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Vitamin E deficiency in rabbits receiving a high PUFA diet with and without a non-absorbable antioxidant II. Incorporation of ^{14}C-labelled glycine and L-leucine into liver and plasma proteins

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Summary: As determined by in vivo studies using [1–^{14}C] L-leucine and [1–^{14}C] glycine, vitamin E deficiency in young rabbits caused a higher turnover rate of liver proteins and of plasma albumin and globulin fractions. This effect was most clearly and consistently observed in animals fed a diet containing 10 mg of the non-absorbable polymeric antioxidant Anoxomer per g of fat in the diet.

Zusammenfassung: Eine erhöhte Turnover-Rate der Leberproteine, des Plasmaalbumins und der Plasmaglobulinfraktionen in Vitamin-E-frei ernährten jungen Kaninchen wurde durch In-vivo-Untersuchungen mit [1–^{14}C]L-Leucin und [1–^{14}C]Glycin festgestellt. Dieser Effekt trat am deutlichsten auf und war am besten reproduzierbar, wenn das Futter der Tiere 10 mg des nicht resorbierbaren polymeren Antioxidans Anoxomer pro g Fett enthielt.

Key words: vitamin E, protein turnover, liver proteins, plasma proteins, antioxidant, autoxidation, peroxides, rabbit

Introduction

The biological function of vitamin E is still not fully understood. Tappel's hypothesis (1) that vitamin E acts primarily or exclusively by preventing peroxide formation in vivo, has provided a stimulus to much further research. What has emerged in the past twenty years is the realization that the initiation and control of peroxidation reactions have far-reaching biological consequences, which we are only beginning to understand (2).

Studies of the role of vitamin E in the prevention of peroxidation have left some questions unanswered, and have led to alternative hypotheses of its biological function (3, 4). Some evidence indicates that vitamin E is involved in genetic regulation at the level of protein synthesis. Olson proposed that vitamin E may serve as a corepressor for the synthesis of specific proteins and enzymes required in the differentiation or adaptation of given tissues (5). While an increased turnover rate of skeletal muscle proteins (6) and of muscle creatine kinase (5) of vitamin E-deficient rabbits has been established, conflicting evidence exists concerning the influence of vitamin E on the turnover of liver proteins. Catignani et al. (7) demon-

strated an elevated rate of synthesis of xanthine oxidase in liver of vitamin E-deficient rabbits and found evidence for an increased rate of general protein synthesis in the deficient liver under in vivo conditions. Bonetti et al. (8) found no significant effect of dietary vitamin E on in vitro incorporation of ^{14}C-leucine into liver proteins of rats, while Reiss and Tappel (9) reported decreased in vitro incorporation of ^{14}C-phenylalanine into liver proteins of rats fed vitamin E-deficient diets. Incorporation of ^{14}C-glycine, leucine and lysine into liver proteins of rabbits seemed to be elevated in vitamin E deficiency, but the difference was not statistically significant (10).

Plasma proteins, with the exception of the γ-globulin fraction, are synthesized in the liver, and vitamin E deficiency was shown to increase the rate of incorporation of ^{14}C-glycine into total plasma proteins (11) and of ^{14}C-leucine into plasma protein fractions of rabbits (12). However, the diets used in these studies may have contained high levels of peroxides, and it was not clear to what extent the observed effects were due to vitamin E deficiency or to the consumption of dietary peroxides. Choosing diets free of unsaturated fatty acids, in order to minimize peroxide formation, would have been unrealistic in view of dietary recommendations demanding a high ratio of unsaturated to saturated fatty acids in human nutrition. Preventing peroxide formation by addition of a synthetic antioxidant to the diet was also unsatisfactory, because this would make it difficult or impossible to differentiate between in vivo effects of vitamin E and the added antioxidant. As described in the preceding publication (6) a new approach to this problem was made possible by the availability of the non-absorbable polymeric antioxidant Anoxomer. The aim of the present work was to study the effect of vitamin E deficiency on amino acid incorporation into liver and plasma proteins of rabbits in the presence and absence of Anoxomer in the diet.

Materials and Methods

Diet and animals

Preparation of the vitamin E-free diet and animal care were as described (6).

Study of [1-^{14}C] leucine incorporation

Sixteen White New Zealand rabbits initially weighing about 600 g were placed on the vitamin E-free diet without Anoxomer. Eight were supplemented with vitamin E. When symptoms of muscular weakness appeared in the unsupplemented animals after about 25 to 28 days, both unsupplemented and supplemented animals were injected intraperitoneally with 100 μ Ci (3.70 MBq) of [1-^{14}C] L-leucine per kg of body weight (specific activity 29.4 mCi (1.09 GBq)/mmole; New England Nuclear, Boston, Mass.). Two supplemented and two unsupplemented animals were killed after 2, 10, 24 and 48 hours. Blood was collected in a heparinized beaker. The liver was removed and immediately cooled on ice. Proteins of homogenized liver and of blood plasma were obtained by the trichloroacetic acid (TCA) method, as previously described for muscle proteins (6). Leucine in the TCA-soluble fraction and in the acid hydrolyzate of liver proteins and plasma proteins was isolated by ion exchange chromatography and determined by the ninhydrin method, as described for glycine from skeletal muscle (6). Radioactivity was also determined as described (6).

Plasma protein fractions were isolated by electrophoresis and their specific activity was determined in the following way:

The Rapid Electrophoresis Chamber of Gelman Instrument Co., Chelsea, MI, was used with Sepraphore cellulose acetate strips and barbiturate buffer pH 8.6, ionic strength 0.055. Five µl of plasma were applied in duplicate. After 2 h of electrophoresis (initially 250 V, 7.2 mA) the strips were removed, blotted to remove excess buffer, permitted to dry for 15 min at ambient temperature, stained in a saturated solution of bromophenol blue in 10 % acetic acid, washed 3 times with acetic acid. The blue bands were cut out, dissolved in 3 ml of dioxane:conc. ammonia:water, 100:5:10. Optical density was read at 600 nm and protein was quantified by comparison with known amounts of bovine albumin run on parallel electrophoresis strips. The stained protein solution was subsequently transferred to nickel-plated planchets, dried, and counted in a windowless continuous gas-flow counter.

Study of [1-^{14}C] glycine incorporation

The rabbits used in this study were the same as those used in the previously described skeletal muscle incorporation study, second series (6). Eighteen animals received the vitamin E-free diet without Anoxomer (8 supplemented with vitamin E, 10 unsupplemented), 8 received the diet with 10 mg Anoxomer per g fat (3 supplemented, 5 unsupplemented). When symptoms of muscular weakness appeared in unsupplemented animals after 25 to 28 days (no Anoxomer), 35 to 37 days (10 mg Anoxomer/g fat), 40–57 days (50 mg Anoxomer/g fat), both unsupplemented and supplemented animals were injected with 50 µCi (1.85 MBq)[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, blood was collected (heparinized) and the liver was removed. Plasma proteins and liver proteins were prepared by the TCA method as described. Radioactivity in liver proteins and in the TCA supernatant of liver was determined in duplicate samples by liquid scintillation counting as described for skeletal muscle (6).

The following method for determining radioactivity in plasma protein fractions was used:

Triplicate samples with 10 µl of plasma were separated on 25 × 170 mm cellulose acetate strips (Cellogel) in a Shandon Model 600 Electrophoresis Chamber. Electrophoresis was carried out for 75 min at 350 V using a 0.05 M sodium veronal buffer, pH 9.5 at a temperature of 5 °C. The gel strips were stained with Ponceau S (0.5 % in 5 % trichloroacetic acid), destained with 5 % acetic acid and made transparent by immersion for 30 s in methanol followed by 1 min in a mixture of methanol:glacial acetic acid:glycerol, 85:14:1 v/v. The transparent strips were dried at 50 °C in a few min, and in two strip fractions corresponding to albumin, globulins 1,2 and 3 were cut out. The gel sections were combusted in an automatic sample oxidizer (Oxymat, Intertechnique), the produced $^{14}CO_2$ being absorbed with phenetylamine. The third strip was combusted unsectioned as a total control. The scintillation cocktail consisted of 320 ml phenetylamine (scint. grade, freshly dist.), 220 ml methanol (absolute), 400 ml toluene (scint. grade), 50 ml dist. water and 7 g of butyl 2-phenyl-5-(4-biphenylyl)-1,3,4-oxadiazol (butyl PBD). Liquid scintillation counting was performed in a TriCarb (Packard).

The protein distribution was determined by electrophoresis of triplicate samples with 3 µl of plasma employing identical conditions. Two samples were sectioned after destaining, dissolved in 80 % acetic acid and the absorbance measured at 520 nm. The third sample was made transparent and recorded by a Schoeffel SD 3000 dosimeter in remission at 490 nm for control purposes. Total protein content in the plasma samples was measured by the Lowry procedure (13) as modified by Peterson (14).

The estimation of the protein distribution by this electrophoretic procedure was checked by the use of a control serum (Precinorm U, Boehringer Mannheim), the obtained values being in good agreement with the nominal ones.

In a further control experiment the distribution of radioactivity obtained by combustion analysis was found to be similar to that obtained by autoradiography of cellulose acetate strips with separated plasma (incubation for 5 weeks with a Kodak XAR film).

Results and Discussion

Typical electrophoresis patterns of one vitamin E-deficient and one control rabbit are shown in Figure 1. The albumin peak is lower, the globulin peaks are higher in vitamin E deficiency. Globulin-1 obviously consists of a double peak, and some authors differentiate between α_1 and α_2 globulin. However, for the measurement of radioactivity intended in the present study, the two were too close together to justify separate determination. In the deficient animals, specific activity of albumin and of the 3 globulin fractions was higher at 2 and 10 h after injection of [1–^{14}C] L-leucine, but then declined faster (Fig. 2). However, the difference between the two treatment groups was not as pronounced as seen in an earlier study where [1–^{14}C] glycine had been used (11). The explanation is apparent from some of the data presented in Table 1. The vitamin E-deficient animals had a higher concentration of free leucine in the liver (line 3 of Table 1), resulting in greater dilution of the tracer and therefore in a lower specific activity of the free leucine pool in liver (line 5). The same has been observed by Catignani et al. (7) in liver of vitamin E-deficient rats. The failure of Bonetti et al. (8) to find a significant elevation of leucine incorporation into liver proteins of rats is probably due to the same phenomenon. Since the initial specific activity of plasma protein fractions (Fig. 2) and of

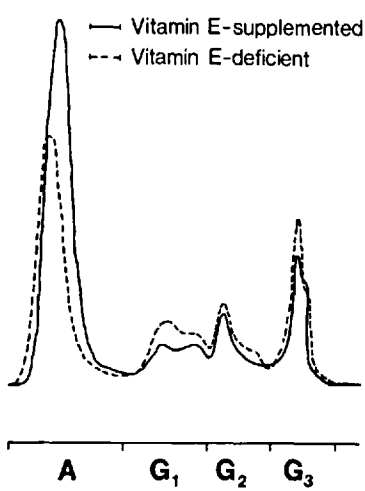

Fig. 1. Optical density scan of plasma proteins from one vitamin E-deficient and one control rabbit. Cellulose acetate strip stained with bromophenol blue; A = albumin, G_1, G_2, G_3 = globulins 1, 2, 3.

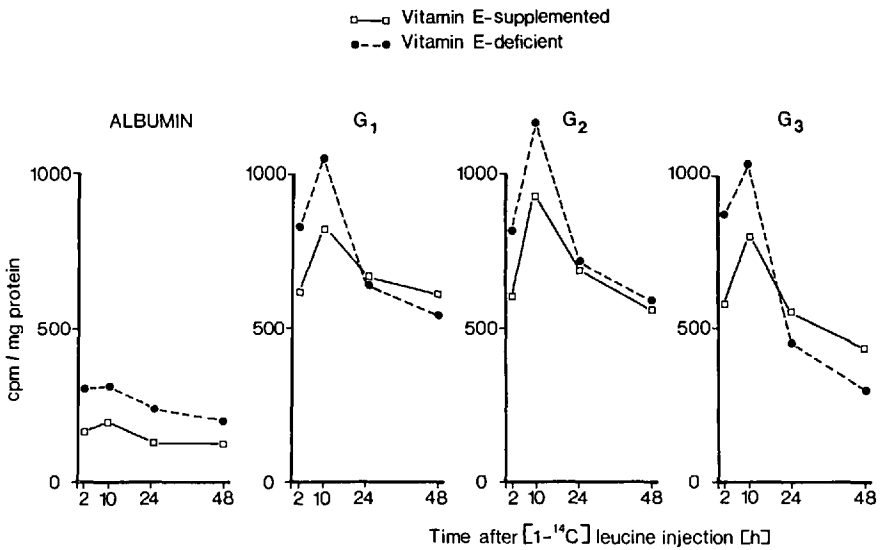

Fig. 2. Specific activity of plasma protein fractions in vitamin E-deficient and control animals at various time periods after injection of [1–^{14}C]L-leucine. Each experimental point is the mean obtained from 2 animals.

leucine isolated from plasma proteins (line 6 of Table 1) is higher in the vitamin E-deficient animals, in spite of a lower activity of the precursor pool, the turnover rate of plasma proteins must actually be even more

Table 1. Effect of vitamin E-deficiency on some properties of liver and plasma; number of animals in parenthesis. Arithm. means ± SD when more than 2 animals, individual values when 2 animals per group.

	Vitamin E-supplemented	Vitamin E-deficient
1 TCA-precipitable protein in liver tissue, mg/g wet weight	155 ± 21 (8)	164 ± 26 (8)
2 TCA-precipitable protein in plasma, mg/ml	47.7 ± 4.6 (8)	50.3 ± 7.7 (8)
3 Free leucine in liver tissue, µmoles/g wet weight	1.19; 1.32 (2)	2.43; 2.59 (2)
4 Leucine content of plasma proteins, µmoles/g protein	660; 730 (2)	640; 732 (2)
5 Specific activity of free leucine in liver tissue 2 h after inj. of ^{14}C-leucine, cpm/µmole	3570; 2710 (2)	1232; 1360 (2)
6 Specific activity of leucine isolated from hydrolyzate of plasma proteins 2 h after inj. of ^{14}C-leucine, cpm/µmole	272; 232 (2)	540; 615 (2)

Table 2. Specific activity of TCA-precipitable liver proteins and of the TCA-supernatant of liver in vitamin E-deficient and vitamin E-supplemented rabbits at 2 h after injection of [1-^{14}C] glycine. Arithm. means ± SD, number of animals in parenthesis, P = probability that differences between vitamin E-deficient and supplemented groups were due to chance.

Diet	Liver proteins, cpm/mg		P	TCA supernatant, cpm/g liver tissue		P
	Vitamin E-supplemented	Vitamin E-deficient		Vitamin E-supplemented	Vitamin E-deficient	
No Anoxomer	148 ± 52 (5)	202 ± 63 (5)	> 0.1	83 300 ± 41 300 (5)	87 600 ± 37 800 (5)	> 0.1
10 mg Anoxomer/g fat	279 ± 56 (3)	551 ± 180 (5)	< 0.05	73 300 ± 17 800 (3)	104 800 ± 30 200 (5)	> 0.1
50 mg Anoxomer/g fat	264 ± 58 (3)	463 ± 102 (5)	< 0.05	98 200 ± 30 100 (3)	134 600 ± 54 500 (5)	> 0.1

Table 3. Effect of vitamin E deficiency on protein levels in plasma protein fractions of rabbits; number of animals in parenthesis.

	Total plasma proteins mg/ml	Albumin %	Globulin 1 %	Globulin 2 %	Globulin 3 %
Vitamin E-supplemented (11)	53.6 ± 4.4	72.0 ± 3.5	10.8 ± 2.4	8.3 ± 1.9	8.8 ± 2.1
Vitamin E-deficient (17)	52.7 ± 7.0	64.1 ± 6.3	13.4 ± 3.1	11.1 ± 2.3	11.3 ± 3.7
P	> 0.1	< 0.001	< 0.025	< 0.005	< 0.05

elevated in vitamin E deficiency than would appear from Figure 2. The other data presented in Table 1 show no significant difference between the two treatment groups with regard to the levels of total liver proteins (line 1), plasma proteins (line 2) and leucine content of plasma proteins (line 4).

When compared with the earlier work in which [1–^{14}C] glycine had been used (11) this preliminary study with [1–^{14}C] L-leucine, while confirming elevated plasma protein turnover in vitamin E-deficient rabbits, showed a less pronounced difference between the two treatment groups with regard to specific activity of plasma proteins. In the main study, which was primarily intended to investigate possible effects of adding the antioxidant Anoxomer to the diet, we therefore returned to [1–^{14}C] glycine as a tracer. Instead of using two animals of each group at different time intervals after injection of the ^{14}C-amino acid, as was done in the ^{14}C-leucine study, using a greater number of animals at only one time after injection was considered preferable, as this would make statistical evaluation of results possible. The laborious isolation of the labelled glycine by ion exchange chromatography was considered unnecessary, because the study of ^{14}C-glycine incorporation into muscle proteins (6) has shown that 80–90 % of the radioactivity was present as glycine, the remainder mostly as serine, and that the ratio of glycine:serine radioactivity was not significantly influenced by vitamin E deficiency.

As shown in Table 2, vitamin E deficiency did not influence radioactivity in the TCA-soluble supernatant, which mostly represents the free amino acid pool of liver. In contrast, the specific activity of liver total proteins was higher in vitamin E deficiency, and this difference was more pronounced in the groups receiving Anoxomer.

While total plasma protein concentration was not significantly affected by vitamin E deficiency (Table 3), albumin levels were lower, globulin levels higher in deficient animals, as observed earlier (Fig. 1). Anoxomer had no effect on the distribution of plasma proteins and the data of groups without or with Anoxomer were therefore pooled.

Specific activity of total plasma proteins and of the plasma protein fractions was higher in the deficient groups, and this effect was most consistent in the groups receiving 10 mg of Anoxomer/g fat in the diet (Table 4). Standard deviations of the means were lower and the statistical significance of the vitamin E effect was higher at this level of Anoxomer. The increase of specific activity of plasma proteins from the "no Anoxomer" to the "10 mg Anoxomer" to the "50 mg Anoxomer" groups, both vitamin E-supplemented and deficient, may well be due to the higher age of the animals fed Anoxomer.

We conclude from these results that vitamin E deficiency in young rabbits leads to a higher rate of synthesis of liver and plasma proteins. This is not an effect of the presence of peroxides in the diet but actually an effect of insufficient vitamin E supply. Similar to what was found earlier with regard to skeletal muscle proteins (6), this effect of vitamin E deficiency was most clearly and consistently observed when the experimental animals received 10 mg of Anoxomer/g fat in the diet.

Although these results may be seen as supporting Olson's hypothesis of a role of vitamin E in the regulation of protein synthesis (5), it remains to be established whether the observed effects are a primary result of vitamin

Table 4. Effect of vitamin E deficiency on specific activity of plasma protein fractions (cpm/mg) at 2 h after injection of [1-^{14}C] glycine; number of animals given in parenthesis.

Diet	Albumin	G_1	G_2	G_3
No Anoxomer				
Vitamin E-supplemented (7)	1.3 ± 0.2	6.9 ± 1.5	5.5 ± 2.0	4.3 ± 1.8
Vitamin E-deficient (9)	3.2 ± 2.5	16.3 ± 11.9	12.0 ± 7.4	7.3 ± 4.5
P	> 0.05	< 0.05	< 0.05	> 0.05
10 mg Anoxomer/g fat				
Vitamin E-supplemented (3)	1.0 ± 0.2	2.3 ± 0.3	4.3 ± 2.3	3.0 ± 0.8
Vitamin E-deficient (5)	5.2 ± 1.7	13.6 ± 4.5	19.7 ± 9.8	16.1 ± 4.3
P	< 0.01	< 0.01	< 0.05	< 0.005
50 mg Anoxomer/g fat				
Vitamin E-supplemented (3)	1.3 ± 0.2	8.5 ± 1.6	6.2 ± 1.6	5.9 ± 1.7
Vitamin E-deficient (5)	7.3 ± 3.2	28.0 ± 17.3	24.2 ± 15.3	23.2 ± 16.2
P	< 0.025	> 0.1	< 0.05	> 0.1

E deficiency or whether they are secondary responses to the muscular dystrophy or to other consequences of an inadequate supply of the vitamin.

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