

Biopotency of vitamin E in barley

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1. Investigations were carried out to establish the total biopotency of the natural vitamin E isomers in barley compared with that of DL- α -tocopheryl acetate.

2. The chick was used as an experimental animal. Prevention of nutritional encephalomalacia (NE) and chick liver-storage and plasma-storage assays of vitamin E were the methods used in the study. The individual tocopherols and tocotrienols, both in the tissue samples and in the grain and barley oil, were analysed using high-pressure liquid chromatography (HPLC) with fluorescence detection. The diagnosis of NE was based on careful clinical and histopathological observations.

3. It can be concluded from the results that full protection against NE in the chicks was obtained with a supplementation level of 7.5 mg DL- α -tocopheryl acetate/kg diet (i.e. a total vitamin E content of 11.20 mg/kg diet) or with a supplement of 8.7 g barley oil/kg diet (i.e. a total vitamin E content of 22.99 mg from barley oil/kg diet). This gave a biopotency factor of 0.49 for barley for prevention of NE of the chicks, as compared to that of DL- α -tocopheryl acetate.

4. Using regression analysis a statistically linear relationship could be observed between the total dietary vitamin E level and the response, as measured by the total vitamin E content in the liver and plasma, both in the groups supplemented with DL- α -tocopheryl acetate and in the groups supplemented with corresponding amounts of vitamin E in barley oil. The liver and plasma responses to the total vitamin E in the barley-oil diet compared with those of the DL- α -tocopheryl acetate reference diet gave identical values for the regression coefficients, i.e. in both liver-storage and plasma-storage assays the value for slopes of dose-response lines was 0.37. This means that the biopotency of the total vitamin E in barley was 37% of that of dietary DL- α -tocopheryl acetate. Thus, barley is not as rich a source of vitamin E as could be supposed on the basis of the chemical determination of its total vitamin E content.

5. It was possible to verify this experimentally established biopotency of 0.37 for the total vitamin E in barley by converting the chemically determined amounts of the vitamin E isomers in barley into DL- α -tocopheryl acetate equivalents by multiplying them with internationally accepted potency factors for the individual natural isomers (DL- α -tocopheryl acetate 1.00, D- α -tocopherol 1.49, D- β -tocopherol 0.60, D- γ -tocopherol 0.15, D- α -tocotrienol 0.37).

6. In spite of the high proportion of α - and β -tocotrienols in the barley-oil diets (about 60% of the total vitamin E content), only traces of these isomers could be detected in the plasma and none could be detected in the liver. On the other hand, calculation of the individual biopotencies for the different isomers in the barley-oil diet by comparing the dose responses, diet:liver, separately for each isomer with those of DL- α -tocopheryl acetate, resulted in biopotency values for α - and β -tocopherol which were twice as high as the internationally accepted conversion factors. These results of the present study tempted the authors to draw the conclusion that there may have been a chemical reduction of the α - and β -tocotrienols to the corresponding tocopherols before entering the liver.

Feed grains, e.g. barley and oats, are vital sources of vitamin E for domestic animals. In many cases, the feed is further supplemented by additives of synthetic vitamin E or other antioxidative substances or both to prevent vitamin E and selenium deficiency diseases. An admixture of Se in commercial feeds is another choice for this prophylaxis. The basis for such supplementation should be a thorough knowledge of the biological activity of natural vitamin E and its isomers in cereals because of the high toxicity of Se. The present knowledge

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of the biopotency of the different natural vitamin E isomers in the animal organism is, however, still insufficient. There is a large scattering of the values for the biological activity of the different isomers (Leth & Søndergaard, 1977; Bieri & McKenna, 1981). The absorption and metabolism of the different vitamin E isomers, especially those of non- α -tocopherols in the living animal are also aspects which require additional investigation. According to our observations in different species, animal tissues contain α -tocopherol as the primary isomer of vitamin E in spite of the wide distribution of all the natural isomers in the feed. The fact that a satisfactory method for separating and determining the individual tocopherols and tocotrienols is relatively recent is probably responsible for part of the inadequacy of available knowledge.

One more aspect that should be studied more thoroughly is whether any substantial biological value can be attributed to non- α -tocopherols in practical feed mixtures. Up until now, the natural non- α -tocopherols have been only sporadically studied with respect to their vitamin E activity and biopotency. Cereal grains, such as barley, oats, wheat and rye, contain significant amounts of natural non- α -tocopherols (Lindberg, 1966; Slover, 1971; Bauernfeind & Cort, 1974; Søndergaard & Leth, 1978; Hakkarainen *et al.* 1983*a, b*) and are thus well suited for such studies.

It is well known that bioassay techniques are useful for evaluating the biopotency of vitamin E and for estimating the vitamin E requirements of humans and animals. The true biological activity of vitamin E can be determined by its ability to prevent or reverse specific vitamin E deficiency symptoms. In our previous studies, the requirements for vitamin E or Se or both in weaned pigs have been established by taking advantage of both prophylactic and therapeutic experimental models (Bengtsson *et al.* 1974, 1978*a, b*; Hakkarainen *et al.* 1978*a, b*).

As far as the chick is concerned the prevention of encephalomalacia under standardized conditions provides a relatively simple means for evaluating the biological activity of vitamin E and its isomers (Dam & Søndergaard, 1964). Another often-used and approved bioassay technique is the liver-storage assay (Scott, 1978; Desai, 1980).

Barley, one of the oldest crops known to man, is also one of the main feed grains for livestock and poultry. At the same time it provides a vital source of vitamin E for domestic animals.

Consequently, the present experiment was designed to use both of the previously-mentioned biological methods to evaluate the total biopotency of the natural vitamin E isomers in barley. The chick was selected as an experimental animal because of its proven usefulness in such studies (Scott, 1978).

Prevention of nutritional encephalomalacia (NE) and chick liver-storage and plasma storage assays of vitamin E were the methods used in the present study. The tocopherols and tocotrienols, both in the tissue samples and in the grain, were analysed using high-pressure liquid chromatography (HPLC) with fluorescence detection.

MATERIALS AND METHODS

Experimental animals and diets

Male White Leghorn chicks (264, 1-d-old) of commercial breeding were purchased as experimental animals. They were divided randomly by weight into twelve groups of twenty-two chicks each. Six chicks from group 12 were killed to establish vitamin E status on day 1.

The chicks were reared in electrically-heated wire batteries. On day 1 the temperature in the cages and in the room was regulated to an optimal 35°. Subsequently, the temperature was lowered by 0.5° every 24 h for 30 d, when it was 20°. Light was provided 24 h/d.

Table 1. *Composition of the basal diet (g/kg)*

Skim-milk powder	195.0
Gelatin	100.0
Casein	85.0
Amino acid mix*	9.5
Fat component†	50.0
Glucose	470.3
Cellulose	30.0
Mineral mix‡	56.0
Vitamin mix§	4.2
Total	1000.0

* Contained (g/kg diet): methionine 5.0, glycine 2.5, arginine 2.0.

† For the composition of the fat component, see Tables 2 and 3.

‡ Contained (/kg diet): $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ 37.23 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 7.60 g, CaCO_3 5.07 g, NaCl 5.00 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.50 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.33 g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.26 g, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 39.3 mg, K_2SO_4 6.4 mg, KIO_3 5.1 mg, $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ 0.33 mg.

§ Provided the following amounts (/kg diet): retinol 18.3 mg, cholecalciferol 27.5 μg , menadione 1.4 mg, cyanocobalamin 0.03 mg, choline chloride 2.0 g, nicotinic acid 55.0 mg, pantothenic acid 25.0 mg, biotin 0.3 mg, riboflavin 12.0 mg, thiamin 10.0 mg, pyridoxine 8.0 mg, pteroylmonoglutamic acid 4.0 mg.

Table 2. *The composition and content of the fat and vitamin E components in experimental diets for different groups of chicks*

Group no.	Fat component (g/kg)		Vitamin E content in diets (mg/kg)							
	Cottonseed oil	Barley oil	DL- α -T-ac*	α -T	α -T3	β -T	γ -T	β -T3	δ -T	Total
1	50.0	—	—	2.33	0.19	0.06	1.12	—	—	3.70
2	50.0	—	2.5	2.33	0.19	0.06	1.12	—	—	6.20
3	50.0	—	5.0	2.33	0.19	0.06	1.12	—	—	8.70
4	50.0	—	7.5	2.33	0.19	0.06	1.12	—	—	11.20
5	50.0	—	10.0	2.33	0.19	0.06	1.12	—	—	13.70
6	50.0	—	12.5	2.33	0.19	0.06	1.12	—	—	16.20
7	45.6	4.4	—	3.48	5.08	0.17	2.43	1.84	0.34	13.34
8	41.3	8.7	—	4.64	9.97	0.28	3.74	3.68	0.68	22.99
9	36.9	13.1	—	5.79	14.85	0.40	5.05	5.52	1.02	32.63
10	32.6	17.4	—	6.94	19.74	0.51	6.36	7.36	1.36	42.27
11	28.2	21.8	—	8.10	24.63	0.62	7.66	9.20	1.70	51.91

T, tocopherol; T3, tocotrienol.

* Vitamin E dry powder 25%; Merck Art. 501618; DL- α -tocopheryl acetate.

The composition of the basal diet is shown in Table 1. The ingredients were premixed before the start of the experiment, except for the addition of molecular-distilled cottonseed oil and DL- α -tocopheryl acetate or extracted barley oil. The final diet mixtures were prepared twice weekly in order to minimize the possibility of vitamin E oxidation. The stability of the vitamin E of the final diets at room temperature was checked for the extent of loss. A daily decrease of 1.7% of the total vitamin E content was noted during an observation period of 2 weeks.

The basal diet contained all the nutrients required by the chicks for normal growth and development, except for vitamin E. The basal diet and final mixtures were stored in the dark at -20° . The composition and content of the fat and vitamin E components in the experimental diets for the different groups are shown in Table 2. A certain amount of polyunsaturated fatty acids was included in the vitamin-E-deficient rations in order to

Table 3. *Fatty acid composition in cottonseed oil and in extracted barley oil used as fat components in experimental diets*

Fatty acid		Composition (g/kg)	
		Cottonseed oil	Barley oil
Palmitic acid	(16:0)	218	224
Stearic acid	(18:0)	27	11
Oleic acid	(18:1)	190	161
Linoleic acid	(18:2)	529	513
Linolenic acid	(18:3)	6	42

produce NE; a trace of $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ was added to the basal diet to secure the prevention of exudative diathesis. The fatty acid compositions of molecular-distilled cottonseed oil and extracted barley oil used as fat components in the experimental diets are shown in Table 3. The purpose of molecular-distillation was to remove the vitamin E from the cottonseed oil.

Experimental procedure

All twelve groups were given the basal vitamin-E-deficient diet *ad lib.* for 10 d to deplete the chicks of their vitamin E stores. A control of the vitamin E status of the chicks during the depletion period was made by killing eight chicks from group 12, at the age of 7 and 10 d respectively. The chicks were weighed at the age of 10 d, and the smallest two in each group were discarded. The experimental dietary treatments were randomly assigned to the cages of chicks.

Group 1 was a control and it was kept on the same encephalomalacia-producing diet during the whole experimental period without any supplementation of vitamin E, as for all the chicks during the introductory depletion period of 10 d.

Groups 2, 3, 4, 5 and 6 received 2.5, 5.0, 7.5, 10.0 and 12.5 mg DL- α -tocopheryl acetate (Vitamin E dry powder 25%; Merck Art. 501618)/kg diet respectively. Groups 7, 8, 9, 10 and 11 were assigned corresponding amounts of natural vitamin E in extracted barley oil. The calculation of the vitamin E activity of the barley oil and consequently the supplementation levels of that oil were based on the results of pilot studies in our laboratory.

The birds were inspected daily for clinical health with special attention to NE. The body-weight gain was noted weekly. Any chick that showed clinical signs of NE was immediately decapitated, otherwise two chicks in each group were killed at 12, 14, 21, 28, 35 and 42 d of age respectively. The remaining chicks in each group were killed at 49 d.

It should be emphasized that the absence of clinical signs of NE did not exclude the possibility of a positive histopathological diagnosis when apparently healthy chicks were killed at regular intervals. The clinical cases of NE were also confirmed histopathologically.

At decapitation, blood was sampled in heparinized tubes. Plasma was separated by centrifugation and stored at -20° until analysis for vitamin E.

All the experimental chicks killed with or without showing clinical symptoms were autopsied without delay. The livers were removed, rinsed in saline (9 g sodium chloride/l) and stored at -20° until vitamin E analysis. The brains were immediately removed and fixed for light microscopy. Specimens from the nervous system were fixed in formalin (100 ml/l), dehydrated and embedded in paraffin wax. Sections were stained with Masson's trichrome, Luxol fast blue, von Kossa, Sudan Black B and Prussian blue.

Isolation of barley oil

The isolation of barley oil for the feeding experiments was started by milling barley in a Retsch Laboratory Mill. Barley flour was extracted in 2-litre Soxhlet extractors. Batches (0.7 kg) of flour were weighed in glass-fibre extraction thimbles and extracted for 21 h using absolute ethanol, containing 5 g ascorbic acid/l. After the extraction, the ethanol volume was reduced to about 500 ml by distillation and then to about 200 ml using a rotary evaporator. The ethanol phase was extracted in a separating funnel with 1 vol. water and 2 vol. hexane. The solvent in the hexane phase was removed in a rotary evaporator and the residual lipid fraction of barley was stored at -20° in a nitrogen atmosphere until the feed was mixed. The total vitamin E content of the isolated barley oil had a mean value of 2.5 g/kg oil. The isomer composition, as well as the fatty acid composition of the isolated oil, was the same as in the unextracted barley.

Analytical methods

The vitamin E content of the basal diet and of the barley was analysed by extracting 20-g-samples with 150 ml absolute ethanol for 5 h in Soxhlet extractors. During the extraction, 1.5 g ascorbic acid provided sufficient protection against spontaneous vitamin E oxidation. The ethanol extract (3 ml) was mixed with an equal volume of distilled water and the solution was extracted four times with 3 ml n-hexane. The pooled hexane phase was concentrated to 1 ml under a stream of N_2 and 10 μ l of the solution was injected on a Perkin-Elmer 10 μ m silica A HPLC-column (250 mm $\times$ 2.6 mm) held thermostatically at 25° . The mobile phase consisted of n-hexane containing isopropanol (99.8:0.2). The flow rate during isocratic elution was 1.2 ml/min.

The Perkin-Elmer HPLC system consisted of a model 3 B pump module, fitted with a Rheodyne 7105 manual valve injector and LC-420 Auto Sampler with a Rheodyne 7010 valve injector equipped with a 10 μ l sample loop.

A Perkin-Elmer 204 S fluorescence spectrophotometer equipped with a microflow-cell unit was used for detection. Wavelength settings were 295 and 330 nm for excitation and emission respectively. The peak areas were calculated by a Sigma 10 B Chromatographic Data Station. The peaks were identified as described previously (Hakkarainen *et al.* 1983 *a*) by comparison of the retention times with those of pure standards (Hoffmann-La Roche, Basel) and with extracts of natural products having a known specific distribution of vitamin E isomers. The peak identification was confirmed by a simultaneous detection of u.v. absorption in the eluate at 254 nm.

Vitamin E analyses of barley oil and cottonseed oil were made directly by dissolving a sample of the oil in n-hexane and by injecting a 10 μ l sample on the HPLC system. Quantitative determination was performed as described previously.

The tocopherol content in 0.25 ml plasma was analysed by precipitation of the plasma proteins with 0.25 ml absolute ethanol followed by extraction of the lipid material with 0.5 ml n-hexane. A portion (0.4 ml) of the hexane phase was evaporated to dryness and dissolved in 50 μ l hexane, followed by a 10 μ l injection on the column. The HPLC procedure was as described previously. The coefficient of variation (CV) for α -tocopherol in plasma was 2.0% and the recovery of added α -tocopherol in samples was 97.7%. The plasma total lipids were analysed according to Epstein *et al.* (1972) and had a mean CV of 3.3%.

Liver samples for tocopherol analyses were prepared according to Taylor *et al.* (1976), including potassium hydroxide saponification in the presence of ascorbic acid but omitting the sulphuric acid treatment, because it was found to be unnecessary when using HPLC analysis. The saponified samples were then extracted with n-hexane. The hexane phase was

concentrated to one-tenth of the initial volume, and a 10 μ l portion was analysed by HPLC as described previously. The mean CV for liver α -tocopherol was 3.6% and recovery was 98.4%.

RESULTS

Biochemical and clinical observations

All the experimental groups showed a normal body-weight gain and development. The weekly controls of the body-weight gain did not show any differences between the groups as a response to the vitamin E supplementation.

During the vitamin E depletion period of 10 d after birth, the liver vitamin E content of the chicks fell steeply from about 350 μ g/g to 5.6 μ g/g. After initiation of vitamin E supplementation at the age of 10 d, the liver vitamin E content showed a clear response to the vitamin E levels in the diets (Fig. 1). The diet for group 1, without a vitamin E supplement, resulted in the lowest vitamin E concentration in the liver. A continual decrease to a level of less than 1.5 μ g/g took place during the first 2 weeks of the experiment, before the onset of clinical symptoms of nutritional encephalomalacia (NE), which first appeared near or below the vitamin E level of 1 μ g/g liver.

Groups 2 and 3 (Fig. 1*a*), receiving 2.5 and 5.0 mg DL- α -tocopheryl acetate/kg diet respectively, as well as groups 7 and 8 (Fig. 1*b*) receiving calculated corresponding amounts of vitamin E in barley oil, showed a similar, continual and rapid decrease in liver vitamin E within the first 2 weeks followed by a levelling off after the third week of the experiment. On the other hand, groups 4, 5 and 6 receiving 7.5, 10.0 and 12.5 mg DL- α -tocopheryl acetate/kg diet respectively, as well as groups 9, 10 and 11 receiving corresponding amounts of vitamin E in barley oil, all showed an immediate and rapid increase during the first 2 d of vitamin E supplementation, followed by a rapid decrease to 14 d before levelling off after 21 d. Thereafter, changes in liver storage with respect to each supplementation level of vitamin E in the diets were small and insignificant to the end of the experimental period of 49 d.

Responses in the plasma vitamin E to the tested dietary vitamin E levels in chicks during the 7-week experimental period are shown in Fig. 2. Changes in plasma vitamin E showed generally the same pattern as liver vitamin E. Thus, the results of the plasma assay confirm the results of the liver storage test. The level of total lipids in plasma was constant in all experimental groups, varying between 4.5 and 4.9 g/l. However, the correlation between plasma vitamin E and plasma total lipids was poor. The correlation between plasma and liver vitamin E was strong and identical both for DL- α -tocopheryl acetate groups ($y = 0.668 + 0.681x$; $r = 0.910$, $P < 0.001$) and for barley-oil groups ($y = 0.721 + 0.675x$; $r = 0.918$, $P < 0.001$).

Clinical signs appeared in group 1 (no vitamin E supplement) at the age of 15 d, after the vitamin E level in the liver had decreased to 1 μ g/g liver or less. Between 15 and 28 d, eleven chicks in this group showed clinical signs of NE. In addition, histopathological alterations typical for NE were observed in the remaining three chicks of this group, when they were killed at 21 and 28 d respectively (Fig. 1).

In group 2 (2.5 mg DL- α -tocopheryl acetate/kg diet), clinical cases of NE started to appear at the age of 19 d, resulting in eleven clinical and three histopathological diagnoses of NE up to 29 d (Fig. 1*a*). The vitamin E level of the chicks was 1.5–2.0 μ g/g liver at the onset of the clinical symptoms.

The first and only clinically diagnosed case of NE in group 3 (5 mg DL- α -tocopheryl acetate/kg diet) was observed on the 26th day of the experiment. In addition, NE was diagnosed histopathologically in five chicks killed without clinical NE signs before the end of the experimental period at 49 d.

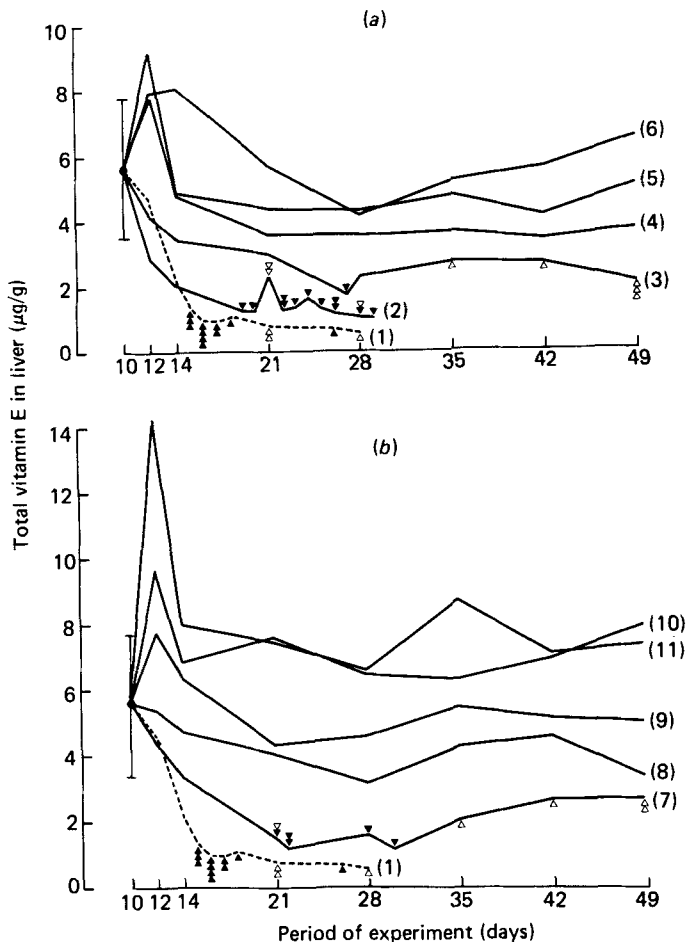


Fig. 1. Total vitamin E content in the liver of chicks, at different supplemental levels of dietary vitamin E in the form of (a) DL- α -tocopheryl acetate or (b) a natural vitamin E mixture from barley oil, during the experimental period from 10 to 49 d of age. Group 1, control, no vitamin E supplement; groups 2-6, 2.5-12.5 mg DL- α -tocopheryl acetate/kg diet; groups 7-11, increasing doses of natural vitamin E in extracted barley oil. The values in parentheses refer to the different dietary groups. (▲), Clinical cases of nutritional encephalomalacia (NE) confirmed histopathologically; (△), cases without clinical symptoms of NE but where the diagnosis was made histopathologically. The experimental lines were based on the mean liver vitamin values on each sampling occasion, with vertical bars representing standard deviations.

In group 7 (receiving calculated vitamin E supplement in barley oil equivalent to 2.5 mg DL- α -tocopheryl acetate/kg diet in group 2), five chicks showed clinical signs of NE, while an additional five chicks were diagnosed after autopsy as histopathological NE cases, between 21 and 49 d (Fig. 1b), when the vitamin E concentration in the liver was about 2 µg/g.

Full protection against NE was obtained in group 4 with a supplementation level of 7.5 mg DL- α -tocopheryl acetate/kg diet (i.e. a total vitamin E of 11.20 mg/kg in the diet), and in group 8 having a supplement of 8.7 g barley oil/kg diet (i.e., a total vitamin E of 22.99 mg/kg). These supplementation levels also gave similar responses in the liver and plasma storage assays. Average concentrations of vitamin E in the livers of groups 4 and

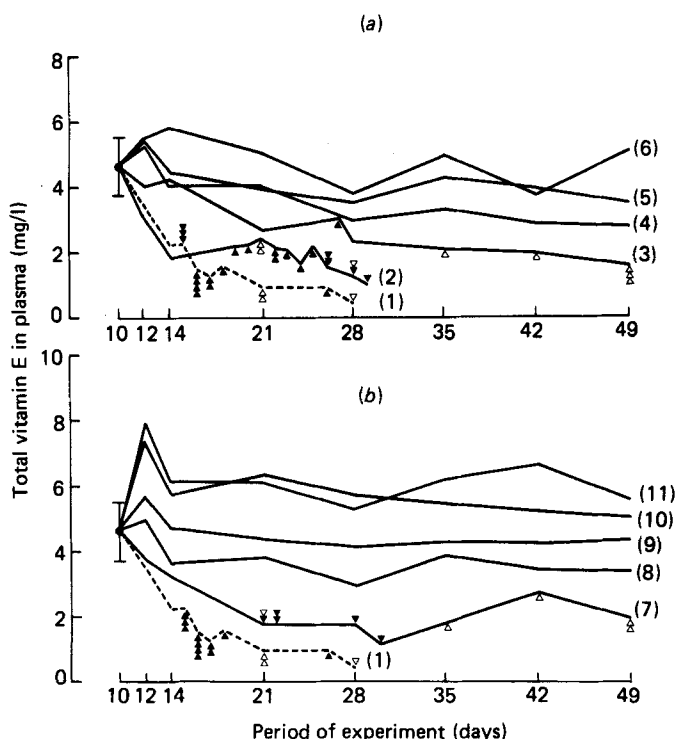


Fig. 2. Total vitamin E content in the plasma of chicks, at different supplemental levels of dietary vitamin E in the form of (a) DL- α -tocopheryl acetate or (b) a natural vitamin E mixture from barley oil, during the experimental period from 10 to 49 d of age. Group 1, control, no vitamin E supplement; groups 2-6, 2.5-12.5 mg DL- α -tocopheryl acetate/kg diet; groups 7-11, increasing doses of natural vitamin E in extracted barley oil. The values in parentheses refer to the different dietary groups. (▲), Clinical cases of nutritional encephalomalacia (NE) confirmed histopathologically; (△), cases without clinical symptoms of NE but where the diagnosis was made histopathologically. The experimental lines were based on the mean plasma vitamin values on each sampling occasion, with vertical bars representing standard deviations.

8 were 3.58 and 3.82 $\mu\text{g/g}$ respectively, and those of plasma vitamin E were 3.11 and 3.48 mg/l respectively.

The content and isomer composition of vitamin E observed in the liver and plasma of chicks maintained on different diets are presented in Tables 4 and 5 respectively.

Cerebellar changes

At the autopsy, discrete macroscopical alterations were found in some animals. The changes were restricted to the cerebellum, which showed soft and friable tissue having a pink colour. Punctate haemorrhages were present on the cut surface and on the surface of the cerebellum in affected areas.

Histopathological changes were found in the cerebellum of all twenty-eight animals having clinical symptoms of NE. The slightest and initial alterations revealed multiple haemorrhages and blood vessels with thick hyalinized walls and fibrin thrombi (Plate 1). These changes were often combined with oedema in the molecular, granular and Purkinje cell layers. The endothelium of the capillaries and venules was swollen and revealed PAS- and Sudan Black-positive granules. These granules were resistant to extraction by lipid solvents and showed a pale yellow colour.

Table 4. *Vitamin E content and isomer composition in the livers from chicks at different supplemental levels of dietary vitamin E*

(Results are expressed as $\mu\text{g/g}$ liver tissue and given as mean values and standard deviations for the chicks killed between 21 and 49 d of the experiment in each group. Group 1, control, no vitamin E supplement; groups 2-6, 2.5-12.5 mg DL- α -tocopheryl acetate/kg diet; groups 7-11, increasing doses of natural vitamin E in extracted barley oil. For further details, see Table 2 and the text)

Group no.	n	Total		α -T		β -T		γ -T	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	11	0.86	0.24	0.81	0.24	—	—	—	—
2	12	1.37	0.49	1.31	0.47	—	—	—	—
3	13	2.39	0.50	2.38	0.50	—	—	—	—
4	13	3.58	0.44	3.50	0.40	—	—	—	—
5	14	4.64	0.69	4.40	0.63	—	—	—	—
6	14	5.70	1.07	5.69	1.08	—	—	—	—
7	14	1.86	0.64	1.43	0.49	0.32	0.15	0.12	0.07
8	12	3.82	0.93	3.27	0.83	0.38	0.11	0.15	0.05
9	13	4.93	0.94	4.42	0.86	0.37	0.16	0.14	0.08
10	14	7.29	1.22	6.62	1.17	0.43	0.13	0.24	0.09
11	14	7.38	1.44	6.86	1.15	0.35	0.08	0.19	0.06

T, tocopherol.

Table 5. *Vitamin E content and isomer composition in plasma from chicks at different supplemental levels of dietary vitamin E*

(Results are expressed as mg/l plasma and given as mean values and standard deviations for the chicks killed between 21 and 49 d of the experiment in each group. Group 1, control, no vitamin E supplement; groups 2-6, 2.5-12.5 mg DL- α -tocopheryl acetate/kg diet; groups 7-11, increasing doses of natural vitamin E in extracted barley oil. For further details, see Table 2 and the text)

Group no.	n	Total		α -T		β -T		γ -T		α -T3		β -T3	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	11	1.22	0.44	1.16	0.43	—	—	0.046	0.015	—	—	—	—
2	12	1.79	0.59	1.76	0.58	—	—	0.035	0.015	—	—	—	—
3	13	2.13	0.56	2.11	0.57	—	—	0.024	0.008	—	—	—	—
4	13	3.11	0.56	3.05	0.56	0.016	0.004	0.042	0.030	—	—	—	—
5	14	3.73	0.39	3.68	0.37	0.022	0.010	0.032	0.013	—	—	—	—
6	14	4.66	0.74	4.61	0.74	0.022	0.007	0.037	0.013	—	—	—	—
7	14	1.88	0.48	1.72	0.45	0.030	0.011	0.115	0.042	0.025	0.026	—	—
8	12	3.48	0.46	3.23	0.41	0.046	0.016	0.168	0.050	0.044	0.020	—	—
9	13	4.23	0.39	3.93	0.33	0.062	0.009	0.168	0.037	0.073	0.034	—	—
10	14	5.40	0.64	4.99	0.55	0.068	0.015	0.193	0.045	0.153	0.106	—	—
11	13	5.76	0.61	5.35	0.57	0.068	0.012	0.184	0.041	0.124	0.057	0.040	0.014

T, tocopherol; T3, tocotrienol.

Severe cerebellar damage included malacia, gliosis and vascular proliferations. The changes often had the appearance of infarction and scar formation (Plate 2). Occluded capillaries and venules were often found in such areas. Mineralized necrotic cells were observed in areas of infarction of the cerebella in nine chicks. The infarcts showed loss of neurons, axons and myelin sheaths and the presence of numerous macrophages and astrocytes which were von Kossa-positive and Prussian blue-positive (Plate 3).

Sixteen chicks which did not exhibit clinical signs of NE nevertheless showed microscopical

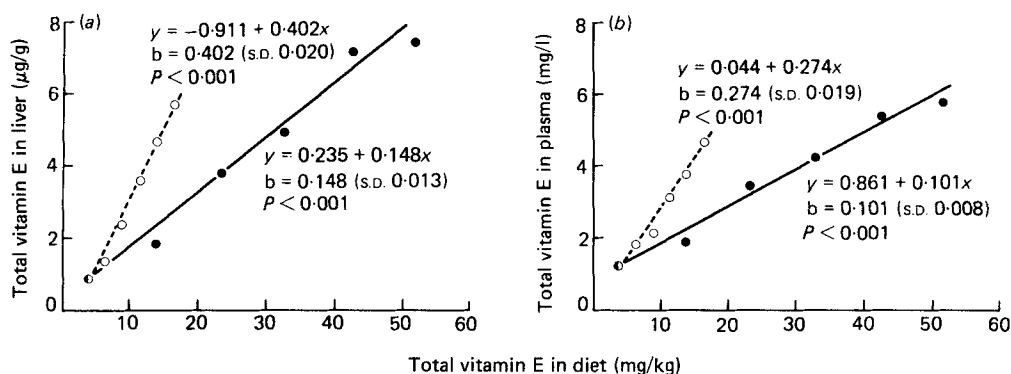


Fig. 3. Total vitamin E content (a) in the liver and (b) in the plasma of chicks as a function of total vitamin E content in the diet. The values represent the mean tissue vitamin E content at each dietary vitamin E level. Group 1, control, no vitamin E supplement (●); groups 2–6, 2.5–12.5 mg DL- α -tocopheryl acetate/kg diet (○); groups 7–11, increasing doses of natural vitamin E in extracted barley oil (●). The dose-response (regression) line for DL- α -tocopheryl acetate groups (----) and for barley-oil groups (—) with corresponding equations are indicated. The slope of the line (b: regression coefficient) with standard deviation and the goodness of fit of the line to the data are included.

cerebellar damage. Haemorrhages, congestion, fibrin thrombi and oedema were general findings in these chicks. On the other hand, severe changes with malacia, gliotic proliferation and calcification could also be found even among these chicks. Pathologic cerebellar lesions of the severe type were observed especially in five chicks without clinical symptoms from group 7 as well as in three similar chicks from each of groups 1 and 2.

Liver and plasma storage assays

The average concentration of vitamin E in the liver and plasma from chicks in the different dietary groups, killed between 21 and 49 d of the experiment with or without clinical signs of NE, are presented in Tables 4 and 5 respectively.

A direct relationship could be observed between the total dietary vitamin E level and the response as measured by the total vitamin E content of the liver, both in groups supplemented with DL- α -tocopheryl acetate and in the groups supplemented with calculated corresponding amounts of vitamin E in barley oil (Fig. 3a). The relationship was found to be linear in both supplementation groups by regression analysis, according to Snedecor & Cochran (1968). The dose response slope for barley oil relative to that for DL- α -tocopheryl acetate gave the ratio $0.148:0.402 = 0.37$. A comparable calculation for the linear responses in plasma vitamin E contents (Fig. 3b) gave the ratio $0.101:0.274 = 0.37$. By both criteria, therefore, the biopotency of vitamin E in barley oil was 37% (1:2.72) of the standard used.

DISCUSSION

Prevention of nutritional encephalomalacia (NE)

NE is a nervous derangement characterized by ataxia, backward or downward retractions of the head with lateral twistings, forced movements, increasing inco-ordination, rapid contraction and relaxation of the legs and, finally, complete prostration and death (Adamstone, 1947; Scott, 1978). The diagnosis is usually confirmed by histopathognomic alterations in the cerebellum (Wolf & Pappenheimer, 1931; Dror *et al.* 1976).

From the previously reported values for the limits of full protection against NE, a biopotency factor of 0.49 for barley oil could be calculated, i.e. DL- α -tocopheryl acetate was

2.05 times more potent than vitamin E in barley according to the present NE prevention study. If the calculations were based strictly on the supplemented amounts of vitamin E and excluding the vitamin E content of the basal diet, the biopotency factor for barley oil was 0.38. Consequently, both the liver-storage and plasma-storage assays and the NE-prevention test gave, from a biological point of view, concordant results.

Calculations of the total biological activity of the vitamin E in barley as DL- α -tocopheryl acetate equivalents

The biological activity of vitamin E is expressed in international units. The international unit (IU) has been defined as the activity of 1 mg DL- α -tocopheryl acetate in preventing resorption-gestation in the female rat deprived of vitamin E, under defined experimental conditions (Desai, 1980; Parrish, 1980). The remaining naturally occurring forms of vitamin E had to be compared against this standard in a bioassay, which in most cases implies utilizing this reproductive performance of female rats. No official or international table of equivalents exists for the natural isomers of vitamin E. The American Pharmaceutical Association (1960) in its National Formulary published an equivalence factor of 1.49 for D- α -tocopherol (1 mg D- α -tocopherol = 1.49 IU; i.e. 1 mg D- α -tocopherol is 1.49 times more potent than 1 mg DL- α -tocopheryl acetate).

During the last few years, there has been a general intention, especially in the USA, to express all vitamin E activity relative to this naturally occurring, most-active form of the vitamin, D- α -tocopherol (International Union of Nutritional Sciences, 1976; Bieri & McKenna, 1981). From the results of numerous studies, the following approximate conversion factors for the other natural isomers in comparison to D- α -tocopherol can be given: β -tocopherol 40%, γ -tocopherol 10%, δ -tocopherol 1%, α -tocotrienol 25%, the activities of the remaining natural tocotrienols are insignificant (Bieri & McKenna, 1981). These values are widely accepted as relevant conversion factors in the international literature when expressing the total vitamin E activity in food or feed.

Leth & S ndergaard (1977), in a thorough and comprehensive study, re-examined the biological activity of the tocopherols and tocotrienols using the rat resorption-gestation test. They obtained the following, from the previously-mentioned conversion factors deviating values (compared with DL- α -tocopheryl acetate 100%): D- α -tocopherol 80%, D- β -tocopherol 45%, D- γ -tocopherol 13%, D- δ -tocopherol < 0.4%, D- α -tocotrienol 13%, D- β -tocotrienol 4%.

All previous conclusions concerning the biological activity of vitamin E in barley oil compared to that of DL- α -tocopheryl acetate were based on the total vitamin E responses in the liver to total vitamin E intake in the feed, i.e. the total vitamin E content was equivalent to the sum of the weights of those natural isomers, tocopherols and tocotrienols, as mg, which were present per kg feed and liver or plasma respectively.

Another way to express the total biological activity of vitamin E in foods and feeds is to determine chemically the individual tocopherol and tocotrienol isomers and convert into DL- α -tocopheryl acetate equivalents on the basis of the previously-determined biological activities of the tocopherols and tocotrienols before summing up (Leth & S ndergaard, 1977).

If the conversion factors of Leth & S ndergaard (1977) are used as the basis for such a calculation, a linear relationship can again be observed between the calculated total vitamin E activity in barley as DL- α -tocopheryl acetate equivalents and the response as measured by the total tocopherol content of the liver (Fig. 4a). The dose-response slope for the calculated biological activity of barley oil as DL- α -tocopheryl acetate equivalents relative to that for the DL- α -tocopheryl acetate gave the ratio 0.770:0.402 = 1.92.

This means that using the factors of Leth & S ndergaard (1977) led to an underestimation

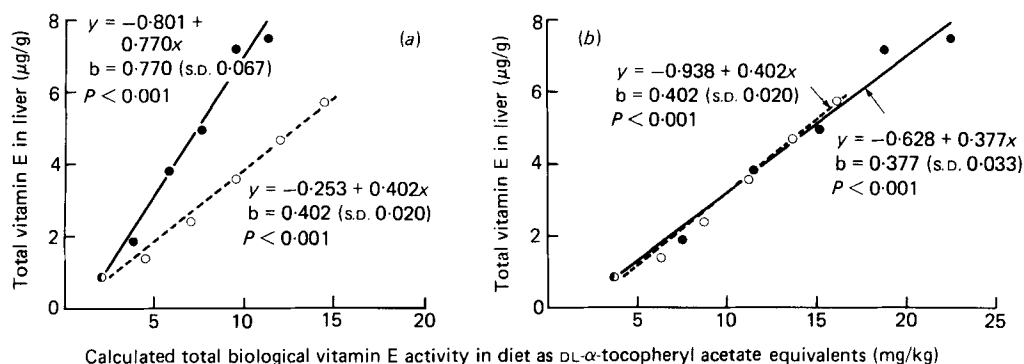


Fig. 4. Total vitamin E content in the liver of chicks as a function of the calculated total biological vitamin E activity as DL-α-tocopheryl acetate equivalents in the diet. Dietary vitamin E activity is calculated by using the conversion factors of Leth & Søndergaard (1977) (a) and the international conversion factors as reported by Bieri & McKenna (1981) (b). The values represent the mean liver vitamin E content at each dietary level of calculated DL-α-tocopheryl acetate equivalents. Group 1, control, no vitamin E supplement (●); groups 2–6, 2.5–12.5 mg DL-α-tocopheryl acetate/kg diet (○); groups 7–11, increasing doses of natural vitamin E in extracted barley oil (●). The dose response (regression) line for DL-α-tocopheryl acetate groups (—) and for barley oil groups (—) with corresponding equations are indicated. The slope of the line (b: regression coefficient) with standard deviation and the goodness of fit of the line to the data are included.

of the biological activity of vitamin E in barley. The disparity may be due mainly to underestimation of the contribution of α-tocotrienol and α-tocopherol fractions to the total vitamin E activity of barley; these fractions could be said to represent quantitative and biopotential dominance respectively in the present calculations.

However, with a corresponding calculation using the conversion factors as reported by Bieri & McKenna (1981), nearly identical slopes for both lines were obtained (Fig. 4b). The ratio of the regression coefficients was in this case $0.377:0.402 = 0.938$ and the lines did not differ statistically from each other (t test: $P > 0.5$). This means that the previously experimentally established biopotency of 0.37 for the total vitamin E content in barley was verified by the recalculation using the international conversion factors according to Bieri & McKenna (1981).

Diet-tissue relationships of vitamin E isomers

The content and composition of vitamin E observed in the liver and plasma of chicks maintained on different diets are presented in Tables 4 and 5 respectively. α-Tocopherol was clearly the dominant isomer in both the liver and plasma in all dietary groups. In the groups receiving DL-α-tocopheryl acetate, the liver and plasma content of α-tocopherol varied between 95 and 99% of the total vitamin E content. The remainder consisted of γ-tocopherol with traces of β-tocopherol.

In the groups receiving barley oil, the amount of α-tocopherol in the liver increased gradually from 80.2% of the total vitamin E in the lowest dietary vitamin E group, to 92.8% in the highest dietary vitamin E group. In plasma, the relative content of α-tocopherol remained constant, at 92–93% of the total vitamin E, for all the barley-oil groups. Since vitamin E and other lipid-soluble compounds in the chick are absorbed via the portal route (Gallo-Torres, 1980), the liver appears to have a stabilizing effect on the circulating vitamin E fraction. It must be emphasized that, in spite of the high percentage of α- and β-tocotrienols in the barley-oil diets (about 60% of the total vitamin E content), only traces of these isomers could be detected in the plasma, and none could be detected in the liver.

Table 6. Regression coefficients (slopes for dose-response lines) in liver and plasma for different vitamin E isomers in barley

Isomer	Slope		Slope ratio to DL- α -T-ac $\times$ 100		Relative biopotency*
	Liver	Plasma	Liver	Plasma	
DL- α -Tocopheryl acetate (DL- α -T-ac)	0.398	0.274	100	100	100
α -Tocopherol	1.163	0.779	292	284	149
β -Tocopherol	0.501	0.089	126	32	60
γ -Tocopherol	0.025	0.020	6	7	15
α -Tocotrienol	—	0.006	—	2	37

* The calculated biopotency factors are compared with the international conversion factors as reported by Bieri & McKenna (1981).

Tentatively, the individual biopotencies of the different isomers in the barley-oil diet were also calculated in the present study by comparing the dose response, diet:liver or plasma level, for each of the isomers separately (Table 6).

The low level of α -tocotrienol response in the plasma gave a diminutive potency factor of 2 for this isomer. On the other hand, using the liver-storage test, the biopotency factors for α - and β -tocopherols were calculated to be twice as high as the international conversion factors. At the same time, no biopotency at all could be calculated for corresponding tocotrienols in the diet. However, it was verified previously that by multiplying the amounts of the vitamin E isomers chemically detected in the barley-oil diet with the international conversion factors, the barley-oil diet had almost the same vitamin E activity in DL- α -tocopheryl acetate equivalents as did the DL- α -tocopheryl acetate reference diet (Fig. 4b).

In the light of the present study a tempting explanation for the absence of tocotrienols in the tissues may be that α - and β -tocotrienols are at least partly reduced to the corresponding tocopherols before they reach the liver.

General aspects

As stated earlier, the requirement for vitamin E may be defined as that amount of the vitamin which is needed to prevent the signs of a deficiency appearing. Similarly, the true biological activity of a natural mixture of vitamin E isomers in a feed should be determined by its ability to prevent or reverse specific vitamin E deficiency symptoms.

However, the biological activity of vitamin E of conventional feeds has very occasionally been established on this basis. Thus, only lucerne (*Medicago sativa*), which is reputedly one of the best natural sources of vitamin E, has been found to have a biological vitamin E activity of only one-quarter to one-third of that of DL- α -tocopheryl acetate as measured by the prevention of encephalomalacia in poultry (Singsen *et al.* 1955; Pudelskiewicz *et al.* 1957) and by the liver-storage assay (Bunnell, 1957).

Although the accuracy of the biopotency determinations in the present encephalomalacia-prevention test was handicapped by the killing of clinically healthy chicks for tocopherol control at regular intervals, the results of the test agreed fairly well with the results of the liver and plasma storage assays.

The total concentration of vitamin E in barley has been determined in our laboratory to vary from 55–65 mg/kg dry matter up to 95–100 mg/kg dry matter at the harvest time depending on the harvest year (Hakkarainen *et al.* 1983b). The results presented here point out the fact that barley is not as rich a source of vitamin E as could be expected on the

basis of the chemical determination of its total vitamin E content. In fact it was established that the total biological vitamin E activity of barley was only about 37% of that of DL- α -tocopheryl acetate. This should be born in mind when composing feed and food rations containing barley.

It must not be forgotten in this connection that the determination of the biological activity of the individual tocopherols and tocotrienols have been based on several of the effects of vitamin E compounds on the organism; resulting in a large scattering in observed values of the biological activity (Dam & Søndergaard, 1964; Leth & Søndergaard, 1977). To complicate matters, different types of bioassays, e.g. erythrocyte haemolysis test, muscular dystrophy test, liver storage test, etc., have given relative potencies frequently significantly different from those obtained by fetal resorption tests in female rats (Mattil, 1954; Bunyan *et al.* 1961; Century & Horwitt, 1965; Brubacher & Wiss, 1972).

These facts make the conclusions concerning the biopotency of individual feedstuffs still uncertain, even if the evaluations are based on the chemical determination of each isomer, but followed by summation for total vitamin E activity on the basis of those discrepant conversion factors. The determination of the true biopotency of vitamin E in a feedstuff, as in the present study, in barley, followed by an application of the international conversion factors for natural isomers for recalculating the results of the chemical isomer analyses thus introduces a new approach of both practical and scientific importance to these issues in animal and human nutrition.

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EXPLANATION OF PLATES

- Plate 1. Histological picture of thrombosed cerebellar capillaries with a haemorrhage and oedema. PAS × 510.
- Plate 2. Cerebellar cortex with an area of infarction and scar formation. Some foci of mineralization are also observed. HE × 80.
- Plate 3. Focal malacia in the cerebellar cortex with proliferation of vessels, macrophages and astrocytes. Some cells are mineralized. HE × 130.

