuring lysine availability (Kuiken and Lyman, '48; Baliga and Lyman, '57; Gupta et al., '58) it should be recognized that this measurement is a reflection of digestion and absorption. It can measure availability only if digestion and/or absorption limit(s) the utilization of the amino acid. Possibly for this reason the observations in table 5 are not in uniformly good agreement with results for availability found by other methods (table 6). The notable example is observed with the value for gluten fed with basal diet B. Only 1% of the lysine from gluten was recovered in the feces, suggesting an availability of 99% but values of approximately 80% were obtained by all other methods. Such a wide discrepancy could indicate that some factor other than digestion or absorption limits the availability of lysine from gluten.

Except with bread a somewhat greater proportion of lysine was excreted in the

TABLE 6

*Availability values for lysine in wheat, flour, bread and gluten, expressed as percentage*[1]

| Performance index | Method of calculation[2] | Basal diet A | | | Basal diet B | | | |
|---|---|---|---|---|---|---|---|---|
| | | Wheat | Flour | Bread | Wheat | Flour | Bread | Gluten |
| Weight gain | (1) | 64 | 67 | 70 | 77 | 82 | 80 | 79 |
| | (2) | 70 | 74 | 71 | 83 | 87 | 83 | 83 |
| | (3) | 72 | 77 | 77 | 87 | 85 | 84 | 83 |
| Empty weight gain | (1) | 59 | 62 | 66 | 70 | 82 | 79 | 78 |
| | (2) | 61 | 67 | 68 | 75 | 85 | 84 | 83 |
| | (3) | 62 | 68 | 69 | 74 | 83 | 83 | 83 |
| Carcass nitrogen gain | (1) | 63 | 62 | 68 | 75 | 84 | 83 | 81 |
| | (2) | 66 | 65 | 68 | 77 | 84 | 84 | 81 |
| | (3) | 75 | 72 | 76 | 78 | 80 | 83 | 80 |

[1] Per cent availability = $\frac{\text{lysine found by rat assay}}{\text{lysine found by microbiological assay}} \times 100$

[2] Methods of calculation: (1) = gain versus % lysine added to diet.
(2) = gain/100 gm food consumed versus % lysine added to diet.
(3) = gain versus grams available lysine consumed.

feces when the samples were fed with basal diet A than with basal diet B. This observation is in agreement with, and may serve as an explanation for, the differences in availability values between basal diets observed for wheat and flour (table 6).

The importance that can be attached to the observations of fecal excretion of lysine is uncertain since the three-day collection period may not have been representative of conditions over the entire experimental period or the results may have been influenced by the varying amounts of roughage presented by the different sample diets.

*Measurement of lysine availability.* Several criteria of performance can be used to measure the response of the test animals to lysine. The possibility was considered that simple weight gain alone may be inaccurate because of differences observed in the amounts of the contents of the gastrointestinal tracts on the different diets. To correct for these differences, empty weight gains were calculated, using the weights obtained after removing the gastrointestinal contents. It was also considered that perhaps the ultimate criterion should be the deposition of body protein as

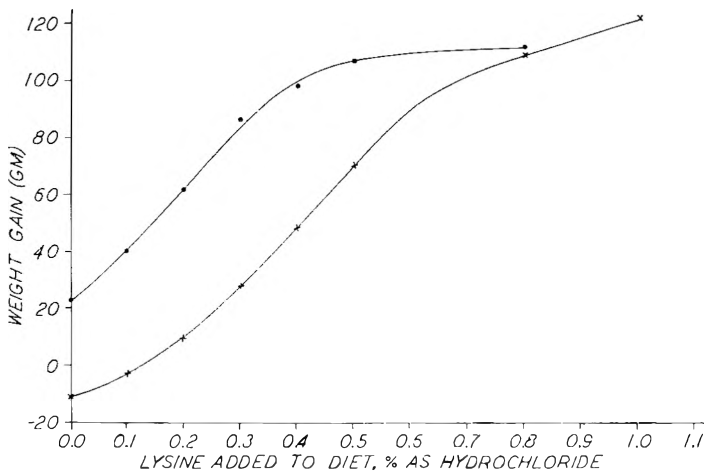


Fig. 1 Weight gain of rats on basal A diets versus added percentage of lysine (●); weight gain on basal B diets versus added percentage of lysine (×).

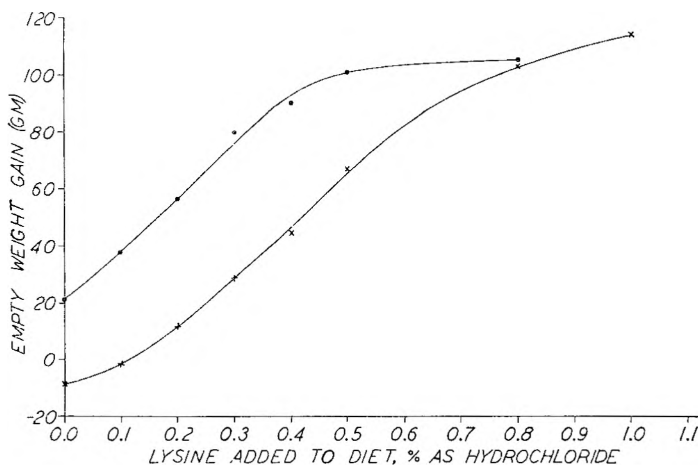


Fig. 2 Empty weight gain of rats on basal A diets versus added percentage of lysine (●); empty weight gain on basal B diets versus added percentage of lysine (×).

measured by the increase in carcass nitrogen. The three performance criteria of weight gain, empty weight gain and nitrogen gain have been used in the calculations for lysine availability.

The three sets of performance data were each subjected to three methods of calculating lysine availability. In method 1, standard curves were constructed by plotting the performance gains versus the percentage of added lysine (figs. 1–3). In each instance the standard curves produced with the basal A diets were similar in shape to those produced by the basal B diets but had slightly different slopes and were displaced because of the lysine contributed by the gluten in basal diet A.

In method 2, a similar set of standard curves was obtained by plotting the performance gains per 100 gm of food con-

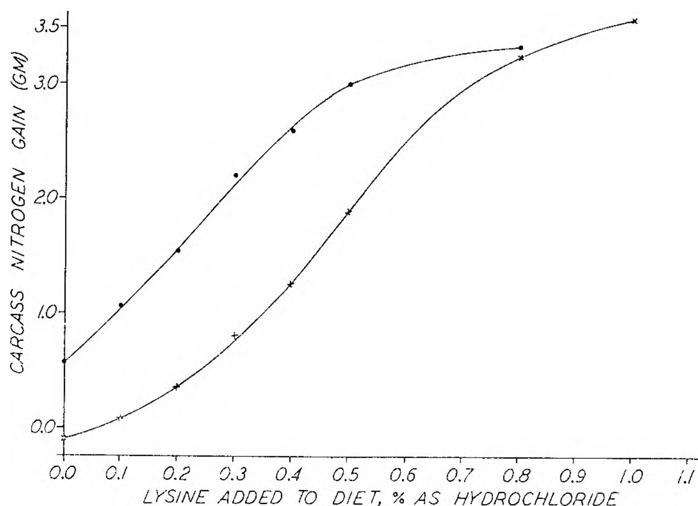


Fig. 3 Carcass nitrogen gain of rats on basal A diets versus added percentage of lysine (●); carcass nitrogen gain on basal B diets versus added percentage of lysine (×).

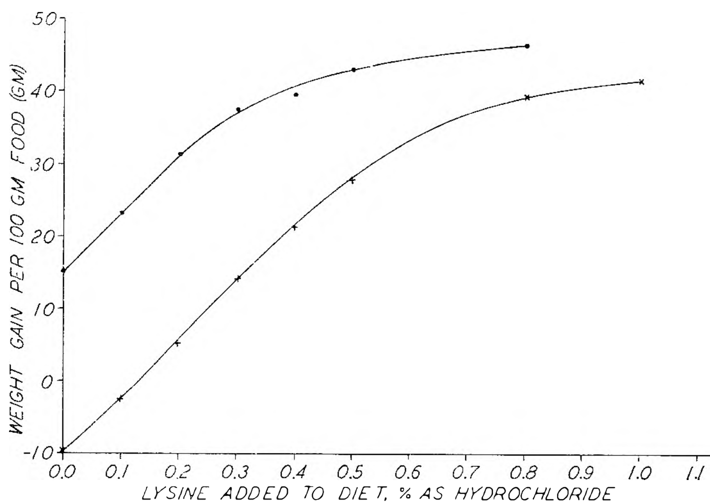


Fig. 4 Weight gain per 100 gm of food consumed of rats on basal A diets versus added percentage of lysine (●); weight gain per 100 gm of food consumed on basal B diets versus added percentage of lysine (×).

sumed (figs. 4–6). Gupta et al. ('58) stated that greater uniformity in availabilities of lysine from various food items from one experiment to another resulted when weight gains were adjusted in this manner. The data in this study yielded curves nearly identical in shape and slope with both basals, and the amount of displacement was more uniform than with method 1, amounting to approximately 0.31% of lysine. Because of the greater uniformity in the standard curves produced by the second method of plotting, one would expect that availabilities calculated on this basis would yield better agreement between the two basals than those calculated without such adjustment (method 1). The results shown in table 6 do not bear out this expectation, however.

Theoretically, the limiting factor in the performance of the animals during the course of the experiment was the total

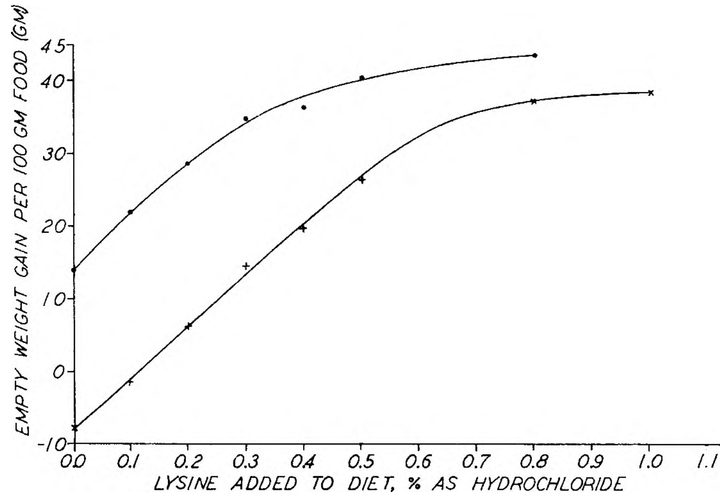


Fig. 5 Empty weight gain per 100 gm of food consumed of rats on basal A diets versus added percentage of lysine (●); empty weight gain per 100 gm of food consumed on basal B diets versus added percentage of lysine (×).

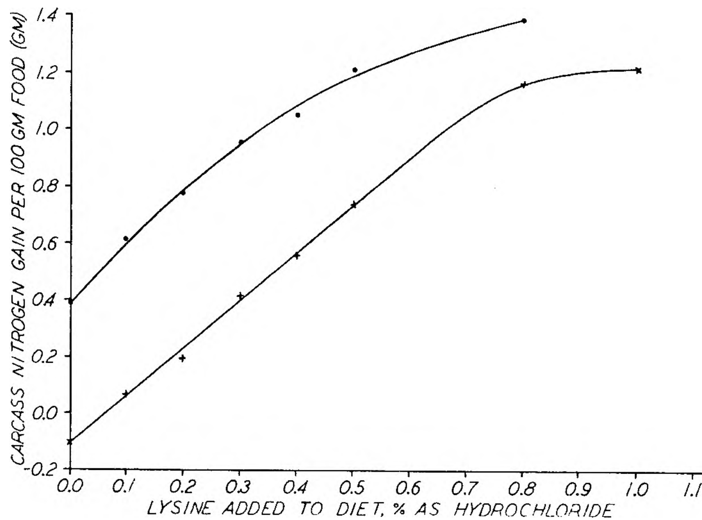


Fig. 6 Carcass nitrogen gain per 100 gm of food consumed of rats on basal A diets versus added percentage of lysine (●); carcass nitrogen gain on basal B diets versus added percentage of lysine (×).

available lysine consumed. In method 3 the performance gains were plotted against the grams of available lysine consumed on the standard curve diets. The basal portion of diets in the second experiment was found to contain 0.008% of free lysine, presumably arising from the amino acid mixture as a contaminant. This amount, together with the increments added, was assumed to provide standard curves based on completely available lysine. The availability of the lysine in the basal portion of the basal A diets was derived from the value obtained with the gluten fed as a sample on the basal B diet. The criterion of nitrogen gain was considered to be the most reliable, yielding a value of 80%. Thus, the available lysine contributed by the gluten of basal A was calculated to be 0.31% of the diet (80% times the 0.39% found by analysis). This value is supported by the amount of displacement of the basal A standard curves shown in figures 4 and 5. The standard curves for experiment 1 (figs. 7–9) were constructed by using the value of 0.31% plus the increments of added lysine in calculating the total available lysine consumed.

The standard curve plots for both basal diets are linear and have similar slopes to about 1.4 gm of lysine when weight gain and empty weight gain are considered (figs. 7, 8). Nitrogen gain (fig. 9) is linear up to approximately 2 gm of lysine with both basal diets and the slopes are nearly the same. This indicates that the deposition of nitrogen was proportional to the amount of available lysine consumed beyond the point where weight gains plateau.

Availabilities calculated by the use of method 3 are given in table 6. The per-

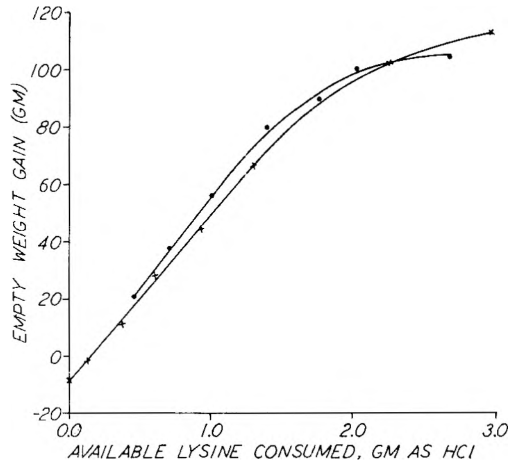


**Fig. 8** Empty weight gains of rats on basal A (●) and basal B (×) diets versus available lysine consumed.

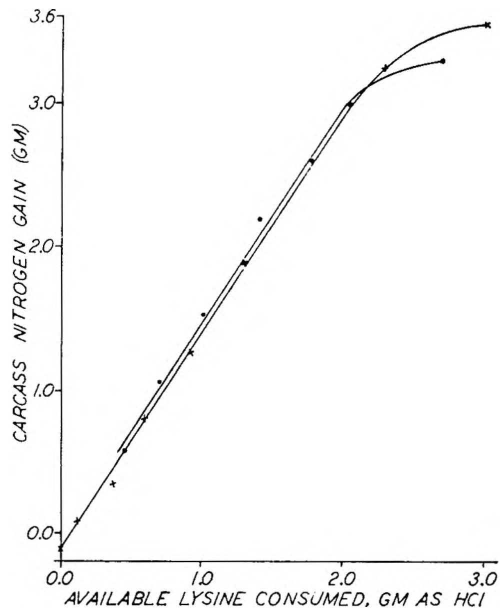


**Fig. 9** Carcass nitrogen gains of rats on basal A (●) and basal B (×) diets versus available lysine consumed.

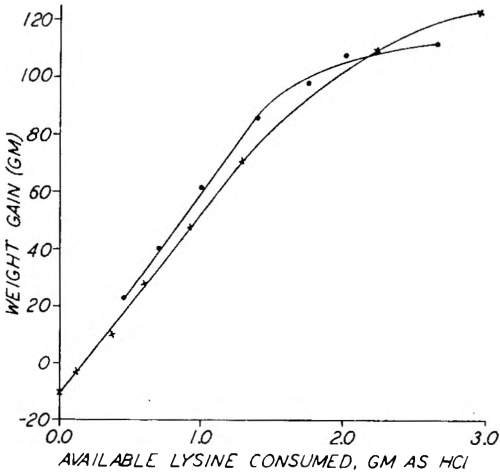


**Fig. 7** Weight gains of rats on basal A (●) and basal B (×) diets versus available lysine consumed.

formance criteria were referred to the appropriate curve to obtain the total available lysine consumed in the diet. From this value was subtracted that amount contributed by the basal diet to give the amount of lysine available from the food supplement alone. Because this method takes into account the differences in actual lysine consumption rather than food consumption, it represents a refinement over adjustment for food intake. In general, results by all criteria of performance yield the best agreement between basal diets when computed against consumed available lysine. Thus this method of calculation seems superior to the other two.

*Comparison of experiments.* The availability values with basal diet B are uniformly greater than those obtained with basal diet A with all methods of calculation. The explanation for this difference is not believed to be the result of a stimulating effect promoted by the protein of the samples because, as shown in figure 1, the increase in carcass nitrogen was proportional to the available lysine consumed in nearly the same degree whether or not protein (gluten) was included in the basal. A possible reason for the differences obtained between experiments is indicated (table 5) by the differences in fecal lysine excretion between basal diets. Except for bread the excretion of sample lysine was less when fed with basal diet B than with basal diet A.

The influence of the method of calculation on the availabilities (table 6) is greatest in the first experiment. This is probably due to the fact that methods of calculation 1 and 2 fail to consider the substantial and variable amounts of lysine consumed from the basal portion of the diets. The results with basal diet B were less affected by the method of calculation used because the amounts of lysine contributed by the basal diet were negligible.

With all methods of calculation the data for weight gain tend to give higher availability values than those for empty weight gain. This is especially evident for wheat and can be explained by the relatively large amounts of food found in the intestinal tracts of these animals. Values for availability obtained with the two basal diets are in best agreement when calculated on the basis of nitrogen gain and this index of performance is believed to be more reliable than weight gain or empty weight gain.

It is concluded that the availability of lysine in the products tested is best represented by the relationship between the increase in carcass nitrogen and the amount of available lysine consumed.

## SUMMARY

The availability of lysine in wheat, flour, bread and gluten was determined by rat growth studies. Two basal diets, one containing 20% of wheat gluten and the other containing an amino acid mixture (omitting lysine) patterned after this amount of gluten were compared. Performance was measured by gain in weight, gain in empty weight and gain in carcass nitrogen over a three-week period. Response to samples was referred to standard curves and the results were compared with those obtained by microbiological assay. Values of lysine availability were calculated by performance versus percentage of added lysine, performance per 100 grams of food consumed versus percentage of added lysine, and performance versus intake of available lysine. Closest agreement between basal diets resulted when carcass nitrogen gain was related to total available lysine consumed. By this method the availability of lysine in wheat, flour and bread was 75, 72 and 76%, respectively, with the gluten basal diet. With the amino acid basal diet, 78, 80, 83 and 80% availabilities were found, respectively, for wheat, flour, bread and gluten.

## ACKNOWLEDGMENTS

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# Pantothenic Acid Deficiency in the Young Guinea Pig[1,2]

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To produce a pantothenic acid deficiency in any species, it has been necessary either to feed simplified rations or to administer an antimetabolite which antagonizes the action of this vitamin. Drell and Dunn ('46) synthesized ω-methylpantothenic acid and studied in detail the effect of the antimetabolite on mice of the Bagg strain (Drell and Dunn, '51). Schinazi et al. ('50) found that the effects produced by the administration of this analogue could be reversed by the simultaneous feeding of pantothenic acid, and that except for interference with the utilization of pantothenic acid, the analogue showed no apparent physiological activity when toxicity tests were made on chicks, rats and mice. More recently, ω-methylpantothenic acid has been used to induce deficiency symptoms in man (Bean and Hodges, '54) and in the calf (Sheppard and Johnson, '57). In the present investigation the effect of ω-methylpantothenic acid was studied using guinea pigs as the test animal.

## EXPERIMENTAL PROCEDURE

Nine pairs of weanling male albino guinea pigs,[4] weighing 138 to 232 gm, were fed a complete basal diet consisting of rabbit pellets[5] supplemented with 10 mg of ascorbic acid per day and one drop of oleum percomorphum per week. One pig of each pair was fed the basal diet plus supplements and designated the control. The other pig of each pair was fed the basal diet plus supplements and the antimetabolite, ω-methylpantothenic acid as 0.15% of the intake, for the first 15 days of the study, as 0.30% for the next 18 days and finally as 0.40% for the remaining 14 days of the study.

The animals were weighed daily and fed their ration and water ad libitum. The analogue was incorporated into the rabbit pellets after they had been ground in a Wiley mill. The ration was then moistened with a small amount of water, repelleted,[6] and dried under an infra red lamp for approximately two hours at a temperature not over 55°C.

After receiving 0.15% of analogue for 15 days, three experimental animals and their littermate controls were killed. The experiment was terminated on the 47th day. The two groups were compared on the following bases: weight gain, ration consumed, food efficiency, red blood cell count, packed red blood cell volume, hemoglobin, serum ascorbic acid, blood pyruvic acid, and condition before and during autopsy. The animals were anesthetized by intraperitoneal injection of a solution of sodium pentobarbital. Blood samples were obtained from the portal vein.

## METHODS

Hemoglobin determinations were made by a modification of the procedure of Wintrobe ('46) which measures oxyhemoglobin. Twenty $mm^3$ of oxalated blood were diluted with 8.0 ml of a 0.5% solution of ammonium hydroxide and read at a wave length of 540 mμ in a Beckman spectrophotometer which had been standardized

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[2] Presented at the meetings of the American Institute of Nutrition, Atlantic City, New Jersey, April, 1956.

[3] Present address: Home Economics Department, University of Missouri.

[4] Obtained from Gopher State Caviary, St. Paul, Minnesota.

[5] Nutrena.

[6] Appreciation is expressed to the Mechanical Engineering Department for use of their plastic mold for repelleting the ration.

by the oxygen capacity method of Peters and Van Slyke ('32,). Since oxyhemoglobin from different sources may differ in both spectroscopic characteristics and in affinity for oxygen (Hawk et al., '51), iron determinations by the Wong procedure (Hawk et al., '51) also were made. These confirmed the hemoglobin values obtained by measuring the oxyhemoglobin formed.

Blood serum ascorbic acid was determined by the Lowry et al. ('45) microadaptation of the dinitrophenylhydrazine method of Roe and Kuether ('43).

Pyruvic acid concentration of the blood was estimated by the micromethod of Tsao and Brown ('50).

Pantothenic acid in the blood and rabbit pellets was determined by microbiological assay using *L. arabinosus* and a basal medium which was essentially that of the American Association of Agricultural Chemists and United States Pharmacopoeia ('45) and the enzyme treatment of Novelli and coworkers ('49).

## DISCUSSION OF RESULTS

Daily weight gain and food intake of the experimental animals and their controls are summarized by the week in figure 1. During the first week, no difference in average daily weight gain, food intake nor food efficiency was observed between the two groups. During the second and third weeks, animals in the experimental group ate 2 gm more of food each day but gained 2 gm less weight each day than those in the control group showing a corresponding decrease in food efficiency. During the 4th week, the pigs receiving the analogue ate 2 gm less of food each day than the controls, while gaining 4 gm less weight each day. In the 5th, 6th and 7th weeks the difference in food intake between the two groups increased to 7, 12 and 11 gm, respectively, whereas the difference in weight gain per day remained constant at 6.6 or 6.7 gm. Since the animals survived for almost 7 weeks, the deficiency which developed was considered to be chronic rather than acute.

The difference in weight gain, food consumption and efficiency of food utilization between the two groups increased proportionally to the level of analogue in the diet. Nevertheless, the experimental animals had fairly good appetites and continued to gain some weight until the termination of the study (table 1). This would indicate that some pantothenic acid in the ration was available to the animals during the entire study. An assay of the rabbit pellets indicated the concentration of pantothenic acid was 47.2 μg/gm. Thus, when the analogue was incorporated at 0.15, 0.30, and 0.40% , the ratio of analogue to pantothenic acid was 32:1, 64:1, and 85:1, respectively. The calculated intakes of pantothenic acid and of the analogue are shown in table 1.

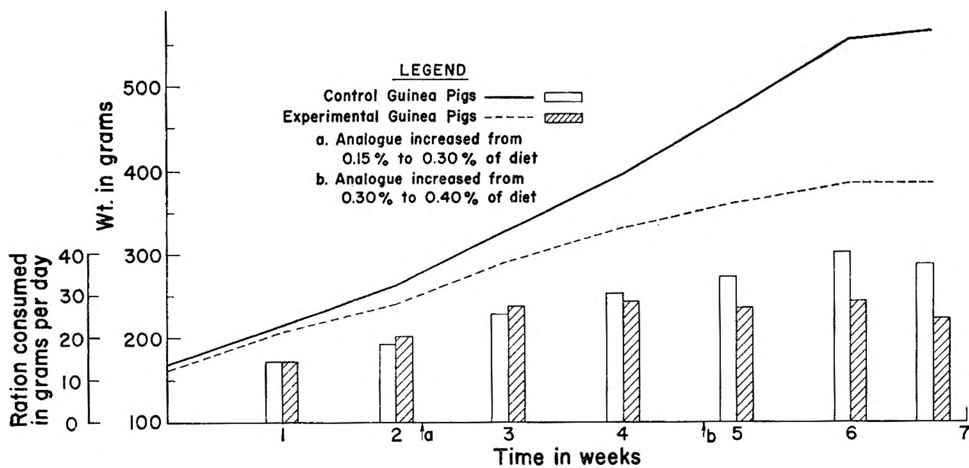


Fig. 1 Average weekly weight and food consumption of guinea pigs receiving ω-methylpantothenic acid and of their litter mate controls.

TABLE 1

*Average weight gain, food consumption, food efficiency, intakes of pantothenic acid and ω-methylpantothenic acid, values of experimental and control animals*

| | Av. gain in weight | Av. food intake | Weight gained/ gm food eaten | Pantothenic acid intake[1] | Omega-methyl-pantothenic acid |
|---|---|---|---|---|---|
| | *gm/day* | *gm/day* | *gm/day* | *mg/day* | *mg/day* |
| During 15 days fed 0.15% analogue | | | | | |
| Controls | 5.9 | 18 | 0.38 | 0.85 | |
| Experimental | 5.0 | 19 | 0.30 | 0.90 | 29 |
| During 18 days fed 0.30% analogue | | | | | |
| Controls | 9.3 | 30 | 0.31 | 1.42 | |
| Experimental | 5.9 | 28 | 0.21 | 1.32 | 84 |
| During 14 days fed 0.40% analogue | | | | | |
| Controls | 8.9 | 38 | 0.24 | 1.79 | |
| Experimental | 1.8 | 27 | 0.06 | 1.27 | 108 |

[1] Ration analyzed microbiologically for pantothenic acid.

Red blood cell counts, hemoglobin levels and packed red blood cell volumes showed essentially no differences between the two groups after 15 days of 0.15% ingestion of analogue, but when the study was terminated, the experimental animals were anemic as indicated by their considerably lowered blood values (table 2). Carter et al. ('45) and Daft et al. ('45) have reported anemia in rats receiving purified diets deficient in pantothenic acid. Carter reported a severe hypochromic anemia, along with other manifestations of pantothenic acid deficiency, in 60% of his animals. When pantothenic acid was administered, the blood picture was restored to normal in only 25% of cases. To be successful, treatment had to be started early in the development of anemia. Daft found that adequate amounts of pantothenic acid, but not of folic acid, prevented the development of anemia and granulocytopenia in his rats. Anemic animals seemed to respond to treatment with pantothenic acid more consistently and rapidly than the granulocytopenic rats; however, to restore the blood picture to normal, therapy with both pantothenic acid and folic acid was required. It is interesting that anemia occurring in scurvy also responds to folic acid therapy (May et al., '50).

The blood serum ascorbic acid of the three experimental pigs, after 15 days on the diet containing 0.15% of analogue, averaged only one-half that of their control littermates, 0.19 versus 0.35 mg%. When the study was terminated, after 47 days, a similar difference in blood serum concentrations of ascorbic acid persisted; the average value for 6 experimental pigs was 0.23%, that for the 6 controls, 0.51 mg%. The range of values did not overlap at either time (table 2). None of the experimental animals showed gross symptoms of scurvy but three pigs showing values of 0.11 or 0.12 mg of ascorbic acid per 100 ml of serum were probably border-line cases. Two of these pigs also tended to be anemic whereas hematological data for the third pig were not obtained. Presnell ('34) believed that changes in the quantity of formed elements in the blood begin in the guinea pig deprived of ascorbic acid before any of the usual gross symptoms of scurvy appear. He found that the blood of scorbutic guinea pigs had a longer coagulation time, a lower hemoglobin concentration and red cell volume and fewer red blood cells than the blood from a healthy guinea pig. In the present investigation, since the animals receiving ω-methylpantothenic acid were fed the same amount of ascorbic acid as their control litter mates, apparently pantothenic acid may be necessary for the efficient utilization of ascorbic acid.

No differences in blood pyruvic acid values (table 2) between the two groups were observed after the first 15 days; how-

TABLE 2

*Blood values of guinea pigs fed ω-methylpantothenic acid and of littermate controls*[1]

| | After 15 days | | After 47 days | |
|---|---|---|---|---|
| | Experimental | Control | Experimental | Control |
| **Erythrocytes, *millions/mm³*** | | | | |
| Average | 4.96(3) | 5.12(3) | 4.09(5) | 5.65(6) |
| Range | 4.29–5.36 | 4.70–5.64 | 3.17–5.04 | 5.46–5.88 |
| **Hemoglobin, *gm/100 ml*** | | | | |
| Average | 12.07(3) | 11.74(3) | 9.33(5) | 13.84(6) |
| Range | 10.29–13.15 | 11.12–12.12 | 7.58–11.14 | 13.28–14.20 |
| **Packed red cell volume, %** | | | | |
| Average | 37.8(3) | 36.7(3) | 29.3(5) | 42.6(6) |
| Range | 32.8–40.8 | 34.5–38.8 | 24.2–34.8 | 41.2–43.2 |
| **Ascorbic acid, *mg/100 ml serum*** | | | | |
| Average | 0.19(3) | 0.38(3) | 0.23(6) | 0.51(6) |
| Range | 0.11–0.23 | 0.26–0.52 | 0.11–0.43 | 0.45–0.64 |
| **Pyruvic acid, *mg/100 ml blood*** | | | | |
| Average | 1.48(3) | 1.50(3) | 4.23(6) | 2.01(5) |
| Range | 0.86–2.01 | 1.30–1.65 | 1.73–8.14 | 1.53–2.71 |
| **Pantothenic acid, *μg/100 ml blood*** | | | | |
| Average | | | | 39(5) |
| Range | | | | 25–58 |

[1] Figures within parentheses represent number of animals included in average.

ever, by the time the study was terminated, pyruvic acid had accumulated in the blood of the experimental animals so that they showed twice as much as the controls. Pantothenic acid values on the blood of the control group ranged from 25 to 58 μg/100 ml of blood (table 2). Determinations on the blood of the experimental group could not be made owing to interference of the analogue.

The condition of the animals before autopsy and observations of the organs during autopsy were recorded. At the termination of the study all 6 control animals were rated good in appearance. One experimental animal was found lying on its side, completely immobile and in a comatose condition early on the morning of the 35th day of the study. After about an hour, the animal got up and moved about, dragging both hind legs behind it. After another hour, its eyes began to water and excessive salivation was observed. The animal dragged itself to the feed cup but lacked the energy to eat. At autopsy a careful examination for signs of infection revealed none.

Another experimental pig had convulsions with head retraction on the morning of the 45th day. After a convulsive attack the animal would lie on its back with all 4 legs extended very rigidly. The attacks, with intermittent rest periods, occurred for about one to one-and-one-half hours. This condition appears to be the same as that described by Sheppard and Johnson ('57) as "tonic-clonic convulsions" which occurred in one calf fed a pantothenic acid-deficient diet. Muscular weakness in the hind legs and a small amount of salivation were observed in this pig also.

By the 47th day, three of the remaining 4 experimental animals were in poor physical condition. They were pale and listless and sat hunched in a corner of the cage. One had soft, woolly fur whereas another had watering eyes. Only one pig remained active but it was pale and had soft woolly fur.

The livers of experimental animals were lighter in color than those of control animals. Adrenal glands of three experi-

mental animals were hemorrhagic. A contrast between the two groups in the color and appearance of the kidneys was quite striking also. The kidneys of one pig were pale and the cortices appeared spongy and swollen whereas the cortices of the kidneys of two other pigs showed hemorrhagic areas. In the three pairs of pigs killed after the first 15 days of the study, the spleens of the two groups were practically the same size. When the study was terminated, the spleens of the experimental animals were larger than those of their litter mate controls. Carter et al. ('45) reported observing splenomegaly along with a severe anemia in about 60% of their pantothenic acid-deficient rats. Large stores of visceral fat were noted in both groups of animals; however, those found in the control animals were more abundant. The gastrointestinal tracts of the experimental animals were either distended, collapsed or filled with fluid. No signs of infection were observed in either group of animals.

## SUMMARY

A pantothenic acid deficiency was produced in guinea pigs by feeding the antimetabolite, ω-methylpantothenic acid along with a natural ration. Except for the decreased concentration of ascorbic acid in the blood serum, the symptoms which were produced by feeding the analogue in this study, have been reported for one species or another as pantothenic acid deficiency. Physical symptoms observed in this experiment included soft woolly fur, pallor, lassitude, salivation, watering of the eyes, muscular weakness of the hind legs, convulsions and coma. Biochemical changes were characterized by anemia, accumulation of pyruvic acid in the blood and lowered serum ascorbic acid levels. Hemorrhagic adrenals and splenomegaly were observed upon autopsy. The deficiency was believed to have been chronic.

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# Experimental Obesity and Weight Reduction in Young Female Rats: Development of Procedures and Calcium and Phosphorus Balance Studies[1]

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Several groups of workers have reported metabolic studies of weight reduction in obese young women (Brown et al., '46a, '46b; Leverton et al., '49, '51; Cederquist et al., '52; Brewer et al., '52; Young, '52a, '52b; Young et al., '53; Young et al., '59a; '59b). Although they have conducted their experiments under varying conditions, all have concluded that during caloric restriction, calcium, phosphorus and nitrogen retention were less than when the caloric intake was adequate. Four investigations in this laboratory have demonstrated that the women were retaining the nutrients in their obese state prior to reduction. After 7 to 10 weeks on the reduction diet, using the same levels of calcium, phosphorus and nitrogen intake, the majority of subjects were definitely retaining smaller amounts of nutrients and often were in negative balance. However, after three or 4 weeks on the post-reduction maintenance diet, the subjects were either in a state of equilibrium or in positive balance with the same level of intake of these nutrients.

In the human studies, the length of experiments have been limited usually to one academic term by the availability of the subjects for that period only. Does the subject eventually come into equilibrium on the reduction diet before she reaches her ideal weight? Or, does she continue to lose nutrients as weight reduction is prolonged? What causes the nutrient losses?

To answer these questions laboratory animals were used as experimental subjects in the current investigations, to permit time for body weights of the obese subjects to be reduced to ideal levels; and, also to make possible, bone density studies of both control and experimental animals after killing. The present report is concerned with the calcium and phosphorus metabolism of obese rats before, during and after weight reduction, by means of balance studies and bone density studies. The obesity was produced by strictly dietary means and forced-feeding to be assured of (1) absolute control of the food intake, particularly calcium, phosphorus and nitrogen; (2) production of obesity; and (3) accuracy of the balance studies.

## SUBJECTS AND METHODS

The general plan was to use animals and methods as comparable in condition as possible to the young women used in previous studies. One problem was that of knowing the nutritional requirements for maintenance of adult female rats. Though numerous studies had been reported, the majority of them concerned young, growing rats or adult rats under special physiological conditions, such as pregnancy and lactation. Usually nutrient requirements are expressed in terms of percentage of the weight of the diet; for our purposes absolute quantities were desired.

Studies were made of 7.5-month-old female Sprague-Dawley rats during the following periods: pre-obesity maintenance; the production of obesity; weight reduction; and post-reduction weight maintenance. Throughout the experiment, female rats of the same age, but kept at relatively constant weight, served as controls. At various stages of the experiment, metabolic studies of the experimental animals were

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made by means of calcium and phosphorus balances and by means of bone density studies of control and experimental animals killed simultaneously. Throughout the entire experiment, both control and experimental animals received intakes of calcium, phosphorus and nitrogen calculated to be constant. The caloric content of the diet for experimental animals was varied with the phase of the experiment.

Thirty-seven rats were used in the entire experiment of 114 days duration. Initially, three rats were selected at random and killed on the first day for controlled data. The remaining 24 rats were divided into groups: control and experimental. The control rats were carried at approximately their initial weights and kept on a maintenance diet throughout the study. For the experimental group, the study was divided into 4 phases:

1. Pre-obesity maintenance period—6 days: all rats were tube-fed basal amounts of calcium, phosphorus and nitrogen plus sufficient calories as determined by previous study, to presumably maintain their weights (48 Cal./day). During the last 4 days, a balance study was conducted on the experimental group.

2. Obesity production period—62 days: the experimental animals were force-fed the basal amounts of nutrients plus extra calories sufficient to produce weight gain (61 Cal./day). Such treatment was continued until body weights were about 30% above their original. Two 4-day balance studies were made with the experimental rats: one at "partial" obesity, when the rats were 15% above their initial weight; the other at "full" obesity, when they were approximately 30% above initial weights. On the last day of the obesity production period, three control and three experimental rats were killed.

3. Reduction period—34 days: obese rats were fed the basal diet and just enough calories to obtain a satisfactory weight loss until they had attained the weights of control animals (32 Cal., later reduced to 26 Cal./day). Three balance studies were made during the early, middle and late phases of weight reduction. Three pairs of control and experimental rats were killed at mid-reduction; another three pairs on the last day of reduction.

4. Post-reduction maintenance period—12 days: the remaining three experimental rats were fed for 12 days the basal diet plus calories sufficient to maintain their reduced weights (43 Cal./day, successively reduced to 38 and then to 35). During the last 4 days a balance study was made. Then, on the last day of the experiment the remaining three experimental and three control rats were killed.

Young adult female rats were used, of a chronological age comparable to the young women previously studied. The age level was that of the young adult at the end of the growth period when most bone development was complete. According to Asdell ('46), the end of the growth period for the rat, dated from birth, is 6.6 months; for man, this corresponds to 20 years. Poiley (Jayen et al., '53) compiled the weight-age relationships by sex for three rat colonies. For the age range we desired, the figures for female Sprague-Dawley rats would indicate that from 182 days the weight has begun to plateau and probably the rat has reached the mature level. On the basis of these criteria, 6.5-month-old rats having an average body weight of 240 gm were ordered. However, owing to time lost in conditioning the animals to tube feeding and determining maintenance calories, the animals were 7.5 months old when the formal experiment began. They were tube-fed twice daily, 8 to 10 A.M. and 4 to 6 P.M., by a pair of operators using a 10 $cm^3$ Luer-Lok syringe equipped with a 15 gauge short, blunt ended hypodermic needle and "intermedic" polyethylene tube, 2 to 2.25 inches long (outer diameter 0.11 inch; inner, 0.07 inch).

The diet was planned to be similar to that used in the human studies, namely, high protein, moderate fat and calculated to be of constant nitrogen, calcium and phosphorus content. The daily diet consisted of 1.75 gm of lactalbumin; 0.5 gm of U.S.P. Salt Mixture XIV which provided 55 mg of calcium and 33.3 mg of phosphorus, a pure vitamin mixture supplying all known needed vitamins, and corn oil and dextrin. Caloric adjustments were made by varying the amounts of the latter two ingredients without altering greatly the ratio of dextrin to corn oil; the percentage of calories from each of these was

similar to levels from fat and carbohydrate as used in the human diets. In preparation of the diets, the lactalbumin and vitamins were premixed. To these the minerals were added, then the dextrin and corn oil to form small batches of dry mix. Water was added at the time of feeding to make the diet liquid for tube-feeding.

During the 4-day balance periods, fecal material was collected daily and kept in acid alcohol in the cold room until used; urine was withdrawn from the collecting funnel daily and kept under toluene. Samples were analyzed by the method of Kochakian and Fox ('44) for calcium, and of Koenig and Johnson ('42) for phosphorus. The microKjeldahl Winkler-boric acid modification was used for nitrogen determination (Woods and Williams, '50).

At the time of killing, the left femur was used for bone density studies by the method of McCay and associates ('56).

## RESULTS AND DISCUSSION

*Weight changes and caloric intake.* Figure 1 shows the mean weight curves of both control and experimental animals together with the caloric intake of the animals at various stages of the experiment. Timing of the 7 balance periods to which the experimental animals were subjected is also indicated.

During the first 14 days of the experiment, the control rats gained an average of 0.55 gm daily on an intake of 48 Cal./day. Thus the diet was cut to 43 Cal. from that point on. A striking difference existed between the control group and the experimental rats in terms of general health and natural mortality. In general, the control rats were much less healthy; three died before time of sacrifice. Was body growth being retarded by caloric adjustment to maintain weight? Though Poiley (Jayen et al., '53) indicated a plateauing of weight in female Sprague-Dawley ani-

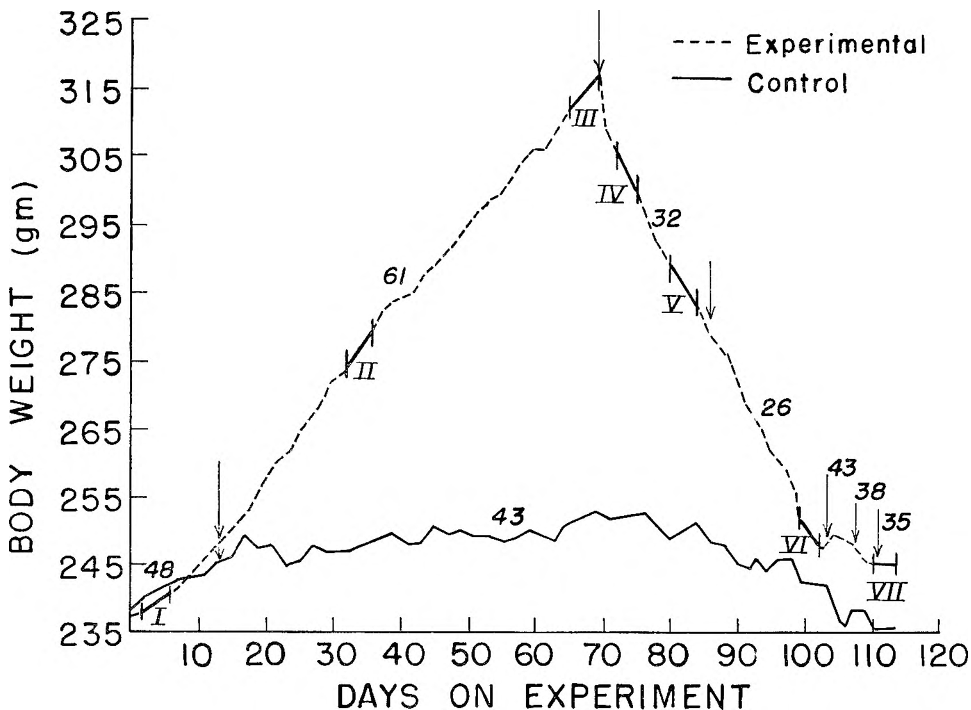


Fig. 1 Weight changes and caloric intake at various stages of the experiment. Numbers above lines are daily caloric intake between points indicated by ↓. Timings of balance studies for experimental animals are represented by Roman numerals within parallels in weight curve.

mals at about 182 days, Bharucha and McCay ('54) demonstrated that adult bone growth occurred even after 280 days of age. Zucker et al. ('41) also indicated that the body weight of a large number of rats they studied continued to increase slowly as late as 70 weeks (490 days) of age. They also gave evidence that the resulting weight increase represented true body growth and not just fat deposition.

In the weight reduction period, during the first 7 days on 32 Cal. daily, the average rate of loss was 2.7 gm/day. During the next 8 days, the rate decreased to 1.6 gm/day. The decrease in rate is similar to that found in previous human studies (Young et al., '59a; '59b). With a reduction in daily caloric intake to 26, the remaining animals lost weight at a rate of 2.02 gm/day during the last 19 days of the period. In the post-reduction period, daily caloric intake had to be reduced from 43 to 38 to 35 Cal. before weight maintenance was achieved. Caloric needs after weight reduction appeared to be less than for the control animals or than for the experimental animals before the production of obesity and subsequent weight reduction.

*Balance studies*

*Calcium.* A summary of the calcium retentions of the 7 balance periods (4 days each) with the experimental rats is presented in table 1. Figure 1 shows the timing of the balance periods in relation to the weight changes. If one uses as an indication of retention or loss of calcium, a retention or loss in excess of 10% of intake, during the pre-obesity period 5 rats were storing calcium; the other 7 were in equilibrium. When the rats were roughly 15% above their initial weights, the calcium retention of several animals was increased considerably: 8 were storing calcium; 4 were in equilibrium. At 30% above initial weight, 8 were in retention, two in equilibrium, and two in deficit. Three rats were then killed for bone density studies.

At the early phase of the reduction regimen when the 9 remaining rats had been on a 32 Cal. diet for 7 days, 7 showed large decreases in calcium retention so that only one rat was in calcium retention, 4 in equilibrium and 4 were losing calcium. One (no. 67) which had been losing calcium previously was the one storing it during this phase.

Toward the middle of the reduction regimen, when the experimental rats had lost 50% of their excess weight, the calcium retention of most of them had improved; 5 rats were storing calcium; two were in equilibrium, and two were losing calcium. At the conclusion of this period three additional animals were killed.

By the end of the reduction period 4 of the remaining 6 rats had deteriorated with regard to calcium balance; two had improved. Hence three were in equilibrium and three were losing calcium. Three animals were again killed. In the post-reduction maintenance period with adequate caloric intake, the calcium retention of the remaining three rats had improved, two rats were in balance and one was storing calcium in large amounts.

In spite of wide variations between individual rats, the same pattern is seen when mean retention figures are examined by period (table 1). Definite calcium losses were observed early and late in reduction. Rats fed caloric levels adequate for weight maintenance or gain showed a mean retention of calcium.

It is interesting to compare these results with rats with those observed in the young women previously studied in this laboratory. Of course, it is recognized that the rate of weight loss was quite different in the two species. The rats lost at the rate of 0.6% of body weight per day; the young women at the rate of 1.1 to 1.2% of body weight per week. If one assumes one day in a rat's life as equivalent to approximately 5.2 weeks in a human life, on an equivalent basis, rats lost only 0.01% of body weight in the same period (one week) in which the young women were averaging a loss of 1.1%. Also, with the animals the opportunity existed to bring them back to nearly pre-obesity weights. At the end of the reduction period, of course, relatively, the animals were considerably older than the young women.

Yet, given these differences, very similar results are apparent for the calcium balances of both the young women and the rats. With the young women usually no balance study was made in an early re-

TABLE 1

*Mean daily retentions of calcium and phosphorus in experimental rats*

| Balance periods | Analyzed intake[1] | Retention by subjects | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 55 | 69 | 80 | 62 | 67 | 75 | 53 | 74 | 57 | 71 | 73 | 56 | Mean |
| | *mg* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* |
| **Calcium** | | | | | | | | | | | | | | |
| 1. Pre-obesity maintenance | 58.00 | 8.51 | 19.16 | 2.55 | 14.78 | 2.96 | 4.98 | −1.16 | 8.54 | 17.66 | −2.65 | 2.17 | −0.14 | 6.45 |
| 2. "Partial" obesity | 58.58 | 13.65 | 6.63 | 5.55 | 5.96 | 16.92 | 4.76 | 3.91 | 15.67 | 3.99 | 14.89 | 16.11 | 21.02 | 10.76 |
| 3. "Full" obesity | 58.03 | −1.94 | 22.19 | 15.56 | 13.01 | −7.02 | 2.10 | 13.52 | 20.96 | −11.20 | 13.84 | 15.98 | 19.74 | 9.73 |
| 4. Early reduction | 57.97 | 0[2] | 0 | 0 | −2.59 | 8.88 | −16.51 | −21.68 | −1.45 | −14.63 | −22.23 | 0.96 | 0.54 | −7.63 |
| 5. Mid-reduction | 64.71 | | | | 5.25 | 22.88 | 14.37 | −14.11 | 7.25 | −4.24 | 14.71 | −9.27 | 16.89 | 5.90 |
| 6. Late reduction | 54.28 | | | | 0 | 0[3] | 0 | 1.78 | −21.26 | −20.96 | −33.92 | 0.90 | 0.01 | −12.24 |
| 7. Post-reduction maintenance | 63.75 | | | | | | | 0 | 0 | 0 | 4.44 | −5.42 | 28.75[3] | 9.26 |
| **Phosphorus** | | | | | | | | | | | | | | |
| 1. Pre-obesity maintenance | 40.0 | 11.13 | 5.70 | 8.92 | 4.10 | 10.95 | 5.90 | 2.00 | 9.10 | 12.40 | 8.52 | 5.67 | 7.10 | 7.62 |
| 2. "Partial" obesity | 48.3 | 25.85 | 8.90 | 18.62 | 14.95 | 23.60 | 10.70 | 14.15 | 16.95 | 23.32 | 20.85 | 15.00 | 5.79 | 16.56 |
| 3. "Full" obesity | 68.0 | 28.57 | 34.80 | 41.50 | 34.40 | 17.80 | 24.00 | 27.80 | 40.80 | 14.50 | 36.20 | 32.10 | 46.47 | 31.58 |
| 4. Early reduction | 44.0 | 0[2] | 0 | 0 | 11.33 | 6.60 | 4.50 | −7.80 | 11.00 | 4.70 | −2.20 | 2.80 | 9.90 | 4.54 |
| 5. Mid-reduction | 54.0 | | | | 13.90 | 23.10 | 2.40 | −3.60 | 10.95 | 7.20 | 8.70 | −0.90 | 12.30 | 8.23 |
| 6. Late reduction | 49.0 | | | | 0 | 0 | 0 | −11.40 | −22.10 | −23.10 | −38.10 | −7.40 | 3.70 | −16.40 |
| 7. Post-reduction maintenance | 51.0 | | | | | | | 0 | 0 | 0 | 7.40 | 5.90 | 20.3 | 11.20 |

[1] Calculated value: calcium = 55.0 mg; phosphorus = 33.3 mg.
[2] 0 = Killed.
[3] + = Sick when killed.

duction phase. But the same pattern is observed: calcium retention or equilibrium in the obese state; some decrease in calcium retention by mid-reduction. As weight reduction was prolonged, a further decrease occurred so that many of the subjects, both human and rats, were in calcium deficit. Even though the rats were carried for a relatively longer time on weight reduction and until they reached an approximation of their pre-obesity weights, calcium equilibrium was not reached; the animals continued to lose calcium as weight reduction was prolonged. However, during post-reduction maintenance period, retentions improved so that in general, the subjects were either storing calcium or were in a state of equilibrium. The occurence of a deficit in two rats during one of the obesity periods is contrary to the usual picture; mean figures for the group showed retention.

Decreases in calcium retention were related usually to increased fecal calcium excretion, which often was accompanied by a small decrease in urinary excretion. We can offer no adequate explanation for the calcium patterns observed. No clear-cut relationship was seen between the rate of weight gain in the obesity period and the calcium retention. Health status did not seem to affect the calcium balance. In fact, when a rat was sick, it appeared to be storing more calcium, if anything. Rat no. 71, the only experimental animal which appeared stressful throughout the entire experiment during the force-feeding process, showed large fluctuations in calcium retentions. Stearns ('55) and Malm ('55) have pointed out an inverse relationship between emotional stress and calcium retention in human subjects under their observation.

*Phosphorus.* The phosphorus intakes and retentions in the experimental animals during the 7 balance periods are presented in table 1. In all of the periods prior to caloric restriction, all animals were either in phosphorus equilibrium or, in most cases, retention. Also, in most cases, with caloric restriction, retention dropped sharply. By the end of the reduction period 5 of the 6 remaining animals showed a severe phosphorus deficit. With relief of caloric restriction in the post-reduction maintenance period, phosphorus retention increased markedly in all of the three surviving rats so that all were in a state of retention. These results are similar to those found in human studies. It is interesting to note that analyses of all diets gave considerably higher values for phosphorus than the calculated daily intake of 33.3 mg.

### *Bone density studies*

In table 2 are given the bone densities of the left femur of experimental and

TABLE 2
*Bone density of left femur of sacrificed experimental and control rats—by period*

| Period in which killed | Age of animals | Experimental | | | Control | | |
|---|---|---|---|---|---|---|---|
| | | Animal no. | Bone density | Mean by period | Animal no. | Bone density | Mean by period |
| | *days* | | | | | | |
| Initial controls | | | | | 54 | 1.2847 | |
| | 225 | | | | 64 | 1.3291 | 1.2885 |
| | | | | | 76 | 1.2527 | |
| Obesity | | 55 | 1.2940 | | 59 | 1.2608 | |
| (+ 30% | 293 | 69 | 1.2272 | 1.2693 | 77 | 1.2555 | 1.2582 |
| weight) | | 80 | 1.2866 | | 52 | —[1] | |
| Mid-reduction | | 62 | 1.1876 | | 58 | 1.0903 | |
| (− 15% | 307 | 67 | 1.1838 | 1.1941 | 65 | —[1] | 1.1913 |
| weight) | | 75 | 1.2109 | | 81 | 1.2922 | |
| Late reduction | | 53 | 1.2039 | | 51 | —[1] | |
| (− 30% | 327 | 74 | 1.2369 | 1.2089 | 61 | 1.1437 | 1.1437 |
| weight) | | 57 | 1.1860 | | 82 | —[1] | |
| Post-reduction | | 71 | 1.2051 | | 68 | 1.1720 | |
| maintenance | 339 | 73 | 1.1680 | 1.1789 | 78 | 1.1685 | 1.1703 |
| | | 56 | 1.1637 | | 72 | —[1] | |

[1] Animal died before end of experimental period.

control animals killed at the end of specified periods of the experiment. Control and experimental animals were paired at the start of the experiment by initial weights. The table shows clearly that death before sacrifice occurred entirely among the control animals. Hence, it is difficult to make good comparisons. No consistent pattern in bone densities was found between paired animals which may be attributed to the production of obesity and subsequent weight reduction or to calcium balance. For all of the animals, bone density appeared to decrease as the experiment was prolonged and as the animal progressed from 32 to 48 weeks of age. This is in agreement with Bharucha's report ('51) that with the Yale strain of albino rats she observed a trend for bone densities to increase with age up to 150 to 180 days and thereafter to decrease. If anything, the maintenance of our control animals at a given weight resulted in lower bone densities than the rigors of the production and reduction of a mild obesity.

## SUMMARY

A study of the effect of the production of obesity, weight reduction, and post-reduction weight maintenance on the calcium and phosphorus metabolism of young adult female Sprague-Dawley rats was made by means of 7 balance studies (4 days each) spaced throughout the various phases of the experiment, and by means of left femur bone density determinations of the 27 paired control and experimental animals (three initial control animals plus 24 experimental and control animals paired by initial weight). The intakes of nitrogen, calcium and phosphorus were calculated to be constant for all animals throughout the experiment; calories were varied with the phase of the experiment. A tube-feeding technique was used with a synthetic diet to insure quantitative control of intake.

Weights of the approximately 240 gm females were maintained on a daily intake of 43 Cal. An average weight gain of 75 gm, which represented an increase of 30% over the initial pre-obesity weight, was produced in 62 days on an intake of 61 Cal./day, the average daily weight gain being 1.2 gm. Fed an average daily intake of 29 Cal. over a 34-day period, the obese animals were reduced at the rate of approximately 2 gm/day to their pre-obesity weights. The rate of weight loss decreased with the prolongation of reduction so that by mid-reduction, caloric intake had had to be reduced from 32 to 26 Cal./day to maintain a satisfactory weight loss.

In the post-reduction weight maintenance period, the animals in which weight reduction had been effected required fewer calories (38) for weight maintenance than controls of similar weight and age and than these experimental rats had required at the same weight prior to the production and reduction of obesity.

In general, during the pre-obesity and obesity periods, the majority of the animals retained calcium. Some were in equilibrium and, just prior to the onset of weight reduction, two were in calcium deficit. In the early phase of the reduction period in the majority of the animals calcium retention or equilibrium tended to shift into equilibrium or deficit. For some unexplained reason, calcium retention improved somewhat in the mid-retention phase. However, late in the reduction period there was a return to the poorer calcium retention found in the early reduction phase. With the elimination of caloric restriction in the post-reduction maintenance phase, calcium retention again improved. These results are similar to those obtained in young female human subjects, although data were not available for the early reduction phase for the human subjects.

No adequate explanation could be offered for the negative calcium balances observed in some of both the rat and human subjects during weight reduction.

Phosphorus retention was noted in practically all of the animals until caloric restriction in the reduction phase, at which time retention decreased. By late reduction over 80% of the animals were in phosphorus deficit; none were retaining phosphorus. With the restoration of calories for weight maintenance in the post-reduction period, all remaining animals returned to a state of phosphorus retention.

Control animals maintained at the relatively constant initial weight of 240 gm were less healthy throughout and several

died before the time scheduled for sacrifice with initially paired experimental rats.

Bone densities of the left femur of paired control and experimental rats showed no consistent pattern with phases of the experiment which might be attributed to the production and reduction of initial obesity or to the calcium balance status in experimental animals. For all animal groups, bone density appeared to decrease as the experiment was prolonged and as the animals progressed from 32 to 48 weeks of age.

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# Potency of Vitamin $K_1$ and Two Analogues in Counteracting the Effects of Dicumarol and Sulfaquinoxaline in the Chick[1,2]

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The reversal of dicumarol-induced hypoprothrombinemia by vitamin $K_1$ and several of its analogues has been widely studied because of its great importance in anticoagulant therapy in humans. For a review of significant contributions in this field with regard to mammals the reader is referred to a recent paper by Mushett et al. ('59). From the literature mentioned in this publication, apparently vitamin $K_1$ is superior to menadione or menadione sodium bisulfite in counteracting dicumarol. Dam and Søndergaard ('53) indicated that a similar situation, at least with regard to menadione, could be observed in the chicken. However, few quantitative data seem to be available for the chicken.

While little doubt exists that dicumarol-induced hypoprothrombinemia will respond to graded levels of vitamin $K_1$ (this being the basis of its use in anticoagulant therapy) the relationship between vitamin K activity and sulfaquinoxaline-induced hypoprothrombinemia is far more obscure. Mushett and Seeler ('47) reversed such hypoprothrombinemia in dogs using vitamin $K_1$ and menadione, the former being 100 to 250 times as active. An increase of the requirement for vitamin K activity in chicks was noted by Frost and Spruth ('55) in the presence of 0.1% of sulfaquinoxaline in the diet. These authors found menadione sodium bisulfite many times more active than menadione in satisfying this requirement. Lack of reversal of sulfaquinoxaline-induced hemorrhages by meadione or alfalfa in the same species was noted by Yacowitz et al. ('55). A reduction of blood clotting time that had been prolonged due to feeding of sulfaquinoxaline was noted by other authors when alfalfa meal or menadione was used (Sweet et al., '54; Shelton et al., '54; Morrison et al., '54). Newberne and Buck ('56) who fed levels up to 0.1% of sulfaquinoxaline to chicks did not observe any hemorrhages, but did not measure blood coagulation. Cuckler and Ott ('55), in an extensive study, reported that no effect was observed on blood clotting or prothrombin times of chicks when they received less than 0.4% of sulfaquinoxaline in practical rations containing sources of vitamin K activity. Finally, an inhibition of prothrombin formation due to oral administration of sulfaquinoxaline and a reversal of this inhibition with compounds having vitamin K activity was noted by Griminger ('57a).

In the present studies the relative efficacy of the feeding of graded levels of vitamin $K_1$, menadione, and menadione sodium bisulfite was measured in the presence and absence of dicumarol and sulfaquinoxaline in young growing chicks receiving a diet low in vitamin K-active compounds. The measurements in the absence of the drugs were to serve as a means of comparison and at the same time permit an estimate of the vitamin K requirement of the growing chick. Previous requirement studies with this species have been reviewed recently by Nelson ('58).

## METHOD

In the first group of experiments, day-old cross-bred chicks were fed a purified diet low in vitamin K-active compounds, known to produce a vitamin K deficiency

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TABLE 1

*Experimental diets*

| Diet A | |
|---|---|
| | % |
| Starch | 20.00 |
| Glucose[1] | 39.35 |
| Soybean protein C-1[2] | 30.00 |
| DL-Methionine | 0.40 |
| Glycine | 0.50 |
| Mineral mixture[3] | 5.30 |
| Vitamin mixture[4] | 0.25 |
| Choline chloride | 0.20 |
| Non-nutritive fiber[5] | 3.00 |
| Corn oil, refined | 1.00 |
| Total | 100.00 |

| Diet B | |
|---|---|
| | % |
| Yellow corn meal | 65.0 |
| Glucose, vitaminized[6] | 1.5 |
| Soybean meal (50% protein) | 30.0 |
| Dicalcium phosphate | 2.0 |
| Mineral concentrate[7] | 1.0 |
| Salt | 0.5 |
| Total | 100.0 |

[1] Cerelose.
[2] Assay Protein C-1, Archer-Daniels-Midland Co., Cincinnati.
[3] Fisher and Johnson ('56).
[4] Containing vitamin levels as listed by Griminger ('57a).
[5] Solka Floc, The Brown Company, Chicago, consisting of 99.5% pure cellulose.
[6] Providing the following vitamin levels (mg/kg diet): riboflavin 2, niacin 10, Ca pantothenate 3, cyanocobalamine 0.01, vitamin A, 1200 I.U., and vitamin $D_3$, 400 I.U.
[7] Lime Crest Mico Concentrate.

in chicks (diet A, table 1). At one week of age the chicks were assigned at random into groups of 8, and graded levels of the following three compounds, added to the diet, were fed for two weeks: 2-methyl-3-phytyl-1,4-napthoquinone (vitamin $K_1$), 2-methyl-1,4-napthoquinone (menadione), and a stabilized preparation of water-soluble menadione, consisting of two thirds of 2-methyl-1,4-napthoquinone sodium bisulfite and one third of sodium bisulfite (menadione sodium bisulfite complex, MSBC).[3] At three weeks of age, blood was drawn by heart puncture and plasma prothrombin time determined essentially as described by Quick ('57). Sodium citrate was used as an anticoagulant, and acetone-dehydrated chick brain powder served as the source of thromboplastin. In these and all experiments reported subsequently the chicks were kept in thermostatically-controlled growing batteries with raised wire floors, and received feed and water ad libitum.

In the dicumarol (3,3′-methylenebis-4 hydroxy coumarin) and sulfaquinoxaline (2-sulfanilamidoquinoxaline) experiments, a practical diet low in vitamin K activity was used (diet B, table 1). In the preliminary dicumarol experiment discussed in this paper 10 one-week old Leghorn chicks were assigned to each level of the drug, which was fed for one week. In all other experiments, two-week old chicks from heavy breeds were used, 6 chicks being assigned to each treatment, and blood was drawn at 4 weeks of age as described previously.

Results of prothrombin determinations were averaged on the basis of the reciprocals of the individual prothrombin times. In all graphs, these averages of reciprocals were plotted against the logarithms (decimal) of the vitamin K dose (Almquist, '54). In indicating the dosage, all forms of vitamin K were expressed as $K_1$, namely, on an equimolecular basis.

## RESULTS

Figure 1 shows the response of chicks receiving a vitamin K-deficient diet to graded levels of vitamin $K_1$, menadione and MSBC. Minimum prothrombin times with the thromboplastin preparation used were in the range of 13 to 14 seconds (769 and 714, respectively, when expressed in 1/second $\times 10^4$, as used in the graphs). Apparently, on continuous feeding, vitamin $K_1$ was approximately 1.5 times as active, on an equimolecular basis, in reducing plasma prothrombin times to normal as was MSBC, and 2.5 times as active as menadione.

The results of one of the preliminary experiments on the effect of dicumarol on chicks are shown in table 2. The diet fed in this experiment (diet B, table 1) had been supplemented with 5 mg of menadione per kg of feed and was fed for one week. This amount of menadione ensured

[3] The authors are indebted to the following for supplies used in this study: Hoffmann-La Roche, Inc., Nutley, N. J.; Abbott Laboratories, North Chicago, Ill.; Merck, Sharp and Dohme and Company, Rahway, N. J.; Heterochemical Corporation, Valley Stream, N. Y.; and Limestone Corporation of America, Newton, N. J.

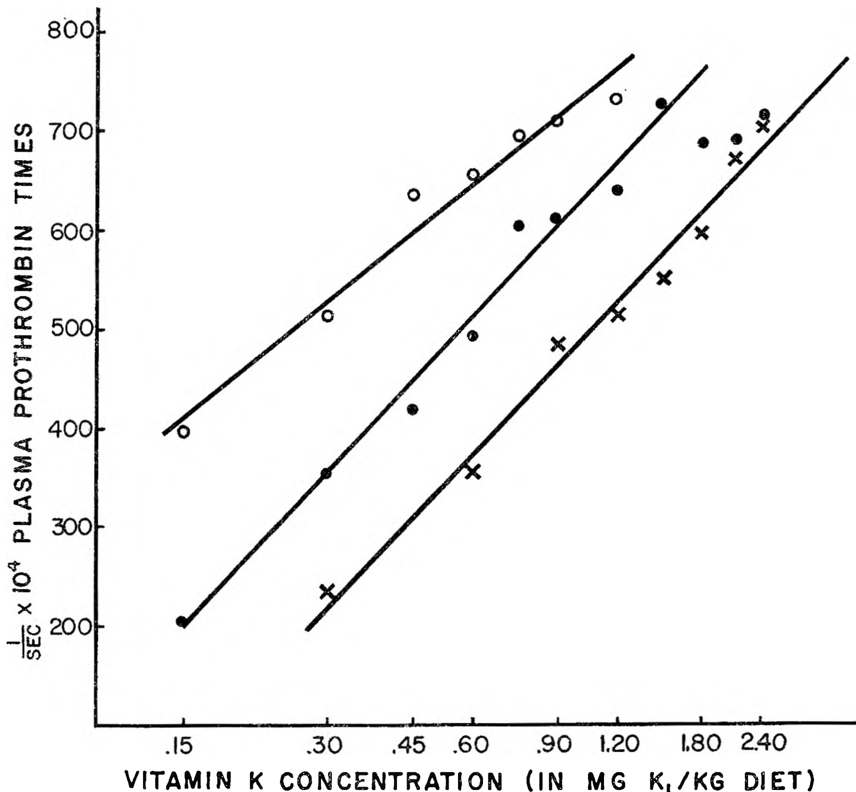


Fig. 1 Effect of continuous feeding of graded levels of vitamin $K_1$ (○), and of equimolecular levels of menadione (×) and MSBC (●) on plasma prothrombin times of three-week-old vitamin K-deficient chicks. Responses to the three highest doses of MSBC were disregarded in the calculation of the equation of the respective regression line.

TABLE 2

*Response of chicks to the feeding of dicumarol for one week when receiving 5 mg of menadione per kg of diet*

| Lot[1] | Dicumarol added | Av. weight at 2 weeks | Av. prothrombin times[2] |
|---|---|---|---|
| | *(mg/kg) diet* | *gm* | *seconds* |
| 1 | 0 | 136 | 13.7 |
| 2 | 50 | 136 | 14.3 |
| 3 | 100 | 145 | 14.7 |
| 4 | 200 | 138 | 17.8 |
| 5 | 400 | 138 | 27.0 |
| 6 | 800 | 133 | 59.9 |

[1] Ten chicks per lot.

[2] Averages on the basis of reciprocals of individual plasma prothrombin times.

normal prothrombin times in the absence of an antivitamin, but appeared to have little, if any, influence on the hypoprothrombinemic effect of dicumarol. Severe hemorrhages were not observed in any of the lots and neither weight gains nor feed consumption seemed to be affected by the doses of the drug used in this instance.

Figure 2 shows the response of 4-week-old chicks, receiving a vitamin K-low basal diet supplemented with 400 mg of dicumarol/kg of diet for two weeks, to graded levels of the three vitamin K-active compounds used in this study. On the basis of oral intake, 1600 mg of vitamin $K_1$ were necessary to overcome the hypoprothrombinemic effect of 400 mg of dicumarol. Levels up to 8 times as much, on an equimolecular basis, of either the water-sobule or fat-soluble form of menadione used in these experiments failed to reduce prothrombin times to less than 16 seconds, a figure indicating, with the system used, a depression of plasma prothrombin below one-half of the normal level. The higher levels of menadione and MSBC were also observed to cause reduced gains, as in the case of two control groups not re-

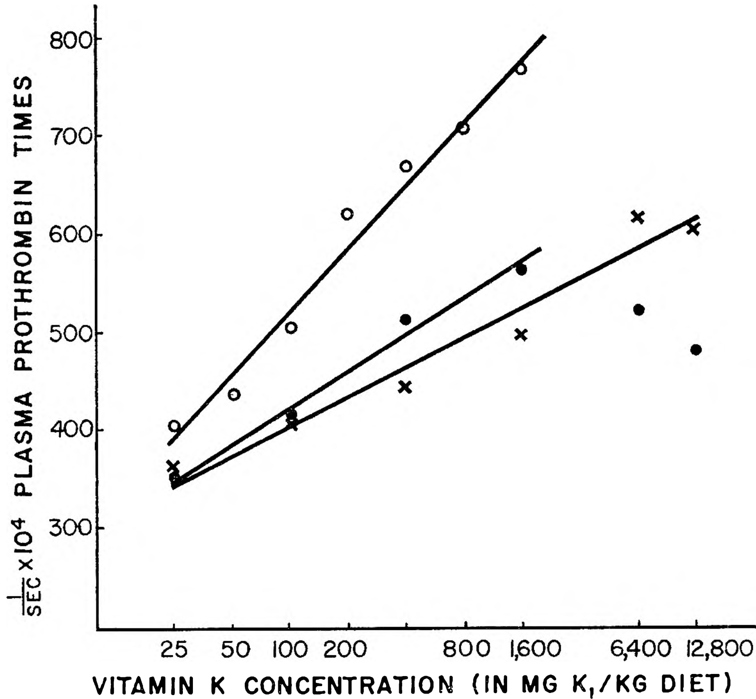


Fig. 2 Effect of continuous feeding of graded levels of vitamin $K_1$ (○) and of equimolecular levels of menadione (×) and MSBC (●) on plasma prothrombin times of 4-week-old chicks receiving a low vitamin K diet supplemented with 400 mg of dicumarol/kg. Responses to the highest level of menadione and the two highest levels of MSBC were disregarded in the calculation of the equations of the respective regression lines.

ceiving any vitamin K. The prothrombin times of these control groups averaged 77 and 66 seconds, respectively, and, owing to the nature of the graph, could not be included in figure 2. This is a more severe deficiency than the one observed with the same level of dicumarol in the short-term experiment shown in table 2. The 6.4 and 12.8 gm levels of MSBC, in fact, increased prothrombin time above that observed at the 1.6-gm level. Owing to these unexpected results, the MSBC part of the original experiment was repeated, with essentially the same results. The values for response to MSBC shown in figure 2 were calculated on the basis of both experiments.

In the last experiment, graded levels of the three forms of vitamin K were added to a diet that had been supplemented with 0.2% of sulfaquinoxaline. Chicks of a control lot, not receiving sulfaquinoxaline or vitamin K, had an average prothrombin time of 41 seconds. The addition of small doses of vitamin K to other control lots reduced the prothrombin time to slightly over 14 seconds. Apparently none of the supplements used in this experiment were effective in reducing prothrombin time to this level (fig. 3). It is clear, however, that in the presence of sulfaquinoxaline substantially higher levels of vitamin K are required to maintain plasma prothrombin at a specific level than in the absence of this drug. On an equimolecular basis, the relative activity of the three forms in the presence of sulfaquinoxaline was similar to that observed without the drug, except that the difference between the two forms of menadione appeared to be magnified.

The chicks receiving the sulfaquinoxaline-supplemented diet gained about 80% of the weight gained by unsupplemented control lots during the two-week experimental period. A similar observation on the weight gain-depressing effect of this drug has been made in another experiment not recorded here.

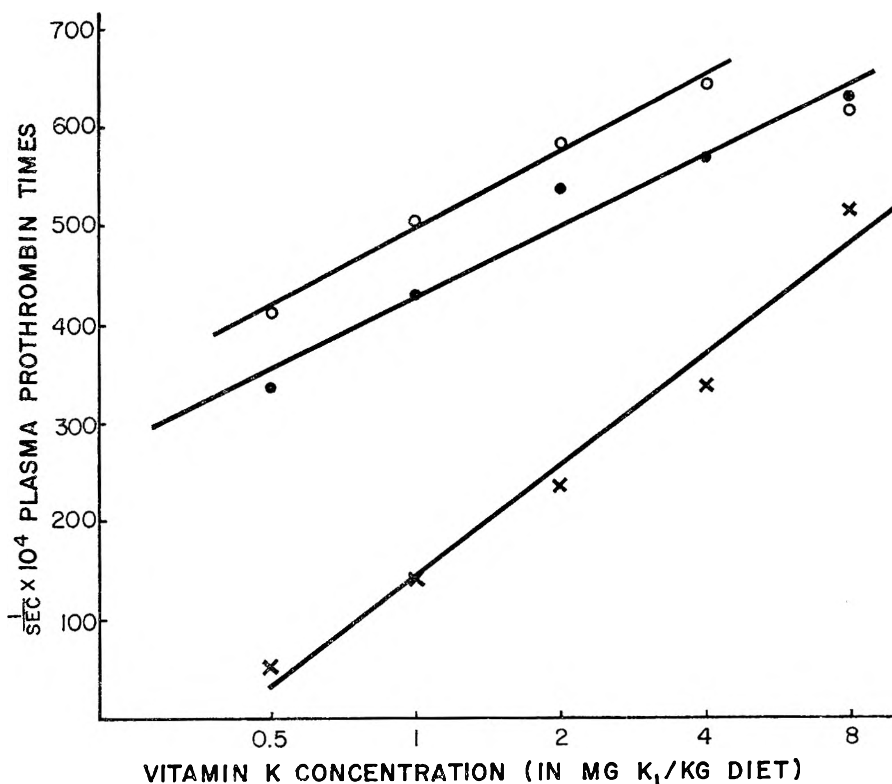


Fig. 3 Effect of continuous feeding of vitamin $K_1$ (○), and of equimolecular levels of menadione (×) and MSBC (●) on plasma prothrombin times of 4-week-old chicks receiving a low vitamin K diet containing 0.2% of sulfaquinoxaline. Response to the highest level of vitamin $K_1$ was disregarded in the calculation of the respective regression line.

## DISCUSSION

The data presented in figure 1 permit an estimate of the requirement of growing chicks for dietary vitamin K if minimum prothrombin time is accepted as the criterion of sufficiency. The potency ratio at the requirement level of vitamin $K_1$, menadione and MSBC was 1:3:1.5; thus menadione was the least active compound on an equimolar basis. In terms of actual weight of the three compounds used, the requirement, per kilogram of feed, was found to be 1 mg of $K_1$, 1.15 mg of menadione or 1.45 mg of MSBC.

Frequently, the phrase "under the conditions of this experiment" is added to conclusions based on experimental evidence. This phrase is most appropriately employed when vitamin K requirements are presented. The elucidation of the vitamin K requirement of any one species is complicated by several factors, such as criteria of sufficiency, bacterial synthesis of the vitamin and the difficulty of maintaining the animals in a state of deficiency, as exemplified by sudden "recovery" in the absence of the vitamin (Luckey et al., '55a, b; Barnes and Fiala, '59). Suffice it to state that Nelson ('58) and Nelson and Norris ('58) obtained a considerably lower requirement under similar conditions but using a different method of assay for sufficiency. Their figures (averages from several experiments) for the requirement per kilogram of feed are 456 μg for $K_1$, 374 μg for menadione and 319 μg for menadione sodium bisulfite, the latter being equivalent to 420 μg of MSBC. These figures are substantially lower than those found in our laboratory. In a determination carried out under conditions similar to those recorded in this report, minimum prothrombin times in growing poults were obtained with approximately 1.75 mg of either men-

adione or MSBC per kilogram of feed (Griminger, '57b).

Essentially, the reaction of plasma prothrombin times to the dietary supplementation with the three forms of vitamin K used in this study was similar in the sulfaquinoxaline-supplemented and the unsupplemented diet, except that menadione seemed to have less activity in the supplemented diet. The reaction is basically different, however, when the diet is supplemented with dicumarol, as shown in figure 2.

Only the natural form, vitamin $K_1$, appeared to give a linear response when the reciprocals of prothrombin times were plotted against the logarithms of the dose, and to overcome entirely the effect of dicumarol. This is not in agreement with the findings of Quick and Stefanini ('48) for the chicken but agrees with observations in other species as reviewed by Mushett et al. ('59).

On the basis of continuous oral intake, approximately 3 molecules of vitamin $K_1$ were required to overcome the hypoprothrombinemic effect of 1 molecule of dicumarol. Whether this ratio would hold over a wide range of dicumarol intake is not known, as absorption might be affected by the dose. The choice of the dose was dictated by the reaction to higher doses of dicumarol (1000 to 3000 mg/kg of diet), consisting of widespread hemorrhages, often fatal, and reduced gains. The reduction in gains seemed to be secondary to the appearance of hemorrhages, and, as in an uncomplicated vitamin K-deficiency, probably a result of the hemorrhages rather than a direct effect. Harms and Tarver ('57), who also fed high levels of dicumarol (up to 3300 mg/kg of diet) did not observe any untoward effects beside prolonged blood clotting times. Their studies, however, were of a short-term nature, being terminated 36 hours after the initiation of feeding of the drug-supplemented diet.

It might be of interest to add that the levels of dicumarol necessary to induce hypoprothrombinemia in laying hens were subsequently shown to be similar to those necessary in growing chicks. Two hens on a low vitamin K diet, receiving 200 mg of dicumarol/kg of diet, had prothrombin times of 21 and 27 seconds; two others, receiving 400 mg, 30 and 54 seconds, and others, with 800 mg, 42 and 112 seconds. Controls were clocked at 17 seconds. Neither weight losses nor lack of appetite could be ascribed to the ingestion of the drug.

As mentioned previously, normal prothrombin times were not obtained in the sulfaquinoxaline experiment; this did not change even when higher doses of vitamin K-active compounds were used in another trial. Possibly other effects of sulfaquinoxaline such as bone marrow changes and anemia (Yacowitz et al., '55; Sanger et al., '56) can account for the occasional failure of vitamin K-active compounds to achieve complete reversal of the coagulation defect caused by continuous feeding of this drug. Using whole blood clotting time as a criterion, Morrison et al. ('54) obtained normal clotting times with chicks consuming a 0.2% sulfaquinoxaline-containing diet with 6.4 mg menadione/kg feed. Whole blood clotting time, however, is not as precise a measurement as plasma prothrombin time (Quick,'57). A comparison of the activity of vitamin K analogues in a sulfaquinoxaline-supplemented diet, however, can also be made at a lower level of prothrombin. To obtain a prothrombin time of 20 seconds, for example, 1 mg of vitamin $K_1$ was required per kilogram of feed; with MSBC, twice as many molecules were required, and between 8 and 9 times as many when menadione was used. Thus, in terms of weights of actual compounds, 1 mg of $K_1$ was equivalent to somewhat less than 2 mg of MSBC and to 3.5 mg of menadione, respectively. Relative to menadione, MSBC was therefore more effective under sulfaquinoxaline stress than without supplementation. Higher effectiveness of MSBC under these conditions has also been reported by Frost and Spruth ('55). There can be little doubt that, as with dicumarol, some quantitative relationship exists between sulfaquinoxaline and the vitamin K required to inactivate it in the steps leading to the formation of prothrombin and/or other factors necessary for normal blood coagulation. Comparing the hypoprothrombinemic effect of sulfaquinoxaline and dicumarol, two differences become apparent: first, when given orally, 4 mg of vitamin $K_1$ counteracted as much of the

hypoprothrombinemic effect of 2000 mg of sulfaquinoxaline as we had been able to overcome in this experiment, or about one molecule of vitamin $K_1$ for each 750 molecules of sulfaquinoxaline, (compared to an estimate of 1:800 obtained in a different type of experiment, using poults and single doses of sulfaquinoxaline and menadione, by Griminger ('57a)). This compares with a 3:1 ratio in the case of dicumarol. Secondly, the feeding of dicumarol did not appear to cause any symptoms other than those secondary to the hemorrhages originated by the vitamin K deficiency (Dessau et al., '58); this is patently not the case with sulfaquinoxaline (Delaplane and Milliff, '48).

It is unlikely that the difference in the levels of vitamin K-active compounds required to counteract dicumarol and sulfaquinoxaline can be explained by extremely poor absorption of the latter. Also, the differential response to vitamin $K_1$ and the two forms of menadione, by chicks made hypoprothrombinemic with these two anticoagulants, points to a basic difference in their antagonistic *modus operandi*.

SUMMARY

When graded levels of vitamin $K_1$, menadione, or menadione sodium bisulfite complex were added to a vitamin K-deficient diet, 1 mg, 1.15 mg and 1.45 mg respectively, per kilogram of feed were required to obtain prothrombin times indicating an optimum level of plasma prothrombin. When the same compounds were fed with a diet containing 400 mg of dicumarol per kg, the requirement of the chicks appeared to be elevated to 1600 mg of vitamin $K_1$. Up to 8 times this level, on an equimolecular basis, of the two menadione compounds did not counteract the hypoprothrombinemic effect of dicumarol. When the diet contained 0.2% of sulfaquinoxaline, vitamin $K_1$ (4 mg/kg diet), as well as the two forms of menadione, counteracted most but not all of the hypoprothrombinemic effect of this drug.

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# Evaluation of Vitamin $B_6$ Nutrition[1,2]

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In a previous paper (Babcock, '59) it was demonstrated with rats that a severe deficiency of vitamin $B_6$ could be detected readily by measuring the increase in serum glutamicoxalacetic transaminase (GOT) after feeding the vitamin. Since this measure of vitamin $B_6$ nutrition may have clinical application, information on its sensitivity and its applicability to human subjects was obtained. These characteristics were studied in rats and human subjects and compared with those of the tryptophan load test which is frequently used as a measure of vitamin $B_6$ nutrition.

## EXPERIMENTAL

To measure the sensitivity of the GOT response, female weanling rats were given purified diets (Babcock, '59) containing 18% of casein and zero, 2.5 µg, 5 µg or excess vitamin $B_6$ each day. After 37 or 43 days, referred to hereafter as the depletion period, each rat was fed 0.1 mg of pyridoxine hydrochloride in water and transferred to the control diet which contained excess vitamin $B_6$. During the depletion period, the vitamin $B_6$ intakes of the 2.5 and 5 µg groups were controlled so that each rat received one of these amounts of pyridoxine mixed in its feed each day, even though total feed consumption and body weights changed during the experiment. In the "excess vitamin $B_6$ group" such daily adjustments were not considered necessary; the vitamin $B_6$ intakes were usually between 100 and 200 µg per day, depending on the total intake of feed which contained 0.0022% of pyridoxine hydrochloride. Throughout the experiment, the total feed intakes were restricted in order to keep the average body weight of each group equal to that of the deficient group. Urine and blood samples were collected at the end of the depletion period and after 1 and 8 days of supplementation. These rat experiments were conducted as described in the previous paper, except that to insure volumes of urine large enough to be collected and measured accurately, 0.5% saline was substituted for the drinking water, starting 10 to 14 days before each urine collection.

In the present experiments, xanthurenic acid excretion was measured by a modification of the method of Satoh and Price ('58). Their method was first scaled down to 1/25th of the specified volumes, but xanthurenic acid was lost when the columns were washed. By using longer columns, less washing and dilute urine samples, satisfactory separations and recoveries were obtained. The revised procedure follows:

Ion exchange columns 2 mm inside diameter by 150 mm long, with constricted tip and a reservior on top, were used. After placing a few ground glass particles in the tip, Dowex 50–X4 ($H^+$) 200 to 400 mesh, with the fines removed by countercurrent washing, was added in water suspension to a height of 60 mm. One milliliter of diluted urine (24-hour rat urines were diluted to 40 ml; human urines to 4000 ml) was mixed with 1 ml 0.2 N HCl in the reservoir and allowed to flow through the column. Air bubbles were

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[2] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Department of Agricultural Biochemistry, New Brunswick.

avoided by inserting a drawn-out glass rod (0.5 mm diameter) which hooked over the edge of the reservoir and extended to the top of the resin bed. This slender rod served also to mix the urine and acid, which were pipetted into the reservoir with the column clamped in a nearly horizontal position. The column was then placed in a vertical position and the sample allowed to flow through. After washing the column with 3 ml 0.2 N HCl the xanthurenic acid was eluted with water. The first 8 ml of water eluate were collected in a calibrated test tube and an equal volume of 50% NaOH solution was added. The tube was then capped with Parafilm and after inverting the tube several times to mix the contents it was placed in a water bath at room temperature. After 40 minutes, floating precipitate was removed and the fluorescence was measured in a Pfaltz and Bauer fluorophotometer with suitable filters (Corning no. 5840 for activation and no. 4308 plus no. 352 for fluorescent light). The instrument was adjusted to zero with a blank containing the same concentration of NaOH. Readings were made promptly because the ultraviolet light caused the fluorescence to fade. The standard curve with pure xanthurenic acid was nearly linear. The columns were regenerated by washing with successive 7 ml portions of 0.1 N NaOH, water, 0.2 N HCl and water.

Serum GOT activities were measured by the modification of the Steinberg, Baldwin, and Ostrow method described in the previous paper (Babcock, '59). To insure reproducibility of the method and to check on possible loss of activity during frozen storage of the serums, reference samples of pooled rat serum were frozen and aliquots analyzed each day that the test determinations were made. These reference serums showed no change in activity when frozen for two months, but with one serum a 6% decrease was observed after 3½ months. Aliquots of 4 reference serums were analyzed in duplicate or triplicate at intervals up to three months. The standard errors of the means of 10 to 20 analyses made on different days were 0.32, 0.42, 0.65, and 0.73. This analytical source of variation is small in relation to the variation between individual rats and the magnitude of the biological response.

To test the applicability of the GOT test for human subjects, college students were given controlled diets consisting largely of natural foods low in vitamin $B_6$ content. Three-day menu cycles were used. The average vitamin $B_6$ content, calculated from literature values and microbiological assay of some of the foods used, were 0.40 mg per day in the study with women and 0.61 mg for the men. Microbiological assay (Atkin et al., '43) of food composites from the study using women indicated 0.46 mg of vitamin $B_6$ per subject per day. Vitamin-free casein was added as needed to bring the protein intake to 1.5 gm per kg body weight per day so that the vitamin $B_6$ requirement would be relatively high. After 49 or 40 days of depletion each subject was given a supplement of 10 mg of pyridoxine hydrochloride, then 5 mg per day for the next 6 days. Blood samples (drawn before breakfast) and 24-hour urine samples were collected at the beginning, near the middle and at the end of the depletion period and one and 6 days after starting the vitamin $B_6$ supplement. At the start of each urine collection period, 5 gm of L-tryptophan were consumed with breakfast. Approximately one-half the subjects had a mild reaction (sleepiness or nausea) to the tryptophan. Physical examinations were made at the beginning, middle and end of the depletion period.

Statistical comparisons were made of the biochemical data obtained during vitamin $B_6$ depletion and supplementation. With the rat experiments, simultaneous comparisons also were made between the group of rats which were severely deficient, the two partially deficient groups and the non-deficient control group. Comparisons of the means were made by the t test without pairing the observations, except as noted below. Both studies, rat and human, were repeated to confirm the findings.

## RESULTS

In the rat experiments severe vitamin $B_6$ deficiency caused retarded growth, acrodynia and high mortality. The stresses of ether anesthesia and heart punctures contributed to the mortality. With higher in-

TABLE 1

*Average xanthurenic acid excretions and serum transaminase (GOT) activities of rats receiving graded levels of vitamin $B_6$ and their responses to vitamin $B_6$ supplementation*

| Experiment and treatment | Vitamin $B_6$ intake during the depletion period | | | |
|---|---|---|---|---|
| | 0 | 2.5 $\mu g$/day | 5 $\mu g$/day | Excess |
| | **Xanthurenic acid[1]** | | | |
| Experiment 1 | | | | |
| Number of rats[2] | 2–3 | 4–6 | 4–5 | 3–5 |
| Depleted[3] | 3.24 ± 1.18[4] | 4.35 ± 0.53 | 2.70 ± 0.77 | 0.51 ± 0.07 |
| Supplemented | | | | |
| 1 day | 0.19 ± 0.06 | 0.21 ± 0.05 | 0.54 ± 0.32 | 0.25 ± 0.10 |
| 8 days | 0.18 ± 0.05 | 0.17 ± 0.02 | 0.13 ± 0.01 | 0.13 ± 0.01 |
| Experiment 2 | | | | |
| Number of rats | 3–4 | 6 | 4 | 5–6 |
| Depleted[3] | 2.72 ± 0.58 | 2.55 ± 0.28 | 1.97 ± 0.35 | 0.08 ± 0.01 |
| Supplemented | | | | |
| 1 day | 0.14 ± 0.03 | 0.11 ± 0.01 | 0.08 ± 0.02 | 0.06 ± 0.01 |
| 8 days | 0.22 ± 0.04 | 0.08 ± 0.02 | 0.13 ± 0.01 | 0.14 ± 0.02 |
| | **Serum transaminase[5]** | | | |
| Experiment 1 | | | | |
| Number of rats | 4–5 | 3–6 | 3–5 | 3–5 |
| Depleted[3] | 35 ± 5.0[4] | 47 ± 4.6 | 64 ± 5.7 | 74 ± 10.8 |
| Supplemented | | | | |
| 1 day | 116 ± 28.2 | 61 ± 11.5 | 65 ± 3.2 | 87 ± 14.0 |
| 8 days | — | 78 ± 5.5 | 78 ± 6.3 | 76 ± 0.7 |
| Experiment 2 | | | | |
| Number of rats | 2–4 | 6 | 3–4 | 5–6 |
| Depleted[3] | 21 ± 2.5 | 26 ± 2.2 | 41 ± 5.8 | 66 ± 5.3 |
| Supplemented | | | | |
| 1 day | 54 ± 4.5 | 48 ± 2.2 | 82 ± 15.6 | 74 ± 2.4 |
| 8 days | 63 ± 5.4 | 83 ± 12.3 | 69 ± 7.4 | 70 ± 4.7 |

[1] Milligrams excreted during the 24-hour period after feeding 30 mg L-tryptophan.

[2] Variation in the number of rats was caused by mortality.

[3] End of the depletion period. The degree of depletion was governed by the vitamin $B_6$ intake; the control group was not depleted.

[4] Standard error of the mean.

[5] Units per milliliter of serum.

takes of vitamni $B_6$ the deficiency was less severe, as indicated by the lower excretions of xanthurenic acid (table 1). In both experiments the difference in xanthurenic acid excretions (after feeding tryptophan) between the control rats and those with an intake of 5 µg of vitamin $B_6$ per day was significant ($P = 0.022$ and 0.0006). The differences in xanthurenic acid excretion between the three depletion levels were not significant. Within the first 24 hours after feeding vitamin $B_6$ there was a considerable decrease in the excretion of xanthurenic acid by the depleted animals, with the result that the differences between the control and deficient groups were no longer significant. Comparisons within each group before and after vitamin supplementation confirm that the changes observed were caused by vitamin $B_6$ deficiency. Within 24 hours after feeding pyridoxine hydrochloride there were significant decreases ($P < 0.05$) in the xanthurenic acid excretions of all groups of rats except the controls.[3]

In both experiments, the serum GOT activities of the depleted rats paralleled the vitamin $B_6$ intakes (table 1). The differences in GOT activities between rats

[3] Because of individual variation in the condition of the severely depleted animals in experiment 1, their xanthurenic acid response was not significant by the unpaired t test, but it was significant when the data were paired (Fisher, '50).

which consumed excess vitamin $B_6$ and those with an intake of 5 μg per day was significant ($P = 0.01$) in experiment 2 but not in experiment 1, where there was greater individual variation. The difference between the 5 and 2.5 μg intakes was significant ($P = 0.04$) in both experiments. The difference between the 2.5 μg intake and no added vitamin $B_6$ was not significant ($P = 0.11$ and 0.13). After one day of supplementation with vitamin $B_6$ there was no longer any significant difference between successive levels of vitamin $B_6$ intake, though in experiment 2 there were still significant differences ($P < 0.01$) between the control group and the two most deficient groups. After 8 days of supplementation significant differences in GOT activities were no longer found between any of the groups. Comparisons within each group before and after vitamin supplementation confirm that the differences in GOT activity were the result of vitamin $B_6$ deficiency. Twenty-four hours after feeding pyridoxine hydrochloride significant increases were noted ($P < 0.05$) in the GOT activities of the severely depleted groups in both experiments and also in the 2.5 and 5 μg groups of experiment 2.

In two experiments conducted previously, rats on vitamin $B_6$-deficient diets containing 9% of casein showed serum GOT responses similar to those on 18% of casein. In one of these experiments the average serum GOT of 11 severely depleted rats increased from 25 to 54 units per ml after one day of supplementation with vitamin $B_6$, and to 69 units per ml after 8 days (7 rats). In the other experiment, the corresponding values for 6 rats were 30, 72, and 76 units per ml. The GOT activities before and after severe depletion were similar in the 9 and 18% casein experiments, although these experiments were conducted at different times.

The human studies confirmed the findings with rats, although the degree of vitamin depletion was relatively much smaller. With the human subjects the aim was to produce only a subclinical depletion of vitamin $B_6$ reserves and to avoid a severe nutritional deficiency. No evidence was noted that the dietary regimen interferred with the academic work of the subjects or had any deleterious effect on their health. The average weight loss was less than two pounds during the study and the only clinical sign observed was dryness and flaking of the skin in two subjects. The biochemical findings for these two subjects were not appreciably different from the group averages.

The mild degree of depletion of our subjects, predicted from the dietary intakes and confirmed by the physical examinations, is further supported by comparison of the xanthurenic acid excretions with similar load-test data reported in the literature. The following normal values after a dose of 5 gm of L-tryptophan or 10 gm of D,L-tryptophan have been reported: 25 to 50 mg (Glazer et al., '51), 4 to 30 mg (Wachstein and Gudaitis, '52, '53), less than 30 mg (Vilter et al., '53), less than 50 mg (Vilter, '55), 12 to 38 mg (Wachstein and Lobel, '56) and less than 50 mg (Maske, '57). Vitamin $B_6$ deficiency produced with deoxypyridoxine usually caused xanthurenic acid excretions to rise to 100 to 500 mg (Glazer et al., '51 and Vilter et al., '53). Greenberg et al. ('49) reported that after 21 days on a puried diet deficient in vitamin $B_6$ the excretion rose to 271 mg for one subject and 515 mg for another. Of our 15 subjects, 6 men and 3 women still remained in the 25 to 50 mg range at the end of their depletion periods. The average excretions were 42 and 88 mg (table 2).

Although our human subjects were not severely depleted, biochemical changes were detectable. In both studies the average xanthurenic acid excretion following a dose of tryptophan increased during depletion and dropped promptly after supplementation with vitamin $B_6$ (table 2). The increases over the total depletion period were statistically significant ($P = 0.021$ for the women and 0.013 for the men), though the intermediate values were not. The decreases in xanthurenic acid during the first 24 hours after supplementation with vitamin $B_6$ were highly significant ($P = 0.0014$ and 0.0002). No further change after 6 days of supplementation was observed. All of the values after supplementation were significantly

TABLE 2

*Average xanthurenic acid excretions[1] and serum transaminase (GOT) activities of college students during depletion of vitamin $B_6$ reserves and their responses to vitamin $B_6$ supplementation*

| Treatment | Xanthurenic acid (mg/24 hour) | | Serum transaminase (units/ml) | |
|---|---|---|---|---|
| | Study 1 (8 Women) | Study 2 (7 Men) | Study 1 (8 Women) | Study 2 (7 Men) |
| Initial | 38 ± 7.9[2] | 27 ± 2.8 | 12.6 ± 0.67[2] | 13.7 ± 1.10 |
| Depleted | | | | |
| 20 days | — | 36 ± 5.0 | — | 12.3 ± 1.19 |
| 28 days | 60 ± 10.6 | — | 12.0 ± 0.63 | — |
| 37 days | — | 42 ± 4.0 | — | 11.9 ± 0.77 |
| 47 days | 88 ± 17.3 | — | 10.8 ± 0.58 | — |
| Supplemented | | | | |
| 1 day | 18 ± 2.3 | 16 ± 1.6 | 12.0 ± 0.63 | 12.6 ± 1.02 |
| 6 days | 14 ± 3.8 | 17 ± 1.2 | 15.4 ± 0.53 | 13.5 ± 1.10 |

[1] Excreted during the 24-hour period after consuming 5 gm L-tryptophan.
[2] Standard error of the mean.

lower ($P < 0.03$) than the initial excretions. This observation does not indicate that the previous vitamin $B_6$ intakes of these students were inadequate to meet their normal requirements, though it does suggest that they were inadequate to meet the higher requirements for metabolizing 5 gm of L-tryptophan plus protein at a level of 1.5 gm per kg body weight.

In both human studies a gradual reduction in the average serum transaminase (GOT) activity during depletion and an increase after supplementation were noted. These changes were analyzed by the t test with the values paired for each subject to minimize the effect of individual variation in GOT levels. The probability values for the decreases over the total depletion period were 0.03 for the women and 0.06 for the men. The corresponding P values for the increases over the 6-day supplementation period were 0.0002 and 0.02. Usually the intermediate changes were not statistically significant. The decreased GOT activity of the subjects after depletion was confirmed by comparison with serum samples drawn a month or more after the termination of each study. The average values obtained after return to normal diets were higher for both males and females than when the subjects were depleted of vitamin $B_6$.

## DISCUSSION

The rat experiments have shown that serum GOT activity reflects the severity of a vitamin $B_6$ deficiency. The human studies with young men and women have also shown that subclinical deficiency of vitamin $B_6$ causes reduction in GOT activity. Since there is appreciable variation in GOT activities of normal individuals, a single measurement of the enzyme would not suffice to detect a subclinical deficiency. However, an increase in serum GOT activity following administration of vitamin $B_6$ provides an indication that the vitamin $B_6$ supply had been inadequate for optimum protein metabolism (or more precisely, for the maximum rate of glutamic-oxalacetic transamination in blood serum). This test of vitamin $B_6$ adequacy would have the advantage, from the clinical point of view, of combining vitamin therapy with the diagnosis. The response can be measured, with a standard clinical method, 24 or more hours after oral administration of the vitamin. While the human studies reported here showed relatively small responses after subclinical vitamin depletion, the rat experiments indicate that larger responses are to be expected when the deficiency is more severe. The rat experiments using 9% of casein indicate also that the GOT response occurs when the protein intake is low. Chinsky et al. ('56) reported that GOT activity is independent of the time of day, relation to meals and previous exercise; but Wang and Appelhanz ('56) found that breakfasts, especially a high-protein breakfast, increased GOT activity. They also ob-

served considerable daily variation in fasting values from the same individuals and suggested that GOT activity may be related to activity or emotional disturbance.

Comparison of the GOT and xanthurenic acid data in tables 1 and 2 shows that, with both rats and human subjects, vitamin $B_6$ depletion and supplementation each had relatively greater effects on xanthurenic acid excretion (following a dose of tryptophan) than on GOT activities. The responses to vitamin supplementation are of particular interest because curative tests offer the most specific evidence of vitamin deficiency and also because they are probably less influenced by individual variation than single measurements of tissue concentration. It is of interest, therefore, to compare the sensitivities of the two methods for evaluating vitamin $B_6$ nutrition when each is used as a curative test. Ranke et al.[4] reported that the two methods gave "similar" responses when applied to older people.

The sensitivity of a test can be expressed by the probability that, for a given degree of deficiency, the response measured was the result of chance variation. Probability values give an overall measure that includes the precision of the laboratory method, the magnitude of the biological response to vitamin supplementation and the variation in response of individuals. The effects of the degree of deficiency and the number of subjects on the probability values may be equalized for two tests by limiting the comparison to samples obtained simultaneously from the same subjects. While 24-hour urine samples cannot be obtained at the same moment that blood samples are drawn, our urine and blood samples were obtained within a day of each other and so represent essentially the same degree of deficiency. The samples obtained immediately following the vitamin supplement are an exception in that the urine samples represent excretion during the first 24 hours, whereas the blood samples give the status at the end of 24 hours. Here also, however, the probability values measure the sensitivities of the two tests as they would be applied in clinical situations.

Table 3 lists the usual probability (P) values obtained when the means before and after treatment with vitamin $B_6$ were analyzed by the paired t test. The lower P values (with one exception) for the xanthurenic acid response to vitamin $B_6$ indicate that this response had greater statistical significance than the GOT response in the same subjects. That is, because of the greater sensitivity of the xanthurenic acid response, this curative test provided more positive evidence of vitamin $B_6$ deficiency than the GOT curative test.

While the curative test with measurement of xanthurenic acid excretion is more sensitive than that with GOT, the choice of method for assessing vitamin $B_6$ nutrition depends also on acceptability of the test doses, availability of the biological samples and difficulty in the analytical methods. In clinical situations, the low palatability of tryptophan, its unpleasant reactions, difficulty in collecting 24-hour urine samples and the special assay

[4] Ranke, E., S. Tauber, B. Ranke, R. Goodhart and B. F. Chow 1958 Pyridoxine deficiency in the aged. Federation Proc., *17:* 490 (abstract).

TABLE 3

*Probability that the response to vitamin $B_6$ supplementation was the result of chance*

| Treatment | Rat experiment[1] | | Human study | |
|---|---|---|---|---|
| | 1 | 2 | 1 | 2 |
| Supplemented for one day | | | | |
| Xanthurenic acid | 0.031 | 0.0003 | 0.0030 | 0.0005 |
| Serum transaminase | 0.20 | 0.0009 | 0.080 | 0.18 |
| Supplemented for 6 to 8 days | | | | |
| Xanthurenic acid | 0.0076 | 0.0004 | 0.0035 | 0.0008 |
| Serum transaminase | 0.020 | 0.0009 | 0.0002 | 0.019 |

[1] During depletion each rat received 2.5 $\mu g$ of vitamin $B_6$ per day.

procedure required for xanthurenic acid may render this method less desirable. These problems are doubly important in curative tests, which require the procurement and analysis of two samples, and for the xanthurenic acid test, administration of two doses of tryptophan.

When the problem is one of deranged vitamin $B_6$ metabolism, rather than simple vitamin $B_6$ deficiency, neither of these tests by itself will provide adequate information. It is then desirable to use several tests to measure the function of various metabolic pathways in which vitamin $B_6$ is involved. The need for more than one test is illustrated by reports that the patterns of xanthurenic acid excretion and GOT activity are quite different in pregnancy than in isonicotinic acid hydrazide therapy. In pregnancy the increase in xanthurenic acid excretion is not accompanied by a change in the activity of whole blood GOT, according to Glendening et al. ('55), although Wroblewski ('58) reported that the mean GOT activity of serum was "somewhat lower" in pregnancy. Isonicotinic acid hydrazide therapy, on the other hand, reduced the GOT activity of whole blood before it affected xanthurenic acid excretion in a study by Sass and Murphy ('58). Price et al. ('57) observed an increase in xanthurenic acid excretion after isonicotinic acid hydrazide therapy. The latter workers concluded, however, that the determination of a single urinary metabolite cannot be relied upon as an indication of abnormal tryptophan metabolism.

These studies were not designed to determine vitamin $B_6$ requirements, but the finding that approximately 0.5 mg per day was inadequate to maintain normal serum GOT activity of young men and women is interesting. The true vitamin $B_6$ value of the diets cannot be stated, since the diets included meat and other natural sources of the vitamin. Lushbough et al. ('59) have shown that the vitamin $B_6$ values of meats obtained by rat assay are approximately twice as high as those obtained by the microbiological assay procedure that we used. Since a large proportion of the total vitamin $B_6$ content of the depletion diet used in our human studies was supplied by meat and milk, apparently the vitamin $B_6$ requirement of young men and women is considerably higher than 0.5 mg per day. Harding et al. ('59) have recently published a note on the human requirement for vitamin $B_6$. They found that 1.93 mg (microbiological assay) per day was inadequate for normal metabolism of a 10 gm dose of DL-tryptophan added to a high-protein diet.

## CONCLUSIONS

The response of serum glutamic-oxalacetic transaminase to oral administration of vitamin $B_6$ provides a measure of the state of vitamin $B_6$ nutrition. This measure is less sensitive than the change in xanthurenic acid excretion (tryptophan load test) after administration of vitamin $B_6$, but it has advantages which may favor it in clinical situations.

The vitamin $B_6$ requirement of young men and women (for maximum serum glutamic-oxalacetic transaminase activity) is greater than 0.5 mg per person per day.

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# Further Observations on Lactose Stimulation of the Gastrointestinal Absorption of Calcium and Strontium in the Rat[1]

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Although many dietary factors influence the gastrointestinal absorption of calcium (Irving, '57), the more important organic substances that increase the intestinal absorption of alkaline earths are vitamin D and lactose. The lactose effect has been known for many years and has been shown to occur in many species and under various situations. Duncan ('55) and Atkinson et al. ('57) have reviewed certain biochemical and physiological aspects of lactose metabolism.

Much attention has been given recently to the physiological site of action of lactose and to the mechanism by which lactose increases calcium absorption. Studies by Fournier and coworkers ('54, '55) had suggested that lactose directly stimulated skeletal ossification and thereby increased the utilization of ingested calcium. However, Wasserman et al. ('57, '59), Lengemann et al. ('59) and Lengemann ('59) presented evidence that lactose had a direct effect at the gastrointestinal site. This conclusion was based on (a) the rapidity of the lactose response, (b) the failure of lactose to increase the deposition of parenterally administered $Ca^{45}$ and $Sr^{85}$, (c) the observation that the fraction of absorbed alkaline earths entering the skeleton was the same in the presence or absence of lactose. Also both Fournier et al. ('54, '55) and Wasserman and Comar ('59) observed that carbohydrates other than lactose can increase calcium and strontium absorption; effective were cellobiose, L-sorbose, D-xylose, raffinose, melibiose, D-glucosamine, D-mannitol and D-sorbitol, and ineffective were D-glucose, sucrose, D-galactose, and D-fructose.

The present experiments were designed to study the mechanism of interaction of lactose and alkaline earths, and specifically to determine whether lactose is effective by acting through the intestinal flora, stimulating intestinal metabolism, or by physicochemical phenomena.

## EXPERIMENTAL

*Influence of various levels of lactose, and effect of neomycin and metabolic inhibitors on alkaline earth absorption.* Calcium absorption was determined by measuring the appearance of $Ca^{45}$ and/or $Sr^{85}$ in the femur of the rat after a single oral dose of a solution containing the radioisotopes and test substance. Previous work (Wasserman et al., '56; Lengemann et al., '59) has shown that the femur content of ingested $Ca^{45}$ and $Sr^{85}$ is a reliable index of alkaline earth absorption under properly defined conditions.

Male albino rats of the Wistar strain and weighing 90 to 110 gm of body weight were used. The rats were fasted for 24 hours and, while under light anesthesia, the dose was given by stomach tube. The dosing solution contained 20 μc of $Ca^{45}$, 2 μc of $Sr^{85}$, 1.8 mg of Ca and 0.84 millimoles of the test substance per 2 ml. At 24 hours after dosing or at the time interval indicated, the animals were killed and the femur content of $Ca^{45}$ and/or $Sr^{85}$ determined by usual procedures (Comar, '55).

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In some of these experiments, the dosing solution was injected directly into a ligated segment of the rat ileum. This segment was used since earlier data from this laboratory had shown that lactose had a greater effect on $Ca^{45}$ and $Sr^{85}$ absorption from the ileum than from the stomach, duodenum, jejunum or cecum (Lengemann et al., '59). The procedure was otherwise identical with the gavage experiment except that the animals were killed at 4 hours after the ileal injection. The results are expressed in terms of "percentage of administered dose per femur."

*Effect of hydrostatic pressure on strontium absorption.* The presence of hypertonic solutions of lactose within the ileum results in the movement of parenteral fluids into the lumen; this causes an increased hydrostatic pressure within the lumen as well as the retention of aqueous fluids. Enhanced calcium and strontium absorption in the presence of lactose could thus have been the result of a Donnan-like effect or the ability of the fluids to maintain the alkaline earth salts in a more soluble state. The following experiment was designed to study these particular physicochemical processes. Young fasted male rats were anesthetized with sodium pentobarbitol[2] and, after an abdominal incision, the proximal end of the ileal segment was cannulated with polyethylene tubing (1.67 mm, i.d.); the opposite end of the tubing was connected to a plungerless 5 ml syringe used here as a small funnel. The test dose was then injected through the distal ileal wall into the lumen and the segment ligated near the ileo-cecal junction. Fluids were added to the syringe and the fluid height maintained by periodic additions of fluid at either 10.5 or 12.0 cm. The fluids used, as indicated, were distilled water, physiological saline or bovine serum ultrafiltrate.[3] The test solution contained 1 mg of calcium carrier and 2 μc of $Sr^{85}$; in the lactose groups, the solution also contained 0.42 millimoles of this disaccharide. The rats were killed at two hours and the $Sr^{85}$ retention by the femur measured.

*Solubility of $CaHPO_4$ in carbohydrate and amino acid solutions.*[4] An experiment was undertaken to determine the ability of lactose and other materials to solubilize an insoluble calcium salt, $CaHPO_4$, since Herrington had reported in 1934 that lactose has some capacity to bind calcium ions and thereby possibly provide a carrier for calcium transport. Fifty milliliters of a 1.5 % ammonium acetate solution adjusted to pH 7.5 was added to a 125 ml Erhlenemeyer flask. To this was added 50 mg $CaHPO_4$ and the test material at levels to yield concentrations that ranged from 0.1 to 0.5 M. Control flasks were carried along with the experimental flasks. The solutions were mechanically shaken for 24 hours at room temperature at which time the solutions were filtered. The calcium content of the filtered solutions was determined by the oxalate precipitation-permanganate titration procedure.

## RESULTS

In the first study, the relationship between concentration of lactose in the dose given by gavage and the gastrointestinal absorption of $Ca^{45}$ and $Sr^{85}$ was determined. These data, shown in table 1, indicate that response was proportional to dose up to 0.4 millimoles of lactose; additional amounts of lactose did not further increase alkaline earth absorption. The strontium-calcium observed ratio (OR)[5] also increased with the addition of up to 0.2 millimoles of lactose. This observation confirms previous findings of Wasserman et al. ('56). By subtracting the $Sr^{85}$ and $Ca^{45}$ concentrations in the femurs of the controls from those of each treated group, it was possible to estimate the comparative effects of lactose on the two alkaline earths. It was interesting to find that, if anything, the $Sr^{85}$ absorption was increased more than that of $Ca^{45}$. This is in contrast to the usual behavior where the gastrointestinal absorption favors calcium over strontium. The change in the observed ratio of $Sr^{85}$ and $Ca^{45}$ in the femur with lactose may be due to (a) the stimu-

[2] Nembutol.

[3] Obtained from Microbiological Associates, Inc.

[4] Carbohydrates and amino acids used in these studies were obtained from Nutritional Biochemicals, Inc., and were used without further purification.

[5] The observed ratio (OR) is defined by Comar et al. ('56) as follows:

$$OR_{femur\text{-}diet} = \frac{Sr^{85}/Ca^{45} \text{ in femur}}{Sr^{85}/Ca^{45} \text{ in diet}}$$

TABLE 1

*Influence of various levels of lactose on the comparative absorption of $Ca^{45}$ and $Sr^{85}$* [1]

| Levels of lactose | Ingested radionuclides in femur | | $OR^2$ | Increased radionuclide content of femur due to lactose | |
|---|---|---|---|---|---|
| | $Sr^{85}$ | $Ca^{45}$ | | $Sr^{85}$ | $Ca^{45}$ |
| *millimoles* | % | % | | | |
| 0 | 2.5 ± 0.4[3] | 4.6 ± 0.5 | 0.54 | — | — |
| 0.1 | 3.9 ± 0.4 | 5.7 ± 0.4 | 0.68 | 1.4 | 1.2 |
| 0.2 | 5.4 ± 0.5 | 7.1 ± 0.2 | 0.76 | 2.9 | 2.6 |
| 0.4 | 6.4 ± 0.3 | 8.1 ± 0.4 | 0.78 | 3.9 | 3.5 |
| 1.6 | 6.9 ± 0.5 | 8.4 ± 0.3 | 0.82 | 4.4 | 3.9 |

[1] Each value represents mean of 5 to 6 animals. Dose contained 20 μc $Ca^{45}$, 2 μc $Sr^{85}$, 1.8 mg Ca and lactose levels indicated.

[2] Observed ratio (OR) defined as $Sr^*/Ca^*$ in femur ÷ $Sr^*/Ca^*$ in dose.

[3] Mean ± standard error of the mean.

TABLE 2

*Influence of antibiotic (neomycin) and lactose on $Sr^{85}$ absorption and pH of rat ileum*[1]

| Group | Injected $Sr^{85}$ in femur | Change in femur $Sr^{85}$ | pH of ileal segment |
|---|---|---|---|
| | % | % | |
| Control | 0.5 ± 0.1 | — | 7.47 ± 0.04 |
| Lactose | 1.5 ± 0.1 | + 186 | 7.27 ± 0.10[3] |
| Neomycin | 1.0 ± 0.1 | + 87[2] | 7.79 ± 0.06 |
| Lactose + neomycin | 1.3 ± 0.2 | + 161 | 7.60 ± 0.08[3] |

[1] Solutions injected in ligated segment of rat ileum. Rats fasted 24 hours prior to dose and killed at 4 hours after dosing. Six rats per group. Values given as mean ± S.E.M. Dose contained 2 μc $Sr^{85}$, 3.6 mg calcium and, where indicated, 0.42 millimoles of lactose and/or 5.5 mg crystalline neomycin.

[2] Value of neomycin group greater than control group but less than both lactose groups at $P < 0.05$.

[3] The pH of ileal contents from lactose-treated groups less than control and neomycin alone at statistical level of $P < 0.05$.

lation of a specific site of intestinal absorption that cannot distinguish between these alkaline earths or (b) the usual pathway of absorption is altered to reduce the differential handling of these elements.

Early theories of lactose action on calcium metabolism were based on the bacterial breakdown of this sugar into acid products and the subsequent beneficial effect of lowered pH on calcium absorption (Bergeim, '26). In the present study, the antibiotic, neomycin, was injected together with the lactose solution to determine whether lactose was still effective in the presence of an antibacterial agent. These data, presented in table 2, showed that neomycin itself had the ability to increase $Sr^{85}$ absorption; however, lactose in the presence of neomycin was more effective than neomycin alone, having about the same effect as lactose alone. Immediately after the animals were killed, the intestinal contents were rinsed from the ileum with minimal amount of distilled water and pH determined on this solution. It was found that the presence of lactose decreased the pH of the intestinal contents slightly ($P < 0.05$); also, the gut contents of the rats that had received lactose plus neomycin were somewhat more acid than those from animals given neomycin alone. By comparing intestinal pH in the various groups, it was indicated that bacterial action and a lowered pH within the intestinal lumen could not account for lactose action. However, a distinct though slight reduction in pH occurred when lactose was present within the gut.

Another possibility was that direct stimulation of intestinal metabolism by lactose could, in some manner, enhance alkaline earth absorption. The influence of various metabolic inhibitors on the effect of lactose was examined; here the inhibitor was

TABLE 3
*Metabolic inhibitors and the effect of lactose on the ileal absorption of radiostrontium*[1]

| Inhibitor | Inhibitor concentration | Injected $Sr^{85}$ in femur | | Lactose + inhibitor |
|---|---|---|---|---|
| | | Control | Lactose | |
| | | % | % | % |
| Phlorrhizin | $10^{-2}$ M | 0.54 ± 0.03 | 1.3 ± 0.1 | 1.5 ± 0.2 |
| Sodium fluoroacetate | $10^{-2}$ M | 0.56 ± 0.05 | 1.1 ± 0.1 | 1.1 ± 0.2 |
| Sodium iodoacetate | $10^{-2}$ M | 0.40 ± 0.05 | 1.1 ± 0.2 | 1.1 ± 0.1 |
| 2,4-Dinitrophenol | $10^{-3}$ M | 0.54 ± 0.03 | 2.3 ± 0.2 | 2.5 ± 0.3 |
| Sodium azide | $10^{-3}$ M | 0.45 ± 0.07 | 1.7 ± 0.2 | 1.8 ± 0.3 |

[1] Solutions injected into ligated segment of ileum of fasted rats. Six rats per group. Values given as mean ± S.E.M. Dose contained 2 $\mu c$ $Sr^{85}$ and 3.6 mg calcium and 0.42 millimoles of lactose plus inhibitor at concentration given.

injected with lactose in the dosing solution. These data are summarized in table 3. In each experiment lactose increased $Sr^{85}$ absorption as expected and neither phlorrhizin, sodium fluoroacetate, sodium iodoacetate, 2,4-dinitrophenol nor sodium azide inhibited the effect of lactose. The sites of action of several of these inhibitors are well-known and the lack of effect of these substances suggest that the common pathways of carbohydrate metabolism via the tricarboxylic acid cycle or the glycolytic cycle are not involved in this phase of lactose action. In the interpretation of these data, it should be taken into consideration that the inhibitor concentration in the injected solution may have been rapidly reduced by dilution with intestinal fluid or by rapid absorption. However, similar studies by other workers (Jervis, et al., '56; Parson et al., '59) have shown that inhibitor levels even lower than those used here were effective in decreasing the gastrointestinal absorption of glucose.

When hypertonic solutions of slowly absorbable solutes, such as lactose, are introduced into the gut, there is a tendency for equalization of the osmotic pressures within the intestinal lumen and the vascular system. During this process, the hydrosatic pressure within the lumen may exceed that of the control group and thus provides an additional driving force for the movement of ions across the intestinal membrane. Also, parenterally available cations, such as $Na^+$ and $K^+$, would move with the intestinal secretion fluid and, thereby, may cause a greater movement of calcium and strontium ions into the body by ion exchange phenomenon. Another consideration here is that the fluid retention within the lumen would provide an aqueous milieu for maintaining relatively insoluble calcium and strontium salts in solution. In order to examine these possibilities, the effect of lactose on $Sr^{85}$ absorption was studied when the hydrostatic pressures in the lactose-treated rats and the control rats were maintained artificially at similar levels. From table 4, it may be seen that when distilled water was used as the hydrostatic fluid in groups 1 and 2 of experiment 1, the presence of lactose still caused a more effective absorption of $Sr^{85}$ in comparison to the non-lactose group. As further controls, two additional groups (groups 3 and 4 of experiment 1) were injected with $Sr^{85}$ with or without lactose but, in this case, the hydrostatic pressure was not controlled. As expected, lactose had its usual enhancing effect; of interest particularly was that, in relation to their respective controls, lactose had the same effect under both experimental conditions as shown by a similar lactose femur $Sr^{85}$/control femur $Sr^{85}$ ratio. In the foregoing experiment, the controls of the cannulated group did not absorb $Sr^{85}$ as readily as the controls that were not cannulated. This difference may have been the result of differences in anesthetics (sodium pentobarbitol vs. ether) or the trauma resulting from cannulation and the maintenance of the hydrostatic pressure. In the second experiment of this series, physiological saline and bovine serum ultrafiltrate were used as hydrostatic fluids in addition to distilled water. By reference again to table 4, it may be seen that none of the fluids, as used, duplicated the effect of lactose.

TABLE 4

*A comparison of the effect of lactose and intralumenal hydrostatic pressure on $Ca^{45}$ and $Sr^{85}$ absorption from the ileum*[1]

| Group | Lactose in injection fluid | Hydrostatic fluid | Femur content $Sr^{85}$ | Ratio lactose/ control |
|---|---|---|---|---|
| | | | *% injected dose* | |
| | | Experiment 1 | | |
| 1 | + | Distilled water | 0.95 ± 0.04 | 3.7 |
| 2 | — | Distilled water | 0.26 ± 0.04 | |
| 3 | + | Not cannulated | 2.06 ± 0.16 | 3.4 |
| 4 | — | Not cannulated | 0.61 ± 0.05 | |
| | | Experiment 2 | | |
| 1 | + | Distilled water | 0.64 ± 0.06 | |
| 2 | — | Distilled water | 0.29 ± 0.03 | |
| 3 | — | Physiological saline | 0.35 ± 0.05 | |
| 4 | — | Serum ultrafiltrate | 0.23 ± 0.03 | |

[1] Each value represents mean ± standard error of the mean of 5 animals. Test solution injected into ileum contained 2 $\mu c$ $Sr^{85}$ and about 1 mg carrier calcium. Sodium pentobarbitol was the anesthesia used for groups 1 and 2 of experiment 1 and for all groups of experiment 2; ethyl ether was used for groups 3 and 4 of experiment 1. For animals that were cannulated the intralumenal hydrostatic pressure was maintained at 10.5 cm $H_2O$ for experiment 1 and 12.0 cm $H_2O$ for experiment 2.

TABLE 5

*Solubility of $CaHPO_4$ in presence of certain carbohydrates and amino acids*[1]

| Test substance | Molar concentration of test substance | | | |
|---|---|---|---|---|
| | 0.0 M | 0.1 M | 0.25 M | 0.5 M |
| | Solubility of $CaHPO_4$ | | | |
| Glucose | 2.8 | 2.5 | 2.8 | 3.0 |
| Xylose | 2.8 | 2.1 | 2.9 | 2.6 |
| Lactose | 2.8 | 2.8 | 2.6 | 2.6 |
| Lysine | 2.8 | 3.7 | 4.6 | 5.5 |
| Glycine | 2.8 | 3.3 | 4.5 | 5.8 |

[1] Experimental flasks contained 50 mg $CaHPO_4$ in 100 ml of 1.5% ammonium acetate buffer adjusted to pH 7.5 and test substances at concentrations indicated. Flasks shaken for 24 hours at room temperature. Values given as mg Ca per 100 ml solution.

These studies indicate that lactose probably does not operate by maintaining a high hydrostatic pressure within the lumen of the gut, or providing the opportunity for elevated alkaline earth absorption by ion exchange mechanisms, or by providing an aqueous medium for better maintaining the calcium salts in a soluble form.

Lactose was shown several years ago to form a lactose-calcium complex (Herrington, '34). It was pertinent, therefore, to estimate the capacity of lactose, glucose, xylose, lysine and glycine to solubilize $CaHPO_4$. The results, presented in table 5, represent the average of duplicate runs of two separate experiments. The data indicate that the carbohydrates did not have any significant effect in solubilizing this relatively insoluble calcium phosphate salt. On the other hand, lysine and glycine distinctly increased the solubility of $CaHPO_4$. This effect of lysine on $CaHPO_4$ must be taken into consideration when explaining the stimulatory influence of lysine on calcium and strontium absorption (Wasserman et al., '56). Apparently, however, the action of lactose and xylose cannot be explained by complexing or chelating reactions.

## DISCUSSION

Although many of the present studies were done under "non-physiological" conditions, it may be well to re-emphasize that the effect of lactose on calcium metabolism has been observed under normal nutritional situations. In this laboratory, young rats raised on complete diets containing 30% of lactose continually absorb (and retain) more chronically ingested $Sr^{85}$ than rats reared under similar conditions on diets containing 30% of sucrose. Also Fournier et al. ('54, '55) reported an enhanced retention of calcium in lactating and rapidly growing rats with the balance-type technique. These latter observations, therefore, indicate that the data obtained by way of the present experimental approach are a true reflection of the physiological action of lactose and cannot be classified as an artifact.

In spite of extensive experimentation on the action of lactose in stimulating calcium and strontium absorption, the mechanisms by which this disaccharide and other effective carbohydrates operate are not clear. These studies and those previously reported eliminate some of the more obvious explanations. It has been shown, for example, that (a) lactose operates physiologically at the gastrointestinal level (Wasserman and Comar, '59; Lengemann et al., '59); (b) the intestinal microflora is not involved in lactose action (Wasserman et al., '57; Fournier, '55; present study); (c) lactose is effective only on ionized calcium and strontium since EDTA will block lactose action (Lengemann et al., '59); (d) vitamin D is not directly involved in lactose action (Lengemann et al., '59); (e) the response is not the result of a delayed gastric emptying time with hypertonic lactose solutions; (f) the rat ileum absorbs proportionally more calcium in the presence of lactose than the stomach, duodenum or jejunum; (g) certain other carbohydrates, such as L-xylose, share this calcium enhancing ability with lactose and that these effective sugars have the common properties of a prolonged residence time in the gastrointestinal tract and absorption by a passive mechanism (Wasserman and Comar, '59); (h) a hormone-like stimulating effect of lactose is not likely since lactose injected into a ligated ileal segment adjacent to the segment receiving $Sr^{85}$ did not cause a greater absorption of this radionuclide, (Lengemann, '59); (i) the higher osmotic or hydrostatic pressures produced by hypertonic solutions of lactose do not alone increase $Sr^{85}$ absorption; (j) lactose and other carbohydrates do not solubilize insoluble $CaHPO_4$ at the pH of the intestinal juices; and (k) certain metabolic inhibitors were unable to inhibit the lactose effect under the present conditions.

Fournier and Digaud ('57, '59) observed recently that diets containing 12% of lactose caused a greater urinary excretion of citric acid, aconitic acid, α-keto-glutaric acid, succinic acid and malic acid in rats consuming this diet contrasted with rats fed similar diets containing starch; pyruvic acid excretion, however, was unchanged. These observations may afford an indication of the mechanism of lactose action but the finding by Harrison and Harrison[6] that calcium absorption per se increased citric acid levels in the blood must be considered in this respect. Fournier et al. ('59) observed also that lactose feeding increased the weight, surface area and hypertrophy of the cecum of the rat; it is felt that these anatomical changes in the digestive tract have no direct bearing on the calcium absorption-lactose interrelationship since single doses of lactose to rats not previously given lactose diets were effective and, too, the enhanced absorption of $Ca^{45}$ and $Sr^{85}$ was observed within hours after administration of this factor (Lengemann et al., '59).

If speculation is permitted in lieu of conclusive evidence, apparently the lactose effect on absorption is a nonspecific membrane phenomenon, resulting in an alteration of the general permeability of the intestinal membrane to alkaline earth cations (and perhaps other cations). This would be conceptually in contrast to acting

[6] Harrison, H. C., and H. E. Harrison 1958 Interrelation of calcium and citrate metabolism. Federation Proc., *17:* 66 (abstract).

as part of or as an accelerator to a specific transport mechanism. Another possibility is that lactose operates through an unknown intermediate that in turn regulates or influences alkaline earth absorption (perhaps phosphate or a phosphorylated compound). Bearing on these hypotheses are observations such as the following: an active transport mechanism has been postulated by Schachter and Rosen ('59) for the absorption of magnesium and calcium but not strontium, and lactose is known to influence the absorption of magnesium, calcium, strontium, barium and radium (Lengemann, '59); the lactose effect is not specific for this disaccharide but is shared by diverse carbohydrates such as D-xylose, arabinose, sorbitol, glucosamine and others; usual physicochemical considerations, such as solubility, complex formation or hydrostatic pressure differences, could not explain the lactose effect. Experiments are being continued concerning the exact mechanism of lactose stimulation, using both *in vivo* and *in vitro* techniques.


## SUMMARY

1. Investigations were performed to study the mechanism by which lactose enhances the gastrointestinal absorption of calcium and strontium using short-term, radioisotope techniques.
2. The absorption of $Sr^{85}$ and $Ca^{45}$ was proportional to the lactose concentration in the test dose up to a maximum response at 0.4 millimoles of lactose.
3. In the presence of an antibiotic, neomycin, the lactose effect was still observed. Neomycin, itself, also was found to enhance $Sr^{85}$ absorption.
4. Metabolic inhibitors, such as phlorrhizin, sodium fluoroacetate, sodium iodoacetate, 2,4-dinitrophenol and sodium azide, did not inhibit lactose action when given as an integral part of the dosing solution.
5. The enhancing effect of lactose could not be duplicated when the hydrostatic pressure within the intestinal lumen was maintained at 10.5 or 12.0 cm with distilled water, saline or bovine serum ultrafiltrate.
6. *In vitro* studies showed that lactose, xylose and glucose at concentrations up to 0.5 M were unable to increase the solubility of $CaHPO_4$; however, the amino acids, lysine and glycine, were able to double the solubility of $CaHPO_4$ at this concentration.
7. These data were discussed in regard to the possible mechanism of action of lactose in enhancing alkaline earth absorption.



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# Efficacy of the FAO Amino Acid Reference Standard for Growth of the Weanling Rat

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Recently, the FAO (Food and Agriculture Organization of the United Nations) Committee on Protein Requirements formulated a reference amino acid pattern for estimating the nutritional value of the proteins in foodstuffs and to serve as a guide in the supplementation of these foodstuffs, one with another and with amino acids. The purpose of the present study is to compare the growth-promoting properties of amino acid mixtures resembling the FAO reference standard with those of casein, mixtures of amino acids patterned after casein and with casein and cottonseed flour supplemented with amino acids, to provide a pattern of essential amino acids identical with that of the FAO standard.

## EXPERIMENTAL PROCEDURE

The experimental diets were made up as designed for man on the basis that, with the exception of D-methionine, only the L-forms of the amino acids are utilized as such. Since arginine and histidine are required for growth of the weanling rat, but are not included in the FAO reference pattern, these amino acids were incorporated into the mixtures in the proportions found in casein. All diets were isonitrogenous, containing 1.8% of nitrogen. At this level small critical changes in essential amino acid content are reflected in the observed growth rates. Non-specific nitrogen was supplied by an equimolecular mixture of L-glutamic acid and DL-alanine.

The 10 diets used are designated as follows and their composition is shown in table 1.

1. Casein
2. FAO Reference Pattern, mixture A
3. FAO Reference Pattern, lysine increased from 270 to 330 mg/gm of nitrogen, mixture B
4. FAO Reference Pattern, tryptophan decreased from 90 to 60 mg/gm of nitrogen, mixture C
5. FAO Reference Pattern, total essential amino acid content increased to that of casein, mixture D
6. Mixture D with tryptophan decreased by one-half, mixture E
7. Amino acid mixture patterned after casein, mixture F
8. Amino acid mixture patterned after casein, total essential amino acid content decreased to that of FAO Reference Standard, mixture G
9. A mixture containing casein supplemented with the essential amino acids to the FAO Reference Pattern, mixture H
10. A mixture containing cottonseed flour supplemented with the essential amino acids to the FAO Reference Pattern, mixture I

The composition of the amino acid mixtures used is shown in table 2.

Groups of 10 male weanling rats of the Holtzman strain were allowed to feed ad libitum for 28 days on diets 1, 2, 7, 9 and 10. Food consumption was determined and a second series of 5 similar groups of animals was pair-fed for 28 days using the same diets, each animal being supplied a quantity of food equal to the average consumption of the group eating the least in the ad libitum experiment. Later diets 3, 4, 5, 6 and 8 were fed ad libitum only. All animals were weighed at 5-day intervals.

## RESULTS AND DISCUSSION

The rat, in many respects, is well adapted for use in testing the nutritive value of a pattern of dietary amino acids for growth. The gain in body weight, for

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TABLE 1
*Composition of diets*

| Diet no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Casein[1] | 12.6 | | | | | | | | | |
| Amino acid mixture A | | 14.3 | | | | | | | | |
| Amino acid mixture B | | | 14.3 | | | | | | | |
| Amino acid mixture C | | | | 14.4 | | | | | | |
| Amino acid mixture D | | | | | 13.4 | | | | | |
| Amino acid mixture E | | | | | | 13.4 | | | | |
| Amino acid mixture F | | | | | | | 14.5 | | | |
| Amino acid mixture G | | | | | | | | 14.8 | | |
| Amino acid mixture H | | | | | | | | | 13.3 | |
| Amino acid mixture I | | | | | | | | | | 19.1 |
| Salt mixture[2] | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| Dextrose | 73.0 | 71.3 | 71.3 | 71.2 | 72.2 | 72.2 | 71.1 | 70.8 | 72.3 | 66.5 |
| Hydrogenated vegetable oil[3] | 8.0 | 8.0 | 8.0 | 8.0 | 8.0 | 8.0 | 8.0 | 8.0 | 8.0 | 8.0 |
| Corn oil | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Linseed oil | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Delsterol[4] | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Vitamin addendum[5] | + | + | + | + | + | + | + | + | + | + |

[1] Labco.

[2] Wisconsin no. 4.

[3] Crisco.

[4] Delsterol (vit. D) 300 units/100 gm of diet; Nopcay (vit. A) 1600 units/100 gm of diet.

[5] Micronutrients mg/100 gm: thiamine, 1.0; riboflavin, 2.0; pyridoxine, 1.0; calcium pantothenate, 10.0; niacinamide, 10.0; inositol, 5.0; choline, 100.0; *p*-aminobenzoic acid, 30.0; biotin, 0.05; folic acid, 0.20; α-tocopherol, 14.2; menadione, 14.2; $B_{12}$ triturate (0.1% trituration with mannitol), 10.0.

TABLE 2

*Composition of amino acid mixtures*

| Amino acid mixture | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| | % | % | % | % | % | % | % | % | % |
| Casein | | | | | | | | 48.1 | |
| Cottonseed flour[1] | | | | | | | | | 70.4 |
| L-Arginine·HCl[2] | 4.0 | 4.0 | 4.0 | 7.1 | 7.1 | 4.4 | 2.4 | 2.3 | —[3] |
| L-Histidine·HCl·$H_2O$[2] | 3.4 | 3.4 | 3.4 | 6.1 | 6.1 | 4.4 | 2.4 | 1.7 | 1.7 |
| L-Isoleucine with alloisoleucine | 6.8 | 6.8 | 6.8 | 12.2 | 12.2 | 11.3 | 6.2 | 2.3 | 2.4 |
| L-Leucine | 3.8 | 3.8 | 3.8 | 6.8 | 6.8 | 9.3 | 5.2 | —[4] | 0.5 |
| L-Lysine·HCl | 4.3 | 5.2 | 4.3 | 7.6 | 7.6 | 10.0 | 5.6 | 0.15 | 1.3 |
| DL-Methionine | 1.8 | 1.8 | 1.8 | 3.2 | 3.2 | 3.2 | 1.7 | 0.6 | 0.7 |
| L-Cystine | 1.6 | 1.6 | 1.6 | 2.9 | 2.9 | 0.5 | 0.3 | 1.5 | 0.7 |
| DL-Phenylalanine | 4.5 | 4.5 | 4.5 | 8.2 | 8.2 | 9.9 | 5.6 | 0.5 | —[5] |
| L-Tyrosine | 2.3 | 2.3 | 2.3 | 4.1 | 4.1 | 5.3 | 2.9 | 0.1 | —[5] |
| DL-Threonine | 4.5 | 4.5 | 4.5 | 8.2 | 8.2 | 8.4 | 4.6 | 1.1 | —[5] |
| DL-Tryptophan | 2.3 | 2.3 | 1.5 | 4.0 | 2.0 | 2.2 | 1.2 | 1.45 | 0.8 |
| DL-Valine | 6.8 | 6.8 | 6.8 | 12.4 | 12.4 | 13.9 | 7.6 | 1.1 | 1.3 |
| L-Glutamic acid | 33.6 | 33.0 | 34.1 | 10.7 | 11.7 | 10.9 | 33.8 | 24.3 | 12.6 |
| DL-Alanine | 20.3 | 20.0 | 20.6 | 6.5 | 7.5 | 6.5 | 20.5 | 14.8 | 7.6 |
| | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| Nitrogen content | 12.5 | 12.5 | 12.4 | 13.4 | 13.4 | 12.4 | 12.2 | 13.5 | 9.4 |

[1] UNICEF Code C-1.

[2] Arginine and histidine are not components of the FAO reference standard but are included here in the proportions found in casein because of their growth stimulating effect in the rat.

[3] Arginine is supplied by cottonseed meal in greater amount than supplied by casein.

[4] Leucine is the amino acid present in greatest excess in casein.

[5] Phenylalanine, tyrosine and threonine are present in equal excess in cottonseed meal.

example, is well correlated with the utilization of dietary amino acids for body protein synthesis (Hegsted and Worcester, '47; Howard et al., '57; and Bender, '59). Such a correlation was recognized by Osborne, Mendel and Ferry, ('19) when they proposed the protein efficiency ratio as the grams gained in body weight per gram of nitrogen intake. Since the magnitude of the ratio varies with the nitrogen intake, these authors and others (Barnes and Bosshardt, '46) recommended the determination of the maximum ratio for each dietary protein as the best single figure for comparative studies. The rate of gain in body weight with respect to nitrogen intake during the first 4 weeks of growth, however, is essentially linear at the lower nitrogen intakes. Thus the slopes of these lines are also correlated with the nutritive

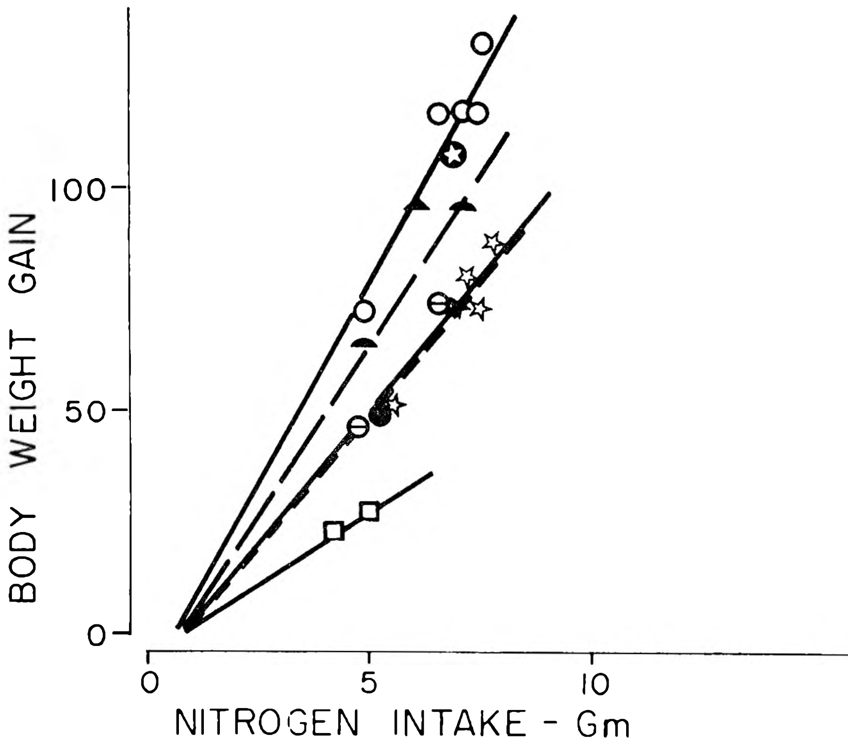


Fig. 1 Body weight gains as a function of nitrogen intake in experiments of 4-week duration. Roman numerals are diet numbers. LEAA = L essential amino acids.

value of the dietary nitrogen source and the rate of change of body weight gain with respect to intake has been called the nitrogen growth index (Allison, '59). Ad libitum feeding can be used to determine the indexes and also the food intake, which is another criterion of nutritive value. The data plotted in figure 1 illustrate such a correlation between nitrogen intake and body weight gain in rats fed different patterns of dietary amino acids.

The open circles record the gain in body weight observed with different intakes of casein nitrogen over the 4-week growth period. The slope of this line (19) and the point at which it intercepts the intake axis is in agreement with extensive studies on the effect of nitrogen intake on body weight gain (Allison, '59; Allison et al., '59). The dotted line drawn through the open stars illustrates the nutritive value of the FAO pattern of amino acids (FAO '57). The slope of this line is 12. Thus the FAO pattern, as fed in this diet, has a lower nutritive value than casein. The circles with cross bar record the data obtained while feeding the mixture containing casein supplemented with the essential amino acids to the FAO reference pattern. These data demonstrate that the amino acids from casein and the free amino acids, added to equal the FAO pattern, resulted in the same gain in weight as the FAO mixture. These results suggest that casein is digested in such a way that the pattern absorbed is approximately the same whether the acids are fed free or combined in part as casein. The open squares in figure 1, on the other hand, illustrate data obtained while feeding cottonseed flour supplemented to the FAO reference pattern with the essential amino acids. The slope of this line is only 4. Possibly the calculated pattern, in this experiment, did not represent the absorption pattern, as provided by the FAO mixture, because of a lower digestion of cottonseed flour. Such a variation in digestion could result in a relatively rapid absorption of the added free amino acids compared with those liberated from the protein, thereby creating an imbalance in the body. More studies are needed to test this possibility. The nitrogen growth index of cottonseed flour without supplementation when used in higher concentration in the diet was determined to be 14 (Allison, '59).

The relatively lower nutritive value of the mixture of amino acid patterned after FAO in comparison with that of casein may be correlated in part with a lower percentage of essential amino acids. Increasing the percentage of essential amino acids from 31.1, provided in the FAO mixture, to 56, an average of that in casein, but retaining the FAO pattern, gave results illustrated by the black triangle. This triangle is on the line describing the casein diet. This mixture, while being utilized as efficaciously as casein, does not support as great a rate of growth. Possibly the greater osmotic effect of the amino acid mixture in comparison with the intact protein restricts food consumption. Similarly, reducing the essential amino acid mixture to 31.1% of the total but retaining the casein pattern of essential amino acids resulted in data recorded by the black circle. This circle is on the line describing the FAO data. These results can be interpreted to mean the rat needs the higher percentage of essential amino acids found in casein. Such a high percentage, however, may not be required under all conditions for growth in the infant (Holt and Snyderman, '56). These results emphasize the necessity of maintaining an essential amino acid intake that is adequate to supply the quantitative as well as the qualitative requirements.

Evidence has been presented that the rat can utilize the D-isomers of tryptophan and phenylalanine (Rose, '38). These amino acids may therefore be present in the FAO pattern as designed for human use in excess of the quantities required for optimum performance. Accordingly, a diet was formulated which contained 56% of essential amino acids in the FAO pattern with the tryptophan content reduced by one-half. Feeding this diet resulted in data illustrated by the star in the black circle on the line describing the casein data. The excess tryptophan present did not decrease the efficacy of the mixture. These and the results obtained by feeding the casein pattern containing 56% of essential amino acids represented by the black semicircles could be interpreted to mean that the FAO pattern may be better

than the pattern provided by casein for growth in rats although the tryptophan content of the reference is higher than need be. It is interesting that the protein efficiencies of the FAO and casein patterns at 31.1% of essential amino acids were identical, but the food intake and consequent rate of growth when these diets were fed ad libitum were much greater with the FAO mixture. The excess of tryptophan in the FAO pattern for growth in rats was also indicated by the experiment represented by the black star on the line with the slope of 12. The star records data obtained while feeding rats the original FAO mixture, with the lower percentage of essential amino acids, but with the tryptophan reduced by one-third. One other change was made in the FAO pattern by increasing the lysine from 270 to 330 mg/gm of nitrogen without altering the other amino acids. Fed this diet, rats ate 8.2 gm of nitrogen and gained 72 gm in body weight, results which demonstrate that such an increase in lysine, at least without other changes in the pattern, had no beneficial effect.

## SUMMARY

In rat growth experiments, the FAO (Food and Agriculture Organization of the United Nations) amino acid reference pattern was found to possess a somewhat greater efficiency than an amino acid mixture patterned after casein at a high essential amino acid content. At a lower essential amino acid content the protein efficiencies were equal but the FAO pattern supported much more rapid growth when the diets were fed ad libitum.

Decreasing the tryptophan content of the FAO pattern by 33 to 50% or increasing the lysine content by 22% did not alter the nutritive value of the mixture.

A mixture containing casein supplemented to the FAO reference pattern with essential amino acids was found to be nutritionally equivalent to the FAO reference amino acid mixture itself. Cottonseed meal so supplemented proved to be inferior to both.

## ACKNOWLEDGMENT

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# Stepwise Weight Reduction in Obese Young Women: Clinical and Metabolic Responses[1]

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Both clinical and metabolic studies of obese young women during weight reduction led to speculation as to the possible psychologic and physiologic advantages of the use of a scheme of stepwise weight reduction in which short periods of weight reduction would be alternated with periods of controlled weight maintenance until the desired objective was achieved (Young, '52a,b; Young et al., '53, '55; '57). It has been observed clinically that a fair number of people can accept rigid caloric restriction over short intervals of time but not for prolonged periods. In some of these individuals if a "rest period" is allowed, restriction will again be accepted. There might, then, be a psychological advantage in planning for such respites in the reduction process. The majority of men subjected to such a process felt so (Young et al., '58). Could there also be physiologic advantage as well? Previous metabolic studies with obese young women had shown that in a pre-reduction weight maintenance period all the women retained nitrogen, calcium and phosphorus. After three to 4 weeks' weight reduction on the same intake of these nutrients, the subjects were either in equilibrium or still retaining the nutrients; however by the 8th to 10th week of the reduction period, the majority of subjects were losing nutrients. Then by the third week, on a post reduction maintenance diet, an almost complete reversal of the downward trend was observed, when the subjects reached a state of equilibrium or retention with regard to all three nutrients. Physiologic advantage might come from the alternation of weight reduction and weight maintenance, since the loss of nutrients occurred only in prolongation of the reduction period.

Also, in studies of limited duration (about 15 weeks) some question has arisen whether the balance variations might be merely ones of adjustment and not due to caloric restriction per se. Since not all subjects respond metabolically in a uniform manner to weight loss, there has been question as to what led to negative balances in some and not in others. The factor seemingly most closely related has been total pounds lost during the experiment (Young et al., '58). However, Ohlson et al. ('55) have suggested that one of the basic needs for research in weight reduction is in the body composition of the overweight as it related to the metabolic response observed.

Hence, the present investigation was undertaken to study three factors: (1) the subjective reaction and metabolic responses to weight reduction achieved by a stepwise process; (2) the effect of caloric restriction per se, by ascertaining the metabolic response of control subjects on maintenance diets of similar nitrogen, calcium and phosphorus content as compared with subjects undergoing weight reduction; and (3) the relation of metabolic response to some objective indications of relative body fatness, namely the measurement of selected fat pads by means of soft-tissue x-rays and certain skinfold thicknesses.

## SUBJECTS AND METHODS

The 10 overweight young women who served as subjects are described in table 1. They were divided into two groups of 5

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**TABLE 1**

*Age, height, weight and weight changes of experimental and control subjects*

| | Subject number | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Reducers | | | | | | Controls | | | | | |
| | 1 | 2 | 3 | 4 | 5 | Mean | 6 | 7 | 8 | 9 | 10 | Mean |
| Age, years | 19 | 25 | 22 | 20 | 19 | 21 | 18 | 20 | 20 | 27 | 25 | 22 |
| Height, in. | 67.0 | 66.5 | 62.5 | 60.9 | 65.0 | 64.4 | 64.0 | 64.5 | 67.0 | 67.0 | 64.5 | 65.4 |
| Initial weight, pounds | 157.25 | 164.25 | 173.0 | 134.0 | 161.5 | 158.0 | 138.75 | 149.0 | 146.5 | 152.5 | 138.0 | 146.7 |
| Standard weight, pounds | 134.0 | 133.0 | 123.0 | 117.0 | 126.0 | 126.6 | 123.0 | 129.0 | 137.0 | 140.0 | 129.0 | 131.6 |
| Excess weight, pounds | 23.25 | 31.25 | 50.0 | 17.0 | 35.5 | 31.2 | 15.75 | 20.0 | 9.5 | 12.5 | 9.0 | 13.2 |
| Excess weight, % | 17.2 | 23.3 | 40.6 | 14.5 | 27.8 | 24.7 | 12.2 | 15.0 | 7.3 | 8.6 | 7.0 | 10.1 |
| Final weight, pounds | 142.25 | 141.0 | 158.25 | 123.25 | 139.75 | 140.9 | 138.5 | 145.5 | 144.75 | 149.5 | — | 144.6 |
| Net weight change, pounds | −15.0 | −23.25 | −14.75 | −10.75 | −21.75 | −17.1 | −0.25 | −3.5 | −1.75 | −3.0 | — | −2.0 |
| Excess weight lost, % | 65.2 | 75.0 | 29.5 | 63.2 | 62.1 | 59.0 | — | — | — | — | — | — |
| Rate of loss, pounds/week | 1.8 | 2.8 | 1.8 | 1.3 | 2.6 | 2.1 | — | 0.4 | 0.2 | 0.4 | — | 0.3 |
| | | | | | | | *Control subjects given maintenance diet during all periods* | | | | | |
| Pre-reduction maintenance, pounds | −1.75 | −1.75 | −2.75 | +1.25 | +1.0 | −0.8 | +0.5 | −1.25 | −0.5 | 0.0 | — | −0.3 |
| Reduction period 1, pounds | −6.0 | −8.0 | −7.25 | −7.25 | −13.5 | −8.2 | +0.75 | −1.25 | −0.5 | −1.0 | — | −0.5 |
| Maintenance, pounds | +1.5 | −1.0 | +2.5 | +1.75 | +0.5 | +0.8 | −0.5 | −1.5 | +2.75 | −4.5[1] | — | −0.9 |
| Reduction period 2, pounds | −6.25 | −6.25 | −5.0 | −4.0 | −6.75 | −7.1 | −1.5[2] | +1.0 | −1.75 | +3.0 | | +0.25 |
| Maintenance, pounds | +1.5 | +0.5 | +1.0 | +0.25 | +1.0 | +1.1 | +0.25 | −1.5 | 0.0 | −1.25 | — | −0.6 |
| Reduction period 3, pounds | −4.0 | −6.75 | −3.25 | −2.25 | −4.0 | −4.1 | +0.25 | +1.0 | −1.75 | +1.75 | — | +0.3 |

[1] Subject no. 9 absent from diet table during this period due to illness.

[2] Subject no. 6 absent from diet table during this period due to emergency.

each: no. 1 through 5, who were subjected to weight reduction in a stepwise fashion; no. 6 through 10, the controls, who were placed on a maintenance diet throughout the entire 16 weeks of study. Those in the reducing group weighed from 17.0 to 50.0 pounds above standard weight (Association Life Insurance Medical Directors) with a mean excess weight of 31.2 pounds. The percentage of excess weight averaged 24.7, with a median of 23.3 pounds. Subjects were screened carefully by interview and by psychological testing to select only those with sufficient emotional stability to cooperate in a 16-week controlled feeding period (Summerskill and Darling, '55).

The plan of the experiment for the subjects to be reduced included, in the following order: (1) a pre-reduction weight maintenance period of 24 days; (2) a 23-day reduction period; (3) a 15-day maintenance period, 10 days of which coincided with the spring semester vacation period; (4) a 22-day reduction phase; (5) a 14-day weight maintenance period; and finally (6) a 14-day reduction period. The 5 control subjects were placed on a maintenance diet throughout the entire 16 weeks of study. Except during the 10-day spring vacation, the women were weighed daily under uniform conditions and consumed a weighed diet prepared and served under the supervision of a dietitian at the special diet table operated for research purposes. Only black tea, black coffee and water were allowed ad libitum and quantities of these consumed were recorded carefully. Throughout the experiment the diets used were calculated to contain protein, 90 gm; calcium, 1.0 gm; phosphorus, 1.5 gm; and amounts of other nutrients to meet the National Research Council's Recommended Dietary Allowances ('58). The caloric content of the reduction diet was 1400 calories per day with 50% of the calories from fat. The maintenance diets were planned to give 2400 calories per day and were adjusted for individual weight maintenance by varying quantities of sugar, butter and a specially prepared candy. During spring vacation the subjects were given suggested guides for food consumption and asked to maintain their weights.

Nitrogen, calcium and phosphorus balances were determined during 4 periods of 7 days each, scheduled for the second week of the initial maintenance period and the last week of each of the reduction periods, for both control and reducing subjects.

During the balance periods samples were taken of all food as served. The 7-day composite reducing and control diets, in each case, were mixed, ground in a food chopper, weighed and a homogeneous sample taken. Food used for additional calories and water were similarly sampled and analyzed. Collections of urine were made for the 7 consecutive days of each balance period using toluene as a preservative. Stools for the balance period were marked by carmine and pooled. All samples were digested on the steam bath for a matter of hours with 2:1 HCl and then made up to volume for later analysis (Stearns, '29). The nutrient contents of urine, feces and food aliquots were determined by the following methods: nitrogen, by the Kjeldahl method (Hawk et al., '47); calcium, by an oxalate precipitation method (Kochakian and Fox, '44); and phosphorus by a vandate photometric method (Koenig and Johnson, '42).

Ideally, either density or total body water determinations would have been used as a measure of relative body fatness. Since at the time suitable equipment and personnel for these were not available, measurements of fat pads from soft-tissue x-rays and of skinfolds were used, being made at the end of the pre-reduction maintenance period and again in the last week of the final reduction period. As studies investigating the selection of anatomical locations giving the most representative picture of subcutaneous fat have been conducted only on men, the 6 locations suggested by Garn ('54) for men and an additional x-ray of the thigh were selected to use with the young women. These included: deltoid insertion fat; medial and lateral lower arm fat, measured at point of maximal deviation of radius and ulna; iliac fat, measured at crest of ilium; trochanteric fat, measured at peak of greater trochanter; medial and lateral thigh fat, measured halfway between the top of the patella and the acetabulum; medial and

lateral leg fat, measured at the maximal muscle diameter; and posterior and anterior leg fat measured at maximal muscle diameter. The left side was used throughout and x-rays were taken posterior to anterior except for the last position which was a lateral to medial view. Pictures were taken at a standardized 6-foot tube-to-film distance, and fat pads were measured on the completed film with a lucite cardinell ruler calibrated to 0.05 cm. Data were recorded to the nearest 0.5 mm. To insure that the measurements of fat pads were made at the same level in both sets of films, the two x-rays at the same location for each subject were superimposed to match anatomical landmarks.

Skinfold measurements were made using the Minnesota caliper which has rectangular jaw faces of 25 $mm^2$ and is calibrated to give a spring tension of 10 $gm/mm^2$ of jaw surface. Skinfolds were grasped between the thumb and index finger, and the caliper was applied about 1 cm from the fingers holding the skinfold and at a depth approximately equal to the thickness of the fold. Three measurements were made at each location with the data recorded as the mean of the three values. Three skinfold measurements suggested by Brozek ('56) were made: (1) the dorsal skinfold measured on the upper left arm (over the triceps) halfway between the tip of the acromial process and the elbow, with the fold parallel to the long axis of the arm; (2) the sub-scapular skinfold measured below the tip of the right scapulae and (3) the lateral waist, halfway between the iliac crest and the costal margin, along the mid-axillary line.

The basal energy requirements per 24 hours were determined for the reducing subjects during the first week of the initial reduction period, and the control subjects were tested during the second week of the same period. Determinations were repeated near the end of the experiment. Each subject spent the night in the infirmary where basal determinations were made the following morning using a Sanborn Metabulator apparatus. The basal metabolic rate was taken as the mean of duplicate determinations made under suitable basal conditions.

At the conclusion of the experiment a questionnaire was given to the subjects to ascertain their previous food intake patterns and their reactions to various phases of the experiment.

## RESULTS AND DISCUSSION

### *Weight loss and caloric intake*

During the entire experiment the subjects on the stepwise reducing regimen lost from 10.75 to 23.25 pounds, with a mean loss of 17.1 (table 1). The median excess weight lost was 63.2%, with values ranging from 29.5 to 75%. With the exception of the heaviest subject, no. 3, the amount lost varied directly with the initial weight of the subjects. Over a 16-week maintenance period, the control subjects showed a mean loss of 2 pounds. In table 1 are given the weight variations of the individual subjects during each of the successive phases of the experiment. Data for control subject no. 10 are not included since she was dropped from the experiment after several weeks for personal reasons.

As observed repeatedly in previous studies, among the reducers the rate of weight loss decreases as caloric restriction is prolonged. In the stepwise process, during the first period of caloric restriction the mean loss was 2.5 pounds/week; during the second, 2.3 pounds/week; during the final period, it dropped to 1.9 pounds/week.

A comparison of the mean caloric intakes and the weight variations in the pre-reduction "maintenance" period, with the caloric intakes and weight changes in the last "maintenance" period after 6 weeks of caloric restriction, showed that, in every instance, those who had been subjected to caloric restriction showed a marked decrease in the number of calories required for maintenance (mean decrease, 637 Cal.). In contrast, the controls. who had not been on caloric restriction, showed some increase in the calories required for maintenance (mean increase, 208 Cal.). The lower calorie maintenance requirement relates to the lesser rate of weight loss as weight reduction progresses. Both are undoubtedly to some extent the result of a reduction in the basal caloric requirements, of lesser activity requirements accompanying the increasing lethargy in

movements noted by the dietitians during the experiment, and of a lesser total mass to be moved. In addition, we believe some physiological adaptation is involved.

### *Basal metabolism*

For the weight reduction subjects at the end of the experiment, the basal caloric requirements for 24 hours showed a mean decrease of 6.8% (range, 2.8 to 12.6%) over that at the beginning of the experiment. These figures compare favorably with the mean decreases of 7.6 and 8.0% reported by this laboratory in previous studies of women in which caloric restriction was extended over a slightly longer interval (Young, '52a; Young et al., '53) and the 8.5% decrease reported when studying men (Young et al., '57). That the decreases probably are not the result of a "training" factor is shown by the fact that the control subjects on maintenance diet for 16 weeks showed a mean increase in basal calories for 24 hours of 2.4%.

### *Relative body fatness*

*Fat pads in soft tissue x-rays.* Though soft tissue x-rays were taken at 7 positions, the sum of the measurements of fat pads at only 5 locations are considered here, namely: trochanteric, deltoid insertion, medial leg, crest of iliac and medial thigh. The first three are the locations suggested by Garn ('54) as showing the greatest correlation with total body fat in men. The latter two (medial thigh fat and iliac fat) were added because they appeared to be measurements important for women. The reducing and control subjects were placed in rank order within each group according to the sums of the 5 fat pad values at the beginning of the experiment and at the end (table 2). The two subjects with the greatest sums of fat pad thicknesses (subjects no. 3 and 2) ranked first and second, respectively, for fat pad thickness in every location measured in this study except one, the medial thigh fat; subject no. 9, with the smallest total fat pad thicknesses, also had the thinnest or next to the thinnest fat pads of any subject for every location measured.

Among the reducers, a comparison of rank orders of subjects according to sums of fat pad thicknesses at the beginning and at the end of the experiment showed that three subjects (nos. 2, 3, and 4) maintained their rank order whereas subjects nos. 1 and 5 shifted relative positions, subject no. 5 having lost considerably more weight than subject no. 1. Among the controls all subjects maintained the same rank order throughout the study.

*Skinfold measurement.* Skinfold measurements were taken over the triceps, below the tip of the scapula and at the lateral waist as described previously. These locations for subcutaneous fat pad measurement have been suggested by Brozek ('56) as being representative of nutritional status of men. However, the totals of these measurements did not prove to relate well

TABLE 2

*Sum of 5 fat pad measurements from soft-tissue x-rays, and rank order of subjects by fat pad thickness*

| Subject no. | Sum fat pad thicknesses | | | | |
|---|---|---|---|---|---|
| | Early in experiment | | Late in experiment | | Difference |
| | *cm* | *rank order* | *cm* | *rank order* | *cm* |
| | Reducing subjects | | | | |
| 1 | 13.15 | 5 | 12.20 | 3 | –0.95 |
| 2 | 19.00 | 2 | 15.75 | 2 | –3.25 |
| 3 | 21.45 | 1 | 18.99 | 1 | –2.46 |
| 4 | 13.30 | 4 | 12.15 | 4 | –1.15 |
| 5 | 14.40 | 3 | 11.60 | 5 | –2.80 |
| | Control subjects | | | | |
| 6 | 13.55 | 2 | 14.65 | 2 | +1.10 |
| 7 | 14.00 | 1 | 15.55 | 1 | +1.55 |
| 8 | 10.05 | 3 | 11.20 | 3 | +1.15 |
| 9 | 10.00 | 4 | 10.20 | 4 | +0.20 |

with either the fat pad measurements from the soft tissue x-rays or the excess weight above standard weight, as measures of relative body fatness. As determined by comparisons of rank order the locations used in the soft tissue x-ray technique compared more favorably with the calculated excess above standard weights. Also, the rank order of the subjects for the changes in the sum of fat pads measured from the x-rays before and after weight loss compared much more favorably with the rank order of the weight loss in pounds than did the rank order of changes in the sum of skinfold measurements. Among the control subjects the relationship between both measures of subcutaneous fat and excess weight was much closer, though that between the locations used in the soft tissue x-rays and excess weight was closer than that between skinfolds and excess weight. It is recognized that with the limited number of subjects involved, rank order comparisons are of limited value.

Obviously, the skinfold measurements suggested as best for use with normal men were not the best for overweight women. They were all located above the waist and in no way evaluated fat pads over the hips, thighs and legs, which may be areas of heavy fat concentrations in women. The greatest weakness in the sites selected for the soft tissue x-rays was the failure to include one of the trunk above the waist. In future studies if either skinfold measurements or fat pad thicknesses from soft tissue x-rays are to be used as indicative of total fatness, changes in locations to be measured would be desirable to be sure that all locations of probable heavy fat concentration in women were represented in each series of measurements. This is not easy, since problems of anatomical landmarks, positioning and interferences with visualization are involved in some of the most important locations.

*Metabolic studies*

In table 3 are presented the mean daily retentions of nitrogen, calcium and phosphorus for both the reducing and the weight maintenance or control subjects at the various phases of the current experiment. Figure 1 presents a summary of the mean retentions of these nutrients by phase of experiment for the 6 studies completed in this laboratory, including the present one. From figure 1 and from table 3 we observe the metabolic response to weight reduction achieved by the stepwise process in contrast to straight reduction and the effect of caloric restriction per se. In addition, in figure 1 we may compare the response of men and of women subjects.

With respect to nitrogen metabolism, and to a lesser extent that of phosphorus, several points seem clear. Obviously, nitrogen deficits are the result of caloric restriction per se, since in all periods for control subjects and in all weight maintenance periods for the reducers (whether pre- or post-reduction), a mean retention of nitrogen is noted. Clearly, then, nitrogen and phosphorus losses are characteristic in certain instances of caloric restriction and do not represent an adaptive or normal fluctuation. In 4 successive balance periods at spaced intervals control subjects always retained these nutrients.

With respect to whether there might be physiologic advantage to the stepwise reduction process over that of an uninterrupted or straight reduction regimen, the evidence for both men and women does not indicate such an advantage. For neither sex did the stepwise reduction alter the essential retention patterns from those established in the straight reduction studies.

A comparison of the sexes with regard to metabolic responses shows that the principal sex difference is the stage of the reduction process at which nitrogen losses occur. In women, whether using the straight or stepwise reduction process, the mean nitrogen loss did not occur until "late reduction" (7th to 10th week); for men, under both regimens, nitrogen deficit was apparent in "early reduction" (third to 4th week).

Another interesting point with respect to sex differences is that only women showed definite mean calcium deficits; this was observed in all studies by "late reduction." Curiously, in the current study, three of the controls lost calcium in the pre-reduction maintenance period. In general, those who were losing calcium had been customarily consuming twice or more

TABLE 3

*Mean daily values for nitrogen, calcium and phosphorus retention, by periods*

| Balance period | Retention by subjects | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Reducers | | | | | Summary | | | Controls—maintenance | | | | | Summary | | |
| | Subject number | | | | | | | | Subject number | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | R[1] | B[2] | L[3] | 6 | 7 | 8 | 9 | 10 | R | B | L |
| | *gm/day* | *gm/day* | *gm/day* | *gm/day* | *gm/day* | *No. subjects* | | | *gm/day* | *gm/day* | *gm/day* | *gm/day* | *gm/day* | *No. subjects* | | |
| **Nitrogen** | | | | | | | | | | | | | | | | |
| 1 Pre-reduction, second week | +0.296B | +0.376B | +2.198R | +1.848R | +0.479B | 2 | 3 | – | +2.115R | −0.237B | +0.509B | +1.911R | +1.624R | 3 | 2 | – |
| 2 Reduction, third week | +0.390B | +0.700R | +1.734R | −0.910L | −1.744L | 2 | 1 | 2 | +3.936R | +1.270R | +0.742R | +1.202R | +1.231R | 5 | – | – |
| 3 Reduction, 6th week | +0.991R | +1.265R | +0.533B | — | −0.909L | 2 | 1 | 1 | +3.269R | +2.067R | +0.381B | +0.583R | — | 2 | 2 | – |
| 4 Reduction, 8th week | −0.815L | +1.026R | −0.594B | −1.227L | −1.069L | 1 | 1 | 3 | +3.017R | +0.456B | +1.457R | +1.350R | — | 3 | 1 | |
| **Calcium** | | | | | | | | | | | | | | | | |
| 1 Pre-reduction, second week | −0.107L | −0.016B | −0.056B | +0.041B | −0.034B | – | 4 | 1 | −0.144L | −0.167L | −0.128L | −0.078B | −0.005B | – | 2 | 3 |
| 2 Reduction, third week | +0.038B | +0.046B | +0.629R | +0.087B | −0.497L | 1 | 3 | 1 | +0.218R | +0.041B | +0.112B | +0.117B | +0.178R | 2 | 3 | – |
| 3 Reduction, 6th week | +0.234R | +0.019B | −0.317L | — | −0.312L | 1 | 1 | 2 | +0.473R | +0.023B | −0.063B | −0.326L | — | 1 | 2 | 1 |
| 4 Reduction, 8th week | −0.325L | −0.252L | −0.720L | −0.348L | −0.658L | – | – | 5 | +0.079B | −0.097B | −0.100B | −0.037B | — | – | 4 | – |
| **Phosphorus** | | | | | | | | | | | | | | | | |
| 1 Pre-reduction, second week | +0.158R | +0.285R | +0.156R | +0.242R | +0.130B | 4 | 1 | – | +0.328R | +0.172R | −0.078B | +0.082B | +0.236R | 3 | 2 | – |
| 2 Reduction, third week | −0.100B | +0.195R | +0.182R | +0.227R | −0.136B | 3 | 2 | – | +0.209R | +0.005B | +0.082B | +0.127B | +0.101B | 1 | 4 | – |
| 3 Reduction, 6th week | +0.059B | +0.018B | −0.113B | — | −0.193L | – | 3 | 1 | +0.521R | +0.240R | −0.029B | +0.166R | — | 3 | 1 | – |
| 4 Reduction, 8th week | −0.073B | +0.102B | −0.234L | −0.008B | −0.105B | – | 4 | 1 | +0.498R | +0.074B | +0.072B | +0.263R | — | 2 | 2 | – |

[1] R = rentention greater than 5% of intake for nitrogen or 10% of intake for calcium and phosphorus.

[2] B = balance: retention or loss less than 5% of intake for nitrogen or 10% of intake for calcium and phosphorus.

[3] L = loss greater than 5% of intake for nitrogen or 10% of intake for calcium and phosphorus.

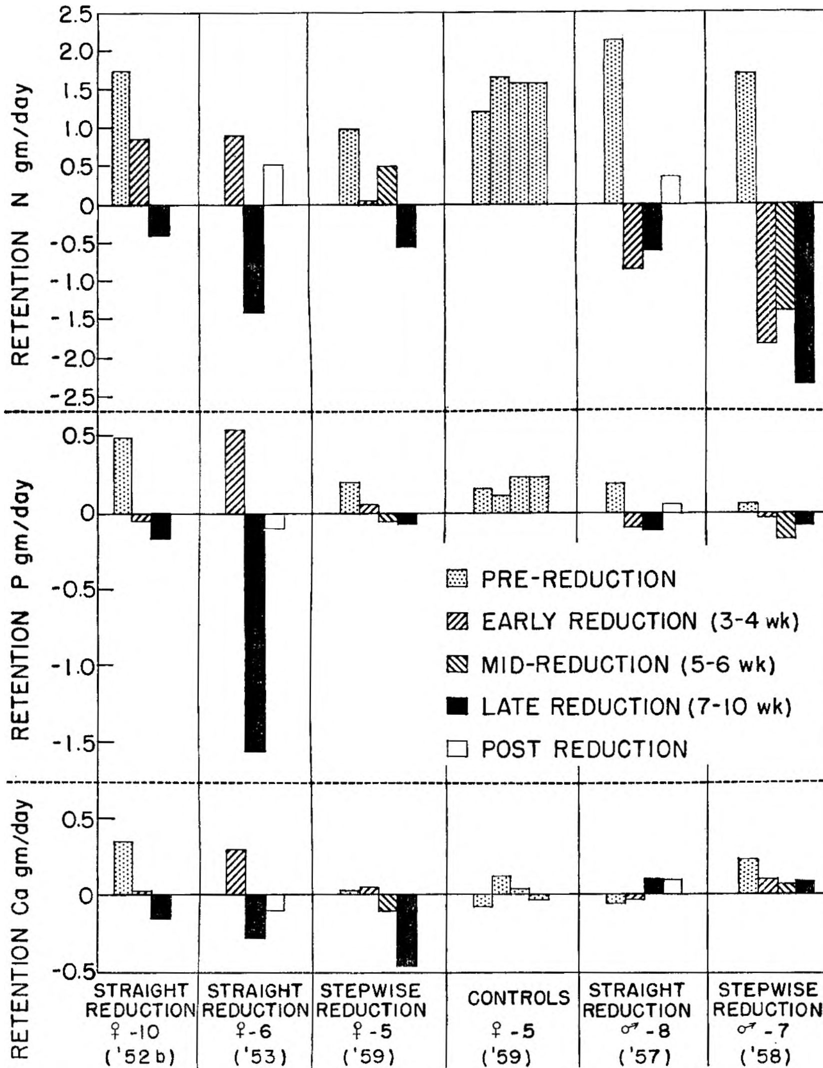


Fig. 1 Mean retentions of nitrogen, phosphorus, and calcium by phases of experiment for 6 metabolic studies; straight reduction with 10 women (Young, '52b); straight reduction with 6 women (Young et al., '53); stepwise reduction with 5 women and 5 women controls (this paper); straight reduction with 8 men (Young et al., '57); and stepwise reduction with 7 men (Young et al., '58).

the quantity of milk which was given on the diet. Among the reducers, as weight reduction progressed, more of the subjects went into negative calcium balance until by the 8th week of caloric restriction all 5 subjects were losing calcium (table 3). In contrast, among the controls, as the experiment progressed, only one subject lost calcium in one period: subject no. 9 in period 3. She had been ill just previous to this period. Hence negative calcium balances appear to be definitely more characteristic of the individuals subjected to caloric restriction than of those not so restricted.

In the current study the percentage of the nitrogen intake in the feces and urine of control subjects remained constant within a narrow range throughout the entire study. During the pre-reduction maintenance period the percentage excreted in the urine and feces of reducers was com-

parable to that of the controls; however, in each of the caloric restriction periods, the percentage of intake excreted in the urine increased and that in the feces decreased for the reducers, in contrast to their own values during weight maintenance or those of the control subjects. Calcium deficits, in general, appeared to be associated with larger fecal excretions of calcium. In the current study, though we have no objective evaluation of emotional stability of the subjects during weight reduction, it is the authors' impression that those who showed the greatest tendency toward instability during caloric restriction were the ones showing negative calcium balances earliest and most consistently.

*Relative fatness in relation to metabolic results.* In previous studies it appeared that a positive relationship might exist between excess body fat and a tendency to stay in nitrogen equilibrium during caloric restriction. In order to investigate this possible relationship further, an attempt was made to obtain a more objective means of evaluating relative fatness, namely, the sum of fat pad thicknesses at 5 locations as viewed by soft tissue x-ray. A comparison of the rank order of the fatness of the reducing subjects, as determined by these measurements, with the nitrogen balances exhibited by the subjects during reduction periods shows a correlation between thickness of fat pads and the tendency of the subject to retain nitrogen or stay in nitrogen equilibrium. The two subjects with the thickest fat pads at both the beginning and the end of the study (nos. 2 and 3) were the only two who never went into negative nitrogen balance with caloric restriction. Subject no. 1 (ranked third in the last measurements) went into negative balance on only the final balance period. Subject no. 5 who ranked 5th or last in the final fat pad measurements was the first subject to lose nitrogen and lost significant quantities throughout caloric restriction. Subject no. 4, who ranked 4th at the last measurements was in negative nitrogen balance for every reduction period for which there are data.

Since the skinfold measurements used in this study did not appear to give a representative ranking of the relative fatness of the women subjects studied, these were not used in relating fatness and nitrogen retention.

Calcium deficits did not appear to be related to body fatness as measured in this experiment. Though it is true that the apparently least fat subject on the basis of fat pad measurements at the end of the experiment (subject no. 5) was in calcium deficit throughout the reduction balance periods, the other subject who showed greatest calcium deficits (subject no. 3) was the fattest of the subjects.

*Questionnaires.* We had thought there might be some psychological advantage to a stepwise reduction scheme and were interested in the subjective reaction of subjects to it. Four of the 5 reducers felt they would prefer the stepwise regimen to an equal number of weeks of uninterrupted caloric restriction. Responses favoring this regimen stressed such points as "a good breather," "something to look forward to," and a "basis for long range weight control." One subject would have favored an uninterrupted reduction period because she missed the satisfaction derived from weight loss during the maintenance period. The percentage favoring a stepwise procedure was almost identical to that reported for men subjects (Young et al., '58). As in the previous report the subject preferring an uninterrupted regimen was the one who tended to find dietary restriction most difficult.

## SUMMARY

The effect of stepwise weight reduction on the nitrogen, calcium and phosphorus metabolism of 5 obese young women was studied by means of the balance technic. Results were compared with those obtained for 5 control subjects receiving the same intake levels for these nutrients but with adequate calories for essentially weight maintenance.

The net weight losses of the reducers ranged from 10.75 to 23.25 pounds, with a mean loss of 17.1 pounds.

The caloric requirement for weight maintenance decreased markedly in subjects who had been subjected to 6 weeks of caloric restriction.

The rate of weight loss on a constant caloric intake decreased from 2.5 pounds/

week early in caloric restriction to 1.9 pounds/week by the 7th and 8th weeks.

The basal caloric requirement at the end of 8 weeks' caloric restriction decreased a mean of 6.8% compared with that in the first week.

During the pre-reduction weight maintenance period all subjects were in a state of equilibrium or retention with regard to nitrogen and phosphorus. With caloric restriction at least some of the subjects lost nitrogen and, to a lesser extent, phosphorus. In contrast, control subjects without caloric restriction lost neither nutrient. Hence, the nitrogen and phosphorus deficits appear to be the result of caloric restriction per se in certain subjects.

There appeared to be no physiologic advantage for the stepwise reducing regimen over straight reduction as measured by elimination of nutrient deficits.

All 5 subjects receiving restricted calories showed calcium deficits by the 8th week of restriction.

Relative fatness as indicated by the sum of fat pad thickness measured on soft tissue x-rays at 5 locations on the body appeared to be related to the ability to stay in nitrogen equilibrium during caloric restriction.

From a psychological standpoint 4 of the 5 women subjected to caloric restriction felt they preferred stepwise reduction to an uninterrupted regimen.

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# The Effect of Non-Specific Nutrients on Absorption and Catabolism of Essential Amino Acids by a Bacterium

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One facet of protein nutrition about which relatively little is understood at present concerns the effect of non-specific nutrients on the efficiency with which essential amino acids are utilized. Experimental evaluation of these problems in higher animals is made difficult by the absence of convenient means of either measuring or controlling the participation of intestinal fermentation, protein digestion and anatomical compartmentalization of metabolism. In the present report a simple microbial system has been used in balance studies designed to reveal the effect of two non-specific nutrients, $NH_3$ and glucose, on the assimilation of three essential amino acids.

## METHODS

The nutritional requirements of mutant no. 1305,[2] derived from *Escherichia Coli* K–12, were satisfied by a medium containing threonine, methionine, leucine, glucose and mineral salts. Each liter of medium contained the following in grams: $K_2HPO_4$, 7.0; $KH_2PO_4$, 3.0; $MgSO_4 \cdot 7H_2O$, 0.1; trisodium citrate, 0.5; and glucose, 2.0; with $(NH_4)_2SO_4$ added at concentrations between 5 and 100 µg of $NH_3$-N/ml. Chromatographically pure L-leucine, L-methionine, and L-threonine were added at concentrations of 40.1, 20.1 and 79.5 µg/ml, respectively. These concentrations of the amino acids were approximately 15% greater than the requirements for optimum growth (number of cells per milliliter) in 16 hours at 37°C. No growth occurred when either leucine, methionine, threonine or ammonia was omitted from the medium under the conditions used here. The growth response to ammonia was linear through 10 µg of $NH_3$-N/ml, maximum at 20 µg of $NH_3$-N/ml and unchanged through at least 100 µg of $NH_3$-N/ml.

Growth was measured turbidimetrically using a Klett-Summerson colorimeter. After separating the amino acids by two-dimensional paper chromatography by the method of Gill[3] (solvent 1: isopropanol-methyl-ethylketone-ammonia-water in volume ratio of 5:3:1:1 and solvent 2: *n*-butanol-glacial acetic acid-water in volume ratio of 4:1:5), the total color of each spot was determined by the procedure of Mortreuil and Khouvine ('54). The values shown in tables and figures are averages of triplicate analyses of triplicate samples. Cells were removed from the cultures by high-speed centrifugation and hydrolyzed at reflux in 6N HCl under $N_2$ for 24 hours. Total nitrogen and ammonia were determined by the procedures of Johnson ('41). Glucose was determined colorimetrically using glucose aero-dehydrogenase (Saifer and Gerstenfeld, '58).

## EXPERIMENTAL RESULTS

Cultures were grown in the medium described using ammonia concentrations ranging from 5.45 to 57.7 µg of N/ml. Analysis of the medium and cells immediately after inoculation and after 17 hours of incubation yielded the data summarized

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[1] Supported by the Polychemicals Department, E. I. du Pont de Nemours and Company, Wilmington, Delaware.

[2] Supplied through the kindness of Dr. J. Lederberg.

[3] Gill, J. W. 1957 The nutritional characteristics and requirements of a *Butyrivibrio*. Virginia Polytechnic Institute thesis.

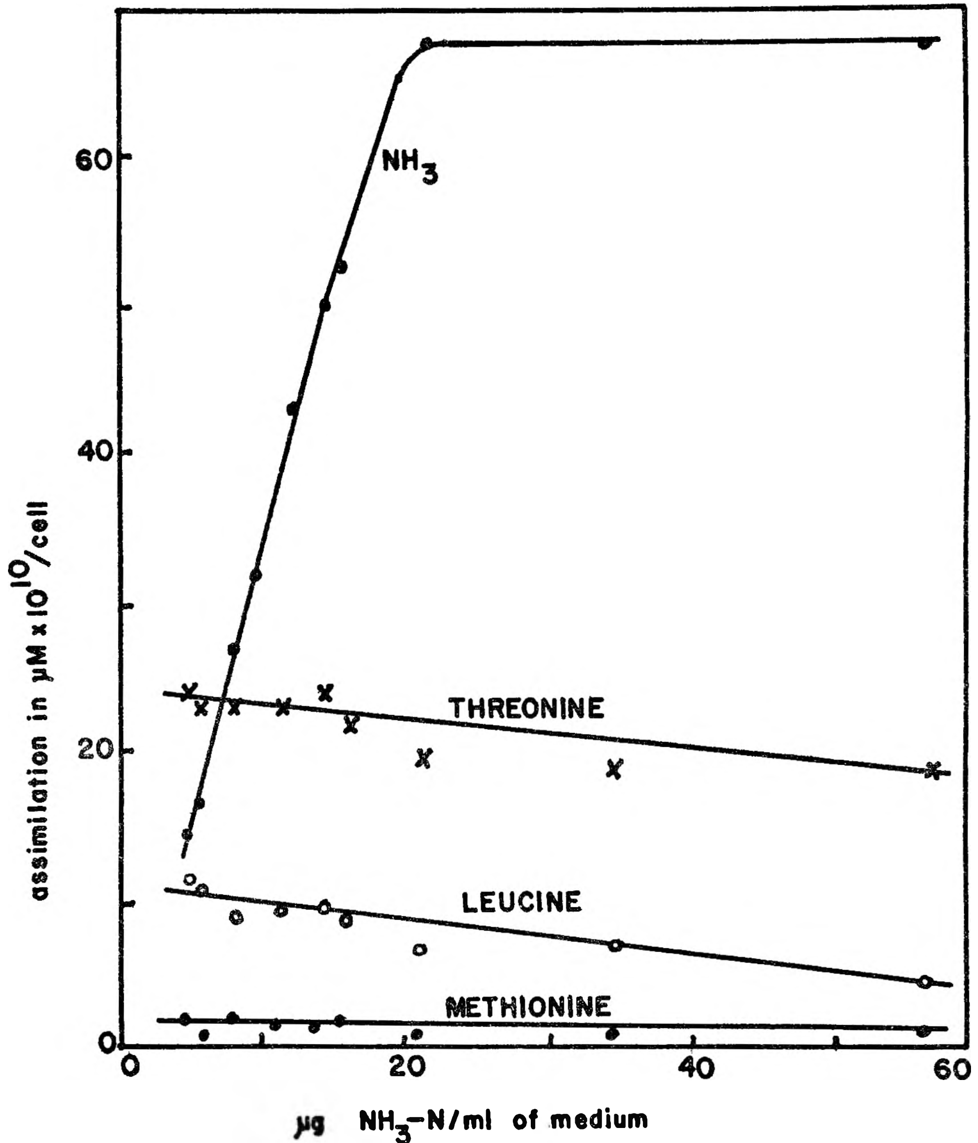


Fig. 1 Effect of ammonia content of medium on assimilation of essential amino acids and ammonia.

in figure 1. Nitrogen balance data from the cultures grown at the highest and lowest concentrations of ammonia are summarized in table 1. Supplemental ammonia exerted a sparing effect on the assimilation of leucine and threonine and apparently had no effect on methionine (fig. 1). The terms "assimilation" and "uptake" are used synonymously. Over the range of ammonia levels studied, the sparing of leucine amounted to about 50% and that of threonine to about 20%.

Each of the three amino acids required by this organism for growth is used in a distinct manner (table 1). The methionine-nitrogen which disappeared from the medium, expressed as nitrogen uptake per new cell, was essentially equal to the methionine-nitrogen recovered from the new cells produced during incubation

TABLE 1

*Nitrogen balance of cultures grown in media containing ammonia at concentrations restricting growth (5.45 μg $NH_3 - N/ml$) and allowing maximum growth (57.7 μg $NH_3 - N/ml$)*

| Supply of $NH_3$ | Nutrient | Nitrogen uptake[1]/ new cell | Nitrogen content/ new cell |
|---|---|---|---|
| | | *μM x $10^{10}$* | *μM x $10^{10}$* |
| Restricted | $NH_3$ | 26.3 | — |
| | Leucine | 12.0 | 4.3 |
| | Threonine | 24.2 | 1.5 |
| | Methionine | 2.4 | 2.3 |
| | Total | 64.9 | 63.6 |
| Ample | $NH_3$ | 67.5 | — |
| | Leucine | 4.6 | 6.2 |
| | Threonine | 18.9 | 1.3 |
| | Methionine | 2.0 | 2.5 |
| | Total | 93.0 | 99.8 |

[1] Nitrogen uptake was calculated by dividing the number of micromoles of nutrient removed from 1 ml of medium by the number of new cells produced per milliliter of medium during incubation.

TABLE 2

*The influence of glucose on nitrogen assimilation*

| | Medium | | | |
|---|---|---|---|---|
| | A | C | B | D |
| Initial $NH_3$, μg N/ml | 5.1 | 5.1 | 51.5 | 51.5 |
| Initial glucose, μg/ml | 94.8 | 4625 | 108.1 | 4387 |
| Growth, K.S. units[1] | 9 | 31 | 9 | 83 |
| Glucose uptake, % of initial | 73 | 27 | 95 | 31 |
| Nitrogen uptake,[2] μM × $10^{10}$/cell | 104 | 60 | 106 | 114 |
| Cell nitrogen, μM × $10^{10}$/cell | 109 | 73 | 114 | 92 |
| Leucine uptake, μM × $10^{10}$/cell | 10.3 | 7.9 | 6.9 | 6.2 |
| Cellular leucine, % of uptake | 43 | 33 | 64 | 66 |
| Threonine uptake, μM × $10^{10}$/cell | 32.9 | 20.1 | 31.9 | 16.0 |
| Cellular threonine, % of uptake | 4.9 | 8.2 | 8.2 | 12.2 |

[1] Klett-Sumerson units.

[2] Calculated as sum of $NH_3$, leucine, threonine and methionine uptakes.

when the medium contained a restricted level of ammonia. There is some suggestion that synthesis of methionine may have occurred in the presence of ample ammonia. The results suggest that this amino acid was largely used per se, whether total ammonia in the medium was ample or restricted. In contrast, leucine appeared to be catabolized extensively when the medium contained restricted ammonia, since only about one-third of the nitrogen disappearing from the medium as leucine was recoverable as leucine form in the new cells. In the presence of ample ammonia, there appeared again to be some evidence of synthesis, although this has not been observed consistently in all trials. Based on the extensive conversion of threonine-nitrogen to other compounds in the cells, evidently this amino acid was largely catabolized (93 to 94%) regardless of the amount of ammonia present in the medium. The increase in total cell nitrogen from 63.6 × $10^{-10}$ μM/cell when nitrogen was restricted to 99.8 × $10^{-10}$ μM/cell in the presence of ample nitrogen represents a shift in the chemical composition of the cells rather than an increase in the size of the cells. This fact was established by direct microscopic measurement of the length and width of 50 cells from each medium.

Using the basic medium described earlier, the maximum growth response to glucose occurred at 0.1 and 0.2% (w/v) at ammonia levels between 5 and 50 μg of N/ml. Using 0.01% of glucose, growth was restricted to less than 35% of that

obtained with 0.1% of glucose regardless of the amount of ammonia available. The influence of restricted glucose on amino acid assimilation was evaluated using media of varying ammonia and glucose levels. The results are summarized in table 2.

At limited nitrogen levels (media A and C), adequate glucose nutriture suppressed the uptake of leucine and exaggerated the degree to which leucine-nitrogen was converted to non-essential compounds. At high levels of ammonia (media B and D) however, the effect of added glucose appeared to be negligible whereas the influence of the ammonia in conserving cell leucine was again evident. In contrast, glucose suppressed the absorption of threonine markedly, regardless of the ammonia level, and also moderately arrested the metabolic wasting of threonine, i.e., its use for non-essential purposes.

## DISCUSSION

Two independent nutritional stresses, restriction of non-specific nitrogen and restriction of the supply of energy and carbon chain precursor, apparently influenced the efficiency of utilization of essential nutrients in ways reminiscent of the experimentally less tractable situation seen in higher animals. Of the three amino acids required, only methionine appeared to be used predominantly per se regardless of the nutritional stress imposed. The extensive conversion of threonine to other compounds was moderately suppressed by both non-specific sources of nitrogen and by glucose, presumably through the latter's dual role as a source of energy and as a precursor for a variety of synthetic processes. Leucine, on the other hand, was spared extensively by non-specific nitrogen but tended to be used less efficiently at high levels of glucose when the supply of non-specific nitrogen was restricted.

## SUMMARY

A mutant strain of *Escherichia coli*, known to require threonine, methionine and leucine, was examined with regard to nitrogen balance between cells and medium under conditions of nitrogen starvation and nitrogen-excess and of energy (glucose) starvation and excess. Each of the three amino acids studied was metabolized in a characteristic manner.

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# The Ratio of Trienoic : Tetraenoic Acids in Tissue Lipids as a Measure of Essential Fatty Acid Requirement[1]

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A recent report has indicated that the essential fatty acid (EFA) requirement of rats increases when the content of saturated fat in a diet increases (Peifer and Holman, '59). Rats fed a diet containing high proportions of saturated fat but deficient in EFA had a lower caloric efficiency than rats fed small amounts of linoleate. High intakes of saturated fat also promoted a pattern of polyunsaturated acids (PUFA), characteristic of EFA deficiency. Hence, the EFA requirement apparently is dependent upon total intake of nonessential fatty acids. If this is true, the consumption of large amounts of fats which have a relatively low EFA content could lead to a chronic low-level EFA deficiency. The present study is an attempt to test the effect of two levels of a relatively saturated fat, an oil rich in EFA and a mixture of the two, using a polyunsaturated fatty acid pattern as the criterion of EFA status.

Total fat consumption by Americans is estimated to be 40% of calories. Of this fat, approximately one-fourth is butterfat. In the experiments reported here, fats were fed at these caloric levels and comparison made between butterfat, cottonseed oil and a mixture of 20% of cottonseed oil and 80% of butterfat. A fat-free diet was used as the deficient control.

## EXPERIMENTAL

Butter was melted and separated into butterfat and aqueous phases; the butterfat was washed twice with warm water, dried and filtered through diatomaceous earth. The sample was analyzed by alkaline isomerization (Holman and Hayes, '58), and found to contain 0.2% of pentaenoic, 0.2% of tetraenoic, 1.4% of trienoic and 1.4% of diencic fatty acids.

Cottonseed oil was found to contain 50.6% of dienoic acid by the same procedure. The 20/80 blend of cottonseed oil and butterfat thus contained 0.16% of pentaenoic, 0.16% of tetraenoic, 0.11% of trienoic and 11.2% of dienoic acids.

Male weanling rats of the Sprague Dawley strain, having an average weight of 41 gm were divided into 7 groups of 8 rats and fed the diets described in table 1. After 89 days, the rats were weighed, given scores, anesthetized by ether and killed by removal of blood from the heart. Polyunsaturated acids were measured in the heart, plasma and erythrocytes by alkaline isomerization of the ethanol-ether extract.

## RESULTS AND DISCUSSION

Within the 89 days of the experiment, rats in the control group fed the fat-free diet developed severe symptoms of EFA deficiency (table 2). The group fed butterfat at 10% of calories exhibited very mild symptoms, but none of the rats in the other groups developed detectable dermal symptoms of deficiency. The failure of development of dermal symptoms in the group fed 40% of calories as butterfat could indicate either that this amount of butterfat contained sufficient EFA to meet the animal's requirement or that the high level of saturated fat masked this symptom of deficiency (Peifer and Holman, '59). In

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[2] Permanent address: Hormel Institute, Austin, Minnesota.

TABLE 1
*Percentage composition of diets*

| | 1 Fat-free | 2 Low butterfat | 3 Low blend | 4 Low cotton-seed oil | 5 High butterfat | 6 High blend | 7 High cotton-seed oil |
|---|---|---|---|---|---|---|---|
| Casein[1] | 18 | 18 | 18 | 18 | 18 | 18 | 18 |
| Sucrose | 74 | 69.7 | 69.7 | 69.7 | 53 | 53 | 53 |
| Butterfat | 0 | 4.3 | 3.44 | 0 | 21 | 16.8 | 0 |
| Cottonseed oil | 0 | 0 | 0.86 | 4.3 | 0 | 4.2 | 21 |
| Wesson salts | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| α-Cellulose | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Vitamins[2] | + | + | + | + | + | + | + |
| Linoleate, *Cal. %* | 0 | 0.14 | 1.12 | 5.1 | 0.56 | 4.48 | 20.2 |

[1] Vitamin-Test Casein.
[2] Each kilogram of diet contained the following in milligrams: ascorbic acid, 500; calcium pantothenate, 70; inositol, 120; 2-methyl-1,4-naphthoquinone, 6; niacin, 60; *p*-aminobenzoic acid, 60; pyridoxine·HCl, 30; riboflavin, 30; thiamine·HCl, 70; folic acid, 11; biotin, 0.005; vitamin $B_{12}$, 0.02; vitamin A, 5; vitamin $D_2$, 100; and tocopherol, 100.

the groups fed 10% of calories as fat, increasing the content of EFA in the diet increased the growth of the rats (table 2). However, at the level of 40% of calories as fat, the blend of cottonseed oil and butterfat induced greater growth than either butterfat or cottonseed oil. This result is comparable to that noted by Thomasson,[3] who observed that certain oils or their fully hydrogenated products do not support growth to the same extent as an optimum mixture of the two.

The patterns of PUFA from rats fed the several diets are shown in figure 1. The pattern with high content of trienoic acid, typical of EFA deficiency, was present in hearts, plasma and erythrocytes of rats fed the fat-free diet. Feeding diets containing 10% of calories as fat decreased the trienoic acid content and increased the dienoic and pentaenoic acid content of heart tissue in proportion to the amount of linoleate in the dietary fat, indicating that the pentaenoic acid may have arisen from the linoleate. Hexaenoic acid was highest in tissues of rats fed butterfat, indicating that it did not arise from linoleate. The same effects were found in the animals fed 40% of calories as fat.

Although the content of PUFA in plasma and erythrocytes is much less than that of heart tissue, the same major changes in pattern are apparent. These results confirm observations that the pattern of PUFA in plasma and erythrocytes reflects the dietary EFA, and may be used to evaluate EFA status of the animal.

In EFA deficiency the content of arachidonate (tetraenoic acid) decreases and the content of eicosatrienoic acid increases in the several tissues. Both of these are

[3] Personal communication 1957 from Dr. H. J. Thomasson, Unilever Research Laboratory, Vlaardingen, the Netherlands.

TABLE 2
*Effects of butterfat and cottonseed oil in the diet of rats*

| Group | Linoleate calories | Final weight | Final dermal score | Triene/tetraene ratio | | |
|---|---|---|---|---|---|---|
| | | | | Erythrocytes | Plasma | Heart |
| | % | *gm* | | | | |
| Fat-free | 0 | 211 ± 12[1] | 4.5 ± 0.5 | 2.29 ± 0.19 | 4.92 ± 0.43 | 3.78 ± 0.31 |
| Low butterfat | 0.14 | 267 ± 10 | 1.2 ± 0.2 | 0.63 ± 0.06 | 1.35 ± 0.05 | 0.99 ± 0.05 |
| Low blend | 1.12 | 284 ± 10 | 0.1 ± 0.1 | 0.14 ± 0.01 | 0.34 ± 0.02 | 0.12 ± 0.01 |
| Low cottonseed oil | 5.1 | 295 ± 8 | 0 ± 0 | 0.07 ± 0.01 | 0.09 ± 0.01 | 0.03 ± 0.01 |
| High butterfat | 0.56 | 276 ± 11 | 0.1 ± 0.1 | 0.25 ± 0.01 | 0.51 ± 0.07 | 0.29 ± 0.02 |
| High blend | 4.48 | 311 ± 11 | 0.1 ± 0.1 | 0.07 ± 0.01 | 0.17 ± 0.02 | 0.12 ± 0.01 |
| High cottonseed oil | 20.2 | 295 ± 7 | 0.1 ± 0.1 | 0.04 ± 0.004 | 0.05 ± 0.01 | 0.05 ± 0.004 |

[1] Standard deviation.

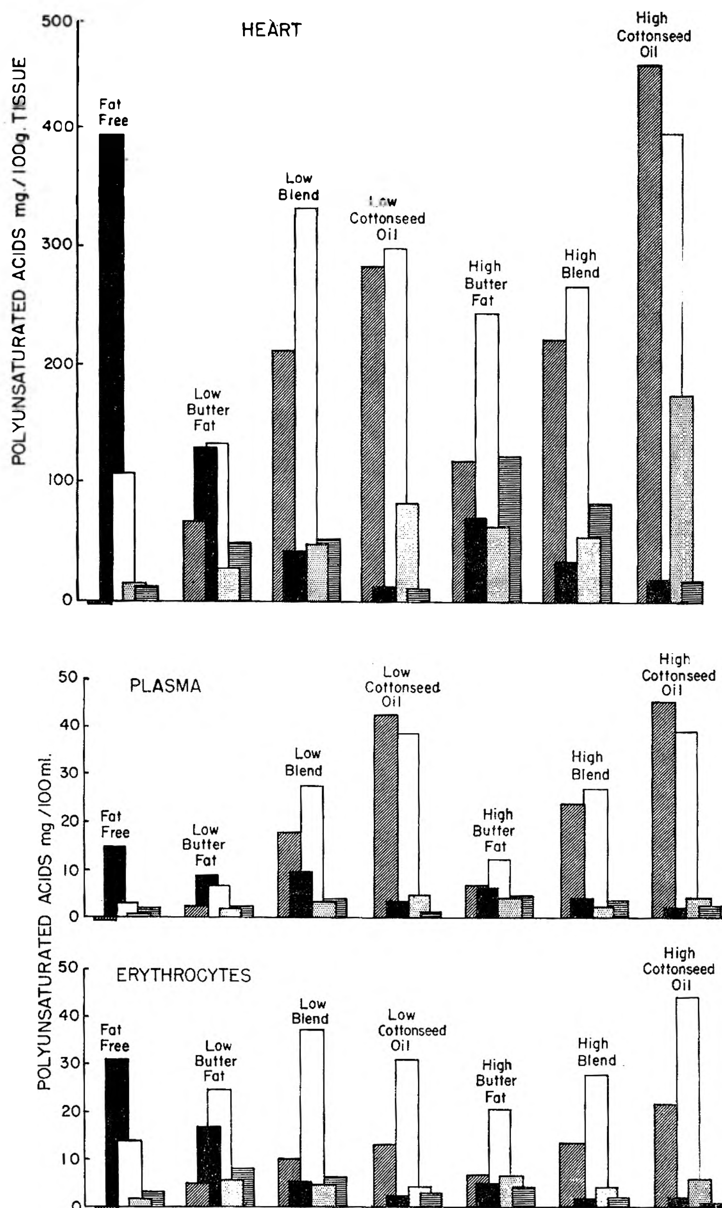


Fig. 1 The effect of butterfat and cottonseed oil upon the pattern of polyunsaturated acids in heart, plasma and erythrocytes of male rats. In each cluster, the bars from left to right are dienoic acid, trienoic acid, tetraenoic acid, pentaenoic acid and hexaenoic acid.

endogenous PUFA, the trienoic acid synthesized from oleic acid (Fulco and Mead, '59) and the arachidonate from linoleic acid (Steinberg et al., '56). The trienoic/tetraenoic acid ratio has proved to be a convenient expression of EFA status. The ratios found in heart tissue, erythrocytes and plasma, shown in table 2, reflected consistently the linoleate content of the diets. If the ratios of triene/tetraene for each group are plotted against the linoleate content of the diet in calories, a hyperbola is obtained in which the maximum rate of change of slope lies near 1% of calories. One leg of the curve represents the condition of deficiency and the other

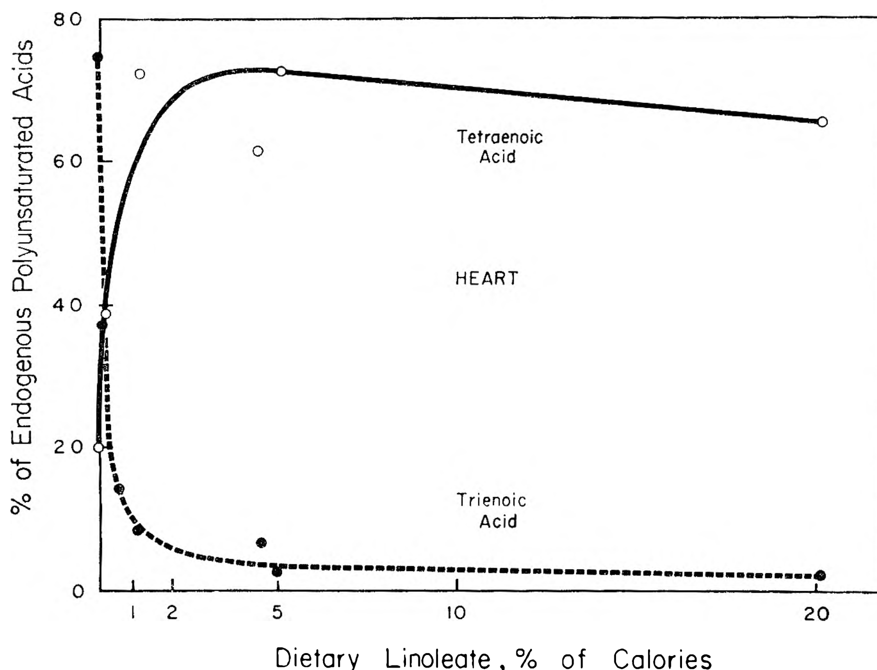


Fig. 2 The effect of level of dietary linoleate upon the trienoic acid and tetraenoic acid content of endogenous polyunsaturated acids of heart tissue.

represents adequate linoleate. Similar curves obtained for heart, plasma and erythrocytes lie so close to each other that they may be used interchangeably (fig. 3).

If the proportion of trienoic acid to total endogenous PUFA is related to the linoleate content of the diet, a similar relationship is seen (fig. 2). At less than 1% of linoleate calories, the content of trienoic acid in endogenous PUFA in heart muscle is high. At more than 1% of linoleate calories, the trienoic acid content remains low. As the linoleate content of the diet is increased, the tetraenoic acid content of the endogenous PUFA of heart increases rapidly until approximately 1% of calories is reached. These same relationships hold for the PUFA in erythrocytes and plasma. The break in the slope of the curves is found at about 1% of the calories as linoleate whether the variable is triene/endogenous PUFA, tetraene/endogenous PUFA, tetraene/total PUFA, triene/total PUFA or triene/tetraene. Data from other recent experiments (Peifer and Holman, '59; Aaes-Jørgensen and Holman,[4]) in which the conditions were somewhat comparable to those of the present experiment, have been plotted on the same curves and gave a reasonable fit in most instances.

Linoleate content of 1% of calories represents the level of EFA below which the normal metabolism of PUFA no longer persists. The synthesis of metabolites from linoleate cannot provide the normal amounts of arachidonate and pentaenoate and the synthesis of eicosatrienoic acid takes place in greater than normal amounts. One per cent of calories is then the minimum requirement of linoleic acid, using the primary metabolic lesion as the indicating end-point. This value has been suggested as a minimum allowance for humans (Food and Nutrition Board, '58). Adam, Wiese and Hansen ('58) found that optimum caloric efficiency of infants was attained when linoleic acid made up 4% of the calories. Hansen et al. ('58) found that as little as 1.3% of calories as linoleate cured the dermal symptoms of

[4] Holman, R. T. and Aaes-Jørgensen, unpublished data.

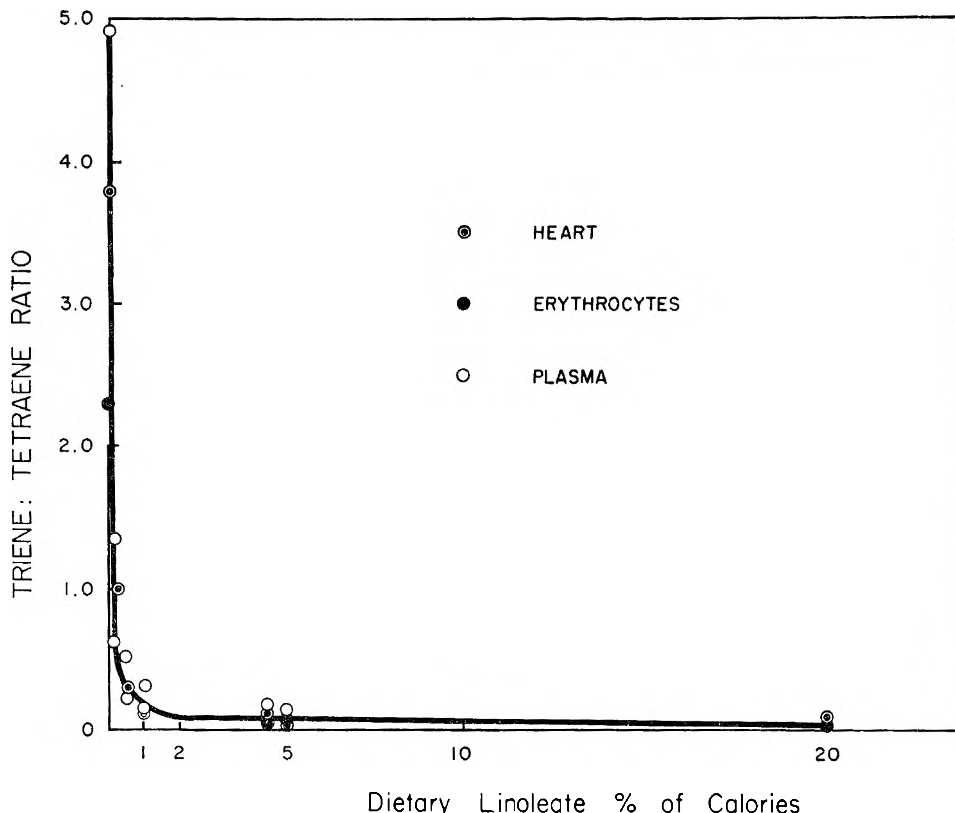


Fig. 3 The relationships between dietary linoleate and the ratio of trienoic acid to tetraenoic acid in the lipids of heart, erythrocytes and plasma.

deficiency in infants. The requirement of linoleate in rats has been usually expressed in milligrams per day. Deuel et al. ('51) found that, using growth of male rats as the criterion, the requirement probably exceeded 200 mg/day, whereas Burr and Burr ('30) originally suggested a minimum requirement of 20 mg/day. The present study indicates that the minimum requirement of linoleic acid, using objective metabolic criteria, is about 1% of calories for male rats. That is, the requirement is not a fixed quantity, but is relative to the amount of calories consumed.

The values for dietary linoleate provided by 10 and 40% of calories as butterfat lie at or near the break in the curves relating tissue trienoic acid to dietary linoleate. Therefore, it is questionable whether butterfat provides the required amount of linoleate when fed as sole dietary fat at either level of calories. Before a judgment can be made, additional trials should be made with other samples of butterfat, for the one used in this investigation appears to be low in its content of polyunsaturated acids. However, it should be noted that the 4:1 blend of butterfat and cottonseed oil amply met the requirement for linoleate.

The data presented here confirm previous observations that as the proportion of saturated fat in a dietary fat is increased, the composition of tissue lipids is altered in the direction of EFA deficiency. A comparison of the polyunsaturated acid patterns for three tissue from animals fed butterfat, butterfat-cottonseed oil and cottonseed oil reveals that phenomenon (fig. 1). This is not a contradiction to the relationship of EFA status to linoleate expressed in terms of calories. The essence of the latter is that carbohydrate and protein calories are equated with non-essen-

tial fat calories in their inability to act as EFA.

Further data presented here suggest that the heart, erythrocytes and plasma may be used equally well for assessing the EFA status of animals. The PUFA content of heart tissue is much greater than that of plasma or red cells, and the data gained is subject to less error. However, plasma may be preferred because of ease in sampling from humans. In either case a triene/tetraene ratio of less than approximately 0.4 indicates that the *minimum* dietary requirement for linoleate has been met. Additional work must be done to determine whether the relationships presented here apply to a wide variety of fats and whether the index may be used to determine the requirement of humans for EFA.

## SUMMARY

Male weanling rats were fed diets containing no fat, 10% of calories as butterfat, cottonseed oil or an 80/20 mixture of these, and 40% of calories as butterfat, cottonseed oil or the 80/20 mixture. Polyunsaturated acids were determined in heart tissue, erythrocytes and plasma after 89 days on the dietary regimes. The polyunsaturated fatty acid pattern indicated a relationship to the content of linoleic acid in the diet. A plot of the percentage of trienoic acid in the endogenous PUFA versus dietary linoleate breaks at about 1% of calories. This is also true of plots of tetraenoic acid which increase to a maximum value as dietary linoleate reaches 1% of calories. A ratio of triene/tetraene in plasma, erythrocytes or heart tissue less than approximately 0.4 indicates that the *minimum* requirement of linoleate (1% of calories) has been met.

## ACKNOWLEDGMENT

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# Acceleration of Essential Fatty Acid Deficiency by Dietary Cholesterol[1]

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A series of studies has been made to find means of accelerating essential fatty acid (EFA) deficiency in the rat. The stress of alloxan diabetes was tried, on the assumption that the catabolism and mobilization of fat is increased in the diabetic condition. Diabetes, indeed, accelerated the onset of EFA deficiency in the rat (Peifer and Holman, '55). Severe deficiency symptoms were developed within one month when weanling rats were fed an EFA-free diet and were made diabetic by an injection of alloxan. The striking results of that experiment prompted the question whether the deficiency was accelerated as a consequence of the hypercholesteremia associated with diabetes.

A high proportion of the fatty acids present in plasma cholesteryl esters are essential fatty acids. Moreover, Beveridge and Lucas ('45), Bromer[2] and Alfin-Slater et al., ('54) have shown that cholesteryl esters accumulate in the livers of animals fed EFA-deficient diets. These findings suggest that cholesterol transport is in some way related to or dependent upon EFA supplies, and it seemed logical, therefore, to explore the effect of dietary cholesterol upon the onset of EFA deficiency.

Cook ('36) noted that dietary cholesterol diminished growth and induced fatty livers in animals. Popjak ('46) fed amorphous cholesterol to rabbits receiving a low-fat diet and found that the unsaturation of plasma phospholipids diminished. These investigators, however, reported no observations that indicated dermal symptoms of EFA deficiency. Bromer[3] made an extensive and thorough study of the relationship between cholesterol and EFA in the rat. Unfortunately, this excellent work was not published, except through an abstract of an oral presentation. Bromer observed that a diet containing 2% of cholesterol and 3% of hydrogenated peanut oil promoted the appearance of EFA deficiency in male rats after 7 weeks and in female rats after 11 weeks. Our initial experiments[4] (Peifer and Holman, '55) demonstrtaed that male rats fed 1% of cholesterol and 1% of hydrogenated coconut oil (HCO) developed EFA deficiency symptoms within one month. Hauge and Nicolaysen ('59) found that dietary cholesterol and HCO accelerates the onset of an EFA deficiency in weanling rats reared from mothers fed a diet containing 10% of HCO and 1% of cholesterol. The present report describes several experiments in which the phenomenon is studied more fully.

## EXPERIMENTAL

Male Sprague Dawley rats, 15 or 21 days old were used in all experiments. Each animal, on arrival at the laboratory, received a subcutaneous injection of 15,000 IU of aqueous penicillin G—potas-

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[1] Supported by grants from The Hormel Foundation, the Office of Naval Research (Contract no. 66218), the National Dairy Council and the National Live Stock and Meat Board.

[2] Bromer, W. W. 1953 Some relationships between the essential fatty acids and cholesterol in the rat. Ph.D. Thesis, Part II, Indiana University. University Microfilms Doctoral Dissertation Series 7429, University of Michigan, Ann Arbor.

[3] Bromer, W. W., and H. Day 1953 Effect of cholesterol intake on the composition of cholesteryl esters in rats on a diet deficient in essential fatty acids. Abstracts, 124th meeting, American Chemical Society, 57C: 140, September 1953

[4] Peifer, J. J, and R. T. Holman 1956 Relation of dietary cholesterol to essential fatty acid deficiency. Federation Proc., 15: 326.

sium. The rats were housed individually or in pairs. Food consumption, weights and dermal scores were recorded weekly. Nath et al. ('59) have observed that 1% of dietary cholesterol increased the skin symptoms and depressed the growth of rats. Dermal scores of EFA deficiency were graded according to the method of Holman and Ener ('54). The extraction and analyses of heart polyenoic acids were performed according to the procedure previously described by Peifer and Holman ('59). Ethyl linoleate used as supplement was prepared from safflower oil by low temperature crystallization, urea fractionation and fractional distillation. Its purity was approximately 95% and the major contaminant was ethyl oleate. Hydrogenated coconut oil,[5] fed as a source of saturated fat, was found to have an iodine number of 2 and to have less than 0.2% of polyenoic acids detectable by alkaline isomerization.

The basic diet used in these studies contained 18% of casein,[6] 70% of sucrose, 4% of cellulose, 4% of Wesson salt mixture, 1% of fat, and 3% of urea. A triturate of vitamins in casein was incorporated so that each kilogram of diet contained the following amounts of vitamins: vitamin A-palmitate, 5 mg; calciferol, 100 μg; α-tocopherol, 100 mg; ascorbic acid, 500 mg; calcium pantothenate, 70 mg; inositol, 1.32 mg; 2-methyl-1,4-naphthoquinone, 6 mg; niacin, 60 mg; *p*-aminobenzoic acid, 600 mg; pyridoxine·HCl, 30 mg; riboflavin, 30 mg; thiamine·HCl, 73 mg; choline·HCl, 13.2 gm; folic acid, 11 mg; biotin, 0.5 mg and vitamin $B_{12}$, 2 mg. EFA was incorporated in the diet as urea complexes of corn oil fatty acid ethyl esters or of ethyl linoleate. These complexes contain 25% of ester and 75% of urea (Holman and Ener, '54). When cholesterol was incorporated in the diet, it was substituted for an equal weight of sucrose. In the fat-free control diet the sucrose content was 71%.

## RESULTS

*Effect of dietary cholesterol upon EFA deficiency.* Weanling 21-day-old male rats were fed a fat-free diet for one week and then distributed randomly into 4 groups and fed one of the following diets: group 1 received 1% of hydrogenated coconut oil (HCO), group 2 received 1% of HCO and 1% of cholesterol, group 3 received 1% of corn oil ethyl esters and group 4 received 1% of corn oil ethyl esters plus 1% of cholesterol. The data in table 1 indicate that EFA deficiency appeared within 5 weeks and that 1% of

[5] Obtained from Durkee Fine Foods Co., Chicago.

[6] Vitamin-Test Casein.

TABLE 1

*Effect of dietary cholesterol upon acceleration of EFA deficiency*

| Group | Diet | Food efficiency[1] | Weight gain[1] | Dermal score 5th week | Testes weight[2] 5th week |
|---|---|---|---|---|---|
| | | *gm gain/ 100 gm food* | *gm* | | *gm* |
| 1 | 1% Hydrogenated coconut oil | 34 ± 2(5) | 102 ± 3 | 4.0 ± 0.5 | 2.46 ± 0.06 (2.30–2.60) |
| 2 | 1% Saturated fat + 1% cholesterol | 21 ± 1(6) | 75 ± 6 | 5.0 ± 0.5 | 2.12 ± 0.25 (1.20–2.60) |
| 3 | 1% Corn oil ethyl esters | 31 ± 2(5) | 90 ± 5 | 0.0 | 2.53 ± 0.05 (2.40–2.60) |
| 4 | 1% Corn oil ethyl esters + 1% cholesterol | 34 ± 2(5) | 103 ± 5 | 0.0 | 2.77 ± 0.04 (2.65–2.85) |

[1] Average food efficiencies and weight gains during last 4 weeks of experiment ± the standard error of the mean. Figures within parentheses represent number of rats used per group.

[2] Mean weight of both testes ± standard error of the mean. The range of weights for each pair of organs is given within parentheses.

TABLE 2

*Effect of dietary cholesterol upon polyunsaturated fatty acids and endogenous ratios of the hearts of young weanling rats after 42 days*

| Group | Diet | Polyunsaturated acids[1] | | | Endogenous polyunsaturated acids[2] | | | Trienoic/tetraenoic ratios |
|---|---|---|---|---|---|---|---|---|
| | | Dienoic | Trienoic | Tetraenoic | Total | Trienoic | Tetraenoic | |
| | | | | | *mg/100 gm tissue* | *% E.P.* | *% E.P.* | |
| 1 | 1% HCO | 37 ± 4(9) | 111 ± 10 | 61 ± 3 | 199 ± 13 | 55 ± 2 | 31 ± 2 | 1.8 |
| 2 | 1% HCO + 1% cholesterol | 51 ± 10(8) | 258 ± 40 | 108 ± 11 | 401 ± 52 | 62 ± 3 | 28 ± 1 | 2.4 |
| 3 | 1% Ethyl linoleate | 100 ± 8(9) | 35 ± 5 | 61 ± 4 | 130 ± 9 | 26 ± 3 | 47 ± 1 | 0.6 |
| 4 | 1% Ethyl linoleate + 1% cholesterol | 105 ± 11(9) | 23 ± 4 | 86 ± 11 | 156 ± 15 | 15 ± 2 | 55 ± 2 | 0.3 |

[1] Concentrations expressed as milligrams of polyenoic acid per 100 gm of tissue ± standard error of the mean. Figures within parentheses refer to the number of rats per group.

[2] Endogenous polyunsaturated acids are those synthesized by the rat. These include trienoic, tetraenoic, pentaenoic and hexaenoic acids.

$$\text{E.P.} = \%\text{ of endogenous polyenoic acids} = \frac{\text{specific polyenoic acid}}{\text{total endogenous polyenoic acids}} \times 100.$$

cholesterol intensified the dermal score, inhibited growth, reduced food efficiency and inhibited development of the testes. In contrast, dietary cholesterol had little influence on rats fed EFA. Dietary cholesterol did not act as a stress factor unless insufficient EFA was provided. Conversely, addition of cholesterol to the EFA-supplemented diet tended to augment EFA activity (see tables 1 and 2). The appearance of severe dermal symptoms within 5 weeks in this experiment was typical. Comparable dermatitis usually appears in rats fed fat-free diets after three months.

Panos and Finerty ('54) have reported that histological changes take place in the testes of EFA-deficient rats. Severe degeneration of spermatogenic tissue was also observed by Holman and Aaes-Jørgensen ('56) in rats depleted by the aid of dietary cholesterol. Gross testis weights, however, had not been found to change even after 5 months of depletion using a fat-free diet. The results seen in table 1 indicate that rats fed an EFA-free diet had significantly lower testis weights than those fed EFA. Some rats fed cholesterol and an EFA-free diet (group 2), showed marked atrophy of the testes. In contrast, dietary cholesterol appeared to enhance testicular development in rats supplemented with EFA (group 4).

*Dermal scores as an index of EFA deficiency.* The grading of dermal scores for the evaluation of EFA-deficiency states in the rat has been standard practice in this laboratory. This assay has the advantage of allowing qualitative studies without the need of specialized equipment or lengthy chemical procedures. However, the severity of symptoms varies with humidity,[7] and experiments conducted at different times cannot be strictly comparable. Nevertheless, the following experiments will illustrate the usefulness of this assay method.

A study was made of the effect of dietary cholesterol upon the utilization of linoleate by 21-day-old weanling male rats in groups of 8 to 10 animals. Group 1 was fed the basic diet containing 1% of

[7] Brown, W. R., and G. O. Burr 1936 Recent studies on fat deficiency. J. Biol. Chem., *114:* xvi (abstract).

linoleate. Groups 2, 3 and 4 were fed the basic diet containing 1% of HCO plus 1% of cholesterol. After 4 weeks, dermal deficiency was severe in groups 2, 3 and 4, and the dietary regimes of groups 2 and 3 were changed to study the effects of linoleate and linoleate plus cholesterol upon the deficiency. Group 1 was continued on 1% of linoleate as a control. Group 2 was fed 1% of linoleate (without cholesterol), group 3 was fed 1% of linoleate with 1% of cholesterol and group 4 was continued on the diet containing 1% of cholesterol plus 1% of HCO. The course of the development of dermal scores during these two dietary periods is shown in figure 1. Group 1 which received linoleate throughout the entire 7 weeks did not develop an appreciable dermal score. Groups 2, 3 and 4 developed significant and equivalent dermal symptoms within 4 weeks. Group 4, which received 1% of cholesterol and 1% of HCO during the second phase of the experiment, developed a more severe dermatitis. Groups 2 and 3, which received linoleate without or with cholesterol, respectively, responded equally to the dietary EFA. The latter observations demonstrate that EFA is utilized even when cholesterol is fed with it.

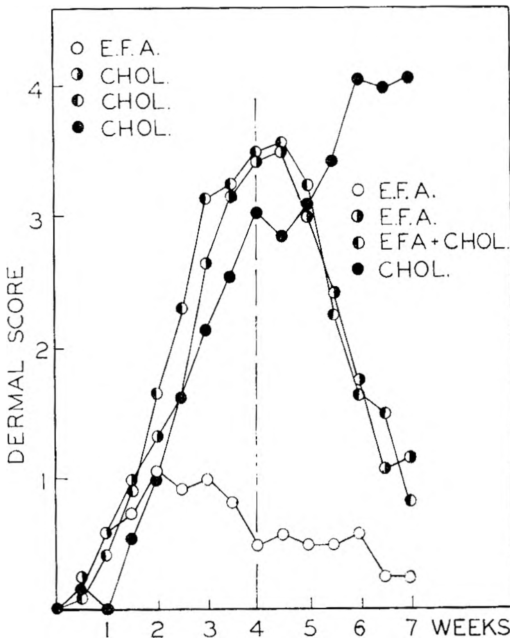


**Fig. 1** Effect of dietary cholesterol on the development and cure of dermal signs of EFA deficiency. Dietary regimes were changed at the end of the fourth week. Group 1, O–O–O, received 1% of linoleate throughout both periods of the experiment. Groups 2, ◑–◑–◑, was fed 1% of saturated fat plus 1% of cholesterol during the first few weeks and 1% of linoleate (no cholesterol) during the last three weeks. Group 3, ◐–◐–◐, was fed 1% of saturated fat plus 1% of cholesterol during the first 4 weeks and 1% of linoleate plus 1% of cholesterol thereafter. Group 4, ●–●–●, was fed 1% of saturated fat during the entire 7 weeks.

A fifth experimental group in the study described above was fed 1% of cholesterol plus 1% of linoleate during both phases of the experiment. These animals did not develop dermal signs of deficiency. In a sixth group which had been fed 1% of cholesterol and 1% of HCO during the first phase of the experiment, the dietary cholesterol was removed during the last three weeks of the experiment. The dermal deficiency symptoms continued to become more severe in these rats even though cholesterol was withdrawn from the diet. This indicates that once the accelerating effect of cholesterol has depleted the animals, further stress of cholesterol is not needed to maintain the deficiency.

Groups 7 and 8 of the same experiment were fed diets containing 1% of HCO plus 2 or 5% of cholesterol respectively, substituted for sucrose. These groups developed EFA deficiency at the same rate as groups fed 1% of cholesterol plus 1% of HCO. This might be expected, for the amount of cholesterol fed in these diets exceeds the limit of cholesterol absorption as reported by Lin et al. ('55). No attempt has been made to find the lower limit of dietary cholesterol effective in accelerating EFA deficiency.

These studies were concluded within 7 weeks, several weeks less than is required to induce a state of deficiency by a fat-free diet severe enough to permit subsequent evaluation of EFA activity. The state of deficiency induced by cholesterol in 4 weeks in this experiment is approximately equal to that usually induced by fat-free diets in three months. This rapid depletion of polyunsaturated fatty acids (PUFA) by dietary cholesterol is

useful for quickly bringing rats to a state of deficiency and for study of factors affecting EFA metabolism. The method offers the advantage of greater speed than feeding mineral oil or restricting water intake. The rapid biological assay method has its greatest use as a screening procedure and quantitation of EFA deficiency should be based on analysis of polyunsaturated fatty acids of the tissues or plasma.

*Influence of age and dietary cholesterol.* Repeated studies in this laboratory have shown that dietary cholesterol acts as a stress factor for rats fed an EFA-free diet. However, the variability of dermal symptoms and growth from experiment to experiment suggested that there is a considerable variation in the EFA stores of rats arriving as 21-day-old weanlings. Of 7 individual experiments studying the effect of dietary cholesterol, two produced no dermal evidence of accelerated EFA deficiency. Studies of the development of the seminal vesicles and testes in rats fed EFA-free and supplemented diets indicate that the young animals varied considerably in their sexual development. The time required for the onset of severe deficiency symptoms (scores of 3 to 4) has been found to vary between 20 and 60 days when 21-day-old rats are fed 1% saturated fat and 1% cholesterol. These experiences prompted the use of younger rats whose EFA reserves should be lower and more uniform.

The initial attempts to use 15-day-old rats were unsuccessful because of high mortality in the groups fed cholesterol (Peifer and Holman, '56). Repetitions under the same conditions met with low mortality, and the younger weanling rats were observed to develop marked EFA deficiency more rapidly than the 21-day-old weanlings. The stress of dietary cholesterol was evident even in rats fed 1% of linoleate, for a transient, yet significant dermatosis developed in such groups. The greater and more uniform response of the young rat could be related to several factors. The younger rat begins depletion with a lower reserve of EFA and has a greater growth potential than the older weanling. The permeability of the intestinal mucosa to cholesterol may be greater in younger animals as is the case with protein absorption. These studies supplement and extend the observations of Barki et al. ('47) and of Aaes-Jørgensen et al. ('58) which indicate that EFA deficiency is more difficult to induce in older anmials than in young ones.

*Changes in heart polyenoic acids due to dietary cholesterol.* Although the measurement of dermal scores is convenient, such data are subjective and are dependent upon low humidity (Brown and Burr, '36) as well as upon the presence of low amounts of fatty substances in the diet (Peifer and Holman, '59). The distribution of polyenoic acids in heart tissue has been demonstrated to be an index of EFA status and offers the advantage of quantitation (Rieckehoff et al., '49; Aaes-Jørgensen and Holman, '58). The effect of dietary cholesterol upon the polyenoic acid pattern of the heart tissue of 15-day-old weanling male rats fed various diets for 43 days is shown in table 2. The results demonstrate that dietary cholesterol promotes the accumulation of endogenous polyenoic acids in the hearts of the EFA deficient groups. The concentration of all polyenes in the heart was greatest in the group receiving 1% of HCO plus 1% of cholesterol. However, the trienoic acids represented a greater proportion and the tetraenes, a smaller proportion of the total endogenous polyenes in the heart muscle. When linoleate was fed, dietary cholesterol induced a relatively greater accumulation of tetraenoic acid and a reduction in trienoic acid (see table 2).

The development of EFA deficiency has been shown to be accompanied by a relative increase in eicosatrienoic and palmitoleic acids and a relative decrease in tetraenoic acid (Rieckehoff et al. '49; Mead, '57). The most obvious effect of dietary cholesterol upon EFA-deficient rats was the increase in total endogenous polyunsaturated acids (PUFA) in heart tissue. The effect of dietary cholesterol in rats fed linoleate was much smaller. *In EFA-deficient rats dietary cholesterol induced a higher proportion of trienoic acid and a lower proportion of tetraenoic acid, effects indicating intensified EFA deficiency.* This is illustrated best by con-

sidering trienoic/tetraenoic acid ratios (Holman, '60) in table 2. In rats fed linoleate, dietary cholesterol decreased the trienoic acid content and increased the tetraenoic acid content, effects which agree with the augmenting of EFA activity by dietary cholesterol.

Accumulation of polyunsaturated fatty acids in cardiac tissue has also been observed in rats fed high proportions of saturated fat (Peifer and Holman, '59). The stress of dietary cholesterol and that of excessive dietary saturated fat are also alike in that they induce hyperlipemia and accelerate or intensify EFA deficiency. The accumulation of PUFA in the heart possibly indicates an increased mobilization of PUFA rather than an increased synthesis, because the required dietary precursors were not supplied to the animals.

If the composition of plasma lipides is to remain reasonably constant, the increased transport of one component of the lipides requires that the other components be synthesized or mobilized to accompany it. Therefore, increased transport of cholesterol would require among other things an increased amount of PUFA as components of lipoprotein complexes. Increased transport of cholesterol over a prolonged period of time could reduce the tissue stores of PUFA. If an adequate supply of precursors of PUFA is not provided by the diet, depletion of PUFA could take place resulting in an EFA deficiency. This condition is accompanied by increased synthesis of other unsaturated acids such as palmitoleic and 5,8,11-eicosatrienoic acids which require no dietary PUFA precursors. Dietary cholesterol and high levels of dietary fat have been demonstrated to be stresses accentuating or accelerating EFA deficiency, possibly through a mechanism such as the one outlined.

## SUMMARY

The inclusion of 1% of cholesterol in an EFA-free diet accelerates the appearance of dermal signs of essential fatty acid deficiency in rats. This acceleration has been found to be more uniformly produced if depletion is begun with 15-day-old rats rather than with 21-day-old rats. Linoleate fed with the cholesterol prevents or cures dermal symptoms. Feeding 2 or 5% of cholesterol induces accelerated deficiency equal to that induced by 1% of cholesterol. That dietary cholesterol accentuates EFA deficiency is indicated by a lowered food efficiency, lower weight gain, retardation of testicular development and an accentuated polyunsaturated fatty acid pattern of heart lipides indicative of EFA deficiency. The proportion of trienoic acid increases and the proportion of tetraenoic acid decreases in the endogenous PUFA of heart tissue from rats having an accelerated EFA deficiency. The pattern of polyunsaturated acids in cholesterol-fed EFA-deficient rats is similar to that found in deficient rats fed a high proportion of saturated fats.

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# GUIDE FOR CONTRIBUTORS

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|---|---|---|---|
| kg | kilogram | km | kilometer |
| gm | gram | m | meter |
| mg | milligram | cm | centimeter |
| $\mu$g | microgram | mm | millimeter |
| m$\mu$g | millimicrogram | $\mu$ | micron |
| $\mu\mu$g | micromicrogram | m$\mu$ | millimicron |
| $m^3$ | cubic meter | $\mu\mu$ | micromicron |

| *Volume* | | *Area* | |
|---|---|---|---|
| $cm^3$ | cubic centimeter | $m^2$ | square meter |
| $mm^3$ | cubic millimeter | $cm^2$ | square centimeter |
| l | liter | $mm^2$ | square millimeter |
| ml | milliliter | | |

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| | | | |
|---|---|---|---|
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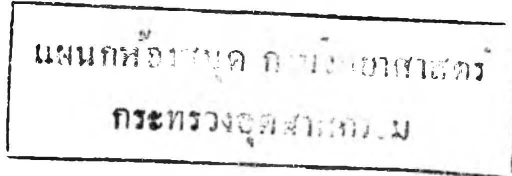

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