THE INTERACTION OF VITAMIN A AND METHIONINE

by

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ABSTRACT

The effects of vitamin A upon a methionine deficiency were studied. Doses of beta-carotene from 50 to 100 micrograms per day improved feed efficiency and weight gain corrected to a common food consumption. A methionine deficiency significantly decreased plasma methionine and inorganic sulfate, and reduced nitrogen and amino acid retentions. Elevated levels of dietary vitamin A, 5000 or 10,000 micrograms per kilogram of diet, improved amino acid retentions almost to normal levels, but decreased nitrogen retention, indicating endogenous protein destruction and increased amino acid degradation. The plasma sulfate concentration was increased, indicating increased oxidation of sulfur-containing amino acids or hydrolysis of sulfated mucopolysaccharides. These results supported the hypothesis of J. T. Dingle, that vitamin A stimulates the release of hydrolytic enzymes from the lysosomes of tissue cells.

INTRODUCTION

Although vitamin A has been recognized as a required nutrient since 1913, its biochemical role, other than in the visual process, has remained a mystery. Its chief known function is the maintenance of certain epithelial structures, bones, and teeth. The observed effects of vitamin A are related to the amount administered. Too little vitamin A causes normal epithelium to be replaced by keratinized cells and decreased proliferation of cartilage cells. An excess of vitamin A causes mucous secretion from a thickened epithelium and accelerated bone growth resulting in numerous fractures (Fell and Mellanby 1952, 1953).

Three hypotheses have been presented relating vitamin A to these symptoms. Varandani, Wolf, and Johnson (1960) proposed that vitamin A was involved in the activation of inorganic sulfate in the production of sulfated mucopolysaccharides. Balakhovsky and Drozdova (1957) proposed that vitamin A hinders keratinization by blocking the copper-catalyzed oxidation of cysteine to cystine. This effect would involve both the folding of intracellular proteins and the blocking of sulfate production by inhibiting the initial oxidation step. The third hypothesis is that vitamin A functions in the hydrolysis of proteins and protein-polysaccharide complexes either as an enzyme

activator (McElligot 1962) or as an enzyme releaser altering the permeability of the lysosomal membrane (Dingle 1963, Lucy and Dingle 1962).

All three of these hypotheses relate vitamin A to sulfur metabolism, either in proteins or polysaccharides. Because methionine and cysteine are the major sources of sulfur, the effects of vitamin A upon a deficiency of these amino acids was of interest.

The role of methionine in metabolism is threefold: (i) incorporation into proteins, (ii) conversion to cysteine and subsequent oxidation to sulfate, and (iii) donation of methyl groups. Retardation of growth, anemia, and liver necrosis are the major indications of a methionine deficiency. Other indications of a deficiency will be discussed later.

This thesis reports the results of four experiments undertaken to study the interrelationship between sulfur amino acid metabolism and vitamin A or carotene.

EXPERIMENT ONE

Introduction

Previous work by Kemmerer and Samonds (unpublished) indicated that optimum growth and feed efficiency of chickens were dependent on the level of methionine and cysteine in the diet and the daily consumption of vitamin A, supplied as beta-carotene. At low levels of methionine growth was improved by doubling the daily carotene dose.

Experimental Design

When characterizing a response to two or more independent variables, a convenient method is the Response Surface Design (Box 1954, Hader et al 1957). In this design, carefully selected combinations of the treatments are tested, the response measured, and a functional relationship postulated of the form:

$$Y = f(X_i), \quad i = 1, 2, \ldots, k. \tag{1}$$

Unfortunately the true functional relationship is unknown and some approximation is required in its place. For a problem dealing with the response to only two variables equation (1) takes the form of the second-order polynomial,

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{12}X_1X_2. \qquad (2)$$

This functional relationship may be visualized as a three-dimensional surface generated in the space having the coordinates X_1, X_2, and Y. The first three terms represent a plane, while the remaining terms add the characteristics of a quadratic surface.

Various experimental designs have been proposed to facilitate the estimation of the β_i's in equation (2) and thereby plot the surface, examine its shape, and evaluate the significance of the contributions of each of the variables and their interaction. The most advantageous of these is the central composite rotatable design (Box and Hunter 1954) in which the combinations of the levels of X_1 and X_2 are selected in such a way as to locate them on the circumference of a circle in the X_1X_2 plane. The design insures that the reliability of the estimated response at any point (X_1, X_2) will depend only upon its distance from the center of the circle and not upon its direction from the center. With two variables the design furnishes eight such combinations of levels of X_1 and X_2. The center point is replicated five times as an estimate of experimental error. The entire design may be replicated several times to increase over-all precision.

The solution of the complicated set of least-squares equations may be extremely tedious, but is greatly simplified by the judicious selection of the treatment combinations.

Experimental Procedure

Treatment combinations were chosen in such a way that the methionine and cysteine concentration varied from .525% (the minimum obtainable using purified soy bean protein) to 1.325%, coded to the mean, 0, by the equation

$$\text{concentration} = .9 + .2829\ X_1 , \qquad (3)$$

where X_1 is the coded value for methionine. The daily carotene dose ranged from zero to 100 micrograms according to the equation

$$\text{concentration} = 50 + 35.36\ X_2 , \qquad (4)$$

where X_2 is the coded value for carotene. Treatment levels were determined by substituting the coded values ± 1.414, ± 1, and zero into the above equations. The conversion of these coded values to the actual experimental levels is recorded in Table 1. The treatment numbers and the combinations of administered treatment levels are recorded in Table 2.

The eight star-point treatments, 1 to 8, and the five center-point treatments, 9 to 13, were randomly assigned to cages in each of three controlled environment batteries. Eight one-day-old Vantress x Arber Acre chicks were randonly assigned to each cage and were fed the basal diet ad libidum for fourteen days, then fed the X_1 diets for twenty-four

Table 1. Treatment Levels Determined from Coded Values

Variable \ Coded Values	-1.414	-1	0	+1	+1.414
X_1, Methionine %	.500	.6171	.900	1.1829	1.300
X_2, Carotene, micrograms per day	0	14.64	50.0	85.36	100.0

Table 2. Treatment Numbers and Coded Values

Treatment Number \ Coded Values	X_1, Methionine	X_2, Carotene
1	-1	-1
2	0	-1.414
3	1	-1
4	1.414	0
5	1	1
6	0	1.414
7	-1	1
8	-1.414	0
9 through 13	0	0

days. The X_2 carotene capsules were administered daily. The batteries were maintained at 90°F. for the first ten days, then at room temperature for the remainder of the experiment. Weight gain and food consumption for each group was recorded weekly. At the end of the twenty-four day period the birds were sacrificed by exsanguination and their livers were removed and frozen in dry ice. Stored liver vitamin A was determined by a modification of the method of Dugan, Frigerio, and Siebert (1964).

Response surfaces were examined for weight gain, food consumption, and weight gain corrected for food consumption by covariance analysis.

Diet Preparation

The diet chosen for this experiment, and as a basal diet for subsequent experiments, contained 36.5% cerelose, 40.0% ADM protein, 3% solka floc, 8% corn oil, 4% vitamin premix (free of vitamin A), 8% mineral mix, and .5% of choline chloride (25%). This diet was calculated by computer to supply 32.8% protein, 8.2% fat, 3.04% fiber, 1076.9 calories of productive energy per pound, or 1338.6 calories of metabolizable energy per pound, sufficient amounts of all minerals and vitamins (excluding vitamin A), and .525% methionine plus cysteine. Further information concerning diet composition is recorded in Tables 9 and 10, Appendix A. Microkjeldahl analysis of this diet indicated a protein content of 28% of dry weight, and amino acid analysis indicated a methionine-plus-cysteine content of .54%.

Capsule Preparation

Beta-carotene was recrystalized from chloroform by addition of methanol and washed several times with methanol. Because of the difficulty encountered in dissolving the carotene directly in corn oil, one gram was first dissolved in a minimum of chloroform. The dissolved carotene was added to approximately 200 milliliters of corn oil and the mixture was heated to drive off as much of the chloroform as possible. The solution was cooled and one gram of alpha-tocopherol was added as an antioxident. After diluting to one liter, this solution was used to prepare solutions of the desired concentrations. One-tenth milliliter of the various carotene solutions was added by syringe to number two gelatin capsules. These capsules were stored at -3°C. until time of administration.

Liver Analysis

Eight livers from each treatment group were homogenized in a Waring Blendor and a pooled sample was used for the analysis. Ten to fifteen grams of the liver homogenate were mixed with approximately twenty grams of anhydrous sodium sulfate to remove excess moisture, and were extracted with one-hundred milliliters of chloroform for one hour. After filtering, a thirty milliliter sample was evaporated to dryness in successive ten milliliter aliquoits and redissolved in one milliliter of chloroform. The solution was reacted with five milliliters

of a 2:1 mixture of triflouroacetic acid and chloroform, and the resulting blue color was read at 620 millimicrons. The concentration of vitamin A was determined by comparison with a standard curve.

Results

Weight gain, food consumption, and liver vitamin A storage are recorded in Table 3. Computer analysis of the weight gain data produced the equation:

$$Y = 430.06 + 4.0X_1 + 64.6X_2 - 8.8X_1^2 - 44.6X_2^2 + 1.8X_1X_2 \quad (5)$$

which generated the surface represented by the contour pattern shown in Figure 1. Comparison of the regression coefficients with their standard errors indicated that the linear and quadratic response to vitamin A and the quadratic response to methionine were significant (Analysis of Variance, Appendix B, Table 11). Analysis of the food consumption data produced the equation

$$Y = 681.0 + 4.64X_1 + 89.7X_2 - 11.3X_1^2 - 88.7X_2^2 - 9.1X_1X_2 \quad (6)$$

which generated a surface similar in shape to that generated by weight gain (Figure 2) as would be expected with a correlation between weight gain and food consumption of .928 (Analysis of Variance, Appendix B, Table 12). Because food consumption was not held constant from

Table 3. Mean Values of Several Chick Characteristics for Combinations of Dietary Methionine and Carotene

Treatment		Growth	Total Food Consumption per Chick	Corrected Growth	Feed Efficiency	Vitamin A Storage	
Met + Cys %	Carotene, μg/day					μg/gm	μg/liver
		gm	gm	gm	%		
0.642	14.64	336.4	609.0	337.0	67.5	.51	4.32
0.925	0.00	225.4	382.9	329.6	67.4	.82	5.27
1.208	14.64	331.0	530.7	368.7	76.2	.41	3.43
1.325	50.00	414.3	642.2	400.0	75.4	.55	5.70
1.208	85.36	445.0	694.9	406.7	74.8	1.41	12.67
0.925	100.00	434.6	632.3	424.8	75.5	1.19	12.43
0.642	85.36	443.3	724.6	391.4	71.0	1.00	11.36
0.525	50.00	388.9	699.7	348.4	64.7	.69	6.63
0.925	50.00	441.7	690.7	405.3	77.2	.62	6.55
0.925	50.00	412.4	655.0	390.5	68.4	.70	7.05
0.925	50.00	417.9	670.2	390.8	73.7	.59	6.26
0.925	50.00	440.1	694.7	401.8	74.0	.71	7.30
0.925	50.00	440.7	722.7	389.7	71.5	.48	4.95

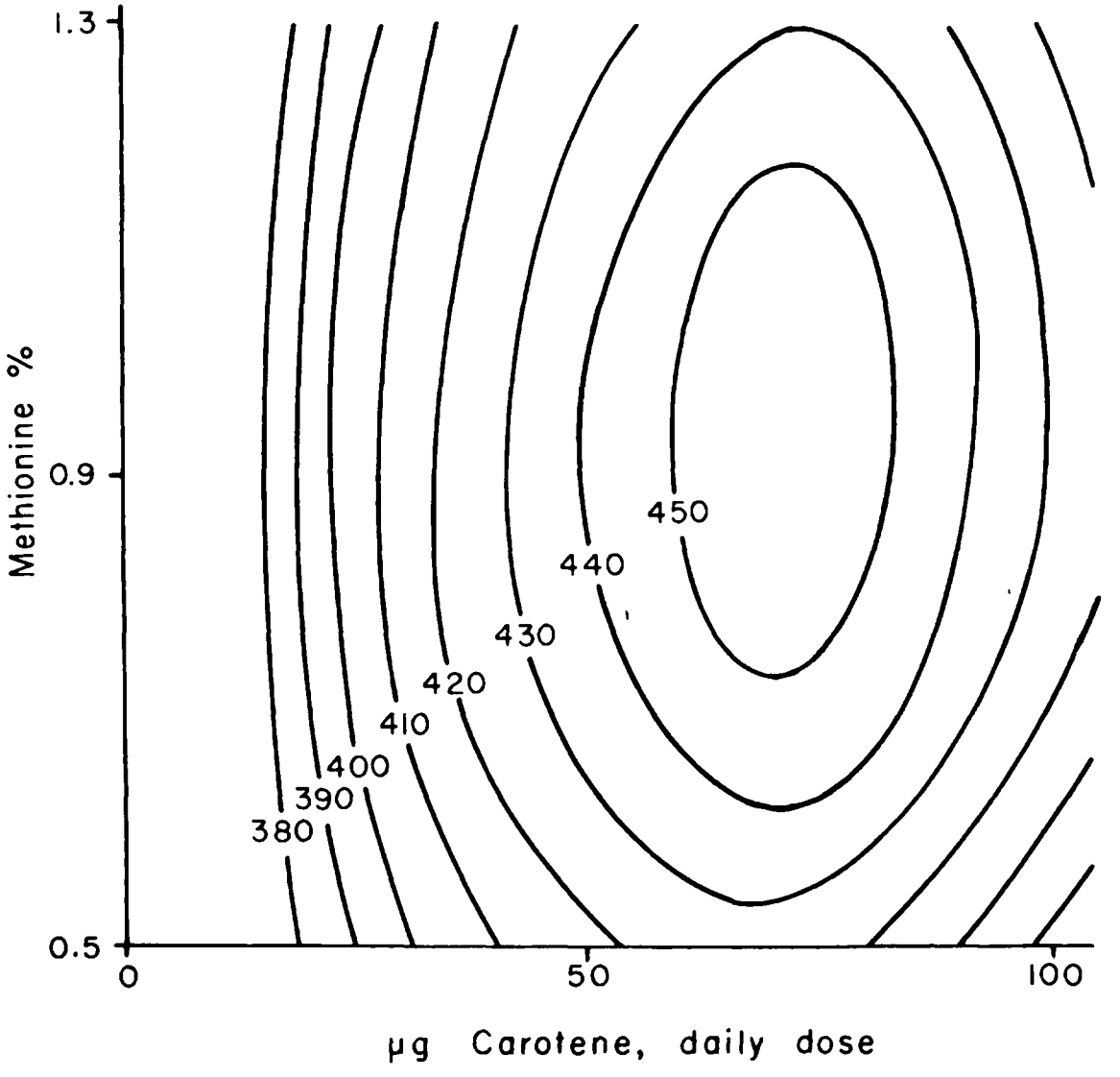

Figure 1. Response of Weight Gain (grams in 24 days) to Methionine and Carotene

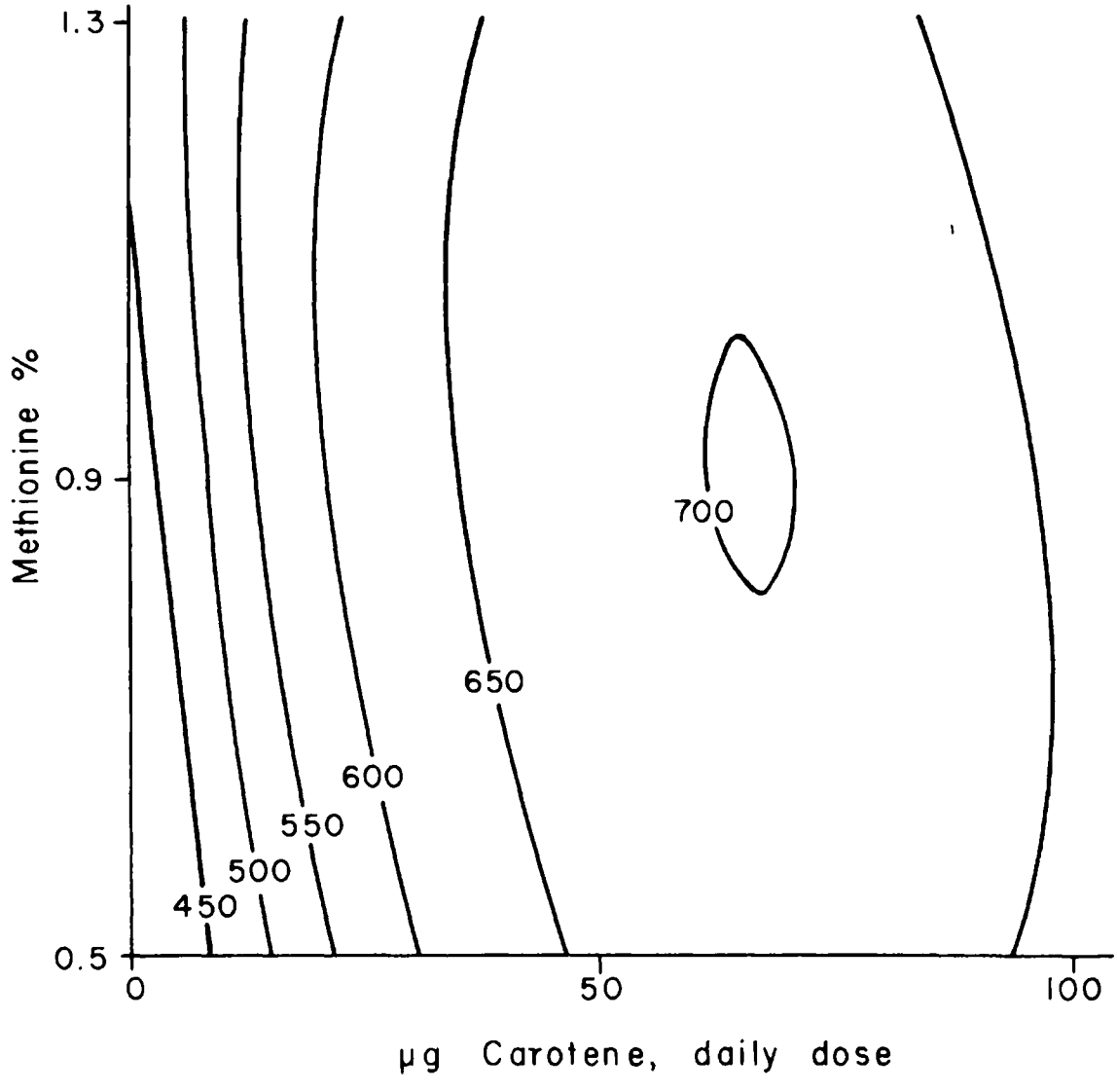

Figure 2. Response of Food Consumption (grams/chick in a 24 day period) to Dietary Methionine and Carotene

treatment to treatment, the weight gains were corrected by covariance analysis to a mean total food consumption of 4888 grams. Corrected weight gains are recorded in Table 3. An F test of the unadjusted weight gain data gave a highly significant F value of 26.4 with 15 and 40 degrees of freedom (Appendix B, Table 14). The same analysis performed on the corrected data gave an F value of 18.55 with 15 and 29 degrees of freedom, which was still highly significant. The equation describing the response of weight gain corrected for food consumption to the variables was

$$Y = 395.7 + 15.0X_1 + 28.4X_2 - 10.7X_1^2 - 9.2X_2^2 - 4.1X_1X_2 \quad (7)$$

which generated the surface shown in Figure 3. In equation (7) the linear and quadratic functions of both variables and the interaction between the two were significant (Analysis of Variance, Appendix B, Table 13). Correcting weight gain data for food consumption rotated the axes of the surface, changed its shape, and moved its maximum point. Examination of the contour diagram for the corrected data showed that the corrected weight gain at low levels of methionine and high levels of vitamin A equaled that at normal methionine and normal vitamin A, indicating a partial compensation for a methionine deficiency by increased beta-carotene consumption.

Block effects (variation caused by raising the chickens in three different batteries) were non-significant but Block x Treatment

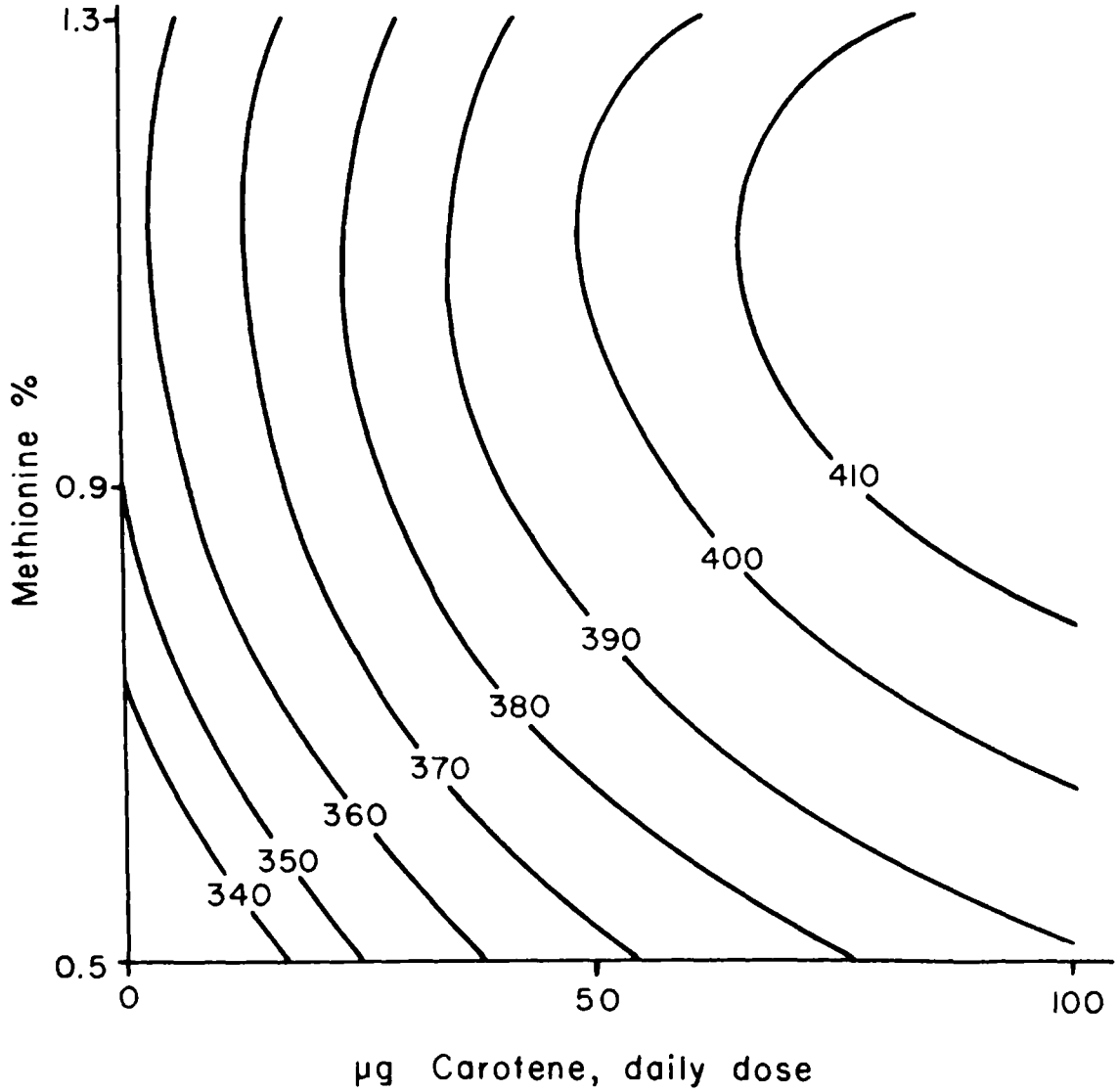

Figure 3. Response of Weight Gain (grams in 24 days corrected to common feed consumption) to Dietary Methionine and Carotene

interactions were significant, indicating that the treatments did not perform uniformly from battery to battery. Generation of response surfaces for the data from each individual battery produced surfaces of similar shape, but with their maxima at different positions. Values of these maxima, however, were similar.

Feed efficiency, calculated as grams of chicken produced per gram of food consumed, was determined for each treatment. These efficiencies, recorded in Table 3, ranged from 64% to 75%, the lowest values occuring in methionine and vitamin A deficient groups and the highest occuring in groups receiving an above normal level of methionine. Feed efficiency depended more upon the amount of methionine in the diet than upon the amount of carotene consumed.

Liver vitamin A storage for all treatment groups was much lower than that for control chickens fed stabilized vitamin A. The control chickens stored an average 55.48 micrograms per gram of liver while the treatment values ranged from .41 to 2.03 micrograms per gram. The highest value occurred in the groups receiving a large dose of carotene and a low level of methionine, while the lowest value occured in the group receiving a small dose of carotene and a high level of methionine. Random error, however, was so high that the analysis of variance (Appendix B, Table 15) indicated a non-significant F value of 1.62 with 15 and 30 degrees of freedom, even though the treatment

averages of the three replications appeared to generate a second-order response surface.

EXPERIMENT TWO

Introduction

In the previous experiment the growth response to methionine and vitamin A was studied over a period of twenty-four days. In the following experiment more information was desired about the change in feed efficiency and the change in random variation as growth proceeds when feeding a purified diet containing a minimum amount of methionine.

Experimental Procedure

Eight one-day-old Hubbard chicks were randomly assigned to each of two treatment groups. The first group received the basal diet used in the previous experiment with 0.4% added DL methionine which was equivalent to the optimum level found in the response surface analysis. The second group received the basal diet with no added methionine. To both diets 1750 micrograms of beta carotene were added per kilogram of diet making the daily intake of carotene (based on the average daily consumption of the previous experiment) equivalent to the optimum daily dose determined in that experiment. The carotene was added as a 700 microgram per gram solution in corn oil. This

solution, with alpha-tocopherol added as an antioxidant, was stored at 3°C. until it was added to the diets. Residual food was stored at 3°C. to retard oxidation of the carotene. The diets were fed ad libidum, and food consumption was recorded weekly. The individual chickens were weighed on Tuesday and Friday of each week for a period of eight weeks.

Results

The growth curves of the two treatments are plotted in Figure 4 (Analysis of Variance of Data from the 52nd Day is recorded in Appendix B, Table 16). For the first three weeks the growth curves were very similar. After three weeks the growth rate was reduced 30% in the deficient group, and in the final weeks of the experiment it was reduced 80%. For the first six weeks both groups consumed the same amount of food per week, but after that the weekly consumption of the deficient group fell sharply. For the first three weeks the methionine-deficient group showed no significant difference in feed efficiency compared to the normal group. After the third week the methionine deficient group demonstrated poorer utilization. The overall efficiency for the normal group in the eight week period was 60.3% compared to 43.6% for the deficient group.

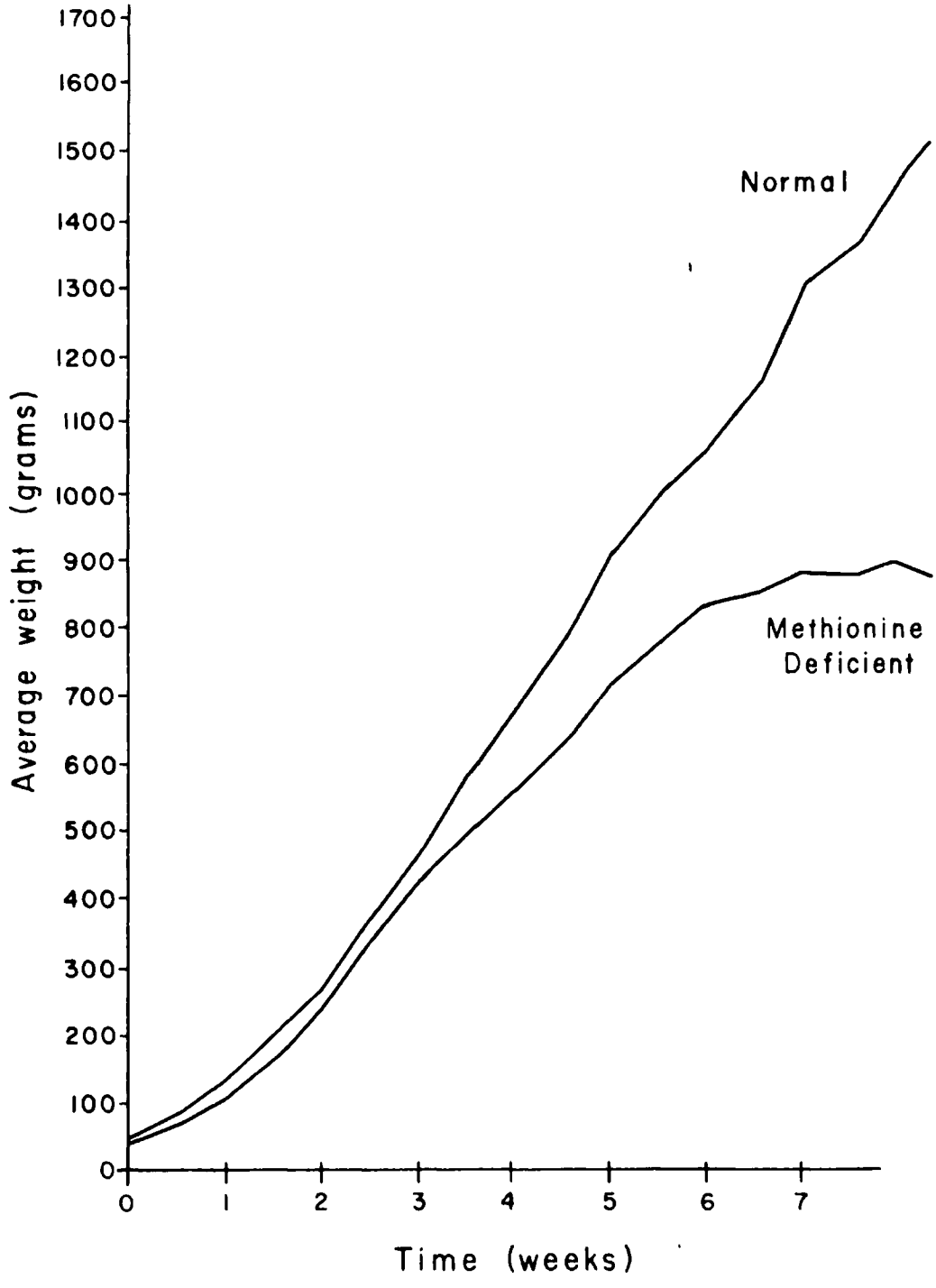

Figure 4. Growth (grams) of Normal and Methionine Deficient Chicks

EXPERIMENT THREE

Introduction

In the response surface analysis, liver vitamin A storage had been much less than expected, assuming at least 60% conversion of beta carotene to vitamin A and subsequent storage of residual vitamin A. An experiment was designed to study liver storage at double and quadruple the optimum level of carotene determined in the response surface analysis. Also, changes in liver storage as growth proceeded were studied.

Experimental Procedure

Twelve one-day-old Hubbard chicks were assigned to each of two groups. Two diets were prepared, both composed of the basal diet with .4% added DL methionine. Beta carotene in corn oil was added to the diets to supply 3,500 and 7,000 micrograms of carotene, respectively, per kilogram of diet. The carotene solution was prepared and added to the diets as in Experiment Two. The diets were prepared in small batches, stored at 3°C. and fed ad libidum. Weekly growth and food consumption were recorded and the feed efficiency was calculated. After the third week and at the conclusion of each week thereafter, two

chickens were selected at random from each group, killed, and their livers removed. The livers were immediately frozen and stored for subsequent analysis for vitamin A. Individual livers were analyzed rather than a pooled sample.

Results

Growth, food consumption, and feed efficiency of both groups were similar to those of the control group in Experiment Two. No reduction in growth was evident at these high carotene levels. Feed efficiencies for the two groups were, respectively, 53.3% and 59.2% for an eight week period, which were lower than the efficiency of the control group in Experiment Two for the same period of time. The individual values of vitamin A storage were highly variable from chicken to chicken, and no conclusions could be drawn concerning the effects of growth upon storage. Average vitamin A storage is recorded in Table 4. The amount of ingested carotene, however, did have a significant effect. The groups which received four times the normal vitamin A dose stored three to ten times as much vitamin A as did the group which received twice the normal dose.

Table 4. Mean Vitamin A Storage for Two Levels of Dietary Carotene for Six One-Week Intervals

Week \ Vitamin A	micrograms per gram		micrograms per liver	
	3500 μg/kg	7000 μg/kg	3500 μg/kg	7000 μg/kg
3	0.71	6.41	8.60	77.0
4	1.00	4.15	16.49	75.33
5	0.58	1.97	11.79	39.10
6	0.34	3.43	5.02	86.80
7	0.83	3.08	19.90	71.00
8	1.11	1.21	35.4	32.20

EXPERIMENT FOUR

Introduction

Rodahl, Issekutz, and Shumen (1965) tested the previously mentioned hypothesis of Balakhovsky and Drozdova and obtained contradictory results. Their results, based on massive overdoses of vitamin A and studies of urine-secreted sulfate, indicated that vitamin A did not inhibit oxidation of S^{35}-labeled cysteine to sulfate, but seemed to have a proteolytic effect, increasing breakdown of body proteins or removal of sulfate from mucopolysaccharides. These results favored Dingle's hypothesis that vitamin A acted as an enzyme releaser.

Zimmerman and Scott (1965) and Dean and Scott (1966) studied the effects of amino acid deficiencies on the plasma concentrations of free amino acids. They found that the concentration of the limited dietary amino acid dropped sharply while the concentrations of many others rose. Use of plasma free amino acid concentrations was found to be a good, though time-consuming, indicator of amino acid balance.

Experimental Procedure

Changes in serum sulfate and an imbalance of plasma free amino acids were chosen as indicators of the severity of a methionine

deficiency. The effects of vitamin A on these indicators was examined by combining vitamin A and methionine concentrations in diet treatments.

An experiment was designed with four treatments and three replications, a total of twelve groups, with eight one-day-old Hubbard chicks per group. Group 1, the control group, was fed the basal diet with .4% added DL methionine and 1000 micrograms of gelatin-protected vitamin A per kilogram. Group 2 received the methionine deficient basal diet with no added methionine, but with 1000 micrograms of vitamin A per kilogram. Groups 3 and 4 received the methionine deficient basal diet with 5000 and 10,000 micrograms of vitamin A added per kilogram, respectively. These diets were fed ad libidum for forty days. Individual chickens were weighed every fifth day, and group feed consumption was measured every tenth day. From the fourteenth through the twenty-seventh day .3% chromium oxide was added to the diet as a non-digestible marker. Feces samples were collected on the 28th, 29th, and 30th day and pooled for each of the four treatment groups. The samples were dried, then analyzed for Kjeldahl nitrogen, chromium oxide, and amino acid content.

On the 40th day the food trays were removed for seven hours, then returned for one hour to insure simultaneous food intake. The food trays were again removed for 1 1/2 hours, and ten milliliter blood sample taken by heart puncture from the chicken in each group whose weight most closely approximated the mean weight of that

group. Blood plasma was obtained from these twelve samples and was analyzed for unbound sulfate and free amino acids.

Feces and Feed Analysis for Nitrogen and Amino Acid Retention

Triplicate samples of feed (three grams) and feces (one-and-one-half grams) were analyzed for chromic acid content. The undigestible chromic oxide in the feces was compared to that found in the feed to determine the amount of food eaten to form one gram of feces. Percent retention of various dietary components was determined by analyzing both the feed and feces for these components, then correcting the feces concentrations for the concentrating effects of digestion.

To the weighed samples of feed and feces ten milliliters of concentrated nitric acid were added and permitted to stand for two days to facilitate digestion of organic matter. Then the mixtures were boiled almost to dryness and cooled. Fifteen milliliters of a digestion mixture composed of sodium molybdate, sulfuric acid, and perchloric acid were added to each flask. The samples were heated and digested for approximately one hour, or for fifteen minutes after the color change from green to yellow-orange. After cooling the solutions were diluted to 100 milliliters and allowed to stand overnight to permit settling of undigested material. The percent transmission of aliquoits of these solutions was measured at 444 millimicrons and compared to a standard curve to determine chromate concentration. The chromate concentrations

of the four feed and the four feces samples are recorded in Table 5. Dry matter retention, calculated from these chromate values is also recorded in Table 5.

Kjeldahl nitrogen percents were determined for the feed and feces samples, and the nitrogen retention values were calculated. These values are recorded in Table 5.

Weighed samples of feed and feces were hydrolyzed with forty milliliters of 6N Hydrochloric acid for twenty-four hours at reflux temperature. The solutions were cooled, filtered, and evaporated to dryness under vacuum. The hydrolized amino acids were dissolved in pH2.2 acetate buffer and diluted to 100 milliliters. Appropriate dilutions of these solutions were made and they were analyzed on the Beckman Amino Acid Analyzer according to the procedure used in this laboratory.

Plasma Analysis

Plasma was obtained by centrifugation of the freshly drawn blood samples in heparinized centrifuge tubes. Six milliliters of each plasma sample were deproteinized by treatment with eighteen milliliters of 0.4% uranyl acetate and centrifugation. The deproteinized samples were immediately frozen for later inorganic sulfate and free amino acid analysis.

The sulfate in the plasma was precipitated as benzidine sulfate and the benzidine in the precipitate was quantitated photometrically by

Table 5. Weight Gain, Efficiency, Plasma Sulfate, and Retention Studies, Experiment 4

	Treatment Number			
	1	2	3	4
Weight gain, gms	1024.3	915.0	907.7	897.3
Efficiency %	62.02	57.29	57.94	57.92
Plasma Sulfate (mg/100 ml)	6.87	6.83	6.85	6.86
Feed Chromate %	.2404	.2392	.2453	.2454
Feces Chromate %	1.4044	1.2620	1.2339	1.3252
Feed Nitrogen %	27.7	28.3	28.6	24.2
Feces Nitrogen %	68.8	73.9	76.0	74.4
Nitrogen Retention %	57.5	50.5	47.3	43.1
Dry Matter Retention %	82.82	81.05	80.12	81.49

reaction with sodium beta-naphthoquinone 4-sulfonate according to a modification of the method of Kleeman, Taborsky, and Epstein (1956). Two milliliter aliquoits of the plasma supernatants were treated with 0.5 milliliter of glacial acetic acid, 4.5 milliliters of 1% recrystalized benzidine in ethanol (prepared fresh each day), and a pinch of powdered glass to accelerate crystalization and to form a solid pellet during subsequent centrifugations. Two standards containing ten micrograms of sulfate and two blanks were also prepared. The tubes were stoppered and placed in the refrigerator overnight. In the morning they were centrifuged for twenty minutes, the supernatant drawn off by reverse-tipped capillary, and the tubes inverted to drain for a short time. The precipitate was washed consecutively with one milliliter of ethanol, ten milliliters of 2:1 ethanol-ether, and .5 milliliter ethanol to remove excess benzidine. One-half milliliter of 1% sodium borate in .1N sodium hydroxide was added to each tube, and they were heated in a 60°C. water bath for five minutes. After cooling, five milliliters of distilled water and one-half milliliter of .1% sodium beta-naphthoquinone 4-sulfonate in water (prepared fresh daily) were added, the tubes shaken, and allowed to stand for exactly five minutes. To conclude the reaction one milliliter of acetone was added, and the tubes centrifuged. The supernatant was transferred to colorimeter tubes. The red-orange color was read at 485 millimicrons and compared to the standards and blanks to determine the original sulfate concentrations. This method

reportedly obeys Beer's Law up to concentrations of 7.5 milligrams of sulfate per 100 milliliters of solution, and has a reproducibility of approximately plus or minus five per cent (Henry 1964).

Plasma free amino acids were determined by evaporating 15 milliliters of the deproteinized plasma samples to dryness and dissolving in five milliliters of pH 2.2 acetate buffer. These solutions were analyzed on the Beckman Amino Acid Analyzer. The concentrations of seventeen amino acids, as micromoles and micrograms per milliliter, and the molar and gram ratios to leucine, tyrosine and methionine were calculated by computer.

Results

Average weight gains and efficiencies are recorded in Table 5. A methionine deficiency caused a significantly lower weight gain and efficiency, as had been demonstrated in the previous experiments. The relatively large dose of vitamin A seemed to reduce growth, but not significantly. The mortality rate of those chickens in Treatment 4, receiving 10,000 micrograms of vitamin A per kilogram of diet, was much higher than normal, 17% versus 5% for Treatment 1, the control group. Autopsy of several of these Treatment 4 chickens revealed general tissue damage including capillary fragility, frequent hemorrhages, scar tissue in the muscles, and an excess of fluid in the

cranial cavity. Several instances of gangrene of the colon and enlarged liver were also noted.

Results of the amino acid retention study are recorded in Table 6. Treatment 2, the methionine deficient group with normal vitamin A, demonstrated a significant drop in retention of all amino acids. Increased vitamin A in Treatments 3 and 4 improved amino acid retentions almost to the normal level. Decreased and increased retention values were not selective; all seventeen amino acids followed the same trends. Valine retention demonstrated the largest drop during a methionine deficiency.

Average inorganic sulfate concentrations are recorded in Table 5. Analysis of variance of these values (Appendix B, Table 18) indicated that treatment differences were not significantly different at the .05 level. Duncan's Multiple Range Test of the treatment means indicated that Treatment 2 had a significantly lower sulfate concentration. Increasing vitamin A accompanying a methionine deficiency increased plasma sulfate to approximately the normal level. Whether this .6% decrease in plasma sulfate can be given any biological significance was not determined.

Plasma amino acids, as micromoles per milliliter and molar ratio to leucine, are recorded in Tables 7 and 8, respectively. Analysis of variance of the twelve values of each of the seventeen amino acids indicated that only methionine showed a significant response to the

Table 6. Per Cent Retention of Amino Acids as Affected by Dietary Methionine and Vitamin A

Amino Acid	Treatment Number 1	2	3	4
Lysine	93.4	83.1	91.0	88.2
Histidine	94.5	87.9	93.1	91.5
Arginine	95.7	87.1	93.2	93.0
Aspartic Acid	93.5	84.7	89.6	89.8
Threonine	89.2	71.2	80.4	81.5
Serine	91.6	76.2	84.7	88.1
Glutamic Acid	95.0	88.9	93.0	91.4
Proline	92.9	78.8	88.8	86.6
Glycine	91.3	86.0	88.6	90.1
Alanine	91.8	79.3	87.2	86.8
Cysteine	90.0	89.3	91.5	94.3
Valine	93.1	65.7	84.2	83.5
Methionine	96.0	82.2	89.5	88.0
Isoleucine	92.2	78.4	82.4	--- *
Leucine	93.3	82.2	88.7	87.8
Tyrosine	94.0	85.3	89.6	88.7
Phenylalanine	94.7	80.1	87.4	84.4

* Isoleucine chromatograph peak obscured by contaminants.

Table 7. Plasma Amino Acids, Micromoles Per Milliliter

	Treatment Number			
Amino Acid	1	2	3	4
Lysine	.852	.899	1.277	.813
Histidine	.225	.216	.256	.200
Arginine	.416	.403	.476	.377
Aspartic Acid	trace	trace	trace	trace
Threonine	.878	1.814	2.444	1.732
Serine	2.000	2.258	3.062	2.578
Glutamic Acid	.126	.189	.138	.148
Proline	.720	.794	.881	.834
Glycine	.900	.989	1.266	1.158
Alanine	.926	.975	1.128	1.096
Cysteine	.062	.038	.045	.049
Valine	.612	.495	.669	.554
Methionine	.191	.068	.077	.058
Isoleucine	.324	.315	.324	.322
Leucine	.422	.410	.451	.378
Tyrosine	.281	.335	.327	.254
Phenylalanine	.178	.184	.199	.185

Table 8. Plasma Amino Acids, Molar Ratios to Leucine

	Treatment Number			
Amino Acid	1	2	3	4
Lysine	2.018	2.193	2.831	2.151
Histidine	0.533	0.527	0.568	0.529
Arginine	1.092	0.982	1.055	0.997
Aspartic Acid	-	-	-	-
Threonine	2.080	4.424	5.419	4.582
Serine	4.739	5.507	6.789	6.820
Glutamic Acid	0.298	0.461	0.306	0.391
Proline	1.706	1.936	1.953	2.206
Glycine	2.133	2.412	2.807	3.063
Alanine	2.194	2.378	2.501	2.899
Cysteine	0.147	0.093	0.099	0.130
Valine	1.450	1.207	1.483	1.466
Methionine	0.453	0.166	0.171	0.153
Isoleucine	0.768	0.768	0.718	0.852
Leucine	1.000	1.000	1.000	1.000
Tyrosine	0.666	0.817	0.725	0.672
Phenylalanine	0.422	0.449	0.441	0.489

treatments (Appendix B, Table 19). Plasma methionine levels were significantly lowered in conjunction with a methionine deficiency. Threonine, serine, proline, glycine, and alanine concentrations were increased in a methionine deficiency similar to the response noted by Dean and Scott (1966) in an amino acid deficiency. Sample-to-sample variation was so high, however, that the analysis of variance indicated that the treatments had no significant effect. The plasma valine concentration was lower in conjunction with a methionine deficiency which was in agreement with the theory that methionine aids valine absorption (Christensen 1963).

CONCLUSIONS

Several conclusions can be drawn from the preceding experiments. First, beta-carotene replaces vitamin A in maintaining growth in the chick, but does not provide enough vitamin A for appreciable liver storage. Since carotene is converted to vitamin A as it is absorbed through the intestinal wall, the absence of residual vitamin A in excess of the amount required to sustain growth may be attributed to the absorption mechanism. The presence of sufficient vitamin A in the blood stream may inhibit the oxidative splitting of carotene.

Second, a methionine deficiency significantly reduced plasma methionine and increased the concentrations of several other amino acids, a pattern previously noted in deficiencies of other amino acids. It decreased nitrogen retention and the retention of all amino acids, probably to avoid antagonizing the existing amino acid imbalance. It should be noted that the retention (absorption) of methionine did not increase during a methionine deficiency, as might be expected as a simple means of alleviating the imbalance. The reduced feed efficiency demonstrated by methionine-deficient groups can be partially explained by the poorer absorption of amino acids. The reduction in plasma inorganic sulfate, the end product of sulfur oxidation, is also

explained by the reduction in methionine and cysteine retentions because these two amino acids are the primary source of sulfur.

Third, increased vitamin A intake in conjunction with a methionine deficiency increased the absorption of amino acids, almost to normal levels in some instances. Nitrogen retention, however, decreased with increased vitamin A dosage indicating that these absorbed amino acids were not participating in protein synthesis, but were being degraded and excreted as uric acid. The decreased nitrogen retention also indicated the degradation of endogenous protein.

A scheme for these effects of vitamin A, only partially substantiated by this work, might be described as follows: ingested vitamin A is carried via the blood stream to the tissue cells where it is absorbed. It alters the permeability of the lysosomal membranes releasing hydrolytic enzymes into the cell which hydrolyzes endogenous protein to amino acids which are excreted into the blood stream. The release of these amino acids into the blood stream of an amino acid deficient animal would only aggrevate the imbalance already in existence, and thereby stimulate amino acid degradation. Increased degradation would lower the plasma levels, as would protein synthesis which also would be occurring, but probably at a slower rate than normal. Release of methionine by endogenous protein hydrolysis would make more of it available for protein synthesis in other cells. Thus we find an increased

absorption and degradation of amino acids accompanied by a net loss of endogenous protein.

Vitamin A also increased plasma sulfate. Whether this was accomplished by the release of lysosomal enzymes which hydrolyze sulfated mucopolysaccharides or simply by the increased oxidation of the better absorbed sulfur-containing amino acids is difficult to determine. Increased mortality and extensive tissue damage at high levels of vitamin A also may be explained by the release of hydrolytic enzymes into tissue cells.

APPENDIX A

Table 9. Composition of the Basal Diet

	%
Assay Protein C-1[1]	40.0
Solka Floc[2]	3.0
Cerelose 2001[3]	36.5
Corn Oil[4]	8.0
Choline Chloride[5]	0.5
Vitamin Mix[6]	4.0
Mineral Mix[7]	8.0

[1]Skidmore Enterprises

[2]Cellulose, Brown Company

[3]Dextrose sugar, Corn Products Company

[4]Mazola Corn Oil, Corn Products Company

[5]25%, Vitren Corporation

[6]Supplies per kilogram of diet: 961.0 IU, Vitamin D3, 8.73 ICU Vitamin E, .03 mg Vitamin B12, 6.61 mg Vitamin K, 12.00 mg Riboflavin, 88.1 mg Niacin, 15.24 mg Pantothenic Acid, 50.0 mg Santoquin, 4.0 mg Thiamin, 0.91 mg Folic Acid, 1.8 mg Pyridoxin, and .09 mg Biotin.

[7]Supplies per kilogram of diet: 1.0% Calcium, 1.1% Phosphorus, 0.85% Available Phosphorus, 86.25 ppm Zinc, 124.0 ppm Manganese, .06% Magnesium, .225% Sodium, .383% Potassium, and .08% Iron.

APPENDIX A

Table 10. Amino Acid Per Cent of Diet Protein and the Requirements for Growing Chicks

Amino Acid	% of Protein in Diet	% of Protein Required by Growing Chicks *
Lysine	6.80	4.50
Histidine	2.60	0.75
Arginine	8.31	6.00
Threonine	3.90	3.00
Glycine	4.10	5.00
Valine	5.50	4.00
Methionine	1.00	4.00
Cysteine	0.60	may supply up to 1.75% of the 4% required Methionine
Isoleucine	6.50	3.00
Leucine	7.50	7.00
Tyrosine	3.40	4.50
Phenylalanine	5.00	8.00

* Nutritional Research Council, Subcommittee on Nutrient Requirements of Growing Chicks, 1960

APPENDIX B

Table 11. Analysis of Weight Gain Response Surface

$B_0 = 430.6$

$B_1 = 4.027 \pm 5.02$

$B_2 = 64.589 \pm 5.02$

$B_{11} = 8.776 \pm 5.39$

$B_{22} = 44.565 \pm 5.39$

$B_{12} = 1.755 \pm 7.11$

Source	df	SS	MS	F
First order terms	2	335.03	167.52	82.93 **
Second order terms	3	138.91	46.30	22.92 **
Lack of Fit	3	19.30	6.43	3.18 NS
Error	4	8.08	2.02	

APPENDIX B

Table 12. Analysis of Food Consumption Response Surface

$B_0 = 681.0$

$B_1 = 4.64 \pm 11.78$

$B_2 = 89.70 \pm 11.78$

$B_{11} = -11.26 \pm 12.63$

$B_{22} = -88.79 \pm 12.63$

$B_{12} = -9.12 \pm 16.66$

Source	df	SS	MS	F
First order terms	2	645.47	322.74	29.076 **
Second order terms	3	551.72	183.91	16.568 *
Lack of Fit	3	120.51	40.17	3.619 NS
Error	4	44.40	11.10	

APPENDIX B

Table 13. Analysis of Corrected Weight Gain Response Surface

$B_0 = 395.7$

$B_1 = 14.99 \pm 2.60$

$B_2 = 28.38 \pm 2.60$

$B_{11} = 10.72 \pm 2.79$

$B_{22} = 9.22 \pm 2.79$

$B_{12} = 4.10 \pm 3.68$

Source	df	SS	MS	F
First order terms	2	82.41	41.20	76.296 **
Second order terms	3	13.00	4.33	8.019 *
Lack of Fit	3	2.91	0.97	1.800 NS
Error	4	2.16	0.54	

APPENDIX B

Table 14. Analysis of Variance: Average Weight Gain, Experiment One

Source	df	SS	MS	F
Blocks	2	1,952.88	976.440	.963 NS
Treatments	15	401,067.61	26,737.841	26,379 **
Error	40	40,543.68	1,013.592	

Table 15. Analysis of Variance: Liver Vitamin A Storage (μg/gm), Experiment One

Source	df	SS	MS	F
Blocks	2	0.53	.27	NS
Treatments	15	11.38	.759	1.625 NS
Error	30	14.02	.467	

APPENDIX B

Table 16. Analysis of Variance, Weight Gains, 52nd Day, Experiment Two

Source	df	SS	MS	F
Treatment	1	722,952	722,952	61.60 **
Error	14	164,326	11,737	

Table 17. Analysis of Variance and Duncan's Multiple Range Test, Weight Gains, Experiment Four

Source	df	SS	MS	F
Blocks	2	173,687	86,844	12.346 **
Treatments	3	245,663	81,888	11.642 **
Blocks x Treatments	6	91,590	15,265	2.170 NS
Error	85	597,923	7,034	

Duncan's Multiple Range Test:

Treatment Number	1	2	3	4
Treatment Mean	971.2	875.1	856.6	841.3

Underlined means do not differ significantly.

APPENDIX B

Table 18. Analysis of Variance and Duncan's Multiple Range Test, Plasma Sulfate (mg/100 ml), Experiment Four

Source	df	SS	MS	F
Treatments	3	.0018	.0006	4.00 NS
Blocks	2	.0117	.0058	38.67 **
Error	6	.0009	.00015	

Duncan's Multiple Range Test:

Treatment Number	1	4	3	2
Mean	6.87	6.86	6.85	6.83

Underlined treatment means do not differ significantly.

Table 19. Analysis of Variance, Plasma Free Methionine (micromoles/ml), Experiment Four

Source	df	SS	MS	F
Blocks	2	.001065	.000532	0.98 NS
Treatments	3	.034643	.011548	21.23 **
Error	6	.032610	.000544	

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