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Intestinal Absorption and *in vivo* Formation of Lipoperoxides in Vitamin E Deficient Rats

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Vitamin E deficient and supplemented rats were fed diets containing 10 % methyl esters of fatty acids from cod liver oil. Larger amounts of peroxides were found in adipose tissue of vitamin E deficient animals after feeding fresh esters than when autoxidized esters were given.

Methyl linoleate hydroperoxide was given intragastrically to thoracic duct cannulated vitamin E deficient rats. No lipoperoxide was found in the lymph, but the linoleate hydroperoxide was partially recovered as hydroxyoctadecadienoate. The recoveries were of the same order of magnitude as have been found in animals fed a normal diet.

It is concluded that the peroxides found in adipose tissue of vitamin E deficient animals have been formed by autoxidation *in vivo*.

The fate of orally ingested lipoperoxides has been studied by Glavind and Tryding.[1] Peroxidized olive oil was split by pancreatic lipase with the formation of peroxide-containing fatty acids. When peroxidized ethyl linoleate or olive oil was given perorally to thoracic duct cannulated rats, no lipoperoxide was found in the thoracic duct lymph. The hypothesis was advanced that the ultimate "deperoxidation" takes place in the intestinal mucosa. Andrews *et al.*[2] and Nishida and Kummerow [3] arrived at similar conclusions.

The absorption of methyl linoleate hydroperoxide in the thoracic duct cannulated rat was reinvestigated by Glavind and Sylvén.[4] Hydroxyoctadecadienoate, formed by reduction of linoleate hydroperoxide, was present in the triglyceride fraction of the lymph lipids, but relatively small recoveries were obtained. The results indicated that dietary lipoperoxides and the corresponding hydroxy compounds are to a large extent metabolized in the intestinal mucosa.

Since lipoperoxides are not absorbed, it has been generally assumed that the peroxides found in adipose tissue of vitamin E deficient rats fed large

amounts of highly unsaturated fatty acids are formed *in vivo*, *i.e.*, that vitamin E acts as an antioxidant *in vivo*. A novel hypothesis has, however, been put forward by Bunyan *et al.*[5] They postulate that lipids do not become peroxidized in the adipose tissue of vitamin E deficient rats. The peroxides found in the adipose tissue are supposed to be of exogenous origin, and it is claimed that vitamin E acts by reinforcing a barrier to their absorption by the gut.

It was decided, therefore, to continue the study on intestinal absorption of lipoperoxides with experiments including vitamin E deficient rats. Fresh cod liver oil methyl esters and esters oxidized to a high peroxide content were administered orally to rats, and their effect on the level in the adipose tissue was compared. Furthermore, the absorption and lymphatic transport of methyl linoleate hydroperoxide in the vitamin E deficient rats were studied.

EXPERIMENTAL

Forty-five three weeks old male Wistar rats were distributed into four groups, 15 animals in group 1 and 10 in each of the other groups. They were given the basal vitamin E deficient diet of Bunyan *et al.*[6] supplemented as indicated in Table 1. The tocopherol acetate was given from the beginning of the experiment, the supplements of esters were added after four weeks. Cod liver oil methyl esters were prepared as described by Green *et al.*;[7] only the distillation was omitted. The esters were stored at −30°C in closed bottles. The peroxide content was lower than 1 μequiv./g. Peroxidized cod liver methyl esters were prepared by exposing the esters to atmospheric oxygen on shallow trays. The peroxide content was controlled daily by the iodometric macromethod. The exposure was interrupted when a level of 600 μequiv./g had been reached. The esters were then transferred to glass bottles and deaerated with nitrogen. The bottles were closed and also stored at −30°C.

Table 1. Supplements given to basal vitamin E deficient diet of rats.

Supplement	g/kg diet	Group number 1	2	3	4
Tocopherol acetate	0.1			+	+
Cod liver oil methyl esters,					
peroxide content 0 μequiv./g	100	+		+	
peroxide content 600 μequiv./g	100		+		+

The animals were weighed and inspected weekly. The depletion of vitamin E was followed by the dialuric acid induced hemolysis test of György and Rose.[8] After the rats had been 20 days on the low-fat diets, blood samples were taken from a number of animals in the vitamin E deficient groups 1 and 2. There was complete hemolysis 30 min after incubation. No hemolysis was observed in 4 h in the blood samples from the groups 3 and 4.

The occurrence of peroxide in the adipose tissue was studied by the removal of 50–200 mg samples from the inguinal subcutis of the rat in ether narcosis. Lipid extracts were made by the method of Bunyan *et al.*[6] They were carefully evaporated and weighed, and peroxide determinations were made by the colorimetric thiocyanate method [9] in the absence of oxygen.

After a feeding period of 8 weeks on the diets supplemented with cod liver oil esters, a number of animals from group 1 were taken for thoracic duct cannulation. The rest of the animals of group 1 together with those of the other groups were kept for another

two weeks on the experimental diets. They were then killed and carefully autopsied, and small samples of adipose tissue from the inguinal region were taken for peroxide determination.

Thoracic duct cannulation was carried out as described previously.[10] Lymph was sampled in heparinized bottles under nitrogen. The samples were immediately frozen until extraction. Fasting lymph was collected during the first 15 h after the cannulation. The rats were then intragastrically given 0.2 ml methyl linoleate hydroperoxide together with 0.4 ml triolein followed by 2 ml isotonic saline. Lymph was again collected during the first 6 and the following 18 h.

The lymph samples were extracted according to Bunyan *et al.*[6] The extracts were evaporated, rapidly weighed and dissolved in 10 ml peroxide-free hexane (analytical degree, suitable for spectroscopy). Two ml of each solution was used for the determination of peroxide by the colorimetric thiocyanate method. Samples containing about 0.4 mg lipid were taken for thin-layer chromatography, which was carried out exactly as described previously.[4] Aliquot samples were diluted to a suitable concentration with ethanol and examined spectroscopically in the range 224 – 240 nm against blanks containing the same amounts of α-tocopherol as the samples in ethanol solution. The E(1 cm, 1 %) at 232 nm and the content of conjugated diene were calculated as in our previous paper.[4]

RESULTS

The supplementation of the diet with 10 % of peroxidized cod liver oil (peroxide content 600 μequiv./g) resulted in a diminished growth rate in comparison with the feeding of fresh esters, but no sign of a specific toxicity of the peroxide was noted. The digestive tube appeared normal, and no difference with respect to the appearance of skin and tail was noted between the four groups. Yellow-brown discoloration of the adipose tissue was almost absent in the vitamin E deficient group supplemented with peroxidized esters, but dental depigmentation was rather more pronounced than in the group fed fresh cod liver oil methyl esters. The weight growth of the four groups of rats together with a summary of some observations at inspection or autopsy are presented in Table 2.

Table 2. Influence of diet on growth and deficiency symptoms.

Group number	1	2	3	4
Weight gains in 14 weeks, g.				
Mean values with their standard errors	253 ± 7	225 ± 6	280 ± 8	232 ± 13
Yellow-brown discoloration of subcutaneous fat.				
No. of rats with discolorated tissue/total number autopsied	5/5	1/10	0/9	0/10
Depigmentation of teeth.				
No. of rats with completely depigmented teeth/total number	4/15	6/10	0/9	1/10
No. of rats with normal teeth/total number	2/15	0/10	9/9	5/10

The results of the determinations of peroxide in the adipose tissue of the four groups are presented in Table 3.

No coloured spot was produced when chromatograms of the lymph lipids were sprayed with the leucodichlorophenolindophenol-hemin reagent for

Table 3. Peroxide content of rat adipose tissue, μequiv./g fat. Mean values with their standard errors.

Weeks fed cod liver oil methyl esters	Group number 1	2	3	4
7	6.2 ± 0.4	1.6 ± 0.3	0.66 ± 0.09	0.45 ± 0.05
8	6.7 ± 1.3	2.4 ± 0.3	0.34 ± 0.66	0.31 ± 0.03
10	17 ± 3	2.8 ± 1.4	0.75 ± 0.09	0.33 ± 0.04

peroxides.[11] A chromatographic plate sprayed with 50 % sulfuric acid followed by charring is depicted in Fig. 1. The results of the physico-chemical examination of the lymph lipids are presented in Table 4.

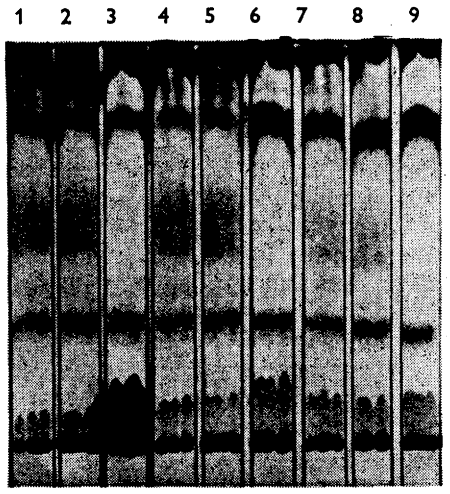

Fig. 1. Thin-layer chromatography of thoracic duct lipids of vitamin E deficient rats fed methyl linoleate hydroperoxide. Silicagel G. Solvent system: petrol ether-ether-chloroform (6 : 3 : 1); development twice. Designations of samples as in Table 4. Location of zones: polar lipids (R_F-value 0 – 0.15), free cholesterol (R_F 0.3), hydroxy acid-containing triglycerides (R_F 0.5 – 0.6), α-tocopherol (R_F 0.8), triglycerides (R_F 0.95 – 1.0).

DISCUSSION

The animals were given diets without added fat until the dialuric acid hemolysis test of the erythrocytes indicated a depletion of vitamin E in the deficient groups. The diets were then, from the fifth week, supplemented with methyl esters from cod liver oil. An accumulation of peroxides in the adipose tissue of the vitamin E deficient rats, especially those of group 1, was noted after another seven weeks (Table 3). When the groups were compared at the end of the experiment, after ten weeks of feeding the fatty acid esters, significant differences were observed. The peroxide content was higher in the vitamin E deficient groups than in those supplemented with tocopherol acetate, and higher when peroxide-free methyl esters of cod liver oil were fed than when esters having a peroxide content of 600 μequiv./g were given. The higher peroxide content of adipose tissue in the vitamin E deficient groups is, according to the concepts of Bunyan *et al.*[5] of the exogenous origin of the

Table 4. Recovery of methyl linoleate hydroperoxide in lymph after feeding to vitamin E deficient rats. Bracketed figures mean that no absorption maximum about 232 nm was registered, and consequently no reliable estimate of conjugated dienes could be made.

Chromatogram No.	Rat No.	Lymph			Lymph lipids		
		Collection period, h before (b) or after (a) feeding	Weight g	Weight mg	Peroxide μequiv./g	E(1cm,1%) 232 nm	Conjugated diene %
1	1	15 b	26.6	78.1	5	(3.8)	
2	1	6 a	6.9	40.1	0.5	25	2.9
3	1	18 a	24.6	172.8	2.5	21	2.5
4	2	15 b	25.4	89.2	4	(1.5)	
5	2	6 a	4.2	52.5	0	30	3.5
6	2	18 a	18.3	275.1	3.5	30	3.5
7	3	15 b	1.65	16.2	0	(7)	
8	3	6 a	3.4	45.2	0	31	3.6
9	3	18 a	17.6	196.4	2.5	25	2.9

peroxides of adipose tissue, explained by the failure of a barrier to the absorption of lipid peroxides which is influenced by vitamin E and overcome by prolonged feeding of a high dietary concentration of a suitable lipid. However, the low peroxide content of adipose tissue of rats fed a vitamin E deficient diet containing 10 % highly peroxidized cod liver oil esters compared with the much higher level when peroxide-free esters were fed, is hard to reconcile with the existence of such a barrier.

A number of rats of group 1, all of which had shown the presence of peroxides in samples of adipose tissue taken by biopsy, were thoracic duct cannulated. Methyl linoleate hydroperoxide was given intragastrically, exactly as in the experiments on thoracic duct cannulated normal rats reported in our previous paper.[4] Lymph was collected and examined in exactly the same way with only one exception: tocopherol was added, in the amounts prescribed by Bunyan *et al.*,[6] in order to protect against autoxidation. Compensation for light absorption about 232 nm due to the presence of tocopherol in the extracts was made by the use of blanks containing the same amounts of tocopherol.

The examination of the lymph extracts gave exactly the same result as that of lymph from normal rats.[4] Neither by the chemical method nor by thin-layer chromatography could peroxide be recovered in the lymph after feeding. In thin-layer chromatograms (Fig. 1), the added tocopherol appeared as a zone slightly more polar than the triglycerides. Otherwise the chromatograms were virtually identical with those obtained from the lymph extracts of normal rats (Glavind and Sylvén,[4] Fig. 1). The most conspicuous feature was, in both cases, the appearance of a broad zone with an R_F-value about 0.5 – 0.6 after the feeding of methyl linoleate hydroperoxide. Spectroscopic evidence that the zone was due to triglycerides containing hydroxyoctadecadienoic acid was presented in the previous paper.

An estimate of the amount of the conjugated hydroxy-compounds was made from the optical densities of the extracts at 232 nm (Table 4). No conjugated diene could be estimated in the extracts of fasting lymph, but 2.5–3.5 % was found after feeding linoleate hydroperoxide, the same level as was found in lymph from normal rats (2.0–4.0 %) after the same treatment. The ingested linoleate hydroperoxide contained 82 % conjugated diene, corresponding to 27 % after dilution with two parts of triolein. Only a low recovery of conjugated diene in comparison with oleic acid was thus obtained. The recovery was of the same order of magnitude in normal and vitamin E deficient rats and must presumably be due to the same cause. A probable explanation for the low recovery would be a high metabolism of the lipoperoxides in the intestinal mucosa.[4]

The conclusion must be drawn that methyl linoleate hydroperoxide is reduced in the intestinal mucosa to hydroxyoctadecadienoate, which can be partially recovered in the lymph. Autoxidized soy bean oil is similarly converted,[2] and it can scarcely be doubted that reduction of the hydroperoxido-group to a hydroxygroup in the intestinal mucosa is the general mechanism of the organism for "deperoxidation" of lipoperoxides of the food.

If the peroxides occurring in the adipose tissue are not exogenous they must be formed *in vivo*. They are formed when large amounts of highly unsaturated fatty acids from, *e.g.*, cod liver oil or linseed oil are given to vitamin E deficient animals. It seems reasonable to assume that the formation takes place as it would do *in vitro*, *i.e.*, by autoxidation, and that tocopherol prevents the peroxide formation in the same manner as *in vitro*, *i.e.*, as an antioxidant.

The concept that vitamin E acts as an antioxidant *in vivo* is under dispute. One trend, the biological antioxidant theory for the function of vitamin E, explains all the manifestations of vitamin E deficiency diseases as being due to the occurrence of chain reactions and the formation of lipoperoxides in the absence of the antioxidative protection by vitamin E.[12] The opposite view is represented by Green and Bunyan [13] who deny any antioxidant function *in vivo* of the vitamin.

It is concluded from our experiments that the drastic rejection of any function of vitamin E as a biological antioxidant is untenable.

As regards the biological antioxidant theory, it offers the advantage of a coherent theory able to account for all the manifestations of vitamin E deficiency diseases. The experimental evidence for the theory is, however, indirect and circumstantial. The claims for the occurrence of lipoperoxides are mainly based on estimates of the production of malonic dialdehyde in incubation experiments which estimate only the formation of peroxides *in vitro*. Direct attempts to demonstrate the occurrence of lipoperoxides *in vivo* are fewer and have scarcely been conducted with suitable methods.

As long as the occurrence of products of autoxidation is established only in adipose tissue and is contested in other tissues affected by vitamin E deficiency, it cannot be settled whether vitamin E functions solely as an antioxidant. The alternative will be that it acts as an antioxidant in adipose tissue but has also some other specific or general metabolic function.

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