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THE EFFECTS OF VITAMIN A ON THE FATE OF ^{35}S-METHIONINE IN METHIONINE-DEFICIENT CHICKS

by

Kenneth Wayne Samonds

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I hereby recommend that this dissertation prepared under my direction by Kenneth W. Samonds

entitled The Effects of Vitamin A on the Fate of ^{35}S-Methionine in Methionine-deficient Chicks

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

C R Kemmerer — 4/11/69

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After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

C R Kemmerer	4/11/69
Mitchell G. Vavich	4/11/69
W H McCarthy	4/11/69
Henry Tucker	4/22/69
Robert O Kuehl	4/22/69

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SIGNED: Kenneth W. Samonds

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ABSTRACT

Previous research by this author has shown that inhibited growth in chicks caused by a methionine deficiency could be partially ameliorated by increasing the vitamin A intake above the level required by chicks consuming a balanced amino acid diet. This improvement in growth was accompanied by increased retention of most amino acids and a plasma methionine level which approached that of normal chicks. The cause of this interrelationship is not clear.

Present research utilized ^{35}S-methionine injected by heart-puncture to trace the effects of a methionine deficiency and a vitamin A deficiency or excess upon the fate of the labeled sulfur atom. A flow-through scintillation counter in series with an amino acid analyzer was used to identify and quantitate the radioactive compounds found free in the plasma and tissues, bound in plasma and tissue proteins, and free in the feces.

A methionine deficiency promoted the conservation of the amino acid in its essential form rather than permitting it to be degraded by various pathways. Oxidation of methionine to the corresponding sulfoxide or sulfone and transfer of the sulfur atom to cysteine were

curtailed in the methionine deficient state. The rate of incorporation of methionine into proteins was reduced, however, because the dietary intake of methionine was not sufficient to stimulate normal protein synthesis. The labeled methionine remained in the tissue intracellular space until being incorporated into cellular protein.

A vitamin A deficiency concomitant with a methionine deficiency, destroyed the conservation mechanism by accelerating the oxidation of methionine in the liver where large quantities of free methionine sulfoxide and methionine sulfone were found. This early oxidation of methionine, before it could pass into the tissue reserve, made methionine unavailable for protein synthesis and greatly increased the amount of the label excreted as sulfate; a 3.5-fold increase in the first 6 hours, and a 40% increase over a 48 hour period. More sulfate was also available for incorporation into cartilage mucopoly-saccharides.

INTRODUCTION

The essential amino acid methionine makes three contributions to normal growth and maintenance of the higher organisms. First, an exogenous source of the intact methionine molecule is required for normal protein biosynthesis. The lack of a single amino acid has been shown, in several cases, to retard protein synthesis in vivo and in vitro, and this is also the case with methionine. Secondly, the methionine molecule is a methyl donor. See Figure 1. Methyl groups supplied by methionine are required for the conversion of norepinephrin to epinephrin, acetylserotonin to melatonin, guanidinoacetate to creatine, phosphatidylethanolamine to phosphatidylcholine, and for the in vivo formation of methylated polynucleotides. Thirdly, methionine and cysteine, the other sulfur-containing amino acid, are the major source of sulfur for the biosynthesis of all sulfur-containing compounds. See Figure 1. In the case of a cysteine deficiency, the transfer of the sulfur atom from homocysteine (demethylated methionine) to serine is a possible source of cysteine for protein biosynthesis if the supply of methionine is adequate. A methionine deficiency, however, cannot be alleviated by the reverse of this series of reactions due to the irreversibility of the cystathionine breakdown reaction. The sequential oxidation of cysteine supplies

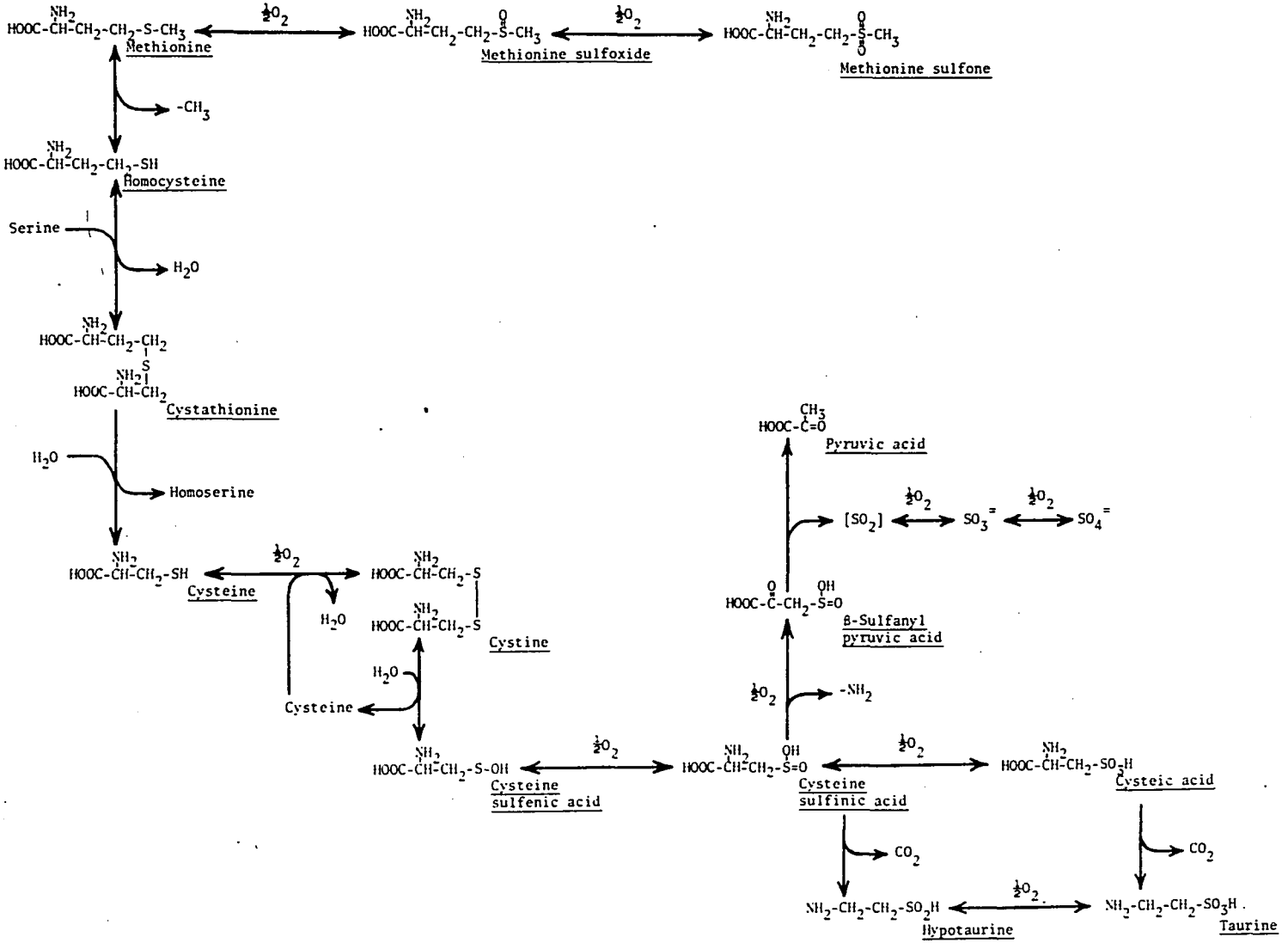

Figure 1. Metabolism of methionine (Krebs, 1964).

a major portion of the sulfate necessary for the formation of sulfated mucopolysaccharides. Tarver and Schmidt (1939) and Daniels and Rich (1918) have shown that the reverse is not true. Ingested inorganic sulfate is poorly absorbed from the intestine and cannot supply the sulfur necessary for cysteine biosynthesis. Some ingested sulfate, however, can help to meet the sulfate requirement for mucopolysaccharide biosynthesis, thus influencing to some extent the requirement for cysteine and methionine (Button et al, 1964; Dziewiatkowski, 1949). The daily requirement for sulfate is unknown. The complex involvement of methionine and cysteine in the above reactions suggests that, in animals receiving a diet deficient in these two amino acids, the body's capacity to fulfill all of the obligations of the methionine molecule is reduced.

A methionine deficiency is characterized by negative nitrogen balance, depressed growth and decreased food intake, anemia, hypoproteinemia, hemorrhagic liver necrosis (Follis, 1958), unbalanced plasma aminograms (Dean and Scott, 1966), altered amino acid retention (Samonds, 1966), inhibited hair growth, and decreased sulfate excretion (Sabry et al., 1965). Kean (1966) and Naito and Kandatsu (1967) have shown that an increased incorporation of S^{35}-methionine occurs in methionine-deficient animals, possibly due to increased activity of the methionine-activating enzyme.

The combined requirement for methionine plus cysteine has tentatively been set at 0.55 grams per day for women (Leverton, 1959) and 1.10 grams per day for men (Rose, 1949). Rose also suggested that as much as 89% of this combined requirement may be met by cysteine. Although individual requirements vary greatly and the criterion upon which to base human requirements has not been resolved, it is evident that the self-selected American diet, while supplying adequate amounts of the other essential amino acids, is only marginal in methionine plus cysteine (Mertz et al., 1952; Futrell et al., 1952). Diets based primarily on vegetable or grain proteins indigenous to the poorer cultures of the world are markedly deficient in methionine. The world-wide extent of sulfur-amino acid deficiency is difficult to measure because it is confounded with severe or marginal protein malnutrition.

Although vitamin A was the first growth-promoting factor isolated, its metabolic role other than in the visual process has remained a mystery. The symptoms of a vitamin A deficiency or excess are so many and varied that the search for a single role which might precipitate all of these multitudinous symptoms has met with failure.

In general, vitamin A affects the formation of mucous-type and connective tissue-type mucopolysaccharides (Gaylor, 1964). The physiological manifestations, in addition to xerophthalmia, are the

disruption and atrophy of mucous-secreting epithelial tissue forming a keratinized layer resembling epidermis and increased activity of osteoblasts. Excess vitamin A causes an increase in the mucoprotein level in the plasma and a differentiation of epithelial basal cells into mucous-secreting goblet cells. The maturation of cartilage cells is accelerated, but the cartilage matrix is depleted due to a loss of chondromucoprotein (Follis, 1958).

Three major hypotheses have been advanced concerning the metabolic role of vitamin A; Balakhovsky and Drozdova (1957) proposed that vitamin A hindered keratinization by blocking the copper-catalyzed oxidation of cysteine to cystine. This effect would involve both the folding and cross-linkage of intracellular proteins and the blocking of sulfate production by inhibiting the initial oxidation step (Balakhovskii and Kuznetsova, 1958). Redfearn and Strangeways (1957) examined the distribution of sulfhydryl and disulfide groups in the epithelium of vitamin A deficient rats. They found that, although sulfhydryl groups were distributed uniformly throughout nonkeratinized and keratinized epithelium, the presence of disulfide groups was found only in the keratinized layers. Additional support for this hypothesis was offered by Smudski and Myers (1963) who found an 85 to 92% increase in sulfate excretion in vitamin A deficient rats. Koyanagi, Tanaka and Takasaki (1964) demonstrated that administration of vitamin A to

hypovitaminotic children significantly decreased their excretion of urinary sulfate and taurine, and altered the incorporation of labeled sulfur administered as sulfate or methionine (1963). Other workers showed that vitamin A treatment improved the availability of methionine and cysteine in liver tissue (Curzio, Rendace, and De Arcangelis, 1967) and in pig bristles, cattle hoofs, and bird feathers (Aizinbudas et al., 1966). Incorporation of methionine into brain, muscle, and kidney protein was improved by a four-fold increase in vitamin A in vitamin E deficient guinea pigs (Shtutman and Chagovets, 1964). Rodahl, Issekutz, and Shumen (1965) have questioned the Balakhovsky proposal by demonstrating that even large doses of vitamin A did not inhibit the oxidation of methionine and cysteine in rats.

Research concerning the effect of vitamin A upon the synthesis of chondroitin sulfate and other sulfated mucopolysaccharides has caused great controversy. Dziewiatkowsky (1954), Wolf and Johnson (1960), Barker, Cruickshank and Webb (1964), and Ushim (1965) found a decrease in the incorporation of ^{35}S- into the mucopolysaccharides of vitamin A deficient rats. Wolf and Varandani (1960) restored the incorporating ability in vitro by the addition of retinol. This effect was traced to the formation of "active sulfate," 3'-phosphoadenosine-5'-phosphosulfate (PAPS) (Varandani, Wolf, and Johnson, 1960; Rao and Ganguly, 1964, 1966) by the enzyme ATP-sulfurylase (Rao, Sastry, and Ganguly, 1963; Levi and Wolf, 1965; Mukherji and Bachhawat, 1966a and b).

Sundaresan (1966) extracted a metabolite of vitamin A from ATP-sulfurylase which was required for the activity of the enzyme. More recent work has cast doubt on this extensive work. Pasternak and Pirie (1964) and Mukherji and Bachhawat (1967), the latter reversing their earlier position, found that a vitamin A deficiency did not impair enzymic activation of sulfate in tissue. Incorporation of ^{14}C-glucose into epidermal mucopolysaccharides (Samsonova, 1967) and of ^{14}C-glucosamine and ^{35}S-sulfate into hyaluronic acid, sulfated hyaluronic acid, heparin sulfate, and chondroitin sulfate of lung tissue (DeLuca and Wolf, 1968) was increased in deficient rats rather than decreased as previously reported.

Others have proposed that vitamin A affects the hydrolytic removal of sulfate from mucopolysaccharides rather than its addition (Fell, Mellanby, and Pelc, 1956). Lucy and Dingle (1962) and Dingle (1963) suggested that the mode of action of vitamin A was the control of the permeability of lysosomes, the reservoirs of degradative enzymes in cells. Guha and Roels (1965) have supported this hypothesis by demonstrating that the activity of arylsulfatase A and B in rat liver tissue was increased in vitamin A deficient animals. Roels, Trout, and Guha (1964) also studied the activity of other enzymes, the acid hydrolases, in rat liver tissue and found that their activities were also dependent upon the vitamin A status of the animal. Rodahl, Issekutz, and Shumen (1965) attributed the increase in sulfate excretion in

^{35}S-methionine pretreated rats, after a massive dose of vitamin A, to the release of proteolytic enzymes from lysosomes, and the subsequent oxidation of the liberated methionine and cysteine. Samonds (1966) and Dua et al. (1966) showed that high levels of vitamin A decreased nitrogen retention and attributed this to increased proteolytic activity. Brown, Button, and Smith (1963), in a bit of armchair biochemistry, proposed a common mode of action for the fat-soluble vitamins A, D, and E in the association of sulfomucopolysaccharides with the cell membrane affecting its permeability. Lucy (1968) proposed a different role for the vitamin A molecule. He envisioned retinol as an integral part of biological membranes controlling their molecular configuration and, in so doing, affecting their permeability by the conversion of the membrane from the bimolecular leaflet configuration to the more porous micellular configuration.

In previous research, vitamin A has been shown to have a protective influence on animals fed a diet low in methionine. Analysis of the growth response of five-week-old chicks to various combinations of administered vitamin A and dietary methionine indicated that additional vitamin A improved food consumption and growth of methionine-deficient birds, but never up to the level of normal birds. The reductions in amino acid retentions and the imbalanced plasma aminograms resulting from a methionine deficiency were favorably altered by

increased vitamin A intake. Bhattacharya and Esh (1964) showed that vitamin A could ameliorate the disorders caused by several amino acid deficiencies, but found no effect on a methionine deficiency.

Understanding the relationship between vitamin A and the sulfur amino acids is of prime practical importance. The frequency of occurrence of a vitamin A deficiency is only slightly less than that of severe protein or calorie malnutrition. The major source of the preformed vitamin is meat and other mamalian products. Green plants supply carotene, but the amount varies over a wide range depending on variety, growth conditions, and the season. Diets mentioned earlier which are based primarily on rice or corn as the protein and calorie source are very low in vitamin A and therefore compound their lack of sufficient methionine and cysteine. Determination of the methionine plus cysteine requirement of any animal may depend greatly upon the vitamin A status of the animal, and vice versa. Therefore, clarification of the interrelationship between vitamin A and the sulfur amino acids is essential. In an attempt to test the various hypothesis for the metabolic role of vitamin A and to elucidate the reasons for its sparing effect on a methionine deficiency the following experiments were performed.

METHODS

Experimental Animals

The chicks used in all experiments were male Hubbards hatched according to standard procedure at the University of Arizona Poultry Farm. Experimental diets were fed from the first day to avoid storage of vitamin A in chicks which later were to be made deficient. All food and water were given ad libitum.

Preparation of Chickens for Injection

To insure that all chickens injected with radioactive material were in a similar metabolic state, a carefully controlled feeding regimen was followed. Fourteen hours before the time of injection, the feed was removed and the chickens were fasted for twelve hours. Feed was then replaced for two hours. Drinking water was supplied ad libitum at all times. After the twelve hour fast the chickens ate their fill quickly insuring synchronous digestion and absorption of nutrients and similar post-absorptive metabolism at the time of injection.

Composition of Basal Diet

The purified basal diet used in all experiments was free of vitamin A and minimal in methionine plus cysteine. The composition of the basal diet is in Table 1. The diet was calculated by computer to supply 32.8% protein, 8.2% fat, 3.04% fiber, 1076.9 calories of productive energy per pound, 1338.6 calories of metabolizable energy per pound, sufficient amounts of all vitamins (except vitamin A), minerals, and amino acids except the sulfur-containing amino acids. The Nutritional Research Council (1960) has listed the requirement of growing chicks for methionine as 4% of the dietary protein. Cysteine may supply up to 1.75% of this requirement. The purified soy bean protein supplies only 1% methionine and 0.6% cysteine and has been shown to cause retarded growth in the third week of feeding. After seven weeks, deficient chicks weighed only 60% of methionine-supplemented hatch-mates (Samonds, 1966).

Free and Protein-bound Compounds in Blood Plasma

Preparation of samples for analysis followed a modification of the method of Block, Markovs, and Steele (1966) who used sulfosalicylic acid as a deproteinizing agent. Approximately five ml of blood drawn by heart puncture was centrifuged in a heparinized teflon centrifuge tube to remove the red cells. One ml of the resulting plasma was mixed with 0.5 ml distilled water and 0.5 ml of 20% sulfosalicylic

Table 1. Composition of the basal diet.

	%
Dextrose[1]	38.5
Soy bean protein[2]	40.0
Cellulose[3]	3.0
Corn oil[4]	8.0
Vitamin mix[5]	4.0
Mineral mix[6]	2.0
Calcium carbonate	.05
Dicalcium phosphate	4.21
Glycine	.25

[1]Cerelose 2001, Corn Products Company.

[2]Assay Protein C-1, Skidmore Enterprises.

[3]Solka Floc, Brown Company.

[4]Mazola Corn Oil, Corn Products Company.

[5]Supplies per kilogram of diet: 960 I. C. U. Vitamin D_3, 8.8 I. U. Vitamin E, 8.8 mg Thiamin, 12.0 mg Riboflavin, 15.2 D-calcium Pantothenate, 4.0 mg Pyradoxine, 1 g Inositol, 88.0 mg Niacin, 0.2 mg Biotin, 2.0 mg Folic Acid, and 6.6 mg Menadione.

[6]Supplies per kilogram of diet: 86.25 ppm Zinc, 124.0 ppm Manganese, 0.06% Magnesium, 0.225% Sodium, 0.383% Potassium, and 0.08% Iron.

acid in a teflon centrifuge tube. The white, flocculent precipitate which resulted was packed by centrifugation. The supernatant was drawn off and used without further treatment for analysis of plasma free amino acids. The precipitate was washed three times with distilled water and centrifuged. After the final washing, the centrifuge tube was placed in a vacuum desiccator to dry the pellet. After seven days in the desiccator the pellet was weighed and pulverized. A portion of the pellet was hydrolized under vacuum with 7 ml of 6 N HCl for 25 hours. The hydrolysate was taken to dryness under vacuum and washed twice with distilled water to remove all traces of HCl. The resulting residues were dissolved in distilled water and diluted to a proper volume for quantitation on a Beckman amino acid analyzer. Normally, samples are diluted with pH 2.2 acetate buffer, but the presence of sulfosalicylic acid in the hydrolysate lowered the pH sufficiently for retention of the amino acids at the top of the resin columns of the analyzer.

The recovery of amino acids using the above method was tested by analysis of one ml of plasma for free and bound amino acids by sulfosalicylic acid precipitation and comparison with the direct HCl hydrolysis of one ml of unprecipitated plasma. Recoveries of seventeen amino acids ranged from 96 to 102%, which is within the 5% reproducibility of the analyzer. None of the amino acids were degraded or altered by treatment with sulfosalicylic acid.

Free and Protein-bound Amino Acids in Tissues

Four g of fresh muscle or liver tissue was blenderized with 20 ml of distilled water and two ml of 20% sulfosalicylic acid. The precipitate was removed by vacuum filtration and hydrolyzed with 6N HCl to determine protein-bound amino acids. The pH of the supernatant was adjusted to 2.2 with sodium hydroxide and used for estimation of free amino acids in the tissue.

Preparation of Excrement Extracts

Excrement was collected in procelain trays containing a shallow layer of water. Solid material was removed by filtration through several layers of cheesecloth, then through filter paper. The cloudy filtrate was taken to dryness under vacuum and dissolved in pH 2.2 buffer. Filtration and dilution to 150 ml yielded a clear, yellow-brown solution of proper concentration for analysis on the Beckman amino acid analyzer.

Quantitation of Radio Active Compounds

The quantitation of isotopically labeled compounds was accomplished with a Packard Tri-Carb Flow-through Scintillation Counter in series with a Beckman Amino Acid Analyzer. The effluent from the resin columns was diverted through a scintillation detector column packed with anthracene, then returned to the amino acid

analyzer for subsequent reaction with ninhydrin and colorimetric analysis.

In the scintillation counter, light energy emitted by the scintillator is converted to electrical pulses and amplified by photomultipliers. The Tri-Carb Flow Monitor, operating in coincidence mode, counts pulses within a selected amplitude range and converts this to a counts-per-minute signal which is recorded on a continuous paper strip chart equipped with a Disc Integrator.

Selection of optimum voltage. Due to the energy distribution of pulses from a beta emitter, a change in amplification caused by increasing the voltage of the photomultipliers affects the distribution of pulses falling between the upper and lower discriminators and thereby causes first an increase, then a decrease, in count rate. Adjustment of the high voltage to the value which maximizes the count rate insures optimum counting efficiency for the chosen window setting (upper and lower discriminator settings) and also minimizes variations due to changing line voltage or aging components.

Determination of the optimizing voltage for ^{14}C and ^{35}S was accomplished by injecting a static sample of labeled material into the scintillator cell. The optimum voltages for balance-point operation using a 100-1000 window were found to be 2.4 for ^{14}C and 2.7 for ^{35}S.

Identification and quantitation of radioactive peaks. As radioactive material passed through the anthracene column, the pen of the paper recorder was deflected an amount proportional to the amount of material in the detector area. After a ten to eleven minute delay, the same material passed through the colorimeter cuvette after having been mixed with ninhydrin and heated. Using the proper time correction, radioactive peaks could be identified by the elution time of their corresponding ninhydrin peaks. Peaks which were not ninhydrin positive were identified by other means. Integration of the area under a radioactive peak gave a quantitative measure of the total amount of radioactive material which passed through the detector, assuming that the flow rate was constant which is the case with the Beckman Amino Acid Analyzer.

Statistical analysis of count data. (Packard Instrument Co. Inc., 1965; Overman and Clark, 1960) Since radioactive decay events occur randomly, no exact value for count rate or total number of counts can be found. Each observation falls within a grouping around the true value of the rate or sum. Count data for radioactive decay is assumed to be distributed according to the Poisson distribution with the mean S and standard deviation $\sqrt{S}$. Because of this relationship between the mean and variance of the Poisson distribution, it is possible to establish a confidence interval about a single observation.

Since the Poisson distribution can be approximated by the normal distribution if S is large, tables of the normal error function may be used to establish a confidence interval. A 95% confidence interval about the observed count summation is

$$S \pm 1.96\sqrt{S}$$

The Packard Tri-Carb does not measure total counts in a unit time, however, which is an assumption made in the above equation. The pulses from the detector are applied to an integrating circuit where the average voltage generated is proportional to the average count rate. This voltage is used to deflect the pen of the chart recorder. The uncertainty in the count rate, therefore, depends not only upon the randomness of the radioactive decay but also upon the time constant of the integrating circuit, t_{RC}, and the accuracy of the recording device. In this case, the standard deviation of a measured rate is

$$\sigma_R = \sqrt{2Rt_{RC}}$$

Integration of the area under a peak is the equivalent of the summation of Poisson-distributed variables, $S_i = 2R_i t_{RC}$, resulting from dividing the peak into i independent segments with widths of $2t_{RC}$. The distribution of T, the total counts under the peak, where $T = \sum S_i$, has mean equal to T and a standard deviation equal to $\sqrt{T}$ because the sum of Poisson-distributed variables is also Poisson with a mean and variance

equal to the sum of the means. Thus, a confidence interval may be established about T and tests performed on differences between single observed values.

Correction for decay of sulfur-35. Because of the relatively short half-life of sulfur-35, 87.1 days, and the delay in the analysis of some samples, correction of count data for decay was necessary. By manipulation of the half-life equation,

$$\frac{A}{A_0} = e^{\frac{-0.693}{T}}$$

where Ao is the original activity, A is the present activity, t is the time which has elapsed, and T is the half-life of the radioisotope. The original activity of samples containing sulfur-35 can be found by multiplying the present activity by the factor

$$F = \text{antilog}\left(\frac{0.693t}{87.1 \times 2.303}\right)$$

or, upon simplification,

$$F = \text{antilog}(.0034t)$$

EXPERIMENTAL PROCEDURE AND RESULTS

Before any experiment could be designed to examine the effects of vitamin A, the techniques of injecting radioactive material and collecting samples required attention. Various studies were conducted to determine the best method for administering the labeled material, the number of microcuries necessary for quantitative measurement of plasma, tissue, and excrement, the most favorable time to collect these samples, and the safest and best quantitative techniques for their analysis.

In these early experiments, ^{14}C-methionine, labeled either in the carboxyl or methyl position, was used. Esophageal injection of a 10 microcurie (μCi) dose proved to be a poor method of administration. No labeled methionine or its metabolic products could be found in plasma samples collected at intervals ranging from one-half hour to eight hours after injection. The small quantity of radioactive material, the gradual absorption, and the high rate of metabolism of methionine discouraged the use of esophageal injection.

Heart-puncture injection of 10 μCi of L-methionine-carboxyl-^{14}C in isotonic saline proved to be much better and supplied satisfactory data for characterization of a methionine deficiency. Chicks were maintained for four weeks on the basal diet, either

deficient in methionine and cysteine or methionine supplemented. Ten μCi of labeled methionine was then injected and plasma was drawn by heart puncture after 10, 20, 40, 60, 90, and 120 minutes. Three peaks were found in the analysis of deproteinized plasma; methionine, methionine sulfoxide, and a peak eluted at 18.5 minutes the same time as urea. Data from the analysis is shown in Table 2. Analysis of excrement indicated a large peak later identified as carbonate, a small ninhydrin reactive peak at 18 minutes, and a small ninhydrin-negative peak at 46 minutes. No methionine peak was noted. The methionine deficient chicken displayed a faster disappearance of methionine from the plasma and less urea formation, indicating a more efficient utilization of the methionine.

The 10 μCi dose produced very small peaks at the limit of resolution from background thermal noise and cosmic interference of the scintillation counter, indicating that a larger dose should be used. Forty microcuries of uniformly labeled methionine was injected in a similar manner and plasma samples were collected at 10, 30, 60, 90, and 120 minutes. Seven peaks were measurable in the plasma free amino acid fraction. Results are compiled in Table 3. The rapid disappearance of methionine, due either to degradation or incorporation into proteins is readily evident. The amount of label incorporated into plasma proteins is recorded in Table 4. Maximum incorporation of methionine occurred in the ninety minute sample.

Table 2. Concentration of radioactive compounds free in the plasma of normal and methionine-deficient chicks after injection of 40 microcuries of L-methionine-carboxyl-^{14}C.

Counts per ml of plasma[1]

Treatment	Time (minutes)					
	10	20	40	60	90	120
Methionine Deficient						
Methionine	730(27)	365(19)	218(15)	173(13)	100(10)	274(17)
Methionine sulfoxide	603(24)	318(18)	244(16)	154(12)	93(10)	79 (9)
Urea	80 (9)	147(12)	94(10)	79 (9)	86 (9)	130(11)
Control						
Methionine	1082(33)	609(25)	387(20)	239(15)	134(12)	200(14)
Methionine sulfoxide	568(24)	301(17)	218(15)	129(11)	102(10)	92(10)
Urea	116(11)	112(11)	192(14)	191(14)	201(14)	175(13)

[1]Numbers in parentheses are standard errors.

Table 3. Concentration of radioactive compounds free in the plasma of a chicken injected with 40 microcuries of uniformly labeled ^{14}C-methionine.

Counts per ml of plasma

	Time (minutes)				
	10	30	60	90	120
Methionine	1535	1174	242	196	281
Methionine sulfoxide	345	221	76	44	29
13 minute peak (CO_3)	48	40	26	10	22
17 minute peak (urea)	41	9	53	59	56
22 minute peak	-	-	-	41	24
24 minute peak	77	-	40	61	71
Unknown V	169	66	46	26	-

Table 4. Incorporation of uniformly labeled ^{14}C-methionine into the plasma proteins of a chicken.

Time (minutes)	Counts per mg of plasma protein precipitate
10	0
30	56
60	98
90	113
120	103

After these preliminary experiments with ^{14}C, ^{35}S-methionine was used to trace the fate of the sulfur atom as affected by a methionine deficiency and vitamin A intake. Ten chicks were fed the methionine-deficient basal diet, with 10,000 IU of vitamin A per kg of diet for two weeks. Half of the chicks were then given a larger supplement of vitamin A, 100,000 IU per kg. After two more weeks the chicks with elevated vitamin A intake exhibited less comb development, poorer growth, and diarrhea. One chicken from each group was selected for injection with ^{35}S-methionine. Four hundred μCi in 1 ml of isotonic saline was injected into the normal vitamin A chicken which weighed 660 grams. Plasma samples were drawn at 10 minutes, 1/2, 1, 2, 4, 12, and 24 hours. Due to the smaller size, 320 grams, and weaker condition of the hypervitaminotic A chicken, only .4 ml, or 160 μCi of radioactive solution was injected, and samples were drawn only at 10 minutes, 1, 2, and 4 hours. Despite the precaution, this chicken died before the 12 hour sample was drawn. A typical pair of chart records indicating radioactivity and ninhydrin reaction optical density versus time is shown in Figure 2. The elution time on the ninhydrin chart has been corrected for the analyzer delay. The number of counts in the peaks of the protein-free plasma supernatant for each bird is listed in Table 5 and the data for methionine, cysteine and sulfates is displayed in Figures 3, 4, and 5, respectively. The amount

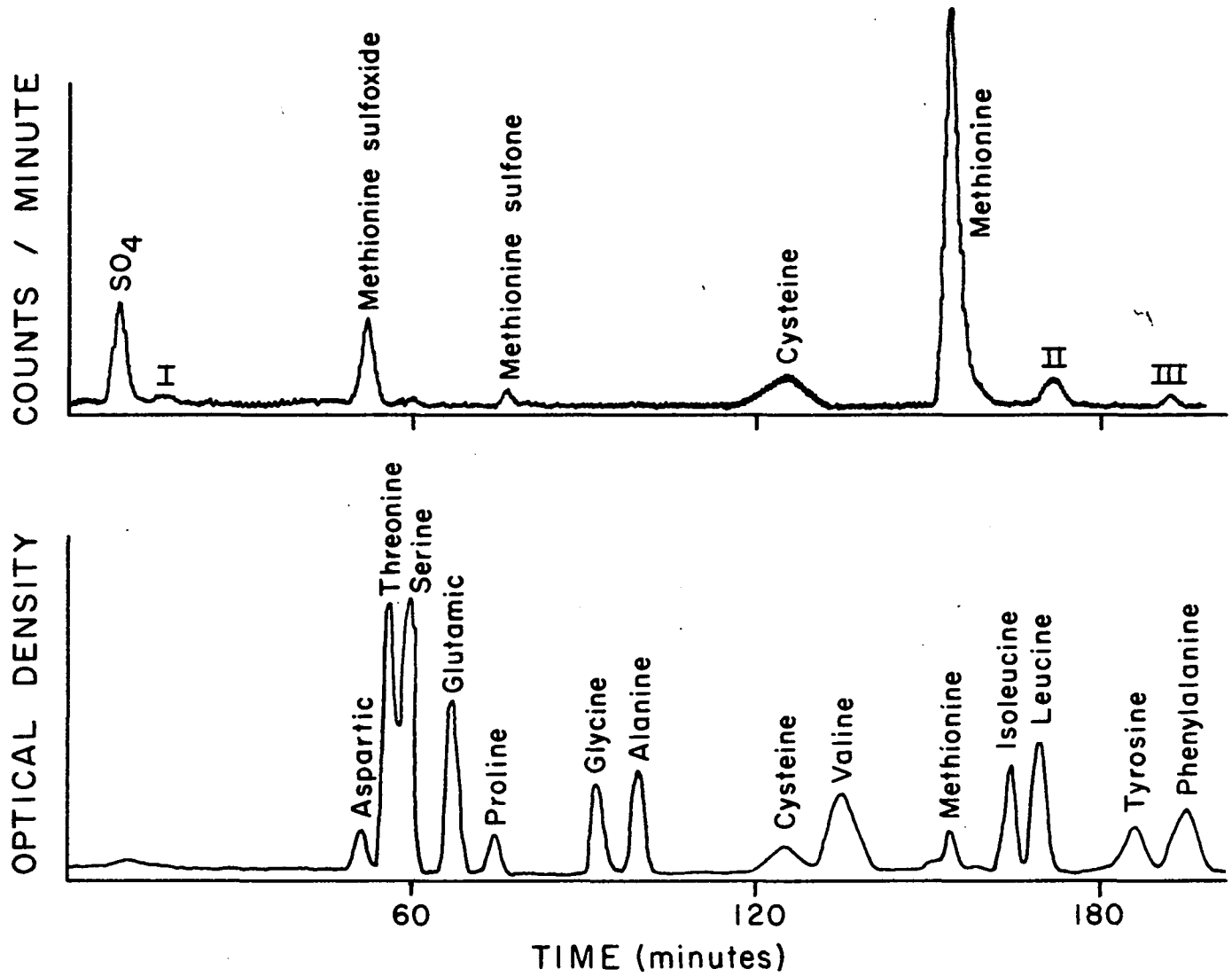

Figure 2. Radioactive and ninhydrin reactive chart records.

Table 5. Concentration of radioactive compounds free in the plasma of normal and hypervitaminotic A chickens after injection of 400 μCi of ^{35}S-methionine.

Plasma compound	Counts per ml of plasma[1]							
	Time							
Treatment	10 min.	30	60	90	2 hrs.	4 hrs.	12 hrs.	24 hrs.
Sulfates								
Normal	25,192 (159)	19,254 (138)	15,380 (124)	14,380 (120)	14,215 (119)	11,326 (106)	11,400 (107)	13,672 (117)
Hypervitaminotic A	5,828 (76)	-	10,910 (104)	-	12,548 (112)	12,587 (112)	-	-
Methionine Sulfoxide								
Normal	15,274 (124)	7,686 (88)	3,860 (62)	2,772 (53)	2,021 (45)	943 (31)	272 (16)	0
Hypervitaminotic A	6,254 (79)	-	6,549 (81)	-	6,226 (79)	4,225 (65)	-	-
Methionine Sulfone								
Normal	1,961 (44)	428 (21)	700 (26)	840 (29)	trace	0	0	0
Hypervitaminotic A	0	-	0	-	0	0	-	-

Table 5--Continued.

Plasma compound	Counts per ml of plasma[1]							
	Time							
Treatment	10 min.	30	60	90	2 hrs.	4 hrs.	12 hrs.	24 hrs.
Cysteine								
Normal	853 (29)	2,389 (49)	2,180 (47)	2,076 (46)	2,027 (45)	1,343 (37)	577 (24)	282 (17)
Hypervitaminotic A	0	-	686 (26)	-	1,434 (38)	1,860 (43)	-	-
Methionine								
Normal	146,997 (383)	48,989 (221)	15,562 (125)	11,886 (109)	9,473 (97)	6,687 (82)	2,829 (54)	1,590 (40)
Hypervitaminotic A	115,229 (339)	-	42,000 (205)	-	13,576 (117)	13,070 (114)	-	-
II Unknown								
Normal	7,697 (88)	5,089 (71)	2,184 (47)	1,506 (39)	854 (29)	383 (20)	153 (12)	0
Hypervitaminotic A	771 (28)	-	4,033 (64)	-	3,574 (60)	2,728 (52)	-	-

[1]Numbers in parentheses are standard errors.

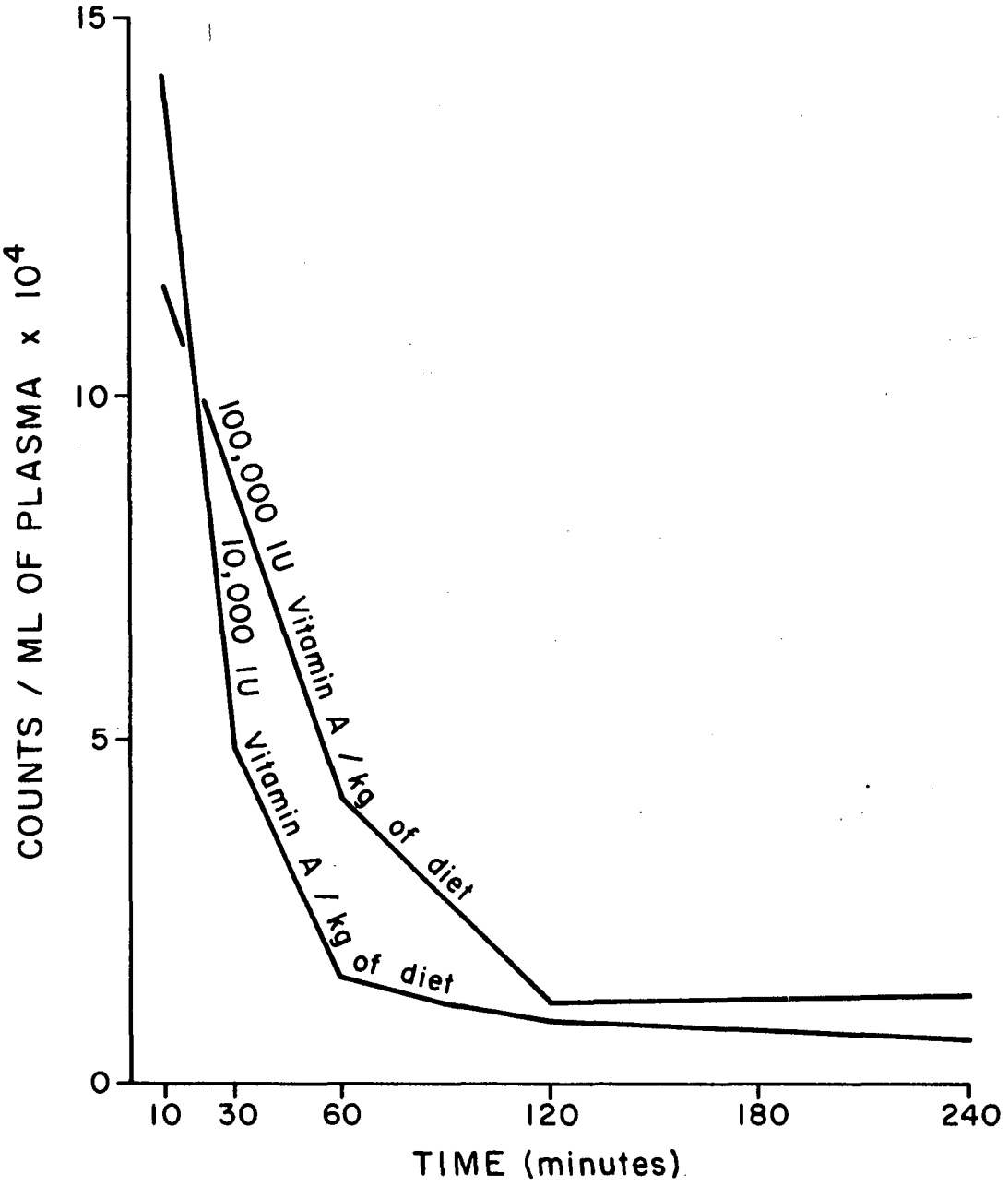

Figure 3. Concentration of plasma free ^{35}S-methionine (counts per milliliter) versus time at normal or elevated vitamin A intake.

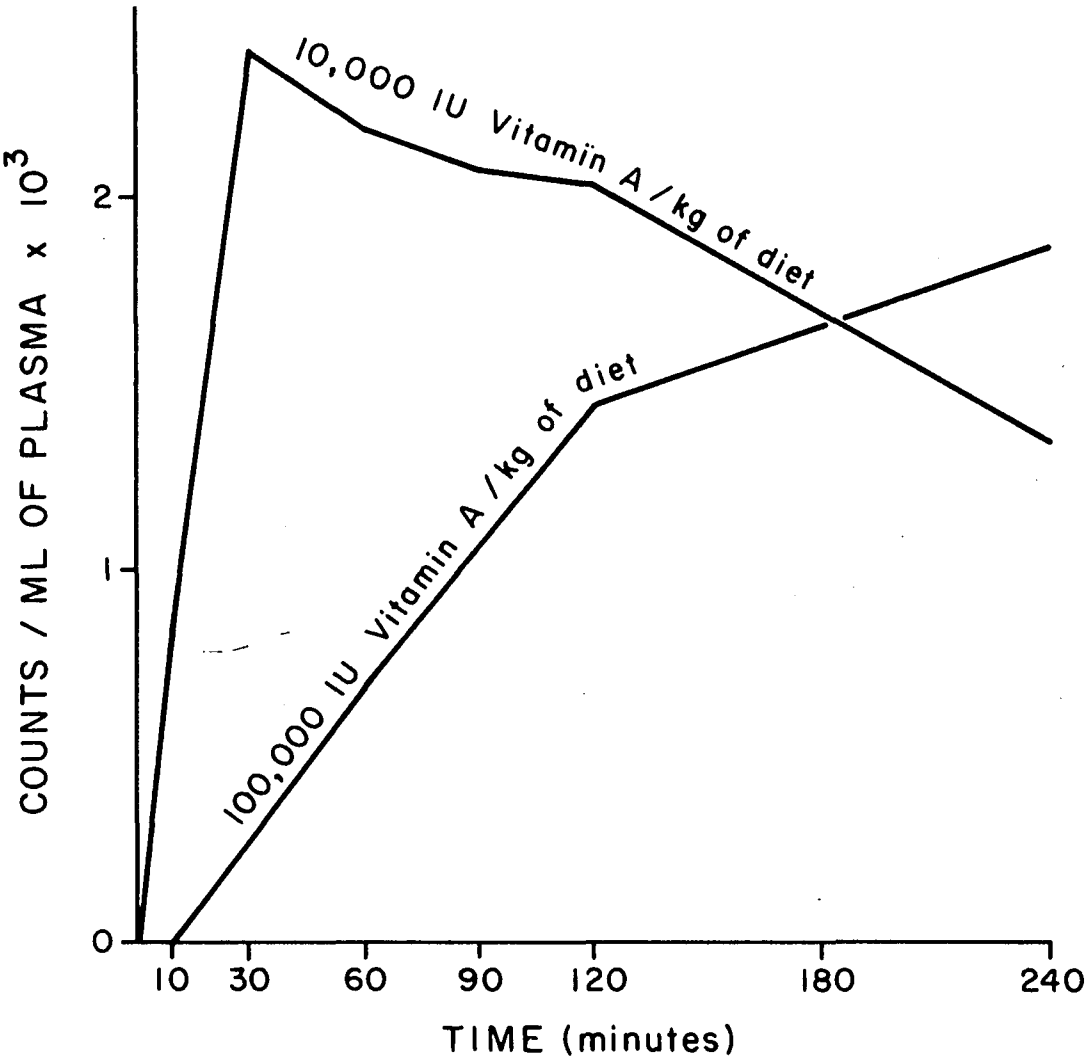

Figure 4. Concentration of plasma free ^{35}S-cysteine (counts per milliliter) versus time at normal or elevated vitamin A intake.

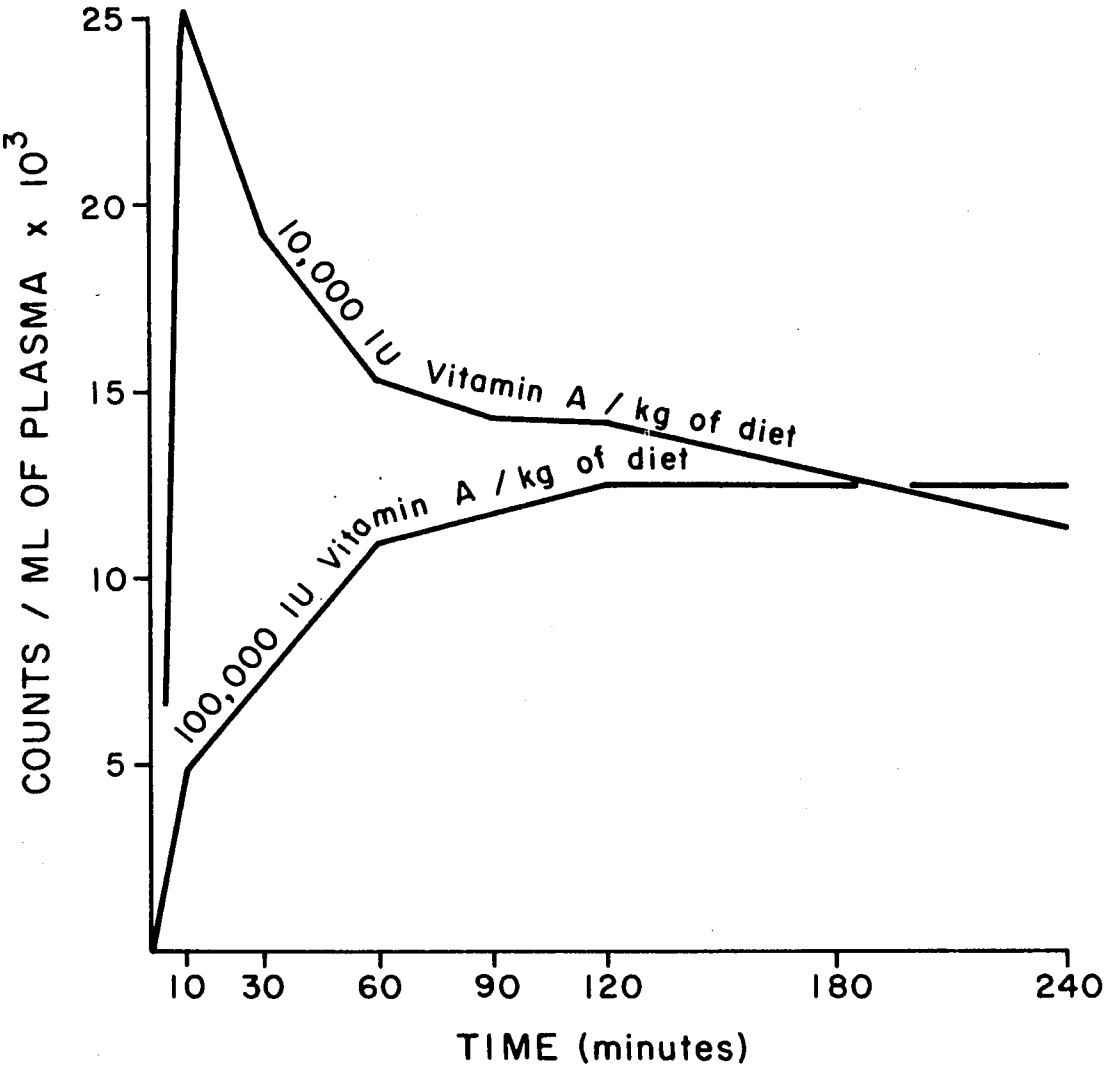

Figure 5. Concentration of plasma ^{35}S-sulfates (counts per milliliter) versus time for normal or elevated vitamin A intake.

of methionine and cysteine incorporated into plasma proteins is in Table 6, and graphed in Figures 6 and 7, respectively.

The unknown peak recorded at 13 minutes was found to be sulfates, both organic and inorganic. These were eluted with the buffer front because of their negative charge and lack of binding to the column. Non-radioactive sulfate ions applied to the column were eluted in the 11 to 16 minute period, as was indicated by the formation of a cloudy precipitate upon treatment of collected samples with saturated barium chloride. Treatment of feces samples with barium chloride or 1% benzidine in ethanol precipitated sulfate ions and removed 64.6% of the radioactive label from this peak, which showed that the majority of the label was in the ionic sulfate form. Treatment of plasma samples with the same reagents, however, removed only 15.2% of the radioactive label indicating that the majority of the sulfur was not in the ionic form. Standard solutions of cysteic acid and homocysteic acid, prepared by peroxide and formic acid oxidation of homocystine, produced ninydrin peaks which were eluted at the same time as the unknown. Thus it was concluded that the major portion of the oxidized sulfur is transported as organic sulfates, but excreted as sulfate ion.

Other unknown peaks were not so easily identified. The small peak, Unknown I, eluted after the sulfate peak may perhaps be sulfated sugars. ^{14}C labeled sugars have been shown to be eluted in this time period, but no positive identification of this peak could be made.

Table 6. Incorporation of ^{35}S-methionine and ^{35}S-cysteine into the plasma proteins of normal and hypervitaminotic-A chickens after injection of 400 μCi of ^{35}S-methionine.

	Counts per mg of plasma protein precipitate[1]							
	Time							
	10 min.	30 min.	60 min.	90 min.	120 min.	4 hrs.	12 hrs.	24 hrs.
Methionine								
Normal	84 (9)	1187(34)	4064(64)	5068(71)	5409(74)	4570(68)	3225(57)	2321(48)
Hypervitaminotic A	41 (6)	-	532(23)	-	1240(35)	2820(53)	-	-
Cysteine								
Normal	0	729(27)	1490(38)	1581(39)	1510(39)	1588(39)	1662(40)	1714(41)
Hypervitaminotic A	0	-	65(8)	-	183(14)	1257(35)	-	-

[1]Numbers in parentheses are standard errors.

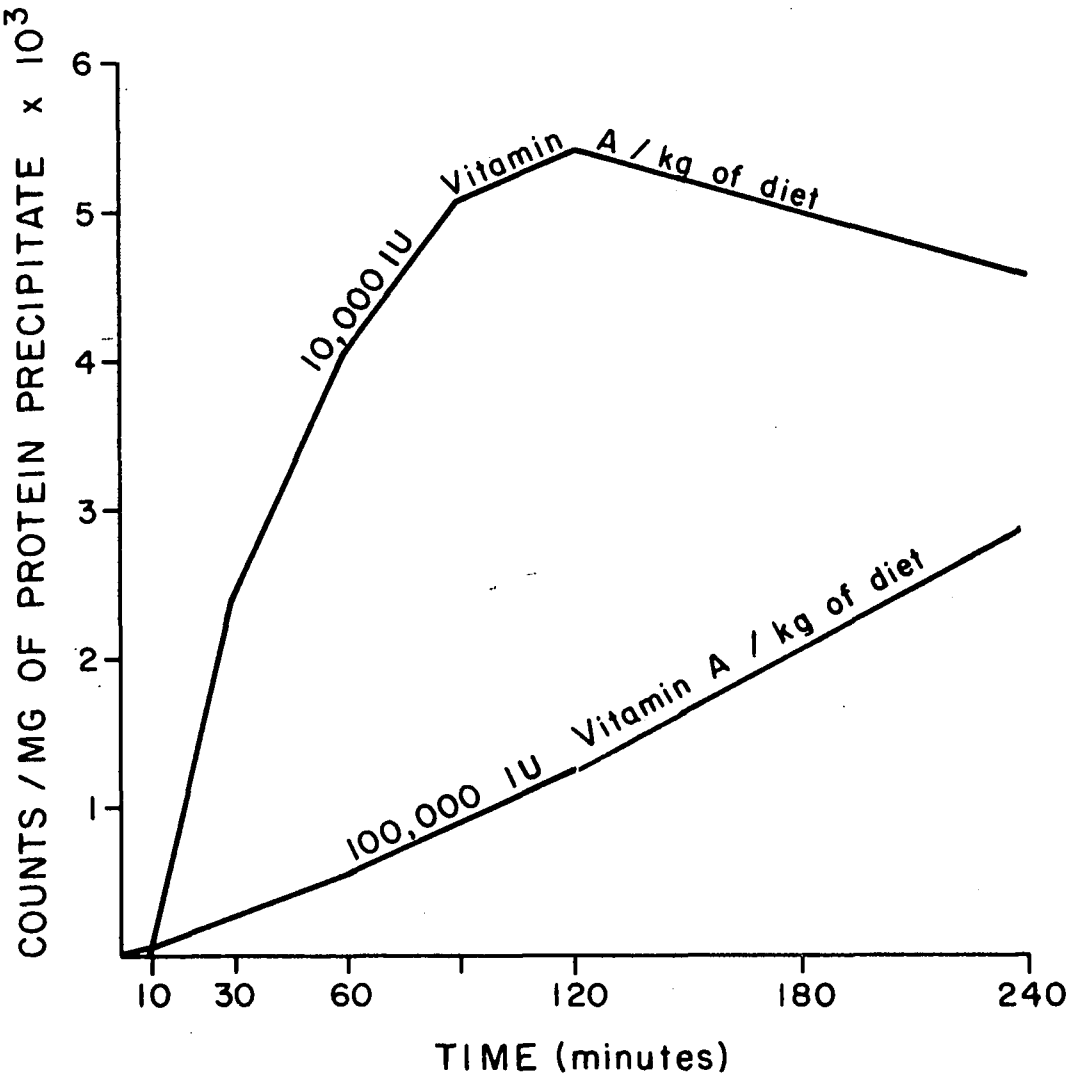

Figure 6. ^{35}S-methionine incorporated into plasma proteins (counts per milligram of protein precipitate) versus time as affected by normal and elevated vitamin A intake.

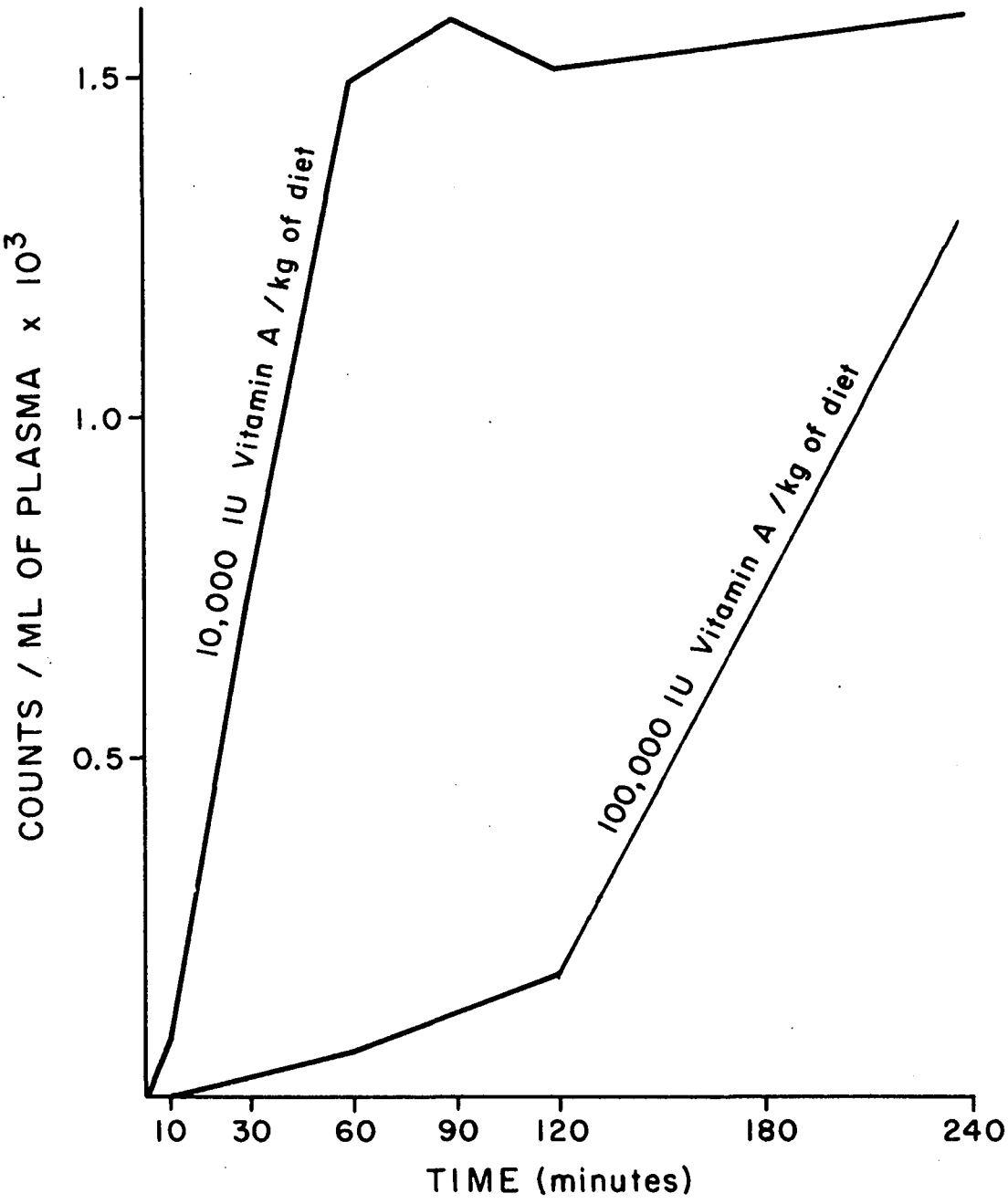

Figure 7. ^{35}S-cysteine incorporated into plasma proteins (counts per milligram of plasma protein precipitate) versus time as affected by normal or elevated vitamin A intake.

Methionine sulfoxide and methionine sulfone, to a lesser extent, are natural contaminants in the purchased methionine. The concentration of the sulfoxide in plasma and its slow clearing time indicated that it may be a natural metabolite of methionine. Care was taken to avoid the oxidation of methionine during storage, and reanalysis of old samples indicated that the sulfoxide was not a product of storage. Two other small peaks, Unknowns II and III, which were eluted after methionine, were not identified. Unknown II was eluted at the same time as Unknown V in the uniformly labeled experiment and was speculated to contain not only the sulfur atom, but also a portion of the carbon skeleton of methionine. Unfortunately, it was eluted at the same time as leucine, and therefore it was impossible to determine whether it was ninhydrin positive or negative.

The early formation of sulfates, within the first 10 minutes, was drastically reduced in the hypervitaminotic bird. Plasma protein incorporation was also severely hindered. More of the amino acid remained in the free form in plasma, twice as much as in the normal bird in the 4 hour sample.

The discovery of this sparing effect on vitamin A upon the oxidation of methionine led to a more thorough examination of methionine metabolism in vitamin A deficient chicks which were also methionine deficient; methionine deficient chicks which were vitamin A supplemented; and normal control chicks, supplemented with both

methionine and vitamin A. One day old males were assigned randomly to groups of eight birds per cage, three cages per diet, to examine weight gain and feed efficiency and to prepare animals for subsequent injection of labeled methionine. Group I received the methionine deficient basal diet supplemented with only 1100 IU of stabilized vitamin A acetate per kilogram, enough to support growth in normal birds. Group 2 received the basal diet supplemented with 8,800 IU of vitamin A acetate per kilogram. Group 3 received the basal diet supplemented with 0.4% crystalline L-methionine and 8,800 IU of vitamin A acetate per kilogram. The diets were fed ad libitum for five weeks. Growth and food consumption were measured weekly.

Data for growth, feed consumption, and feed efficiency $\frac{\text{weight gain in grams}}{\text{grams of feed consumed}} \times 100$, are displayed in Table 7. Analysis of Variance and Duncan's Multiple Range Test indicated that there was no significant difference in growth among the three groups. Supplementation of the basal diet with methionine improved the feed consumption of the control group. As mentioned above, a methionine deficiency of this magnitude begins to show its effect upon growth during the fourth week, and thus, the absence of any outward physiological effect was not unexpected.

After five weeks on the experimental diets, a chick from each group was selected and prepared for injection according to the feeding

Table 7. Average weight, feed consumption, and feed efficiency of chicks on various methionine and vitamin A supplements.

Group number[1]	Week 1	2	3	4	
Average weight (gm)					
1	123	242	380	552	
2	123	235	360	528	
3	128	253	418	590	
Group Feed consumption (gm)					
1	2,678	4,236	6,311	8,043	
2	2,428	4,232	5,205	7,084	
3	2,971	4,608	6,555	8,414	
Feed Efficiency (%)					Overall Efficiency
1	70.0	64.2	50.5	49.1	58.4
2	76.0	61.6	50.5	50.2	59.6
3	66.6	62.6	57.9	47.0	58.5

[1]Group 1: Methionine deficient, minimal vitamin A
Group 2: Methionine deficient, vitamin A supplemented
Group 3: Control, methionine, and vitamin A supplemented.

regimen discussed above. Each chick was injected with 330 μCi of ^{35}S-methionine. Plasma samples were drawn at 10 minutes, 2, 6, 12, 24, and 48 hours. The amount of label found in the various substances free in the plasma and in amino acids incorporated into the plasma proteins is shown, respectively, in Tables 8 and 9. The labeled plasma methionine for the three chickens is shown in Figure 8. Figures 9 and 10 display the data for plasma sulfates and cysteine, respectively. Excrement was collected in porcelain trays at 6, 12, 24, and 48 hours. The amount of label found in the excrement is recorded in Table 10 and the cumulative excretion of ^{35}S-sulfate is displayed in Figure 11. After 48 hours the chickens were sacrificed by exsanguination and the breast muscle and bone were removed. The muscle tissue was minced, weighed, and placed in a vacuum desiccator to determine moisture content. Cartilage from the caudal sternal crest was cleaned of any muscle tissue and dried in the desiccator for a similar determination. Portions of muscle tissue and cartilage were hydrolyzed in 6N HCl and analyzed for the amount of labeled materials they contained. Results are shown in Table 11.

Comparison of chickens from Groups 2 and 3 shows the effects of a methionine deficiency. The deficiency accelerated the disappearance of methionine from the plasma, decreased the amounts of radioactive metabolites, sulfates and methionine sulfoxide, and decreased cysteine formation to one-third that of the normal bird. Less

Table 8. Concentration of ^{35}S-labeled compounds free in the plasma of chicks receiving various methionine and vitamin A supplements.

Plasma compounds	Counts per ml[1] Time					
Group Number[2]	10 min.	2 hr.	6 hr.	12 hr.	24 hr.	48 hr.
Sulfate						
1	15,768 (126)	13,589 (117)	4,605 (68)	2,263 (48)	754 (27)	1,041 (32)
2	5,442 (74)	4,825 (69)	4,021 (64)	3,000 (55)	980 (31)	227 (15)
3	8,703 (93)	8,981 (95)	7,741 (89)	5,229 (72)	1,732 (42)	750 (27)
Methionine sulfoxide						
1	6,080 (78)	1,361 (37)	154 (12)	0	0	0
2	4,547 (68)	649 (25)	148 (12)	0	0	0
3	6,211 (79)	1,098 (32)	254 (16)	0	0	0
Cysteine						
1	2,170 (47)	639 (25)	119 (11)	0	0	0
2	1,102 (33)	402 (20)	283 (17)	0	0	0
3	3,276 (57)	1,213 (35)	484 (22)	283 (17)	81 (9)	0
Methionine						
1	28,770 (170)	2,239 (47)	478 (22)	304 (17)	trace	248 (16)
2	19,834 (141)	777 (28)	441 (21)	520 (23)	197 (14)	200 (14)
3	36,984 (192)	2,553 (51)	646 (25)	453 (21)	225 (15)	131 (11)

Table 8--Continued.

Plasma compounds	Counts per ml[1] Time					
Group Number[2]	10 min.	2 hr.	6 hr.	12 hr.	24 hr.	48 hr.
Unknown II						
1	710 (27)	311 (18)	0	0	0	0
2	931 (31)	193 (14)	0	0	0	0
3	210 (14)	391 (20)	0	0	0	0

[1]Numbers in parentheses are standard errors.

[2]Group 1: methionine deficient, minimal vitamin A
Group 2: methionine deficient, supplemented with vitamin A
Group 3: control, methionine and vitamin A supplemented.

Table 9. The incorporation of ^{35}S-cysteine and ^{35}S-methionine into the plasma proteins of chicks receiving various methionine and vitamin A supplements, counts per milligram[1] of plasma protein precipitate. Group 1: methionine deficient, minimal vitamin A.

Group number[2]	10 min.	Hours 2	6	12	24	48
Cysteine						
1	0	557(24)	680(26)	645(26)	538(23)	444(21)
2	0	1021(32)	925(30)	1003(31)	638(25)	528(23)
3	0	1710(41)	1956(44)	1509(39)	1216(35)	828(29)
Methionine						
1	0	852(29)	752(27)	800(28)	248(16)	251(16)
2	0	1513(39)	1314(36)	822(29)	543(23)	617(25)
3	0	1921(44)	1918(44)	1299(36)	1119(33)	535(23)

[1]Numbers in parentheses are standard errors.

[2]Group 1: methionine deficient, minimal vitamin A
Group 2: methionine deficient, supplemented with vitamin A
Group 3: control, methionine and vitamin A supplemented.

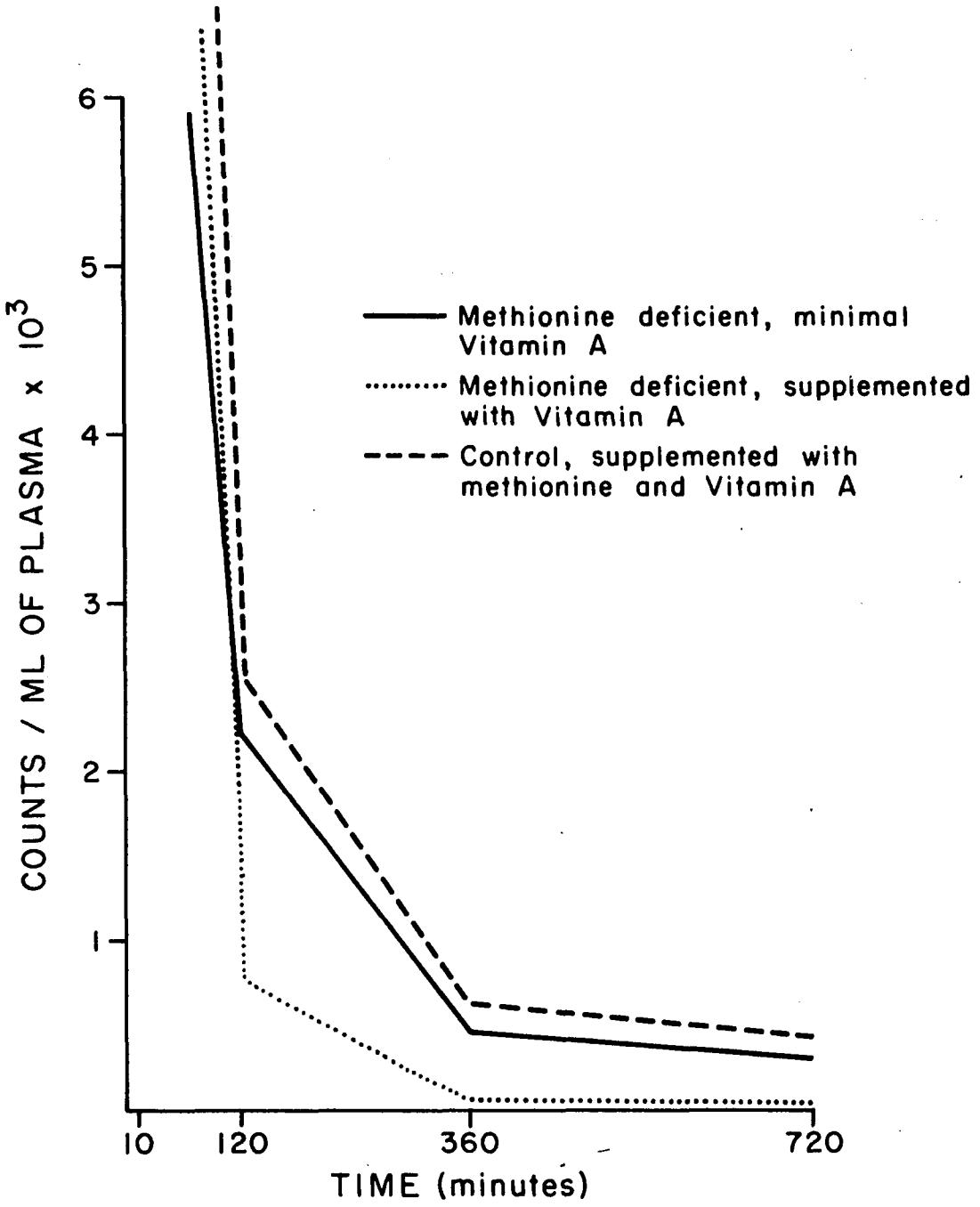

Figure 8. Plasma free ^{35}S-methionine as a function of time for chickens receiving various methionine and vitamin A supplements.

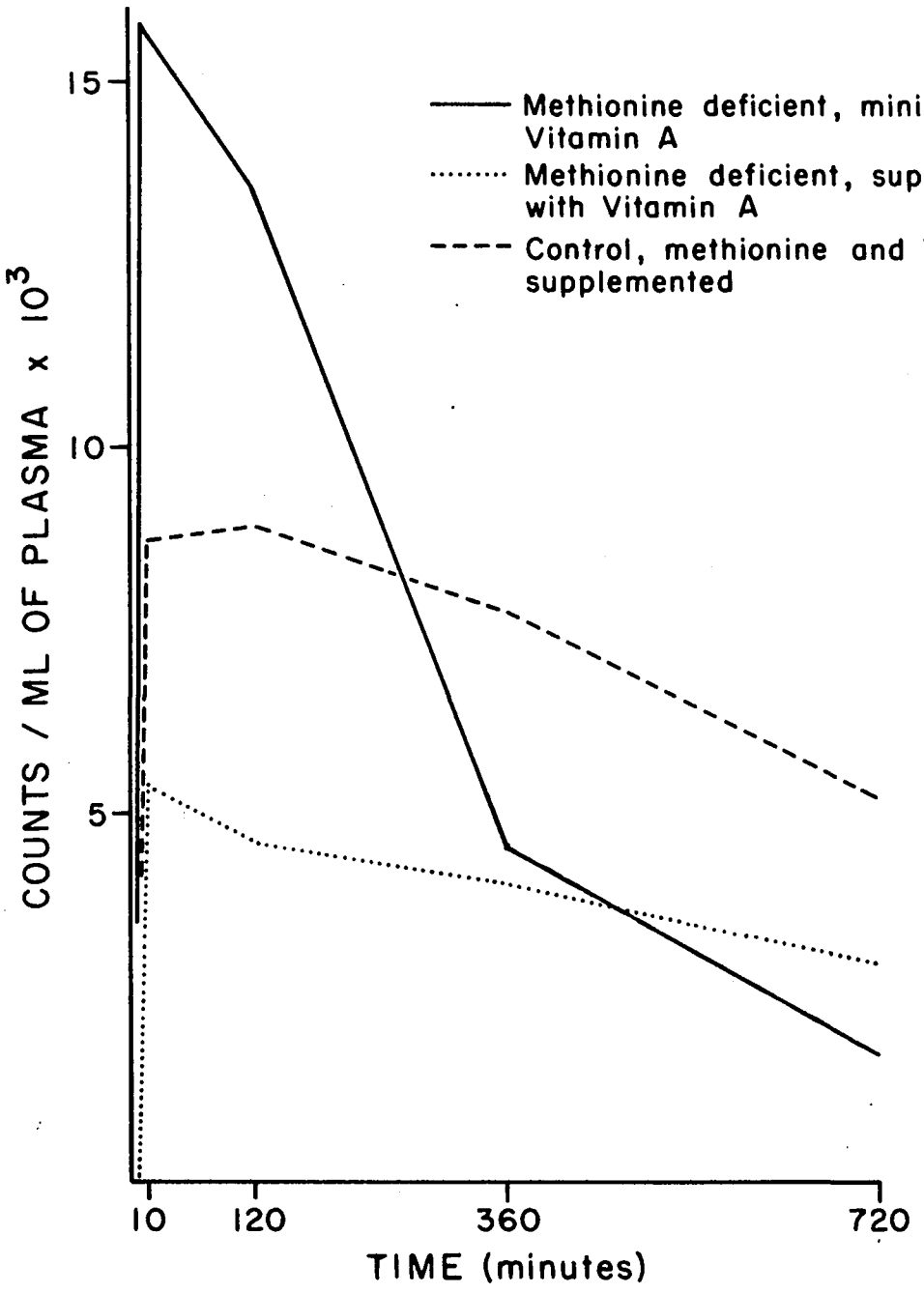

Figure 9. Plasma free ^{35}S-sulfates (counts per milliliter) versus time.

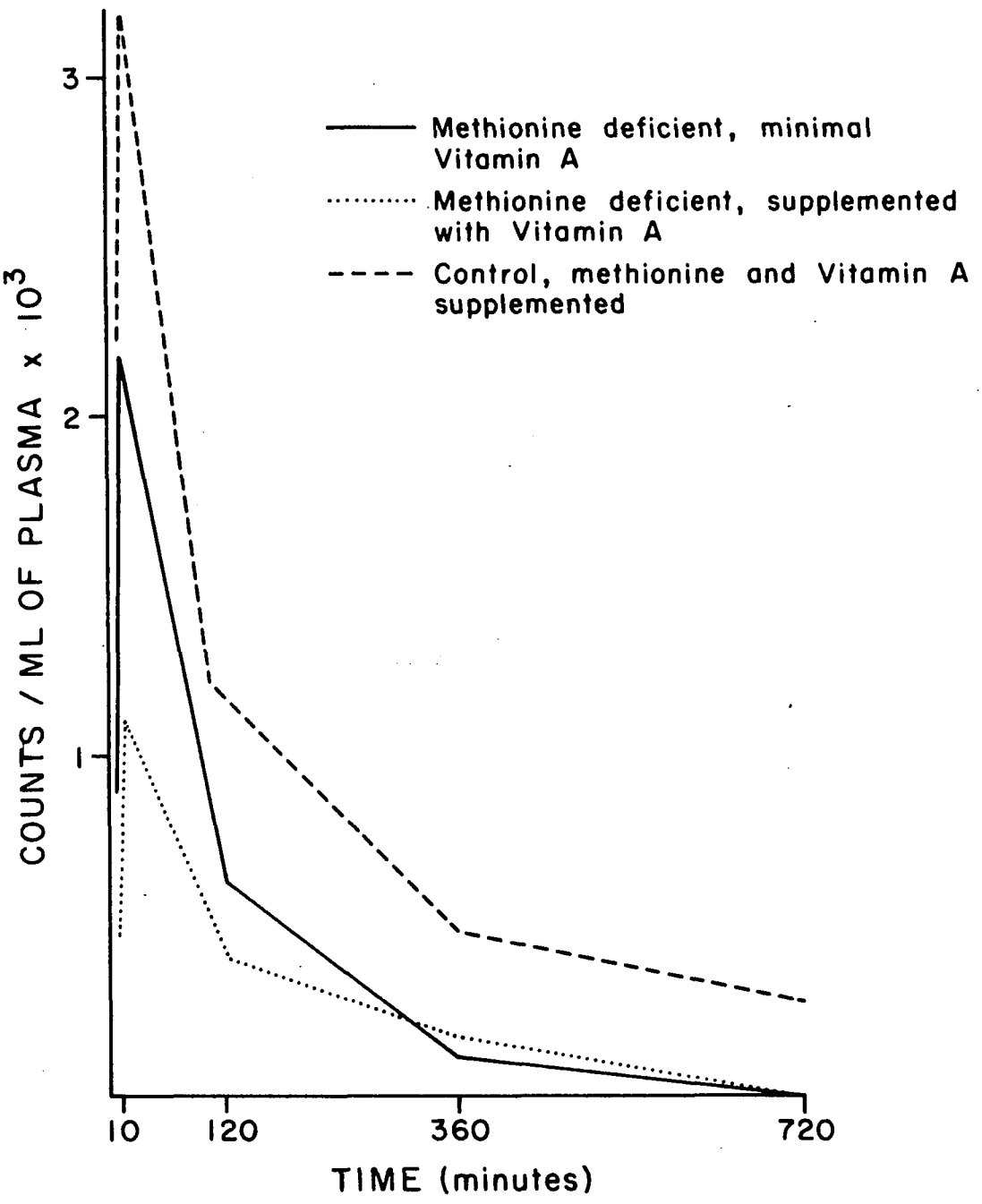

Figure 10. Plasma free ^{35}S-cysteine versus time for chickens receiving various methionine and vitamin A supplements.

Table 10. Total excretion of ^{35}S-sulfate for selected time periods after injection of ^{35}S-methionine by chickens receiving various methionine and vitamin A supplements (counts[1] excreted).

Group[2]	Period (hours)			
	0-6	6-12	12-24	24-48
1	4,919,250	2,505,200	1,688,200	1,180,600
	(27,150)	(19,350)	(15,900)	(13,350)
2	1,408,600	1,585,750	2,973,600	910,800
	(14,550)	(15,450)	(21,150)	(11,700)
3	1,794,250	3,812,600	2,031,200	1,137,250
	(16,350)	(23,850)	(17,400)	(13,050)

[1]Numbers in parentheses are standard errors.

[2]Group 1: methionine deficient, minimal vitamin A
Group 2: methionine deficient, supplemented with vitamin A
Group 3: control, methionine and vitamin A supplemented.

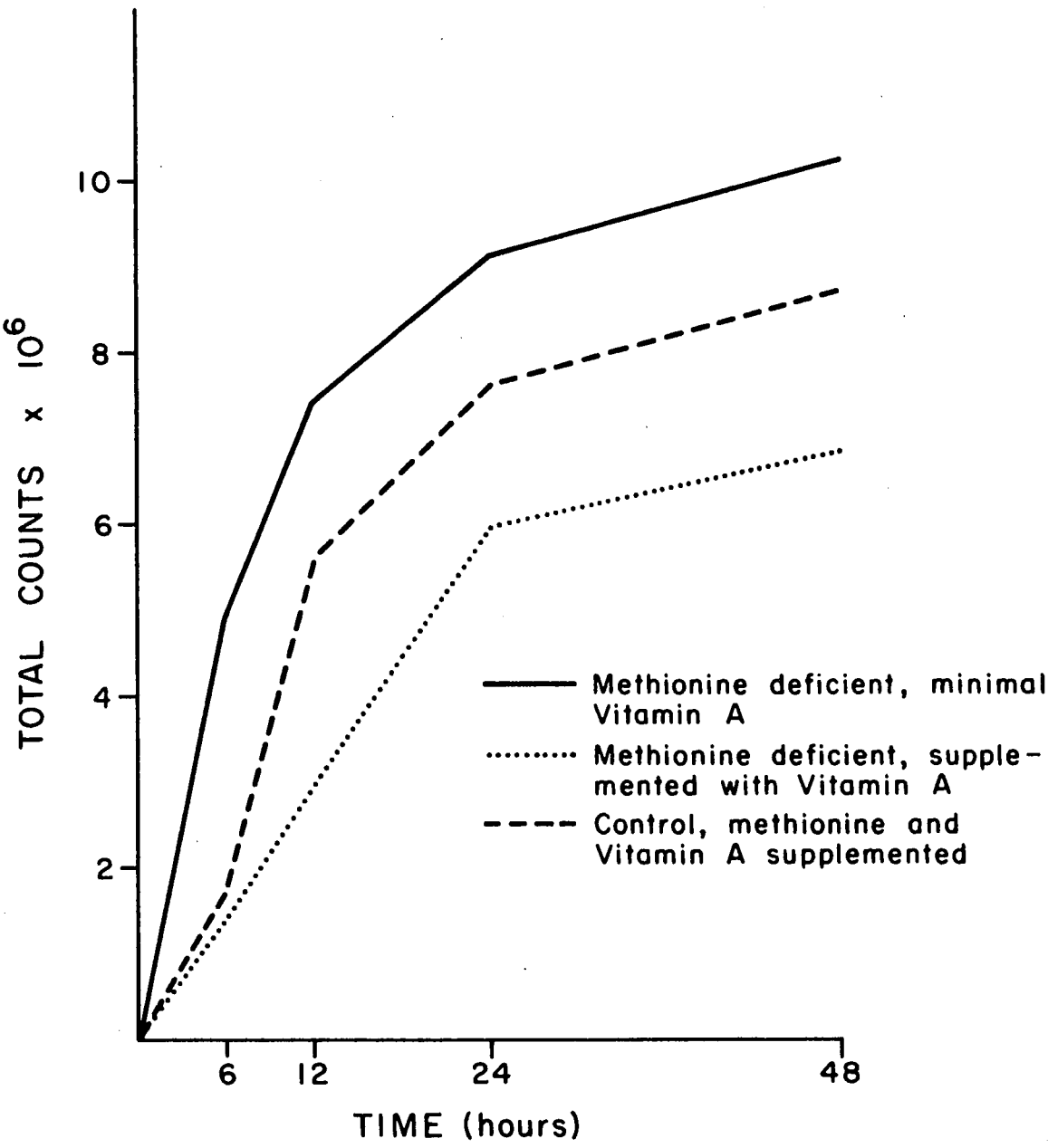

Figure 11. Cumulative ^{35}S-sulfate excretion (counts per total excrement sample) versus time.

Table 11. Incorporation of labeled compounds into tissues of chickens receiving various methionine and vitamin A supplements, counts per gram[1] of wet tissue.

	Group		
	1	2	3
Cartilage Tissue			
Sulfate	6,016 (78)	969 (31)	0
Methionine	4,303 (67)	4,758 (69)	3,540 (59)
Muscle Tissue			
Methionine	18,000	21,300	23,500

[1]Numbers in parentheses are standard errors.

sulfate was excreted. Incorporation of labeled methionine and cysteine into plasma proteins and muscle proteins was retarded, in contrast to the findings of Naito and Kandatsu (1967), and the turnover of plasma proteins, measured by methionine content, was accelerated by a methionine deficiency. More sulfate was incorporated into the cartilage of the deficient birds.

Vitamin A affected the oxidation of the sulfur of methionine in methionine deficient chicks. Comparison of the chickens from Groups 1 and 2 shows that the minimal vitamin A chicken produced three times more sulfates in early plasma samples, excreted three-and-a-half times more sulfate in the first 6 hours, and, over the entire 48-hour period, lost 40% more ^{35}S as sulfate than did the vitamin A supplemented chicken. Six times more sulfate was incorporated into cartilage, but only one-half as much methionine and cysteine were incorporated into plasma proteins in the deficient bird.

Consumption of the basal diet was found to be restricted by a vitamin A deficiency with an ensuing retardation of growth. This was the first outward manifestation of the deficiency. It was feared that this inanition which resulted might be the primary cause of the aberrant sulfur metabolism rather than a primary cause-and-effect relationship, and thus another experiment was designed to corroborate the previous results in chicks which were only recently vitamin A deficient.

Retinoic acid proved to be a perfect tool. Arens and Van Dorp (1946) showed that the carboxylic acid of retinol was biologically active and corrected all lesions of a vitamin A deficiency except those involving reproduction and vision (Howell, Thompson, and Pitt, 1964). The enzymes which form retinoic acid from retinal were identified (Lakshmanan, Vaidyanathan, and Cama, 1964; Futterman, 1962), indicating that the acid was a naturally occurring metabolite. But, in spite of its activity, only small traces could be found in animal tissues and blood (Sharman, 1949; Redfearn, 1960; Jurkowitz, 1962; Krishnamurthy, Bieri, and Andrews, 1963). The ability to grow chicks with retinoic acid replacing vitamin A, but with no vitamin A storage, was of great value. The removal of the retinoic acid supplement induced a vitamin A deficiency quickly and tests could be made on the fate of ^{35}S-methionine before the onset of severe inanition.

The vitamin A-free basal diet was fed to twelve chicks for four weeks. Each chick received a daily oral dose of 50 micrograms of retinoic acid in 0.1 ml of corn oil protected with alpha-tocopherol. After four weeks the chickens were divided into three groups: Group A received a corn oil dose only; Group B received 25 micrograms of vitamin A acetate in corn oil; and, Group C received 250 micrograms of vitamin A acetate in corn oil. These treatments were continued for seven days with daily feed consumption recorded and body weights measured every other day. Feed consumption and body weight data

are displayed in Figures 12 and 13, respectively. Food consumption of the vitamin A-free group dropped markedly after three days. Irregularity of gait appeared on the sixth day and general ataxia on the seventh. The vitamin A-supplemented groups increased their weight and food consumption. On the seventh day, after the previously described fasting schedule, one chicken selected from each group was injected by heart puncture with 300 μCi of ^{35}S-methionine in isotonic saline. Plasma samples were collected at ten minutes and two hours. The chicks were then sacrificed, and breast muscle and liver tissue were frozen for later analysis. The total excrement for the two hour period for each bird was collected and treated as in the previous experiment. Data for radioactive compounds free in the plasma are displayed in Table 12. Plasma protein incorporation, muscle tissue incorporation, and the amount of label appearing in the various free compounds in muscle and liver tissue are displayed, respectively, in Tables 13, 14, 15, and 16. The data corroborates that of previous experiments. A vitamin A deficiency induced the early oxidation of methionine to methionine sulfoxide, methionine sulfone, and other oxidation products, and the resulting incorporation of sulfate into tissues or its excretion.

It is of interest to note that the remaining vitamin A-deficient chicks which had not been selected for injection were kept in that state for five more days. Weakness increased and general

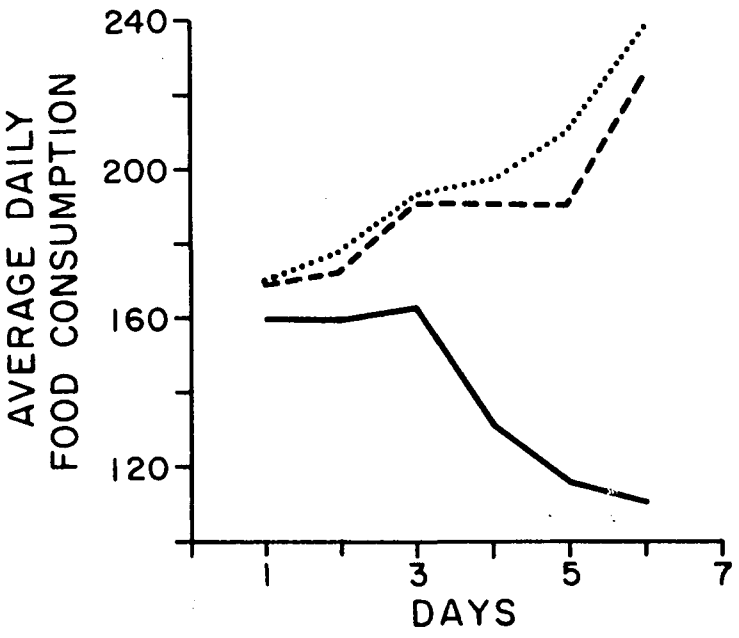

Figure 12. Feed consumption following replacement of retinoic acid with vitamin A.

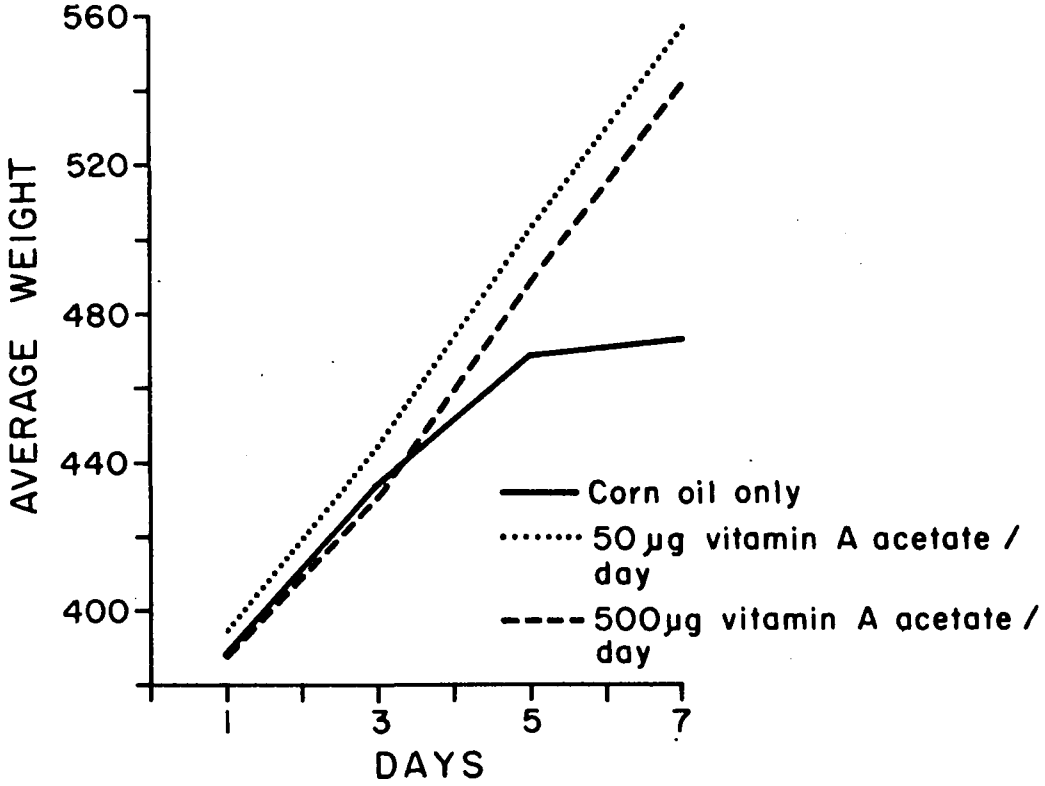

Figure 13. Growth following replacement of retinoic acid with vitamin A.

Table 12. ^{35}S-labeled plasma free components in methionine deficient chicks receiving various vitamin A supplements, counts per milliliter.[1]

Plasma compound / Treatment[2]	Time	
	10 minutes	2 hours
Sulfates		
A	48,428 (220)	39,272 (198)
B	9,098 (95)	4,298 (66)
C	8,810 (94)	5,858 (77)
Methionine Sulfoxide		
A	10,588 (103)	1,204 (35)
B	6,930 (83)	1,366 (37)
C	7,382 (86)	500 (22)
Cysteine		
A	4,864 (70)	250 (16)
B	100 (10)	trace
C	0	0
Methionine		
A	1,052 (32)	1,028 (32)
B	39,814 (200)	3,228 (57)
C	31,028 (176)	706 (27)
II Unknown		
A	128 (11)	trace
B	736 (27)	trace
C	878 (30)	0

[1]Numbers in parentheses are standard errors.

[2]Treatment A: corn oil, only.
Treatment B: 50 µgm Vitamin A acetate/kg of diet
Treatment C: 500 µgm Vitamin A acetate/kg of diet.

Table 13. Plasma protein incorporation of ^{35}S-labeled compounds in methionine-deficient chickens receiving various vitamin A supplements, counts per mg[1] of protein precipitate.

Plasma compound / Treatment[2]	Time: 10 minutes	Time: 2 hours
Methionine		
A	334 (18)	583 (24)
B	trace	1,319 (36)
C	140 (12)	1,440 (38)
Cysteine		
A	897 (30)	950 (31)
B	0	170 (13)
C	0	142 (12)
II Unknown		
A	0	235 (15)
B	0	151 (12)
C	0	41 (6)

[1]Numbers in parentheses are standard errors.

[2]Treatment A: corn oil, only.
Treatment B: 50 µgm Vitamin A acetate/kg of diet
Treatment C: 500 µgm Vitamin A acetate/kg of diet.

Table 14. ^{35}S-labeled compounds free in muscle tissue of methionine-deficient chicks receiving various vitamin A supplements, counts per gram[1] of wet tissue.

	Treatment[2]		
	A	B	C
Sulfate	4051 (64)	1405 (37)	434 (21)
Methionine Sulfoxide	3280 (57)	3738 (61)	1031 (32)
Methionine	9436 (97)	5304 (73)	1031 (32)

[1]Numbers in parentheses are standard errors.

[2]Treatment A: corn oil, only.
Treatment B: 50 µgm Vitamin A acetate/kg of diet
Treatment C: 500 µgm Vitamin A acetate/kg of diet.

Table 15. ^{35}S-labeled compounds bound in muscle tissue of methionine-deficient chicks receiving various vitamin A supplements, counts per gram[1] of wet tissue.

	Treatment[2]		
	A	B	C
Sulfate	2,519 (50)	735 (27)	866 (29)
Methionine	24,994 (158)	83,146 (288)	trace

[1]Numbers in parentheses are standard errors.

[2]Treatment A: corn oil, only.
Treatment B: 50 µgm Vitamin A acetate/kg of diet
Treatment C: 500 µgm Vitamin A acetate/kg of diet.

Table 16. ^{35}S compounds free in liver tissue of methionine deficient chicks receiving various vitamin A supplements, counts per gram[1] of wet tissue.

	Treatment[2]		
	A	B	C
Sulfates	69,615(264)	43,113(208)	26,025(161)
Unknown I	7,772 (88)	3,832 (62)	2,461 (50)
Methionine sulfoxide	262,497(512)	46,021(215)	14,283(120)
Methionine sulfone	31,028(176)	13,367(116)	3,956 (63)
Cysteine	21,848(148)	0	0
Methionine	16,809(130)	13,346(116)	5,531 (74)

[1]Numbers in parentheses are standard errors.

[2]Treatment A: corn oil, only.
Treatment B: 50 µgm Vitamin A acetate/kg of diet
Treatment C: 500 µgm Vitamin A acetate/kg of diet.

debilitation ensued. After they had reached a state of total collapse with uncontrollable tremors, the retinoic acid supplement was returned and within two days the chicks were able to move about with difficulty and had voracious appetites when supported at the feed trays. Five days of supplementation returned these chicks to normal, although stunted, condition.

Table 17. Excretion of S^{35} sulfate in two hours by methionine deficient chicks receiving various vitamin A supplements.

Treatment[1]	Total counts excreted[2]
A	3,239,200 (22,350)
B	1,270,000 (13,800)
C	1,307,600 (14,250)

[1]Treatment A: corn oil, only
Treatment B: 50 µgm Vitamin A acetate/kg of diet
Treatment C: 500 µgm Vitamin A acetate/kg of diet.

[2]Numbers in parentheses are standard errors.

DISCUSSION AND CONCLUSIONS

Methionine injected directly into the heart of a chicken is quickly distributed into the various fluid compartments of the body. The use of Indocyanene Green dye injected in a similar manner indicated that the time required for the injected material to be distributed uniformly in the plasma was less than one minute. Simple calculations of the dilution of labeled methionine in a ten minute period show it to be disappearing at a rapid rate. One μCi of labeled material results in a peak on the scintillator recorder of approximately 238,000 counts. Three hundred μCi, the approximate size of a normal dose, would then give a peak of 71,400,000 counts. The volumes of the fluid compartments, or per cents of body weight, are discussed by Sturkie (1965). For a hypothetical 600 gram chicken the plasma volume is approximately 6% of the body weight, or 36 ml. The homogeneous distribution of a 300 μCi dose would result in approximately 1,983,000 counts per peak per ml of plasma, much greater than the amount actually observed in the ten minute plasma samples. Similar calculations for the distribution of the dose in the plasma plus interstitial fluid (37% of body weight, or 222 ml) results in 322,000 counts per peak per ml of plasma. Distribution of the label in plasma, interstitial fluid, and intracellular fluid (25% of body

weight, or 150 ml) results in a value of 140,000 counts per peak per ml of plasma. The total amount of label found in most ten minute plasma samples, the sum of counts for all labeled peaks, indicated that, generally, the methionine was well distributed throughout the tissues and the entire dose could not be accounted for even in these early samples. Some of the discrepancy may have been caused by the accumulation of the amino acid in the interstitial fluid, the "amino acid pool." The relatively high amount remaining in this unbound state in 2-hour muscle tissue samples indicated that this tissue was indeed accumulating amino acids at the expense of the plasma. This casts some doubt, therefore, on the suitability of plasma amino acid levels as a measure of amino acid metabolism. Levels of amino acids free in the tissue and their disappearance would be better parameters to examine.

The variability in the observed data can be attributed to two sources; variation in the measurement of radioactive decay due to its random nature, and variance caused by biological variation between individual birds. The standard error of measurement can be estimated as the square root of the observed number of counts, as discussed above. Due to the work-load of the analyzing instrument and the high cost of the labeled methionine, only one bird from each treatment was injected, giving no estimate of individual variation. Some conclusions can be reached, however. Examination of the

coefficients of variation (CV) for many physiological phenomena is of interest in making an estimate of the variability one might expect in the data reported here. Review of published and unpublished data[1] reveals a trend in the individual biological variability. Coefficients of variation for plasma amino acid concentrations, plasma protein concentration, and amino acid absorption all fall in a range from 5 to 10%. Incorporation of labeled amino acids in muscle and liver tissue exhibit a CV between 5 and 8%, while excretions of labeled metabolic end-products have CVs of 7 to 12%.

Several of the factors which increase biological variability were minimized in the experiments undertaken. Variations caused by age or diet were eliminated. Variation due to genetic factors, probably the major cause of variation, were controlled by the use of homogeneously in-bred chicks. Variations due to differences in amino acid absorption were avoided by the injection of the labeled methionine directly into the blood stream.

Assuming that external sources of variation are minor and considering the variation reported in the literature, it is not unreasonable to assume a CV for biological variability of approximately 10% in the data reported here. Within the range of count data reported, 100 to 150,000 counts, the CV attributed to measurement error is maximized

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1. Department of Poultry Science, University of Arizona, Tucson.

at 10% at low levels of activity. The combined estimated standard error of a single observation is therefore:

$$S = \sqrt{S_b^2 + S_m^2} = \sqrt{(.1\bar{X})^2 + (.1\bar{X})^2} = .14\,\bar{X}$$

or a combined CV of 14%. If a "t" test for the difference between two means is used, as follows,

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{2S^2}} \quad \text{or,} \quad t = \frac{\%\bar{d}}{1.4CV}$$

then the per cent difference required must exceed (1.4 "t" α CV $\leq \%\,\bar{d}$) to be significant at an arbitrary value of α. By inserting the tabled value for the 0.1 probability of a larger value of "t," 2.9, the $\%\,\bar{d}$ necessary for a significant difference is found to be 57%. At higher activities of approximately 1000 counts the variation due to measurement error becomes insignificant and the $\%\,\bar{d}$ necessary to judge a difference significant is reduced to 41%. Thus a yard stick has been established for the statistical evaluation of the differences observed. If high activity observations differ by 41%, they may be judged significantly different at the 10% level.

Before examining the effect of vitamin A upon a methionine deficiency, it is of interest to look first at the effect a methionine deficiency itself has upon the fate of the sulfur atom of methionine. Considering the results of several of the previously described

experiments, it is evident that methionine is utilized more efficiently in the deficient animal. A smaller amount of ^{14}C-labeled metabolites was found in deficient birds injected with L-methionine-carboxyl-^{14}C and smaller amounts of labeled sulfates, methionine sulfoxide and cysteine were found in the plasma of deficient birds injected with ^{35}S-methionine. Less sulfates were found in the plasma of these birds and their sulfate excretion was reduced by 24%. One might expect that this conservation of methionine by the deficient bird would increase the incorporation of methionine into plasma and tissue proteins. This was not found to be the case. Because of the high specific activity of the labeled methionine, the small amount of methionine injected was not sufficient to accelerate protein synthesis in the birds consuming the unbalanced amino acid diet. The rate of incorporation was diminished but the methionine evidently remained in the intracellular space of the tissues, the "amino acid pool," rather than being degraded. When adequate methionine is supplied, some of it is oxidized, some donates its sulfur atom to homoserine to form cysteine, and some is incorporated into protein. A deficiency, on the other hand, activates some "conservation mechanism" which retards the decomposition of methionine and retains it as the essential amino acid.

A vitamin A deficiency apparently destroys the animal's ability to conserve methionine by opening up an oxidative pathway. The

methionine is oxidized very quickly, possibly on its first passage through the liver before it can infiltrate the tissue intracellular space. This oxidation was especially marked in the chicks which were maintained on retinoic acid and were for all practical purposes, vitamin A free upon the removal of this supplement. The large quantity of methionine sulfoxide and methionine sulfone in the liver tissue of these birds indicate that these are the intermediates by which the methionine is successively oxidized in the vitamin A deficient state. This accelerated oxidation is also reflected in the increased excretion of inorganic sulfate; 50% more in the first two hours and 3.5 times as much in the first 6 hours. The increased availability of sulfate is also demonstrated by the increased amount of sulfate incorporated into mucopolysaccharides in cartilage, 6 times as much in a 48 hour period.

The effect of vitamin A on sulfated-mucopolysaccharide formation and the resulting changes in epithelial and secretory tissues can possibly be explained by the control of vitamin A upon the availability of sulfate for incorporation into mucopolysaccharides rather than a primary involvement in the enzymatic activation of sulfate. If the formation or activation of the enzyme is dependent upon the sulfate concentration, a vitamin A deficiency, by its acceleration of sulfate formation, could account for the changes in secretory ability of these tissues. A high level of vitamin A blocks the formation of sulfates and

in so doing, alters the ratio of unsulfated to sulfated mucopolysaccharides. The formation of mucopolysaccharides with varying degrees of sulfation could account for changes of secretory ability of these tissues also.

No attempt has been made to advance this sparing of methionine as the metabolic role of vitamin A. It does, however, compliment the work of Balakhovsky and Drozdova in showing the involvement of vitamin A in sulfur oxidation. It also agrees with those who have examined the stability of the lysosomal membrane and the release of degradative enzymes, and can explain, in part, the results found by those examining mucopolysaccharide sulfation. The primary purpose has been to explain the cause of the growth-promoting effect of vitamin A upon methionine-deficient birds, and this has been accomplished.

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