Phospholipid fatty acids of brain and liver are modified by α-tocopherol and dietary fat in growing chicks

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Dietary fatty acids modify phospholipid fatty acids in brain and liver of growing chickens post-hatching. The effect of vitamin E deficiency on this process is unknown and may be relevant to the pathogenesis of chick nutritional encephalomalacia (NE). Therefore laying hens received a diet low in vitamin E (10 mg α-tocopherol/kg feed). Resulting chicks were assigned to nine dietary groups each fed with either oleic (18:1*n*-9, 58 g/kg), linoleic (18:2*n*-6, 57 g/kg) or linolenic (18:3*n*-3, 56 g/kg) acid together with 5, 25 or 125 mg α-tocopherol/kg feed. NE affecting the cerebellum only occurred in the group given linoleic acid and 5 mg α-tocopherol/kg. In 1-d-old chicks and after 1 and 2 weeks the phospholipid fatty acid composition of liver, cerebrum and cerebellum (additionally after 3 weeks) was determined. The feed fatty acids were incorporated into the liver very efficiently during the first week of life. Unsaturation of liver membranes decreased in the order dietary linolenic > linoleic > oleic acid. In liver, also, the effect of α-tocopherol supplementation on phospholipid fatty acids was most pronounced. The unsaturation index increased during deficiency, whereas *n*-9 fatty acids decreased. In the chicken brain the alterations were delayed and less distinct. The cerebellum phospholipids were rich in *n*-9 fatty acids and as a whole more saturated in comparison with the cerebrum. Cerebellar unsaturation increased when linolenic or linoleic acid was given. However, NE-producing dietary conditions were not accompanied by specific alterations in cerebellar phospholipid fatty acids due to the α-tocopherol content of the diet. Rather the alterations of membrane fatty acids in the liver seem to play a role in the pathogenesis of NE.

Lipid peroxidation: Encephalomalacia: Vitamin E

A high concentration of polyunsaturated fatty acids in membranes causes susceptibility to peroxidative degradation. This is mediated by free radicals generated during normal cellular events or during metabolism of xenobiotics (Aust *et al.* 1993). Susceptibility to lipid peroxidation is also greatly influenced by tissue concentration of α-tocopherol, which is the most important, if not the only, radical scavenger within membranes *in vivo* (DiMascio *et al.* 1991).

One of the classical vitamin E-deficiency syndromes in the chick is nutritional encephalomalacia (NE). It is readily produced by vitamin E-deficient diets containing polyunsaturated fatty acids of the *n*-6 series (Budowski *et al.* 1987). From the chick (Fuhrmann & Sallmann, 1995) and other species (Bourre & Clement, 1991) it is known that the brain is a poor responder to dietary α-tocopherol. In broiler chickens NE affects only the cerebellum (Hassan *et al.* 1990), leading to ataxia, prostration and death. The disease normally occurs during the second or third week after hatching. It has been proposed that this is related to the cerebellar changes in polyunsaturated fatty acids during the third week of life (Budowski *et al.* 1987). Therefore, the present study was planned to investigate whether α-tocopherol modifies the incorporation of dietary fat into tissue phospholipid fatty acids in such a way as to clarify the role of dietary *n*-6 fatty acids in the pathogenesis

of NE. As an approach to the problem we used 1-d-old broiler chicks obtained from hens with a marginal supply of α-tocopherol. These chicks were reared on diets differing in α-tocopherol content and fat source. A time course was implemented during 3 weeks post-hatching to study in detail the fatty acid pattern in cerebellum in comparison with that in cerebrum and liver.

MATERIALS AND METHODS

To obtain 1-d-old chicks regularly low in tissue α-tocopherol, a breeder flock (Lohmann Meat; Lohmann, Cuxhaven, Germany) received a vitamin E-poor diet (10 mg α-tocopherol/kg) based on barley, wheat starch, cassava and soyabean meal, starting from the 13th week of life. The diet contained 50 g fat/kg with (g/100 g total fatty acids) saturated 33, monounsaturated 34, 18:2*n*-6 19 and *n*-3 fatty acids 11. From the 30th week the eggs were collected for 8 (SD 2) d and incubated. The newly hatched chicks received the basal semi-purified diet (Table 1) without fat and α-tocopherol for 48 h. Thereafter the chicks were randomly divided into groups of 26–29 animals, and each group was randomly allocated to one of the nine dietary treatments. From that time on the animals received the experimental diets containing 50 g fat/kg and the α-tocopherol supplement (Table 1). The compositions of the different dietary fats and the resulting fatty acid patterns are listed in Table 2. The fatty acid compositions of the single fat components are given in Table 3. The tocopherol content of the basal diet and the fat components were measured. The fat mixtures were adjusted to 100 mg α-tocopherol/kg fat with RRR-α-tocopherol. The vitamin E premixes contained 0, 0·5 and 2·5 mg α-tocopherol as all-*rac*-α-tocopheryl acetate/g maize starch to give 5, 25 and 125 mg α-tocopherol/kg in the final feed. The feed (basal diet + supplements) was remixed every week and stored in the dark at −22 °.

Each dietary group was housed in a separate wire cage. The environment in the fully air-conditioned room was kept constant at 32 ± 1° during the first week followed by a weekly reduction by 2–3° down to 25°. Humidity was maintained at 60 ± 5%; light was provided for 24 h. After 1, 2 and 3 weeks one third of the animals were randomly selected for examination. The birds were fasted for 16 h before they were anaesthetized by intramuscular injection of Metomidat-HCl (Hypnodil®; Janssen, Duesseldorf, Germany: 7·5 mg/kg body weight) killed and completely bled. Liver, cerebrum and cerebellum were homogenized in ice-cold 50 mM-KCl (pH 7·4) including butylated hydroxytoluene (BHT) (50 mg/l) for determination of phospholipid fatty acids and protein (Lowry *et al.* 1951). Samples were stored deep-frozen at −80° until further processing. Total lipids were extracted from the 100 g/l homogenates using the Bligh & Dyer (1959) method. A 0·8 ml portion of the sample was quickly thawed and homogenized in 1 ml chloroform containing 150 μg phosphatidylcholine-C17 as the internal standard and 2 ml methanol. The supernatant fraction from the centrifugation step (1000 ***g***, at room temperature for 5 min) was collected and mixed with 1 ml chloroform and 1 ml water. From the chloroform layer the phospholipids were separated by solid-phase extraction on 500 mg aminopropyl columns (Kaluzny *et al.* 1985). The columns were preconditioned with 4 ml hexane, then 0·5 ml of the extract was applied. Neutral lipids were eluted with 4 ml chloroform–isopropanol (98:2, v/v) and free fatty acids with 4 ml diethyl ether–glacial acid (96:4, v/v). Phosphatidylcholine, phosphatidylethanolamine and sphingomyelin were eluted with 4 ml methanol, phosphatidylserine and phosphatidylinositol with methanol–ammonia (250 ml/l) (95:5, v/v). The combined extracts were evaporated under a stream of N_2 and dissolved again in 2 ml chloroform–methanol (25:75, v/v) containing 100 μg BHT in tubes with Teflon-coated screw caps. For direct transesterification (Lepage & Roy, 1986) 200 μl acetylchloride was added slowly under continuous stirring. The tubes were heated for 1 h at 100° with the caps closed. The reaction was stopped in an ice bath by adding, slowly, 4 ml

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

Basal diet	
Maize starch	426
Soyabean meal	344
Mineral mixture*	70
Cellulose	40
Amino acids and vitamins†	20
Supplements	
Fat	50
Vitamin E premixture	50

* Contained (g/kg feed): $CaHPO_4.2H_2O$ 19·6, $CaCO_3$ 20·5, KH_2PO_4 15·2, $NaHCO_3$ 9·6, $MnSO_4.H_2O$ 0·38, $FeSO_4.7H_2O$ 0·54, $MgSO_4$ 3·28, KIO_3 10·0, $CuSO_4.5H_2O$ 0·036, $ZnCO_3$ 0·16, $CoCl_2$ 0·0034, $NaMoO_4.H_2O$ 0·009, $NiCl_2.H_2O$ 0·13, $Na_2SeO_3.5H_2O$ 0·333 mg.

† Rovimix 428 (Hoffmann–La Roche, Basle, Switzerland) without vitamin E.

Table 2. *Composition (g/kg fat) and fatty acid pattern (g/100 g total fatty acids) of the fats used in the experimental diets*

Diet	Fat source	g/kg fat	g/100 g total fatty acids			
			n-3	*n*-6	*n*-9	SFA
Linolenic acid-rich	Linolenic acid 62%*	860	—	—	—	—
	Tallow	140	56	14	18	11
Linoleic acid-rich	Soyabean triacylglycerol†	775	—	—	—	—
	Safflower-seed oil‡	125	—	—	—	—
	Linolenic acid 62%	100	8	57	16	13
Oleic acid-rich	Lard§	670	—	—	—	—
	Oleic acid 82%‖	240	—	—	—	—
	Linolenic acid 62%	90	6	7	58	23

SFA, saturated fatty acids.

* Serva, Heidelberg, Germany.

† Henkel, Düsseldorf, Germany.

‡ Lidl, Hannover, Germany.

§ ICN, Meckenheim, Germany.

‖ Merck, Darmstadt, Germany.

Table 3. *Fatty acid composition (g/100 g total fatty acids) of the fat components of the experimental diets**

Fatty acid	Linolenic acid	Safflower-seed oil	Soyabean oil	Lard	Tallow	Oleic acid
14:0	—	4·6	0·2	1·1	3·7	2·5
16:0	4·5	5·5	9·7	25·6	31·4	4·7
16:1*n*-7	—	—	—	3·3	1·9	0·3
18:0	3·0	2·1	4·5	10·9	24·1	—
18:1*n*-9	16·5	9·7	22·4	50·5	35·2	82·0
18:2*n*-6	15·5	75·5	55·2	7·9	1·6	4·8
18:3*n*-3	59·7	0·9	3·8	0·1	0·1	0·1

* For details of fat sources, see Table 2.

Table 4. *Phospholipid fatty acids (PL-FA; g/100 g total fatty acids) of the liver of chicks as affected by age* (n *68–74*), *dietary α-tocopherol concentration* (n *46–49*) *and dietary fatty acid type* (n *45–49*)

(Values are means with their standard errors for all chicks to whom the characteristic applied)

	Main effects								
	Age			α-Tocopherol			Fatty acid		
PL-FA	Weeks	Mean	SE	mg/kg	Mean	SE	Type	Mean	SE
Σs	0	41·5	0·80	5	39·9	0·20	18:3	40·6^{A}	0·20
	1	39·9	0·20	25	40·4	0·36	18:2	40·4^{A}	0·19
	2	40·5	0·14	125	40·2	0·14	18:1	39·4^{B}	0·14
ANOVA...								$P = 0·0001$	
18:0	0	24·3	0·97	5	24·7^{AB}	0·21	18:3	25·0^{A}	0·23
	1	23·9^{B}	0·16	25	25·4^{A}	0·23	18:2	25·1^{A}	0·21
	2	25·6^{A}	0·14	125	24·2^{B}	0·19	18:1	24·2^{B}	0·21
ANOVA...		$P < 0·0001$			$P = 0·0001$			$P < 0·0001$	
Σ*n*-3	0	14·3	1·89	5	14·8	1·26	18:3	24·5	0·58
	1	12·5	0·80	25	13·8	1·20	18:2	7·1	0·19
	2	15·2	1·10	125	13·1	1·10	18:1	10·0	0·29
ANOVA...		$P < 0·0001$			$P = 0·0011$			$P < 0·0001$	
22:6*n*-3	0	12·7	1·85	5	6·7	0·30	18:3	7·8	0·28
	1	5·7	0·18	25	6·1	0·27	18:2	5·1	0·17
	2	6·7	0·25	125	5·9	0·25	18:1	5·7	0·21
ANOVA...								$P < 0·0001$	
Σ*n*-6	0	35·9	1·10	5	28·1	1·26	18:3	18·6^{C}	0·34
	1	28·0	0·97	25	27·4	1·42	18:2	38·7^{A}	0·37
	2	26·9	1·22	125	26·9	1·21	18:1	24·6^{B}	0·37
ANOVA...								$P < 0·0001$	
20:4*n*-6	0	19·9	0·60	5	11·4	0·71	18:3	5·6	0·23
	1	11·2	0·45	25	10·9	0·77	18:2	16·6	0·32
	2	10·8	0·67	125	10·6	0·67	18:1	10·5	0·24
ANOVA...		$P = 0·0008$			$P = 0·0058$ SED 0·38			$P < 0·0001$	
Σ*n*-9	0	6·8	0·13	5	14·1^{B}	0·75	18:3	13·3^{B}	0·43
	1	15·8^{A}	0·55	25	15·0^{AB}	0·82	18:2	11·3^{C}	0·37
	2	14·6^{B}	0·66	125	16·3^{A}	0·66	18:1	21·4^{A}	0·42
ANOVA...					$P < 0·0001$			$P < 0·0001$	
UI	0	10·50	0·35	5	9·10^{A}	0·12	18:3	9·58	0·10
	1	8·63	0·08	25	8·75^{AB}	0·13	18:2	8·74	0·07
	2	9·02	0·11	125	8·65^{B}	0·10	18:1	8·11	0·08
ANOVA		$P < 0·0001$			$P < 0·0001$			$P < 0·0001$	

Σs, sum of saturated PL-FA; Σ*n*-3, sum of *n*-3 PL-FA; Σ*n*-6, sum of *n*-6 PL-FA; Σ*n*-9, sum of *n*-9 PL-FA; UI, unsaturation index.

$^{A, B, C}$ Mean values (main effects) not sharing a common superscript letter were significantly different ($P < 0·01$, Student–Newman–Keuls).

K_2CO_3 (60 g/l). A 2 μl portion of the supernatant fraction was analysed for fatty acid methyl esters by splitless injection on a Varian 3400 gas chromatograph (Varian, Darmstadt, Germany) equipped with a 30 m Supelcowax 10 fused silica capillary column (0·32 mm i.d.; 0·5 μm coating; Supelco, Deisenhofen, Germany). He gas (15 psi, 1·5 ml/min) served as carrier gas. Injection temperature was 250°, and the temperature of the flame ionization detector was 260°. The oven temperature was kept at 100° for 5 min,

Table 4—*cont.*

Age (weeks)	Interactions with α-tocopherol (mg/kg)*						Interactions with fatty acid*					
	5		25		125		18:3		18:2		18:1	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
1	14·0	1·45	11·9	1·27	11·6	1·41	21·2^{b}	0·45	7·0^{e}	0·28	9·4^{d}	0·41
2	15·6	2·05	15·7	1·99	14·3	1·61	27·5^{a}	0·53	7·2^{e}	0·27	10·6^{c}	0·37
			$P = 0·0056$ SED 0·48						$P = 0·0011$			
1	–	–	–	–	–	–	6·5^{b}	0·30	5·0^{e}	0·24	5·6^{bc}	0·31
2	–	–	–	–	–	–	9·0^{a}	0·31	5·2^{e}	0·24	5·8^{bc}	0·28
									$P = 0·0011$			
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
1	–	–	–	–	–	–	7·0^{e}	0·24	15·1^{b}	0·37	11·3^{c}	0·38
2	–	–	–	–	–	–	4·4^{f}	0·13	18·1^{a}	0·33	9·9^{d}	0·24
									$P < 0·0001$			
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
1	–	–	–	–	–	–	9·18^{b}	0·11	8·53^{c}	0·10	8·13^{e}	0·14
2	–	–	–	–	–	–	9·95^{a}	0·12	8·94^{b}	0·08	8·10^{e}	0·09
									$P = 0·0003$			

$^{a, b, c, d, e, f}$ Mean values (interactions) not sharing a common superscript letter were significantly different ($P < 0·01$, Student–Newman–Keuls).

* For age × tocopherol, n 23–27; for age × fat, n 21–25.

followed by an increase of 30°/min to reach 190°. The temperature was maintained for 5 min and then increased further up to 240° at 5°/min. Peaks were identified by relative retention times in comparison with commercially available standards. Detection limit was 0·5 ng fatty acid on-column. The inter-assay CV (n 6) were < 5·2% and < 10·6% for fatty acids representing more than 2 g and 0·5 g respectively, per 100 g total fatty acids. Results given in g/100 g total fatty acids were calculated using the internal standard method of the Apex data system, version 2.04 (Autochrom Inc., Milford, MA, USA). The sum of the

Table 5. *Phospholipid fatty acids (PL-FA; g/100 g total fatty acids) of the cerebrum of chicks as affected by age* (n *68–74*), *dietary α-tocopherol concentration* (n *46–49*) *and dietary fatty acid type* (n *45–49*)

(Values are means with their standard errors)

	Main effects								
	Age			α-Tocopherol			Fatty acid		
PL-FA	Weeks	Mean	SE	mg/kg	Mean	SE	Type	Mean	SE
Σs	0	48·7	0·92	5	47·6	0·37	18:3	47·9	0·37
	1	47·4	0·28	25	46·8	0·48	18:2	46·9	0·35
	2	46·8	0·33	125	46·9	0·25	18:1	46·5	0·38
18:0	0	18·3	0·46	5	21·6^{A}	0·14	18:3	21·3	0·15
	1	21·1^{B}	0·11	25	21·6^{A}	0·12	18:2	21·6	0·14
	2	21·7^{A}	0·11	125	21·0^{B}	0·13	18:1	21·4	0·12
ANOVA...		$P = 0·0001$			$P = 0·0001$				
Σ*n*-3	0	19·9	1·01	5	18·6	0·63	18:3	21·6^{A}	0·47
	1	18·3	0·34	25	18·2	0·47	18:2	16·3^{B}	0·32
	2	18·6	0·46	125	18·5	0·42	18:1	17·4^{B}	0·34
ANOVA...								$P < 0·0001$	
22:6*n*-3	0	18·6	1·01	5	16·3	0·40	18:3	18·0^{A}	0·32
	1	16·7	0·28	25	16·4	0·36	18:2	15·4^{B}	0·30
	2	16·3	0·29	125	16·6	0·31	18:1	16·1^{B}	0·32
ANOVA...								$P < 0·0001$	
Σ*n*-6	0	15·0	1·12	5	15·0	0·45	18:3	12·2	0·21
	1	15·3	0·22	25	15·0	0·41	18:2	18·3	0·22
	2	14·7	0·41	125	14·9	0·40	18:1	14·4	0·14
ANOVA...		$P = 0·0002$						$P < 0·0001$	
20:4*n*-6	0	9·9	0·13	5	10·0	0·27	18:3	8·4	0·14
	1	10·3	0·12	25	10·0	0·22	18:2	11·7	0·11
	2	9·7	0·23	125	9·9	0·28	18:1	9·7	0·09
ANOVA...		$P < 0·0001$						$P < 0·0001$	
Σ*n*-9	0	12·4	0·12	5	13·7	0·27	18:3	13·2	0·13
	1	13·8	0·16	25	14·4	0·24	18:2	13·2	0·15
	2	14·4	0·23	125	14·3	0·25	18:1	16·1	0·20
ANOVA...		$P = 0·0012$			$P = 0·0015$			$P < 0·0001$	
					SED 0·20				
UI	0	9·52	0·27	5	9·00	0·11	18:3	9·12	0·10
	1	9·06	0·08	25	9·02	0·11	18:2	9·09	0·10
	2	9·04	0·08	125	9·12	0·08	18:1	8·94	0·10

Σs, sum of saturated PL-FA; Σ*n*-3, sum of *n*-3 PL-FA; Σ*n*-6, sum of *n*-6 PL-FA; Σ*n*-9, sum of *n*-9 PL-FA; UI, unsaturation index.

^{A,B} Mean values (main effects) not sharing a common superscript letter were significantly different ($P < 0·01$, Student–Newman–Keuls).

saturated phospholipid fatty acids (Σs) included 12:0, 14:0, 16:0, 20:0, 22:0 and 24:0 besides the 18:0 shown in the tables. The sum of the *n*-3 fatty acids (Σ*n*-3) included 18:3, 20:3, 20:5 and 22:5 besides the 22:6 shown in the tables. The sum of the *n*-6 fatty acids (Σ*n*-6) included 18:2, 18:3, 20:2, 20:3, 22:4 and 22:5 besides the 20:4 shown in the tables. The sum of the phospholipid *n*-9 fatty acids (Σ*n*-9) included 16:1, 18:1, 20:1, 20:3 and 22:1. Dimethyl acetals from plasmalogens are not included in the sums of saturates and monoenes. An unsaturation index (UI) was calculated from the unsaturated fatty acids. It

Table 5—*cont.*

Age (weeks)	Interactions with fatty acid*					
	18:3		18:2		18:1	
	Mean	SE	Mean	SE	Mean	SE
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
1	13·5[d]	0·15	17·3[b]	0·24	15·1[c]	0·18
2	10·9[e]	0·12	19·2[a]	0·23	13·9[d]	0·15
			$P < 0·0001$			
1	9·3[d]	0·09	11·4[b]	0·14	10·2[c]	0·10
2	7·6[e]	0·09	12·1[a]	0·13	9·2[d]	0·08
			$P < 0·0001$			
1	13·0[c]	0·18	13·4[c]	0·24	15·1[b]	0·19
2	13·4[c]	0·18	13·1[c]	0·19	16·9[a]	0·24
			$P < 0·0001$			
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–

[a, b, c, d, e] Mean values (interactions) not sharing a common superscript letter were significantly different ($P < 0·01$, Student–Newman–Keuls).

* For age × fat interactions, n 21–25.

is defined as the sum of the products of % total fatty acids and number of double bonds, divided by the number of C atoms of each fatty acid. Division by chain length was done to correct for the different ratio between double and single bonds in each fatty acid.

Statistical analysis was done by 3-factorial ANOVA for the factors age, dietary α-tocopherol and fat (Friedman, 1986). The grouping due to the fact that the chicks on the same diet were kept in the same cage was not considered. When the Bartlett–Box F test indicated variance inhomogeneity the data were transformed logarithmically. Main effects and interactions were assumed to be significant at $P < 0·01$. In this case means were tested

Table 6. *Phospholipid fatty acids (PL-FA; g/100 g total fatty acids) of the cerebellum of chicks as affected by age* (n *69–75*), *dietary α-tocopherol concentration* (n *68–75*) *and dietary fatty acid type* (n *70–74*)

(Values are means with their standard errors)

	Main effects								
	Age			α-Tocopherol			Fatty acid		
PL-FA	Weeks	Mean	SE	mg/kg	Mean	SE	Type	Mean	SE
Σs	0	46·9	1·33	5	43·8	0·29	18:3	43·8	0·46
	1	43·4	0·25	25	43·4	0·26	18:2	43·3	0·27
	2	42·5	0·26	125	43·5	0·31	18:1	43·8	0·31
	3	45·0	0·28	—	—	—	—	—	—
ANOVA...		$P = 0·0001$							
18:0	0	17·0	0·35	5	19·7	0·09	18:3	19·0	0·10
	1	19·1	0·17	25	19·0	0·41	18:2	19·3	0·12
	2	19·3	0·41	125	19·0	0·12	18:1	19·5	0·44
	3	19·3	0·11	—	—	—	—	—	—
Σ*n*-3	0	17·7	1·66	5	17·2	0·38	18:3	20·8	0·29
	1	18·2	0·29	25	17·7	0·47	18:2	15·3	0·29
	2	18·1	0·42	125	17·1	0·42	18:1	15·7	0·30
	3	15·7	0·46	—	—	—	—	—	—
ANOVA...		$P < 0·0001$						$P < 0·0001$	
22:6*n*-3	0	16·3	1·69	5	14·6	0·29	18:3	$15·7^{A}$	0·26
	1	$15·5^{A}$	0·23	25	14·5	0·29	18:2	$13·5^{B}$	0·27
	2	$14·7^{A}$	0·27	125	13·8	0·30	18:1	$13·6^{B}$	0·27
	3	$12·7^{B}$	0·27	—	—	—	—	—	—
ANOVA...		$P < 0·0001$						$P < 0·0001$	
Σ*n*-6	0	14·6	0·39	5	12·7	0·36	18:3	9·4	0·16
	1	12·9	0·36	25	12·5	0·42	18:2	16·4	0·22
	2	11·9	0·38	125	12·4	0·38	18:1	11·9	0·12
	3	12·9	0·50	—	—	—	—	—	—
ANOVA...		$P < 0·0001$						$P < 0·0001$	
20:4*n*-6	0	8·1	0·33	5	6·7	0·17	18:3	5·1	0·10
	1	7·0	0·08	25	6·6	0·20	18:2	8·3	0·10
	2	6·2	0·17	125	6·6	0·18	18:1	6·4	0·08
	3	6·7	0·24	—	—	—	—	—	—
ANOVA...		$P < 0·0001$						$P < 0·0001$	
Σ*n*-9	0	17·1	0·25	5	20·2	0·24	18:3	20·1	0·19
	1	19·6	0·16	25	20·2	0·27	18:2	19·0	0·21
	2	21·1	0·26	125	20·6	0·26	18:1	22·0	0·26
	3	20·3	0·29	—	—	—	—	—	—
ANOVA...		$P < 0·0001$						$P < 0·0001$	
UI	0	8·63	0·42	5	8·38	0·06	18:3	$8·57^{A}$	0·07
	1	8·65	0·06	25	8·48	0·08	18:2	$8·51^{A}$	0·06
	2	8·55	0·07	125	8·36	0·09	18:1	$8·12^{B}$	0·08
	3	8·02	0·07	—	—	—	—	—	—
ANOVA...		$P < 0·0001$						$P < 0·0001$	

Σs, sum of saturated PL-FA; Σ*n*-3, sum of *n*-3 PL-FA; Σ*n*-6, sum of *n*-6 PL-FA; Σ*n*-9, sum of *n*-9 PL-FA; UI, unsaturation index.

[A, B] Mean values (main effects) not sharing a common superscript letter were significantly different ($P < 0·01$, Student–Newman–Keuls).

Table 6—*cont.*

Age (weeks)	Interactions with α-tocopherol (mg/kg)*						Interactions with fatty acid*					
	5		25		125		18:3		18:2		18:1	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	$43{\cdot}8^{b}$	0·35	$43{\cdot}5^{b}$	0·52	$42{\cdot}8^{b}$	0·43	–	–	–	–	–	–
2	$43{\cdot}3^{b}$	0·40	$42{\cdot}6^{b}$	0·51	$41{\cdot}7^{b}$	0·38	–	–	–	–	–	–
3	$44{\cdot}6^{ab}$	0·43	$44{\cdot}1^{ab}$	0·31	$46{\cdot}2^{a}$	0·58	–	–	–	–	–	–
			$P = 0{\cdot}0005$									
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
1	–	–	–	–	–	–	$20{\cdot}1^{b}$	0·51	$17{\cdot}2^{c}$	0·35	$17{\cdot}2^{c}$	0·35
2	–	–	–	–	–	–	$22{\cdot}1^{a}$	0·44	$16{\cdot}0^{c}$	0·34	$15{\cdot}8^{e}$	0·53
3	–	–	–	–	–	–	$20{\cdot}1^{b}$	0·48	$12{\cdot}5^{e}$	0·24	$14{\cdot}2^{d}$	0·48
									$P < 0{\cdot}0001$			
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
1	–	–	–	–	–	–	$11{\cdot}2^{e}$	0·14	$14{\cdot}7^{c}$	0·15	$12{\cdot}6^{d}$	0·13
2	–	–	–	–	–	–	$8{\cdot}6^{f}$	0·12	$16{\cdot}1^{b}$	0·18	$11{\cdot}0^{e}$	0·13
3	–	–	–	–	–	–	$8{\cdot}6^{f}$	0·14	$18{\cdot}7^{a}$	0·24	$12{\cdot}0^{d}$	0·22
									$P < 0{\cdot}0001$			
1	–	–	–	–	–	–	$6{\cdot}3^{e}$	0·07	$7{\cdot}7^{c}$	0·08	$7{\cdot}0^{d}$	0·08
2	–	–	–	–	–	–	$4{\cdot}7^{g}$	0·06	$8{\cdot}1^{b}$	0·10	$5{\cdot}9^{f}$	0·07
3	–	–	–	–	–	–	$4{\cdot}5^{g}$	0·08	$9{\cdot}3^{a}$	0·14	$6{\cdot}5^{e}$	0·14
									$P < 0{\cdot}0001$			
1	–	–	–	–	–	–	$19{\cdot}2^{de}$	0·23	$19{\cdot}1^{de}$	0·29	$20{\cdot}6^{bcd}$	0·20
2	–	–	–	–	–	–	$20{\cdot}1^{cd}$	0·26	$19{\cdot}7^{cd}$	0·28	$23{\cdot}6^{a}$	0·37
3	–	–	–	–	–	–	$20{\cdot}8^{bc}$	0·41	$18{\cdot}1^{e}$	0·46	$21{\cdot}7^{b}$	0·34
									$P < 0{\cdot}0001$			
1	$8{\cdot}61^{ab}$	0·11	$8{\cdot}64^{ab}$	0·12	$8{\cdot}70^{a}$	0·11	–	–	–	–	–	–
2	$8{\cdot}39^{ab}$	0·11	$8{\cdot}59^{ab}$	0·16	$8{\cdot}65^{ab}$	0·10	–	–	–	–	–	–
3	$8{\cdot}08^{bc}$	0·09	$8{\cdot}24^{ab}$	0·10	$7{\cdot}72^{c}$	0·14	–	–	–	–	–	–
			$P = 0{\cdot}008$									

[a–g] Mean values (interactions) not sharing a common superscript letter were significantly different ($P < 0{\cdot}01$, Student–Newman–Keuls).

* For age × tocopherol interactions, n 19–27; for age × fat interactions, n 22–27.

for significant differences (99% confidence level) by the Student–Newman–Keuls test. When the Student–Newman–Keuls test failed to show significant differences between means despite significant main effects or interactions according to the ANOVA, the standard error of difference between any two means (SED) was calculated. Data from the newly hatched chicks (n 4) were not included.

RESULTS

Body-weight gain and feed conversion values did not indicate any feeding effects (results not shown). The bias produced by keeping the chicks on the same diets in the same cages is considered to be minimal, because the groups were randomly allocated to the treatments and the environmental conditions in the fully climatized room were the same for all groups. Furthermore, the basal diet was prepared in one batch for the whole trial. Thus, possible effects on analysis should be negligible. Beginning at day 9, clinical signs of NE occurred in four out of twenty-nine animals from the group that received the α-tocopherol-deficient, linoleic acid-rich diet.

Phospholipid fatty acid composition of the liver

Dietary fatty acids greatly affected the phospholipid fatty acid composition of the liver (Table 4). Oleic acid-rich fat led to a significantly lower proportion of Σs in liver phospholipids in comparison with linolenic and linoleic-acid feeding. The influence of α-tocopherol on Σs was inconsistent, because 16:0 (not shown) and 18:0 responded differently. Dietary linolenic acid elevated Σn-3 from 14·3 g/100 g in the 1-d-old chick up to 27·5 g/100 g total fatty acids after 2 weeks. This was attributable to 20:5n-3 (0·5 → 12 g/100 g, results not shown). On the other hand 22:6n-3 decreased. Σn-3 was influenced by vitamin E. Feeding linoleic acid to the animals increased Σn-6 in comparison with the level of the newly hatched chick. Σn-6 was not affected by α-tocopherol; 20:4n-6 was the n-6 fatty acid with the most distinctive changes during growth. α-Tocopherol significantly affected 20:4n-6. This was independent of the fat source supplied. Σn-9 varied between 11·3 g/100 g in the linoleic and 21·4 g/100 g total fatty acids in the oleic acid-rich groups. The 125 mg α-tocopherol level led to a higher proportion of Σn-9. This was attributable to the increase of 18:1n-9 (not shown) in liver phospholipids. The UI in liver, as a measure of the unsaturation of membrane phospholipids, decreased after hatching to increase again after 2 weeks when linolenic or linoleic acid was fed. The highest values were found in vitamin E-deficient animals and when linolenic acid was fed.

Phospholipid fatty acid composition of the cerebrum

In the cerebrum the Σs was not affected by dietary conditions and age (Table 5). However 18:0 was reduced by the diet containing 125 mg dietary α-tocopherol/kg. Σn-3 in cerebrum was significantly raised by feeding linolenic acid; 22:6n-3, as the relevant fatty acid of this family, was higher in the linolenic acid groups than in those given linolenic or oleic acid. Σn-6 was affected by age and dietary fat. Only the linoleic acid-rich diet led to a significant increase of this fatty acid family after 2 weeks. The 20:4n-6 decreased during the first 2 weeks when linolenic or oleic acid was supplied. Σn-9 was significantly heightened when oleic acid was the main dietary fatty acid. The cerebral UI was not influenced by the experimental conditions.

Phospholipid fatty acid composition of the cerebellum

In the cerebellum (Table 6) Σs depended on age, with an increase after 3 weeks. This was most pronounced when 125 mg α-tocopherol/kg was supplied. Cerebellar Σn-3 was significantly heightened by feeding linolenic acid especially after 2 weeks. In the linoleic

acid groups the Σ*n*-3 showed a drastic decrease from 17·2 g/100 g after 1 week to 12·5 g/100 g total fatty acids after 3 weeks. This was also valid for the oleic acid groups but to a lesser extent. The level of 22:5*n*-3 (not shown) was raised from the 1-d-old chick; 22:6*n*-3, on the other hand, decreased over all groups. Feeding linoleic acid to the animals for 3 weeks led to a rise of Σ*n*-6 up to 18·7 g/100 g total fatty acids in comparison with the level of the newly hatched chick (14·6 g/100 g). As in liver and cerebrum Σ*n*-6 was not affected by α-tocopherol; 20:4*n*-6 decreased after hatching to increase again, when linoleic acid was fed. Σ*n*-9 and its individual fatty acids increased after hatching. However there was a drop after 3 weeks within the linoleic and oleic acid groups. Oleic acid feeding led to a significantly lower UI after 3 weeks, when 125 mg α-tocopherol/kg was supplied. The UI of cerebellar phospholipids (total mean 8·40, 95% CI 8·32, 8·49) was lower in comparison with cerebrum (UI: 8·83, 95% CI, 8·69, 8·97) and liver (UI: 9·05, 95% CI 8·94, 9·16).

DISCUSSION

Lipid metabolism of the newly hatched chick undergoes a rapid and extensive change (Noble *et al.* 1993). The residual lipid of the yolk is completely taken up by day 4 after hatching (Chamblee *et al.* 1992). During this time normal lipid digestion is established. The chicken diets used in the present experiment were designed to be free from very-long-chain fatty acids in order to study the effect of α-tocopherol on tissue incorporation of the C18 fatty acids. The experimental conditions should give hints as to the influence of α-tocopherol on this process during the first weeks post-hatching.

α-Tocopherol (Fuhrmann & Sallmann, 1995) and tissue fatty acids (Anderson *et al.* 1989, 1990, 1992; Lin *et al.* 1991; Anderson, 1994) of the newly hatched chick are extensively determined by the diet of the laying hen and therefore important for the susceptibility of chicks to NE (Bartov & Bornstein, 1980). The hens' diet was marginally supplied with α-tocopherol in order to produce chickens with low tissue α-tocopherol levels. This was intended to initiate early onset of the disease. On the other hand, 5 mg α-tocopherol/kg was used as the low dose in the chicken diet to avoid high mortality due to encephalomalacia, resulting in the complete loss of the vitamin E-deficient group given linoleic acid. The hens' feed contained 18:2*n*-6 and 18:3*n*-3 from soyabean oil together with long-chain *n*-3 fatty acids from fish meal (11·2 g total *n*-3/100 g, *n*-6:*n*-3 1·7) leading to 14 g/100 g Σ*n*-3 in liver, 20 g/100 g Σ*n*-3 in cerebrum and 18 g/100 g Σ*n*-3 in cerebellum phospholipids. The hen's diet is responsible for the differences in the fatty acid pattern of our 1-d-old chicks in comparison with that in an earlier experiment by Anderson *et al.* (1989). The authors investigated the effect of the hen's diet on tissue fatty acids of newly hatched chicks and reported a comparable fatty acid pattern and *n*-6:*n*-3 in chicks stemming from hens fed on a soyabean-oil diet.

In chicken, Σs of the tissues examined was regulated within a narrow range. However an influence of dietary fat existed in the liver when oleic acid-rich fats were given. Σs was diminished, probably in response to the lower content of polyenoic fatty acids in phospholipids under oleic acid-rich diets. This might help to maintain fluidity of the liver membrane, which is in large part determined by chain length and desaturation of phospholipid fatty acids (Sardesai, 1992). In an earlier study of *n*-3 fatty acid deficiency in chicks Anderson *et al.* (1990) found that dietary 18:3*n*-3 was not very effective in raising levels of 20:5*n*-3 and 22:6*n*-3 after hatching. This is apparently different to the present results in which high levels of 22:6*n*-3 in brain were kept constant by feeding 18:3*n*-3. On the other hand, liver 22:6*n*-3 fell from 12·7 g/100 g total fatty acids at hatching to 6·5 g/100 g after 1 week. This is probably connected with an extensive transfer of long-chain *n*-3 fatty acids from liver to brain. However, at least in the rat, brain endothelia together with astrocytes are capable of desaturation and elongation (Moore *et al.* 1991).

For the synthesis of 20:4*n*-6, Δ6-desaturase is the rate-limiting enzyme. It has gradual preferences for C18 fatty acids in the order 18:3*n*-3 > 18:2*n*-6 > 18:1*n*-9 (Jeffcoat & James, 1984). In addition, *n*-3 fatty acids inhibit the conversion of 18:2*n*-6 to 18:3*n*-6 (Garg *et al.* 1990), resulting in substrate accumulation and product reduction. This obviously led to the higher levels of 22:5*n*-3 (results not shown) and 22:6*n*-3 and reduction of 20:4*n*-6 in all tissues examined in the present study.

It has been stated that the resistance to diet-induced changes of brain 22:6*n*-3 develops after 3 weeks post-hatching (Anderson *et al.* 1992; Anderson, 1994). Our present results and an earlier report (Budowski *et al.* 1987) on the cerebellum with animals fed for 3 weeks confirm that the most prominent changes occur between weeks 2 and 3. However, in the liver the diet-induced changes occurred mainly during the first week. Notable was the intermediate effect of the oleic acid diet on *n*-3 and *n*-6 phospholipid fatty acids probably caused by the balanced *n*-6:*n*-3 value (1·2).

There is considerable evidence that vitamin E influences tissue fatty acids (Infante, 1986). In liver and cerebrum increasing amounts of α-tocopherol led to lower levels of 18:0, as recently described in the liver of rats (Clement & Bourre, 1993). In the cerebellum α-tocopherol had no effect. In liver α-tocopherol affected Σ*n*-3 mainly after 1 week. An increasing effect of α-tocopherol deficiency on Σ*n*-3 has been observed previously in rat liver and brain (Clement & Bourre, 1993). It has been speculated (Witting, 1969) that during vitamin E deficiency the fatty acids are prone to peroxidative loss. The elevated levels observed are thought to be a reflection of an increased turnover of polyunsaturated fatty acids. Others (Buttriss & Diplock, 1988) did not find a rise in *n*-3 fatty acids in α-tocopherol-depleted rats.

According to our results 20:4*n*-6 was influenced by vitamin E, but only in liver. A rise of 20:4*n*-6 due to vitamin E deficiency has also been observed by others (Witting, 1969; Leat, 1983; Cunnane, 1988). This effect has been ascribed to the lack of inhibition of desaturases by α-tocopherol. Others reported no alterations in rats (D'Aquino *et al.* 1985; Buttriss & Diplock, 1988; Clement & Bourre, 1993) and chicken (Klaus *et al.* 1995) or even a decrease that has been attributed to the lack of protection of the polyunsaturated fatty acids (D'Aquino *et al.* 1985; Buttriss & Diplock, 1988). A reduction of α-tocopherol produced lower levels of Σ*n*-9. This effect of α-tocopherol on *n*-9 phospholipid fatty acids was found only in liver and cerebrum. Differences between tissues have been described previously (Clement & Bourre, 1993). The probable underlying mechanism is the influence of Δ9-desaturase activity by α-tocopherol (Okayasu *et al.* 1977).

Under the experimental conditions described here NE began at day 9. In previous experiments (Sallmann *et al.* 1991; Fuhrmann *et al.* 1994) with the same type of linoleic acid-rich fat but commercial chicks the disease started after 16 d. So the onset of the disease obviously depends rather on the α-tocopherol supply of the newly hatched chick than on the alterations of polyunsaturated fatty acids in the cerebellum as stated by Budowski *et al.* (1987).

According to our present results dietary fatty acids did modify tissue phospholipids extensively during the first 3 weeks of life. In liver the changes were most pronounced during the first week, whereas in brain the response was delayed. The UI of the cerebellum increased when both linolenic and linoleic acid were fed. Thus, the occurrence of NE in both deficient groups given fats rich in either 18:2 or 18:3 should be expected, because there is no difference in the tissue α-tocopherol status due to the type of dietary fat (Fuhrmann & Sallmann, 1995).

As an explanation for the occurrence of NE only with dietary linoleic, not linolenic acid, Budowski & Crawford (1985) postulated a protective effect of *n*-3 fatty acids. Budowski *et al.* (1987) later stated that the lack of α-tocopherol in the cerebellum leads to an increased

peroxide tone. In a self-propagating process overproduction of eicosanoids derived from 20:4*n*-6 should result in thrombus formation and ischaemia. The protective effect of *n*-3 fatty acids consists of inhibition of 20:4*n*-6 synthesis and promotion of the production of 20:5*n*-3 eicosanoids. However, according to the present results NE-producing dietary conditions were not accompanied by specific alterations in cerebellar phospholipid fatty acids in response to the α-tocopherol content of the diet. The effect of α-tocopherol on brain fatty acids seems to be limited to the saturated and monounsaturated fatty acids. However, this is not so in liver, where deficiency caused an increase of membrane unsaturation attributable to the pattern of Σn-3 and 20:4*n*-6. The results lead us to the assumption that *n*-6 fatty acids have a pro-oxidative-toxic effect stemming from 20:4*n*-6 of the liver or arising in the cerebellum with its low content of α-tocopherol. Previously Budowski *et al.* (1979) found that oxidation products from *n*-6 fatty acids were encephalopathogenic in vitamin E-deficient chicks. Therefore, the question of why only linoleic acid-rich fats induce the disease should concentrate now on toxic degradation products of *n*-6 fatty acids.

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