

Vitamin E deficiency in rabbits receiving a high PUFA diet with and without a non-absorbable antioxidant

1. Incorporation of [1-¹⁴C] glycine into skeletal muscle proteins

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Summary: Previous studies of metabolic changes in vitamin E-deficient animals were often difficult to interpret because the vitamin E-free, high PUFA diets used in such studies may have contained high levels of peroxides. Presumed effects of vitamin E deficiency could have been effects of peroxide consumption. Addition of the non-absorbable polymeric antioxidant Anoxomer to a semisynthetic vitamin E-free diet containing cod liver oil was found to suppress peroxide formation. Nevertheless, this diet produced necrotizing myopathy, characterized by high urinary creatine excretion, muscular weakness, and increased rate of incorporation of [1-¹⁴C] glycine into muscle proteins. These effects were prevented by vitamin E supplementation. The increased rate of muscle protein turnover is obviously the result of vitamin E deficiency, not the result of consumption of dietary peroxides.

Zusammenfassung: Frühere Untersuchungen über Stoffwechselveränderungen bei Tieren mit Vitamin-E-Mangel waren oft schwer zu interpretieren, da die verwendeten PUFA-reichen Diäten Peroxide in hoher Konzentration enthalten konnten. Vermutete Wirkungen eines Vitamin-E-Mangels hätten Wirkungen des Peroxidverzehr sein können. Durch Zugabe des nichtresorbierbaren polymeren Antioxidationsmittels Anoxomer zu einer semisynthetischen, Vitamin-E-freien, lebertranhaltigen Diät kann die Peroxidbildung unterdrückt werden. Trotzdem verursachte diese Diät nekrotisierende Myopathie, charakterisiert durch hohe Kreatinausscheidung im Urin, Muskelschwäche und erhöhte Rate der Inkorporation von [1-¹⁴C]-Glycin in die Muskelproteine. Diese Effekte wurden durch Vitamin-E-Supplementierung verhindert. Die erhöhte Turnover-Rate der Muskelproteine ist offensichtlich die Folge des Vitamin-E-Mangels, nicht die Folge des Peroxidverzehr.

Key words: vitamin E, muscular dystrophy, protein turnover, antioxidant, autoxidation, peroxides, rabbit

Introduction

Interest in animal models of muscular dystrophy has been renewed by recent observations of ultrastructural and histochemical abnormalities of skeletal muscle in patients with chronic vitamin E deficiency (1). Successful treatment of human myopathies of different origin with vitamin E (2, 3) has also contributed to this renewed interest.

Nutritional muscular dystrophy of experimentally malnourished rabbits and guinea-pigs was first reported in 1931 by Goettsch and Pappenheimer

(4), although a causal relationship to vitamin E deficiency was not recognized until several years later (5). Experiments with many species of animals showed muscular dystrophy to be one of the most common manifestations of vitamin E deficiency (6). More recently it has been suggested that the term "muscular dystrophy" should be restricted to the genetically determined human disorder and that the term "nutritionally induced necrotizing myopathy" be applied to the disorder resulting from tocopherol deficiency (7). The abbreviation NINM will be used in the following.

It was recognized early that diets high in polyunsaturated fatty acids (PUFA) accelerate the onset of symptoms of vitamin E deficiency. In numerous experiments designed to characterize NINM, the animals were fed diets containing cod liver oil either as originally described by Goettsch and Pappenheimer (4) or as modified by others (e.g. 8–12). These studies were criticized because such diets quickly autoxidize; it was argued that results may have been caused by the presence of peroxides in the diet, rather than by the lack of tocopherol. Madsen et al. (13) had even suggested that the essential factor in the production of the myopathy was the toxicity of cod liver oil for herbivora. Miyazawa et al. (14) have observed chemiluminescence in tissues of rats fed autoxidized linseed oil and attributed this to the occurrence of free radical species derived from the autoxidized oil.

After Tappel (15) had proposed that vitamin E acts primarily or exclusively by preventing peroxide formation *in vivo*, the use of a diet rich in peroxides seemed inappropriate for studies designed to test this hypothesis. Preventing autoxidation by addition of an antioxidant to the diet was unsatisfactory, as this would make it difficult or impossible to differentiate between effects of tocopherol and effects of the added antioxidant. A new approach to this problem became possible when Dynapol developed Anoxomer, a non-absorbable polymeric antioxidant (16, 17). The present work was designed to test the suitability of this product for studying NINM uncomplicated by dietary peroxides.

Materials and Methods

Diet

The diet was identical with that used earlier (10) except that cellulose, added as roughage, was replaced by wheat bran. To remove tocopherols, the bran was extracted for 3 h in a Soxhlet with petroleum ether (boiling range 40–60 °C). The diet consisted of 11.15 % casein, 27 % corn starch, 29.5 % saccharose, 25 % wheat bran, 2.25 % each of stripped lard, cod liver oil and salt mix, 0.5 % vitamin mix (vitamin E-free). In some batches of diet the non-absorbable antioxidant Anoxomer was added as described below. Water was added to form a dough which was spread out and dried by an electric fan. The dry, hardened dough was broken into cookie-sized pieces.

When used for animal feeding, the diet was freshly prepared every 2 to 3 weeks and was kept refrigerated until use. To study the effect of added Anoxomer the diet was kept at room temperature for up to 8 weeks. Thiobarbituric acid number (18) and peroxide number (19) were determined periodically.

The antioxidant "Poly AO-79 Anoxomer", a condensation polymer of divinylbenzene-hydroquinone-phenols, was obtained from Dynapol, Palo Alto, CA. To achieve

homogeneous distribution in the diet the required amount was dissolved in the cod liver oil before the latter was mixed with the other ingredients.

Animals

White New Zealand rabbits of either sex, initially weighing about 600 g, were provided with diet and tap water ad lib. Animals of control groups received oral supplements of 2 mg of DL- α -tocopherol (Merck, Darmstadt) dissolved in 0.1 ml of corn oil three times weekly. Animals of experimental groups received the same quantity of corn oil without added α -tocopherol. The rabbits were kept individually in metal cages permitting urine collection, in an air-conditioned room (21 °C, 60 % rel. hum.), with automatic 12 h light/dark cycle. Beginning after 2 weeks, urine samples were collected every other day, and creatine and creatinine were determined by the Folin method (20) with minor modifications. Animals were weighed every 2 or 3 days.

When vitamin E-deficient animals exhibited signs of pronounced NINM (elevated urinary creatine/creatinine ratio, muscular weakness), but well before they appeared moribund, they were injected intraperitoneally with an aq. solution of [1-¹⁴C] glycine. Vitamin E-supplemented control animals were treated in the same way. At specified times after injection the animals were stunned, decapitated and allowed to bleed for 1–2 min. Thigh muscles were quickly excised. To isolate total proteins, weighed portions of muscle were homogenized with an equal volume of 10 % trichloroacetic acid (TCA), heated in a boiling water bath for 10 min, centrifuged, and the precipitate was successively washed with cold 5 % TCA, hot methanol, methanol-ether 1:1, and twice with ether (21). Combined TCA extracts were kept refrigerated until use for determination of free glycine. Dried total proteins were used for determination of incorporated [1-¹⁴C] glycine.

First series of [1-¹⁴C] glycine incorporation studies

Twenty-nine animals were fed the diet without added Anoxomer, 14 supplemented with vitamin E, 15 unsupplemented. When symptoms of NINM appeared in the unsupplemented rabbits after about 26 days, the animals (supplemented and unsupplemented) were injected i.p. with 100 μ Ci (3.70 MBq) of [1-¹⁴C] glycine per kg of body weight (specific activity 4.84 mCi (179 MBq)/mmole; New England Nuclear, Boston, Mass.), and killed after 30 min (5 supplemented, 5 unsupplemented), 1 h (2 suppl., 4 unsuppl.), 2 h (3 suppl., 2 unsuppl.), 4 h (1 suppl., 1 unsuppl.), 12 h (1 suppl., 1 unsuppl.), 24 h (2 suppl., 2 unsuppl.). Samples of total muscle proteins (TCA precipitate) were hydrolyzed with 6 N HCl for 20 h at 110° in sealed tubes, and excess acid was removed under reduced pressure in a rotary evaporator. The amino acids were separated by ion exchange chromatography, essentially as described by Moore, Spackman and Stein (22). Duplicate aliquots of eluate fractions were taken for determination of amino acids by the ninhydrin method, and for determination of radioactivity by drying on metal planchets and counting in a windowless continuous gas-flow counter.

TCA-extracts were freed of TCA by extraction with ether and concentrated under reduced pressure in a rotary evaporator. After separation by ion exchange chromatography amino acids and radioactivity were determined as described.

Radioactivity was also determined, without prior amino acid separation, in the TCA-extract and in the unhydrolyzed proteins. Concentrated TCA-extracts were directly dried on planchets, while proteins were dissolved in 0.2 N NaOH and this solution was transferred to planchets and dried for counting. Counts were corrected for self-absorption when necessary.

Second series of [1-¹⁴C] glycine incorporation studies

Eighteen animals received the diet without added Anoxomer, 8 of these were supplemented with vitamin E (= group A), 10 were unsupplemented (= group A'); 8

animals received the diet with 10 mg Anoxomer per g fat, 3 supplemented (= group B), 5 unsupplemented (= group B'); 8 animals received the diet with 50 mg Anoxomer per g fat, 3 supplemented (= group C), 5 unsupplemented (= group C'). When symptoms of NINM appeared in the unsupplemented animals, both supplemented and unsupplemented rabbits were injected i.p. with 50 μ Ci (1.85 MBq) of [$1-^{14}$ C] glycine per kg of body weight (specific activity 55.4 mCi (2.05 GBq)/mmole, Amersham-Buchler, Braunschweig). Two hours later the animals were killed and thigh muscle total proteins were prepared as described. For measurement of radioactivity 200 mg of dry protein were mixed with 0.2 ml of water and 2 ml of Protosol (New England Nuclear, Boston, Mass.) and heated in a 50 °C water bath until completely dissolved. After cooling, 18 ml of scintillation cocktail (6 g PPO in 1 l toluene) were added, and radioactivity was determined by liquid-scintillation counting.

Radioactivity in the protein-free TCA supernatant was determined by mixing 5 ml TCA extract with 15 ml of scintillation cocktail (892.7 ml dioxane, 7.0 g PPO, 0.3 g POPOP, 100 g naphthalin) and counting in the same counter.

Results and Discussion

The first series of [$1-^{14}$ C] glycine studies showed a faster rate of incorporation into the total thigh muscle proteins of animals not supplemented with vitamin E, and also a faster disappearance of the label. This is obvious from the specific activity of the proteins (Fig. 1a) and of glycine isolated from the hydrolyzate of the proteins (Fig. 1b). Specific activity at 30 min after injection was at the limit of detectability and was therefore not

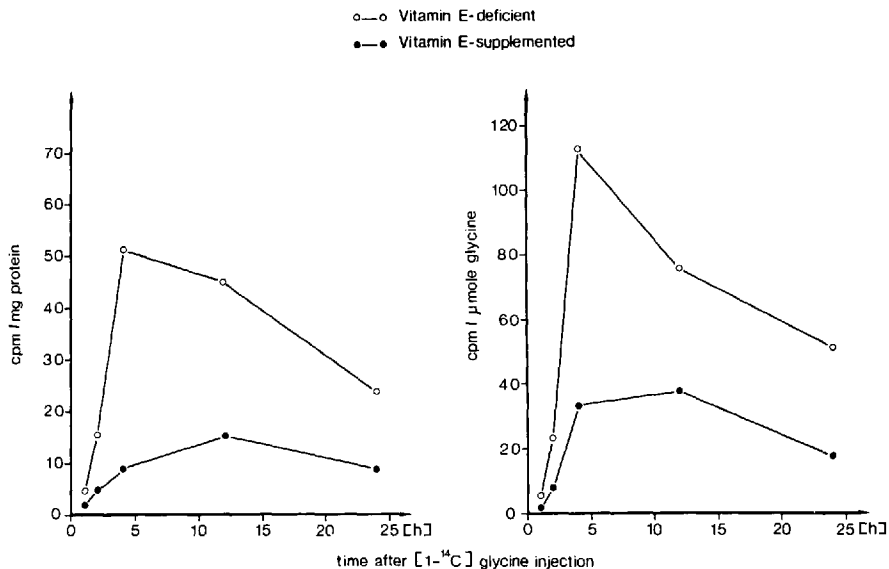


Fig. 1. Effect of vitamin E deficiency on incorporation of [$1-^{14}$ C] glycine into total proteins of rabbit skeletal muscle at different time periods after injection. 1a: Specific activity of protein. 1b: Specific activity of glycine isolated from protein hydrolyzate. For number of animals from which the indicated mean values were obtained see Methods section ("First series").

Table 1. Some properties of skeletal muscle of vitamin E-supplemented and vitamin E-deficient animals. Diet without Anoxomer; n = number of animals; P = probability that differences between vitamin E-supplemented and unsupplemented groups were due to chance.

	Vitamin E-supplemented (n = 14)	Vitamin E-deficient (n = 15)	P
Free glycine in muscle tissue (μ moles/g wet wt.)	4.1 $\pm$ 0.9	3.7 $\pm$ 0.9	> 0.1
TCA-precipitable protein in muscle tissue (mg/g wet wt.)	209 $\pm$ 8	164 $\pm$ 20	< 0.05
Glycine content of muscle proteins (μ moles/g dry weight wt.)	514 $\pm$ 64	536 $\pm$ 91	> 0.1

presented in Figure 1. Determination of radioactivity in all fractions of the eluate obtained by ion exchange chromatography showed that 80–90 % of the total radioactivity was present in the glycine peak, 10–15 % in the serine peak. Vitamin E deficiency had no effect on the distribution of ^{14}C between glycine and serine. To save space, only the glycine values are presented.

A high rate of amino acid incorporation could be indicative of a high rate of protein synthesis – but it might also be caused by a high specific activity of the precursor pool. If, for instance, the vitamin E-deficient animals had a lower concentration of free glycine in the muscle tissue, the ^{14}C -labeled glycine would be less diluted with unlabeled glycine. As shown in Table 1,

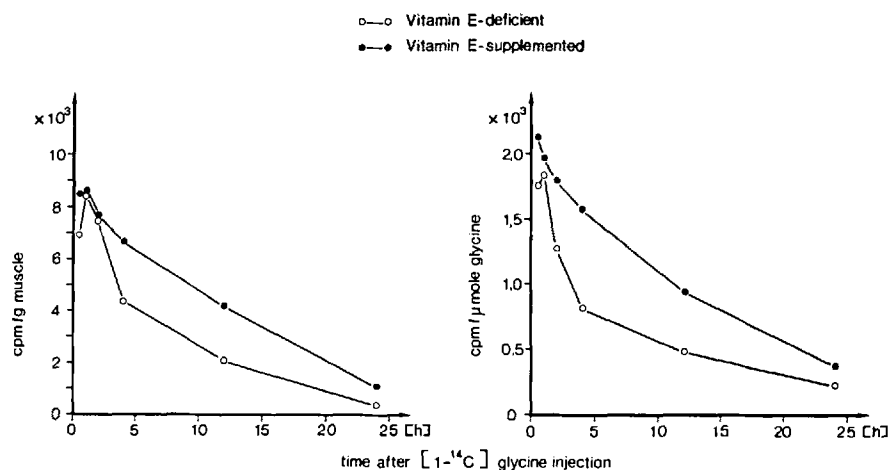


Fig. 2. Effect of vitamin E deficiency on free glycine radioactivity in rabbit skeletal muscle at different time periods after injection of $[1-^{14}\text{C}]$ glycine. 2a: ^{14}C -activity in trichloroacetic acid extract of muscle tissue. 2b: Specific activity of glycine isolated from trichloroacetic acid extract of muscle tissue.

however, the concentration of free glycine in muscle tissue was not significantly different in supplemented and unsupplemented animals. The other data in Table 1 indicate that NINM muscle contains significantly less total protein per g tissue, while the concentration of bound glycine in muscle proteins is not significantly affected by NINM.

During the first hour after injection of [$1-^{14}\text{C}$] glycine the label appeared at about the same rate in the free amino acid pool of supplemented and unsupplemented animals, as indicated by ^{14}C -activity in the unfractionated TCA extract (Fig. 2a) or by the specific activity of isolated free glycine (Fig. 2b). At later time periods, lower ^{14}C -glycine levels were observed in the unsupplemented animals. The higher rate of incorporation into muscle proteins of the vitamin E-deficient animals is in good agreement with earlier observations (23, 24). This higher rate of amino acid incorporation was also observed when muscle homogenates obtained from vitamin E-deficient animals were incubated in vitro (25), which strengthens the conclusion that the higher rate of incorporation reflects a higher rate of muscle protein synthesis in the vitamin E-deficient animals, and not a higher specific activity of the precursor pool. The myopathy is clearly not caused by a defect in protein synthesis. The observed faster disappearance of the labeled glycine from the free amino acid pool in muscle of vitamin E-deficient animals (Fig. 2a, b) may be related to effects of vitamin E deficiency on amino acid transport in skeletal muscle (26, 27).

At this stage of the investigation the question remained, whether the observed phenomena depended on the presence of peroxides in the diet. The second series of [$1-^{14}\text{C}$] glycine studies was carried out to answer this question with the aid of the nonabsorbable antioxidant Anoxomer. As indicated in Figures 3 and 4, diet prepared without added Anoxomer exhibited the earliest increase in peroxide number and TBA number and reached the highest peak values of these indicators of autoxidation. Increasing concentrations of Anoxomer exerted increasing protection. Addition of 10 mg Anoxomer per g of fat prevented autoxidation for about

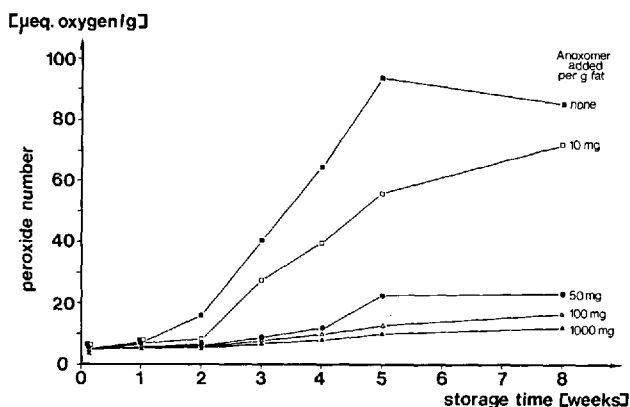


Fig. 3. Peroxide number of vitamin E-free semisynthetic diet, with different amounts of Anoxomer added, after various periods of storage at ambient temperature.

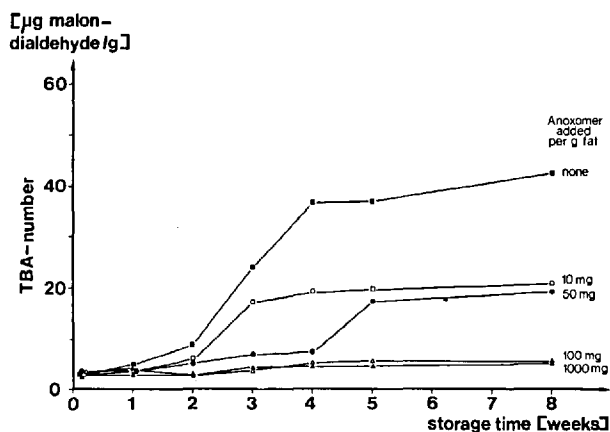


Fig. 4. Thiobarbituric acid number of vitamin E-free semisynthetic diet, with different amounts of Anoxomer added, after various periods of storage at ambient temperature.

2 weeks, while 50 mg protected for about 4 weeks. In this context it is of interest that Ke et al. (28) found Anoxomer effective in the protection of high PUFA mackerel lipids. It is also noteworthy that the Food and Drug Administration has cleared Anoxomer as an antioxidant in food at a level of not more than 5 mg/kg fat (29). For the diet used here, this level probably would have been insufficient.

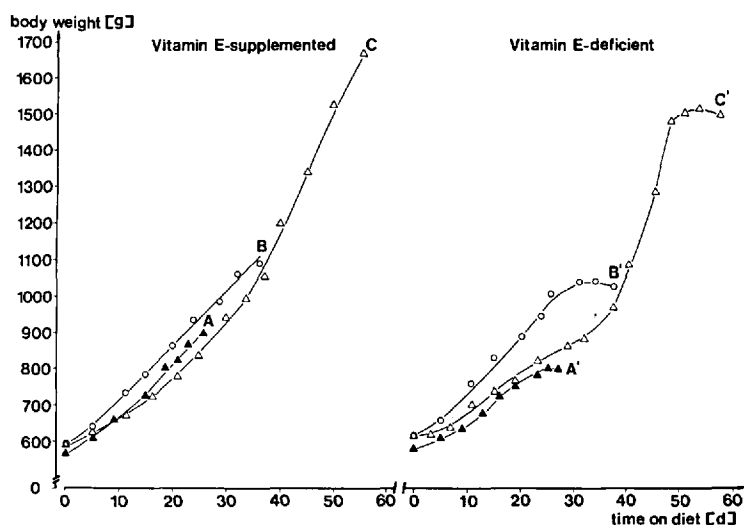


Fig. 5. Growth curves of rabbits receiving different levels of Anoxomer in the diet. Mean body weights of animals in diet groups A, B, C (Fig. 5a), and A', B', C' (Fig. 5b). For explanation of diet groups and number of animals see Table 2.

In order to establish whether suppression of peroxide formation in the diet would influence the results of $[1-^{14}\text{C}]$ glycine studies, the second series of experiments was carried out with groups of animals receiving different levels of Anoxomer in the diet. Rabbits showed better growth with diet B (10 mg Anoxomer/g fat) than with diet A (no Anoxomer) or C (50 mg Anoxomer/g fat), both in the groups supplemented with vitamin E (Fig. 5a) and in the unsupplemented groups (Fig. 5b).

The vitamin deficiency syndrome led to a plateau and finally to a slight decrease in body weight, first in group A', then B' and finally C' (Fig. 5b). The myopathy is associated with steeply increasing urinary creatine excretion (30), expressed in Figure 6 as the ratio of urinary creatine/creatinine. Muscular weakness becomes increasingly evident at this stage: when placed on its side the animal can right itself less quickly, and when this procedure is repeated several times, the animal tires earlier than controls supplemented with vitamin E.

When pronounced muscular weakness was noted after 25 to 28 days in group A', 35 to 37 days in group B' and 40 to 57 days in group C', the animals (and at the same time those in groups A, B, C) were injected intraperitoneally with $[1-^{14}\text{C}]$ glycine and sacrificed 2 h later. As indicated in Table 2, TCA-soluble radioactivity per g thigh muscle was not significantly affected by vitamin E deficiency or Anoxomer in the diet. In contrast, specific activity of thigh muscle proteins was approximately doubled in group A' as compared to group A, and more than doubled in the groups fed Anoxomer-containing diets B' and C' as compared to B and C. The apparent increase in protein specific activity from A to B to C, and A' to B' to C' may be related to the higher age and body weight reached by animals in the groups fed Anoxomer.

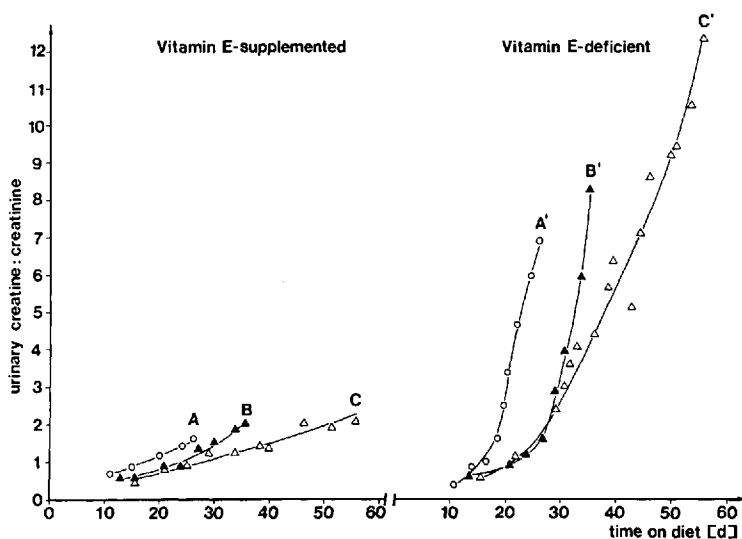


Fig. 6. Urinary creatine/creatinine ratio of rabbits receiving different levels of Anoxomer in the diet. Mean values in diet groups A, B, C (Fig. 6a) and A', B', C' (Fig. 6b).

Table 2. Effect of vitamin E deficiency on incorporation of $[1 - ^{14}\text{C}]$ glycine into total proteins of thigh muscles; arith. mean $\pm$ standard deviation. Letters in parentheses indicate diet group, figures in parentheses indicate number of animals per group. P = probability that the differences between vitamin E-supplemented and deficient groups were due to chance (Student's t-test); cpm = counts per min

Diet	Spec. act. of muscle proteins, cpm/mg protein			Radioactivity in protein-free supernatant, cpm/g tissue wet wt.		
	Vitamin E-supplemented	Vitamin E-deficient	P	Vitamin E-supplemented	Vitamin E-deficient	P
No Anoxomer	(A: 8) 13.2 ± 8	(A': 10) 30.1 ± 14.1	< 0.01	(A: 8) 24 300 $\pm 12 150$	(A': 10) 26 160 $\pm 9 190$	> 0.1
10 mg Anoxomer/g fat	(B: 3) 15.2 ± 3.0	(B': 5) 50.0 ± 6.8	< 0.001	(B: 3) 21 560 $\pm 6 350$	(B': 5) 25 390 $\pm 8 410$	> 0.1
50 mg Anoxomer/g fat	(C: 3) 22.5 ± 7.8	(C': 5) 57.3 ± 12.3	< 0.005	(C: 3) 30 610 $\pm 13 710$	(C': 5) 28 870 $\pm 12 330$	> 0.1

In contrast to the experiments of the first series, no attempt was made in this second series to isolate glycine by ion exchange chromatography. Comparison of Figure 1a with 1b and 2a with 2b shows that the same conclusions can be drawn without the laborious isolation of glycine.

The results of the second series show clearly that the myopathy and the higher rate of glycine incorporation into muscle proteins were a result of vitamin E deficiency, not a consequence of dietary peroxide intake.

Since 10 mg of Anoxomer/g fat in the diet resulted in better weight gain than 50 mg, and gave sufficient protection against autoxidation, it is suggested that this level is best suited to study effects of vitamin E deficiency uncomplicated by possible effects of peroxide ingestion. With regard to studying the possible role of vitamin E in the regulation of protein synthesis, it is noteworthy that the comparison of groups B and B' (i.e. those receiving 10 mg Anoxomer/g fat) showed the greatest effect on glycine incorporation, both numerically and with regard to statistical significance ($P < 0.001$). A high PUFA diet protected in this way by a non-absorbable antioxidant should be a useful tool also in other studies designed to elucidate the biological function of vitamin E.

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