Rapid modulation of the n–3 docosahexaenoic acid levels in the brain and retina of the newly hatched chick

Gregory J. Anderson,[1] William E. Connor, Julie D. Corliss, and Don S. Lin

Section of Clinical Nutrition and Lipid Metabolism, Department of Medicine, The Oregon Health Sciences University, Portland, OR 97201

Abstract The newly hatched chick obtains its fatty acids almost completely from the lipids of the egg yolk as these are transferred to the developing embryo during its 21-day period of incubation. Since the diet of the laying hen greatly influences the fatty acid composition of the egg lipids, and presumably also the fatty acid composition of the resulting chick, we tested how quickly and to what extent varying the amount of n-3 fatty acids in the diet of the hen would modulate the level of n-3 fatty acids in the brain and retina of the newly hatched chick. White Leghorn hens were fed commercial or semi-purified diets supplemented with 10% fish oil, linseed oil, soy oil, or safflower oil. Eggs, together with the brain, retina, and serum of newly hatched chicks, were then analyzed for fatty acid composition. The fatty acids of egg yolk responded quickly to the hen's diet with most of the change occurring by 4 weeks. There was a linear relationship between the linolenic acid content of the diets and levels of this fatty acid in egg yolk and chick serum. In chicks from hens fed the fish oil diet, the total n-3 fatty acids, including 22:6(n-3), were elevated twofold in the brain and retina and sevenfold in serum relative to commercial diet controls. The safflower oil diet led to a very low n-3 fatty acid content in egg yolks and only 25% of the control n-3 fatty acid content in the brain and retina of chicks. However, 5'-nucleotidase activity in chick brain homogenate was unaffected by the various diets. This study demonstrates that the n-3 fatty acid content of neural tissue in newly hatched chicks can be quickly and conveniently manipulated through the diet of the laying hen.—**Anderson, G. J., W. E. Connor, J. D. Corliss, and D. S. Lin.** Rapid modulation of the n-3 docosahexaenoic acid levels in the brain and retina of the newly hatched chick. *J. Lipid Res.* 1989. **30:** 433–441.

Supplementary key words docosahexaenoic acid • n-3 fatty acids • brain • retina • chick

N-3 fatty acids from fish oils are the focus of increasing research interest. These fatty acids in the diet are antithrombotic and hypolipidemic and can be metabolized to the 3-series prostaglandins (1, 2). The consumption of even modest quantities of marine foods rich in n-3 fatty acids is associated with decreased risk of cardiovascular disease (for recent reviews see references 3, 4). N-3 fatty acids cannot be synthesized by animals and birds and are required in the diet. They have important structural functions, since the membrane phospholipids of some tissues, such as the brain, retina, and sperm are rich in docosahexaenoic acid (22:6 n-3) (5–7). In addition, the activity of at least one membrane-bound enzyme, 5'-nucleotidase (8), has been reported to depend on dietary linolenic acid (18:3 n-3).

Despite this circumstantial evidence for essentiality, the consequences of a deficiency of n-3 fatty acids are subtle in most animal species except for fish (for recent reviews see references 9, 10). However, recent work with rhesus monkeys that were deprived in utero and raised from birth on a liquid formula deficient in n-3 fatty acids has demonstrated a substantial decrease of visual acuity and an abnormal electroretinographic response (11, 12). The continuing interest in the topic of n-3 fatty acid essentiality is of particular importance since some commercial infant formulas contain very low amounts of n-3 fatty acids, and none contain the fatty acid 22:6(n-3) which is present in human milk. Experimental constraints, such as the expense of primate studies and the need for multigeneration work with rats (13), have slowed progress in this field. We reasoned that the large lipid flux through the laying hen (5.5 g of fat per day) should allow for rapid diet-induced modulation of the fatty acid profile of the hen and her progeny. We wish to report here that the laying hen/newly hatched chick is a convenient model for the study of n-3 fatty acid metabolism and for the development of dietary n-3 fatty acid deficiency.

METHODS

Animals and diets

Single-comb white Leghorn laying hens (commercial strain) were housed individually under 16 hr light/day and

[1]To whom correspondence should be addressed.

fed oil-supplemented diets designed to manipulate tissue levels of n-3 fatty acids in the progeny. Diets 1-3, intended to show the effect of normal to large amounts of n-3 fatty acids in the diet, consisted of: 1) commercial chow for laying hens, consisting principally of corn mash with 2.9% fat, (control diet); 2) this same chow supplemented with 100 g linseed oil/kg chow (linseed oil diet); and 3) chow supplemented with 100 g MaxEPA fish oil/kg chow (fish oil diet). The linseed oil diet offered a very high concentration of linolenic acid (18:3 n-3), while the fish oil diet contained substantial amounts of the chain elongation/desaturation metabolites of linolenic acid, namely eicosapentaenoic (20:5 n-3) and docosahexaenoic (22:6 n-3) acids. Diets 4 and 5 were intended to demonstrate the effects of a low intake of n-3 fatty acids, and, therefore, required the use of a semi-purified fat-free basal mix (Teklad, Madison, WI). This base was supplemented with 100 g soy oil/kg diet (soy oil diet) or 100 g safflower oil/kg diet (safflower oil diet), respectively. The safflower oil diet had very little 18:3(n-3) and in addition contained a high concentration of 18:2(n-6). This high ratio of n-6/n-3 fatty acids is known to exacerbate the resulting n-3 fatty acid deficiency induced by the diet, since the two families of fatty acids compete for the same delta-6 desaturase enzyme (14). The soy oil diet served as both a source of moderate quantities of dietary linolenic acid and as an additional control for the semi-purified deficient diet. In preparing the diets, the various oils were combined directly with the powdered basal mixes. Diets were stored at 4°C and made fresh weekly, while the feed cups were changed daily. The composition of the semi-purified diet is shown in **Table 1**, while the fatty acid composition of all the diets is shown in **Table 2**. The fatty acid composition of the stored diets did not change after 1 week.

TABLE 1. Composition of semipurified diet for laying hens

	g/100 g
Dextrose monohydrate	62.53
Fat-free casein	20.78
L-Arginine HCl	0.39
DL-Methionine	0.39
Cellulose	3.33
Salt mix[a]	12.20
Vitamin mix[b]	0.38
Soy or safflower oil	10.00

[a]Salt mix (per 100 g diet): 6.82 g, $CaCO_3$, 3.78 g $CaHPO_4 \cdot 2H_2O$, 0.77 g potassium citrate H_2O, 0.57 g NaCl, 0.15 g MgO, 66.7 mg ferric citrate, 17.1 mg $MnSO_4 \cdot H_2O$, 20 mg $ZnCO_3$, 6.67 mg $CuSO_4 \cdot 5H_2O$, 3.22 mg CrK $(SO_4)_2 \cdot 12H_2O$, 1.0 mg KIO_3, 0.28 mg $Na_2MoO_4 \cdot 2H_2O$, 50 μg $Na_2SeO_3 \cdot 5H_2O$.

[b]Vitamin mix (per 100 g diet): 0.35 g choline bitartrate, 3.33 mg niacin, 5.56 mg vitamin B_{12}, 1.67 mg calcium pantothenate, 2.22 mg thiamine · HCl, 1.78 mg menadione sodium bisulfite complex, 1.67 mg pyridoxine, 1.67 mg riboflavin, 0.44 mg folic acid, 60 μg biotin, 11.11 mg vitamin E acetate (500 U/g), 3.33 mg vitamin A palmitate (500,000 U/g), 0.67 mg vitamin D_3 (500,000 U/g).

Every other night a white Leghorn rooster was placed in the cage with the laying hens for insemination. Fertile eggs were collected daily, stored at 40°F, and set in groups of approximately seven eggs each in a Marsh Roll-X automatic incubator at 98-99°F. The fatty acid composition of the yolks of unincubated eggs was monitored weekly and become stable after 4 weeks of the experimental diets fed to the laying hens. The various experimental diets produced no differences in egg production, fertility, or hatchability.

Chicks were killed within 12 hr of hatching and were not fed. Blood was collected by heart puncture immediately after exposure to a lethal dose of ether. Total lipids were extracted from brain, retina, and egg yolk with chloroform-methanol 2:1. Fatty acids were liberated by incubation of a dried aliquot of extract with 2 ml of 6% ethanolic KOH for 1 hr at 37°C. After addition of water, acidification, and recovery of the fatty acids by hexane extraction, methyl esters were prepared by heating the dried extract with 1 ml of 12% BF_3 in methanol for 10 min at 100°C in tightly sealed tubes with Teflon-coated screw caps (15). Serum fatty acids were liberated directly by incubation of 0.1-ml aliquots with ethanolic KOH. All solvent evaporations were carried out under a gentle stream of nitrogen to prevent lipid peroxidation. Fatty acid methyl esters were separated on a 30-meter Supelco SP 2330 fused silica capillary column (column temperature 185-190°C) attached to a Perkin-Elmer Sigma 3B gas chromatograph equipped with an HP 85 computer/3390A integrator. Results are reported as percent of total fatty acids, by weight. Lipid class composition was not determined in this study, since others have found it not to change after similar modification of the fatty acid composition of rat brain (16).

Diet-induced differences in levels of selected fatty acids were examined by multiple *t*-tests using the Bonferroni inequality (17) to control the overall alpha level.

5'-Nucleotidase assays

Brains from freshly killed chicks were frozen at −70°C and assayed within 1 month. Brain homogenate was prepared in 0.1 M Tris-HCl, pH 7.4, containing 0.25 M sucrose (7 ml buffer per gram tissue). Protein concentration was estimated by the method of Hartree (18) with bovine serum albumin as standard. 5'-Nucleotidase activity in the homogenate was measured generally according to Bosmann and Pike (19) at 37°C in 1.0 ml final volume containing 0.1 M Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 3 mM 5'-AMP, and 0.7 mg protein. The reaction was stopped by the addition of 0.5 ml 9% $HClO_4$ and the precipitated protein was removed by centrifugation. A 1.0-ml aliquot was removed and the inorganic phosphate was determined according to Fiske and SubbaRow (20), but including 0.4% Triton X-100 for greater sensitivity (21). Rates were calculated from five time points (15-35 min), each with its own enzyme blank. 5'-AMP (3 mM) was added to each blank, af-

TABLE 2. Fatty acid composition of the control and experimental diets

Fatty Acid	Control Diet	Fish Oil Diet	Linseed Oil Diet	Soy Oil Diet	Safflower Oil Diet (n-3)-Deficient
			percent of total fatty acids		
16:0	11.5	15.4	7.1	10.7	5.7
18:0	1.9	2.8	2.8	3.9	1.7
Total saturated	13.5	25.3	10.1	16.1	7.8
18:1(n-9)	25.0	14.7	22.7	22.5	8.8
Total monounsaturated	25.3	27.1	23.3	23.0	9.3
18:2(n-6)	58.2	12.5	29.4	54.0	81.7
20:4(n-6)	-[a]	0.9	-	-	-
Total (n-6)	58.8	15.2	31.3	54.1	82.0
18:3(n-3)	1.4	1.2	34.6	6.5	0.2
20:5(n-3)	-	13.1	-	-	-
22:6(n-3)	-	8.5	-	-	-
Total (n-3)	1.4	27.7	34.6	6.5	0.2
(n-6)(n-3) ratio	37	0.55	0.9	8.3	400

[a]Dash indicates not detected.

ter stopping the reaction with $HClO_4$, in order to control for any free phosphate in the substrate as well as to standardize the interference caused by AMP.

RESULTS

Transfer of n-3 fatty acids from hen to egg yolk

Egg yolks from hens consuming the fish oil and linseed oil diets high in n-3 fatty acids contained more than ten times the total n-3 fatty acids as those from the chow control group (**Table 3**). However, individual n-3 fatty acids did not appear in the egg yolk in the same relative proportions as found in the diet. For example, in egg yolks from fish oil-fed hens there was a substantial increase in the proportion of 22:6(n-3) and a substantial decrease in the proportion of 20:5(n-3) relative to the concentration of these fatty acids in the fish oil diet. This preferential metabolism of 20:5(n-3) to 22:6(n-3) was also apparent in egg yolks from hens fed linseed oil and soy oil. Although the increased

TABLE 3. Effect of hen's diet on egg yolk fatty acid composition

Fatty Acid	Control Diet (6)	Fish Oil Diet (12)	Linseed Oil Diet (6)	Soy Oil Diet (2)	Safflower Oil Diet (8)
			percent of total fatty acids		
16:0	26.1 ± 1.3	27.1 ± 0.9	18.7 ± 0.6	22.4 ± 0.2	24.0 ± 3.0
18:0	9.5 ± 0.5	10.7 ± 1.0	12.5 ± 0.3	11.3 ± 1.5	10.8 ± 0.7
Total saturated	36.3 ± 1.3	39.2 ± 0.8	31.9 ± 0.5	34.0 ± 1.3	34.9 ± 2.3
18:1(n-9)	40.3 ± 1.9	35.6 ± 1.1	33.0 ± 1.1	33.1 ± 0.4	25.0 ± 1.8
Total monounsaturated	44.4 ± 2.3	39.9 ± 0.9	35.0 ± 1.2	35.2 ± 0.7	27.7 ± 1.7
18:2(n-6)	14.7 ± 2.1	8.2 ± 0.6	17.5 ± 0.5	23.4 ± 0.8	30.0 ± 2.6
20:4(n-6)	2.1 ± 0.2	0.7 ± 0.1	1.4 ± 0.3	2.1 ± 0.5	3.2 ± 0.3
22:5(n-6)	0.5 ± 0.1[a]	0.08 ± 0.07[b]	-[b]	-[b]	0.9 ± 0.2[c]
Total (n-6)	17.8 ± 2.1[a]	9.6 ± 0.5[b]	19.7 ± 0.5[a]	25.2 ± 2.3[c]	35.9 ± 2.9[d]
18:3(n-3)	0.4 ± 0.1[a]	0.4 ± 0.1[a]	9.4 ± 1.2[b]	1.7 ± 0.3[c]	0.06 ± 0.04[d]
20:5(n-3)	-[a]	2.1 ± 0.1[b]	0.3 ± 0.04[c]	-[a]	-[a]
22:6(n-3)	0.6 ± 0.1[a]	6.3 ± 0.2[b]	2.2 ± 0.2[c]	1.8 ± 0.1[c]	0.2 ± 0.1[d]
Total (n-3)	1.0 ± 0.2[a]	10.1 ± 0.8[b]	12.6 ± 1.0[c]	3.1 ± 0.5[d]	0.3 ± 0.1[e]
Total (n-6) + (n-3)	18.8 ± 1.5	19.7 ± 0.7	32.3 ± 0.8	28.3 ± 1.7	36.2 ± 2.0
(n-6)/(n-3) ratio	17.8	1.0	1.6	8.1	120

Values are shown as mean ± SD. Results with unlike superscripts were found to be different at an overall $P < 0.01$; dash indicates not detected; number of eggs analyzed per diet in parentheses.

amount of 18:3(n-3) in these diets relative to chow controls led to significantly higher levels of 22:6(n-3), the final desaturation-elongation product of 18:3(n-3) in the yolks, there was almost no build-up of 20:5(n-3), one of the intermediates in this conversion.

Even very high levels of dietary 18:3(n-3), such as in the linseed oil diet, were not as effective in raising the concentration of 22:6(n-3) in yolk as the relatively modest amount of pre-formed 22:6(n-3) contained in the fish oil diet (Table 3). In addition, the quantity of 18:3(n-3) found in the soy oil diet (one-fifth of that in the linseed oil diet) increased egg yolk levels of 22:6(n-3) to the same degree as the linseed oil diet. Clearly, there was a block in the conversion of 18:3(n-3) to 22:6(n-3) by the hen.

Feeding the semi-purified diet very low in 18:3(n-3) and very high in 18:2(n-6) (safflower oil diet) resulted in almost no 18:3(n-3) and very little 22:6(n-3) in the egg yolk, there being a 70% depletion of total n-3 acids relative to control. When all the diets were examined with regard to transfer of 18:3(n-3) from hen to egg, a linear relationship was seen between 18:3(n-3) in the diet and the amount of 18:3(n-3) appearing in the egg yolk (r = 0.99).

Dietary fatty acids: effect upon the fatty acid composition of the chick tissues

The fatty acid compositions of the brain, retina, and serum of chicks, hatched from eggs laid near the same time as the eggs whose yolk fatty acids were analyzed, are shown in Tables 4-6. There was a linear relationship between 18:3(n-3) in the diet and 18:3(n-3) in the serum of newly hatched chicks from all diets groups (r = 0.99) (**Table 4**). There was no detectable 18:3(n-3) in the serum of the n-3-deficient (safflower oil) group, and other n-3 fatty acids were barely detectable, the total being 0.5%. Eicosapentaenoic acid (20:5 n-3), which was much lower in the "fish oil" egg yolk relative to the other n-3 fatty acids in the fish oil diet, was present in the serum of chicks hatched from "fish oil" eggs at a higher ratio relative to 22:6(n-3) than in the egg yolk. As seen in previous studies with fish oil feeding (22, 23), the great increase in serum n-3 fatty acids to 16.4% of total fatty acids [mainly due to increased 20:5(n-3) and 22:6(n-3)] led to a substantial reduction in serum total n-6 fatty acids. With linseed oil feeding, however, a similar increase in total n-3 fatty acids [predominantly 18:3(n-3)] in egg yolk and chick serum occurred without appreciably lowering total n-6 fatty acids, probably because of the higher n-6 fatty acid content in linseed oil vis-a-vis fish oil. This disparity between the effects of fish oil and linseed oil on tissue n-3 fatty acids is also seen when chicks are fed directly (24-26).

Dietary fatty acids had a great effect upon the fatty acid composition of the lipid-rich chick brain and retina (**Table 5** and **Table 6**). Very high levels of 22:6(n-3) were found in the brain and retina of chicks from hens fed the fish oil diet, while low levels were found in "safflower oil" chicks. Accompanying the decrease in 22:6(n-3) from the safflower oil diet was an increase in 22:5(n-6), the elevation of which is characteristic of n-3 fatty acid deficiency (14). All of the n-3 fatty acid-supplemented diets produced higher levels of n-3 fatty acids in the brain and retina than did the control diet. Nevertheless, the level of 22:6(n-3) in ethanolamine glycerophospholipids of control brain (25% of fatty acids, data not shown) was within the range seen in variety of species, including the rat and primates (5), showing

TABLE 4. Effect of the hen's diet on serum fatty acid composition of newly hatched chicks

Fatty Acid	Control Diet (3)	Fish Oil Diet (7)	Linseed Oil Diet (9)	Soy Oil Diet (4)	Safflower Oil Diet (7)
			percent of total fatty acids		
16:0	18.0 ± 0.9	19.6 ± 3.0	15.3 ± 0.3	17.9 ± 0.9	9.5 ± 3.5
18:0	12.0 ± 0.3	10.8 ± 1.0	14.0 ± 1.2	13.6 ± 0.5	15.7 ± 0.8
Total saturated	30.7 ± 1.1	31.7 ± 2.4	30.0 ± 1.2	32.5 ± 0.7	25.8 ± 2.8
18:1(n-9)	28.0 ± 1.0	29.1 ± 3.3	22.2 ± 3.0	20.4 ± 0.8	16.1 ± 1.9
Total monounsaturated	29.5 ± 1.3	31.8 ± 3.0	23.0 ± 3.0	21.5 ± 0.9	16.7 ± 1.9
18:2(n-6)	24.1 ± 0.7	14.0 ± 1.7	26.9 ± 1.2	27.7 ± 0.6	32.1 ± 1.8
20:4(n-6)	9.4 ± 1.0	2.8 ± 0.5	5.4 ± 0.4	10.6 ± 0.6	17.4 ± 1.9
22:5(n-6)	1.1 ± 0.2^a	0.1 ± 0.1^b	0.04 ± 0.05^b	0.4 ± 0.1^c	2.9 ± 0.8^d
Total (n-6)	36.2 ± 1.4^a	17.7 ± 1.8^b	33.5 ± 1.3^c	40.4 ± 0.3^d	55.8 ± 2.0^e
18:3(n-3)	0.3 ± 0.06^a	0.3 ± 0.1^a	6.0 ± 0.5^b	0.8 ± 0.05^c	–^{d}
20:5(n-3)	0.1 ± 0.06^a	5.9 ± 0.9^b	1.5 ± 0.2^c	0.4 ± 0.3^a	–^{a}
22:6(n-3)	1.5 ± 0.3^a	8.4 ± 1.1^b	3.5 ± 0.6^c	3.3 ± 0.1^c	0.4 ± 0.3^d
Total (n-3)	2.2 ± 0.2^a	16.4 ± 2.0^b	12.0 ± 0.7^c	4.8 ± 0.4^d	0.5 ± 0.3^e
Total (n-6) + (n-3)	38.4 ± 1.0	34.1 ± 1.9	45.0 ± 1.0	45.2 ± 0.4	56.3 ± 1.4
(n-6)/(n-3) ratio	16.5	1.1	2.8	8.4	112

Values are reported as the mean ± SD. Values with unlike superscripts were found to be different at an overall $P < 0.02$; dash indicates not detected; number of animals per diet in parentheses.

TABLE 5. Effect of the hen's diet on the brain fatty acid composition of newly hatched chicks

Fatty Acid	Control Diet (7)	Fish Oil Diet (8)	Linseed Oil Diet (9)	Soy Oil Diet (10)	Safflower Oil Diet (15)
			percent of total fatty acids		
16:0	26.8 ± 0.9	20.6 ± 3.8	25.0 ± 2.0	23.4 ± 4.4	22.7 ± 3.8
18:0	15.7 ± 0.7	17.7 ± 1.3	16.7 ± 5.8	19.0 ± 1.7	19.2 ± 0.8
Total saturated	44.4 ± 1.4	39.9 ± 4.5	44.6 ± 1.9	44.1 ± 4.2	42.9 ± 3.3
18:1(n-9)	15.2 ± 1.0	16.9 ± 1.7	15.1 ± 4.5	13.9 ± 1.1	13.2 ± 1.7
Total monounsaturated	18.4 ± 1.1	19.5 ± 2.2	18.2 ± 2.3	16.3 ± 1.3	16.0 ± 2.1
18:2(n-6)	2.0 ± 0.3	0.20 ± 0.13	2.9 ± 0.4	2.1 ± 0.4	2.6 ± 0.3
20:4(n-6)	10.2 ± 0.5	3.6 ± 1.4	7.3 ± 0.6	10.8 ± 1.3	13.3 ± 1.6
22:4(n-6)	2.7 ± 0.2	0.50 ± 0.28	1.5 ± 0.1	2.8 ± 0.4	5.4 ± 0.9
22:5(n-6)	3.7 ± 0.4^a	0.31 ± 0.31^b	0.30 ± 0.11^b	1.2 ± 0.2^c	13.6 ± 2.1^d
Total (n-6)	19.1 ± 2.1^a	7.4 ± 1.9^b	13.3 ± 0.8^c	17.7 ± 2.4^a	37.3 ± 3.6^d
20:5(n-3)	-^{a}	2.1 ± 1.0^b	0.91 ± 0.32^c	0.12 ± 0.07^d	-^{a}
22:6(n-3)	13.6 ± 0.9^a	24.6 ± 3.6^b	18.7 ± 1.3^c	19.6 ± 3.7^c	2.6 ± 0.7^d
Total (n-3)	13.8 ± 0.9^a	29.3 ± 4.4^b	21.9 ± 1.6^c	20.2 ± 3.8^c	2.7 ± 0.7^d
Total (n-6) + (n-3)	32.9 ± 1.6	36.7 ± 3.4	35.2 ± 1.3	37.9 ± 3.2	40.0 ± 2.6
(n-6)/(n-3) ratio	1.4	0.3	0.6	0.9	14

Values are shown as mean ± SD. Results with unlike superscripts were found to be different at an overall $P < 0.02$; dash indicates not detected; number of animals per diet in parentheses.

that the chick brain has a representative fatty acid composition. The lower level of retinal 22:6(n-3) seen in "linseed oil" chicks versus "soy" chicks (Table 6) is difficult to explain. We believe that it is not an artefact, however, because similar results were obtained in the follow-up study (data not shown).

The time course of the changes in chick brain 22:6(n-3) and 22:5(n-6) during feeding of the safflower oil diet to the hen is shown in **Fig. 1**. A severe deficiency of brain 22:6(n-3) was evident in the progeny just 2 weeks after the hen began to consume the n-3 fatty acid-deficient safflower oil diet. By about 4 weeks, this deficiency had reached ca. 80% relative to chow controls and there was little change in chick brain 22:6(n-3) and 22:5(n-6) subsequently.

When 18:3(n-3) was the only n-3 fatty acid in the diet (soy and linseed oils), the level of total n-3 fatty acids [principally 22:6(n-3)] in the chick brain was increased by increasing the amount of 18:3(n-3) in the diet of the hen.

TABLE 6. Effect of the hen's diet on the retinal fatty acid composition of newly hatched chicks

Fatty Acid	Control Diet (6)	Fish Oil Diet (4)	Linseed Oil Diet (7)	Soy Oil Diet (5)	Safflower Oil Diet (13)
			percent of total fatty acids		
16:0	23.6 ± 1.6	10.5 ± 4.5	24.3 ± 6.0	14.0 ± 5.8	10.5 ± 5.7
18:0	20.6 ± 1.4	21.7 ± 1.0	19.1 ± 2.1	23.1 ± 1.8	22.5 ± 2.3
Total saturated	47.8 ± 3.4	33.6 ± 3.6	46.8 ± 7.9	40.1 ± 3.5	37.8 ± 7.2
18:1(n-9)	13.9 ± 1.3	14.9 ± 1.5	13.3 ± 0.8	14.1 ± 1.0	13.5 ± 3.7
Total mono	16.7 ± 1.4	16.9 ± 2.0	17.3 ± 1.5	17.4 ± 1.3	16.7 ± 4.3
18:2(n-6)	1.5 ± 0.5	1.3 ± 0.2	2.1 ± 1.3	1.6 ± 0.3	2.1 ± 0.4
20:4(n-6)	8.9 ± 1.2	4.5 ± 0.6	5.9 ± 1.3	9.2 ± 0.7	11.1 ± 2.5
22:4(n-6)	2.7 ± 0.4	1.0 ± 0.4	1.3 ± 0.4	2.8 ± 0.4	6.4 ± 2.0
22:5(n-6)	4.1 ± 0.7^a	-^{b}	0.44 ± 0.18^c	1.7 ± 0.4^d	17.1 ± 4.9^e
Total (n-6)	18.2 ± 3.0^a	8.0 ± 0.5^b	11.3 ± 1.9^c	17.0 ± 1.3^a	39.4 ± 9.1^d
20:5(n-3)	-^{a}	3.7 ± 1.7^b	0.90 ± 0.22^c	0.43 ± 0.15^d	-^{a}
22:6(n-3)	15.1 ± 2.2^a	33.0 ± 5.0^b	17.5 ± 3.7^a	23.7 ± 2.1^c	3.7 ± 1.3^d
Total (n-3)	15.8 ± 2.6^{a,e}	39.3 ± 6.4^b	20.2 ± 3.6^{c,e}	24.6 ± 1.8^c	4.1 ± 1.1^d
Total (n-6) + (n-3)	34.0 ± 2.8	47.3 ± 4.5	31.5 ± 2.9	41.6 ± 1.6	43.5 ± 6.5
(n-6)/(n-3) ratio	1.2	0.2	0.6	0.7	9.6

Values are shown as mean ± SD. Results with unlike superscripts were found to be different at an overall $P < 0.03$; dash indicates not detected; number of animals per diet in parentheses.

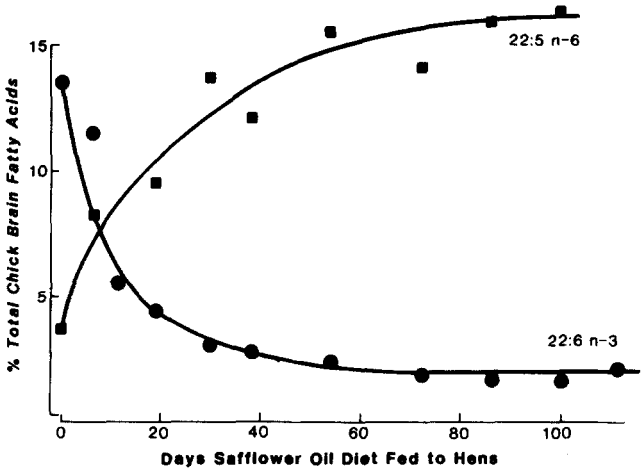

Fig. 1. Reciprocal replacement of 22:6(n-3) by 22:5(n-6) in the brains of newly hatched chicks from hens that were changed from the control diet to the safflower oil diet. The brain fatty acid values are from chicks whose mothers were fed the safflower oil diet for periods of time (0 to 120 days).

However, large amounts of dietary 18:3(n-3), as found in the linseed oil diet, were no more effective in raising brain n-3 fatty acid levels than the modest amounts of 18:3(n-3) found in the soy oil diet. This relative ineffectiveness of linseed oil versus soy oil was also seen in the chick retina, although linseed oil did increase n-3 fatty acid levels in egg yolk and chick serum more than soy oil did.

Brain 5'-nucleotidase

The functional consequences of these changes in brain fatty acid composition were examined by assaying brain 5'-nucleotidase, an enzyme that has been reported to be much lower in rats fed an n-3-deficient diet (8). As can be seen in **Table 7**, however, brain 5'-nucleotidase activity in newly hatched chicks did not vary significantly over the more than tenfold range of brain n-3 fatty acid concentrations after the five different diets.

DISCUSSION

The results presented here indicate that the levels of n-3 fatty acids in the plasma, brain, and retina of the newly hatched chick can be quickly and conveniently modified over a more than tenfold range by feeding appropriate diets to laying hens. Within a month of initiating a given experimental diet to the hens, eggs were laid which gave rise to newly hatched chicks whose serum and tissues, including the brain and retina, reflected completely the fatty acid composition of the hen's diet.

The effects of various oil supplements fed to laying hens on egg and chick fatty acid composition has been examined previously (27-31, review 32), but generally either before adequate methodology was available or before the recent interest in the metabolism of n-3 fatty acids. For instance, it has long been known that the fatty acid composition of egg yolk responds to that of the hen's diet. In fact, safflower oil fed to hens has been shown to lower n-3 fatty acid levels in egg yolk and chick plasma and heart (30, 31). What has not been appreciated is the dramatic effect that short-term changes in the hen's diet, such as switching to a low n-3 fatty acid diet based on safflower oil, can have on the fatty acid composition of the brain and retina of the newly hatched chick. We found that the n-3 fatty acid content of these tissues could be reduced by 75-80% within 4 weeks of changing the hen's diet. Thus, a severe n-3 fatty acid deficiency can be induced quickly and inexpensively in the nervous tissue of newly hatched chicks. Such chicks are immediately available for further experimentation, including dietary manipulation. This is in contrast to a mammalian model such as the rat, where the brain of the suckling newborn is subjected (indirectly) to the mother's stores of n-3 fatty acids. This presents problems in the production of n-3 fatty acid deficiency, since n-3 fatty acids are notoriously difficult to deplete once they have been incorporated into the brain (33). A severe deficiency could be achieved with the laying hen/newly hatched chick model in much less time than in comparable experiments with rats (23, 34), and, because of the prodigous reproductive capacity of the laying hen, the potential exists to produce large numbers of deficient animals in order to test the consequences of the deficiency.

The n-3 fatty acid content of the newly hatched chick brain and retina could also be dramatically increased by feeding fish oil to the hen. For example, total n-3 fatty acids in the brain and retina of the chick rose to 29 and 39%, respectively, relative to control. Thus, the n-3 fatty acid content of newly hatched chick nervous tissue could be manipulated over a more than tenfold range by feeding the laying hen an n-3 fatty acid-deficient diet (safflower oil diet) on the one hand, or a fish oil diet (containing large amounts of n-3 fatty acids) on the other.

Regardless of diet, it is interesting to note the biomagnification of 22:6(n-3) which is evident as egg yolk

TABLE 7. 5'-Nucleotidase activity in the brain of newly hatched chicks

Hen's Diet	5'-Nucleotidase Activity	Brain n-3 Fatty Acid Content[a]
	μmol phosphate · hr^{-1} · mg protein^{-1}	
Fish oil	1.31 ± 0.14 (6)[b]	29.3 ± 4.4
Linseed oil	1.41 ± 0.32 (8)	21.9 ± 1.6
Soy oil	1.34 ± 0.32 (4)	20.2 ± 3.8
Control	1.46 ± 0.11 (4)	13.8 ± 0.9
Safflower oil	1.60 ± 0.27 (4)	2.7 ± 0.7

[a]These data are from Table 5.
[b]Number of animals in parentheses.

fatty acids are mobilized into chick serum, brain, and retina. This relative enrichment of nervous tissue with n-3 fatty acids is a general phenomenon (5) and may be due in part to preferential uptake of n-3 fatty acids by the developing brain (35). Thus, irrespective of the 22:6(n-3) content of the diet, the hen passed 22:6(n-3) to the egg, suggesting that the hen can draw on stores to supply a need for this fatty acid by the developing chick.

With respect to n-3 fatty acid deficiency in the chick, it should be noted that the commercial chow (control) diet of the hen contained a high proportion of linoleic acid and relatively little linolenic acid, the type of diet which can lead to n-3 fatty acid deficiency. A hallmark of n-3 fatty acid deficiency is the appearance in the brain and retina of significant levels of the fatty acid 22:5(n-6) (7, 12, 14). This fatty acid was in fact present at almost 4% of total fatty acids in the brains of chicks fed commercial chow, suggesting that the chow diet may not supply adequate linolenic acid.

A question of interest that this chick model may address is the effect of specific n-3 fatty acids in the diet, such as 18:3(n-3) versus 20:5(n-3) versus 22:6(n-3). α-Linolenic acid [18:3(n-3)] is the precursor for the ultimate formation of 20:5(n-3) and 22:6(n-3) through the desaturation-elongation pathway. Our results from feeding large amounts of 18:3(n-3) to laying hens are in agreement with human studies (36-39, review 40) showing dietary 18:3(n-3) to be less efficient in increasing serum and tissue levels of 20:5(n-3) and 22:6(n-3) than oils containing these preformed fatty acid species directly. However, other studies with rats (41) and even chicks (42) have found high tissue levels of 20:5(n-3) after feeding relatively high levels of 18:3(n-3). These studies may not be generally applicable, since the rats (41) were fed a diet lacking in n-6 fatty acids, while the chicks (42) were fed a diet lacking in vitamin E. Nevertheless, the quite high tissue levels of 20:5(n-3) that were obtained by these workers [16.6% of total fatty acids in the chick livers (42) and 16.4% in the rat kidneys (41)] indicate that more research is needed to determine under what conditions dietary 18:3(n-3) can enhance tissue levels of 20:5(n-3) and 22:6(n-3).

Another question is the fate of dietary 20:5(n-3). We found that feeding a laying hen a fish oil-supplemented diet, containing 20:5(n-3) and 22:6(n-3) at 13% and 9% of total fatty acids respectively, yielded egg lipids with 2% 20:5(n-3) and 6% 22:6(n-3). That is, the ratio of 20:5(n-3) to 22:6(n-3) in egg lipids was much reduced relative to the ratio in the diet. Three simple explanations for this relative depletion of 20:5(n-3) are possible: *1*) differential transfer of these two fatty acids to the egg; *2*) greater retroconversion or oxidation of 20:5(n-3) than would occur for 22:6(n-3); or *3*) desaturation and elongation of most of the dietary 20:5(n-3) to 22:6(n-3) by the hen. The first alternative is unlikely, since Marion and Edwards (43) and Menge, Calvert, and Denton (31) found a much reduced ratio of 20:5(n-3) to 22:6(n-3) (vis-a-vis the diet) in the liver and plasma, respectively, of hens fed fish oil. Thus, the relative depletion of 20:5(n-3) is already evident in the hen before transfer of lipids to the egg. As for the second alternative, we found no evidence that the 20:5(n-3) was retroconverted. Since 20:5(n-3) is an intermediate in the synthesis of 22:6(n-3) and is positioned after the rate limiting step [the Δ^6 desaturation of 18:3(n-3)], it is likely that the 20:5(n-3) was converted to 22:6(n-3). This model would be useful to study further the metabolism of different n-3 fatty acids in the hen-chick system and the influence of diet thereon.

It has been proposed that there is a biochemical mechanism for the maintenance of a certain amount of unsaturation in brain lipids (23). This hypothesis is based partly on the observation that the brain responds to a shortage of n-3 polyunsaturated fatty acids by increasing the levels of n-6 polyunsaturates, in order to maintain an approximately equivalent amount of total unsaturation. However, it is not clear whether this homeostatic mechanism is fully operative in both directions. For instance, is there an upper limit to brain unsaturation or n-3 fatty acid content? White and co-workers (22) and Miller and White (44) have found that the n-3 fatty acid content of chick brain can be increased dramatically by feeding fish oil to newly hatched chicks for 3 weeks. The laying hen/chick model could be used to explore this question further in a controlled fashion.

As a functional test of the consequences of manipulating tissue n-3 fatty acid levels over a tenfold range, we assayed 5'-nucleotidase activity in newly hatched chick brain. The activity of this membrane-bound enzyme has been reported to rise in liver plasma membranes of fish oil-fed rats (45), while dietary supplements of 18:3(n-3) were necessary to restore full activity in brain homogenates of rats reared on fat-free diets (8). However, we saw no effect of any of the diets on chick brain 5'-nucleotidase activity, indicating that this enzyme does not respond simply to the membrane concentration of n-3 fatty acids. Our observation that n-3 fatty acid deficiency per se does not lower enzyme activity confirms results by Tinoco et al. (16) and argues that the lowering of activity seen by Bernsohn and Spitz (8) was probably connected to the feeding of a fat-free diet.

This work was supported by National Institutes of Health Training Grant HL-07295, National Heart, Lung, and Blood Institute Grant HL-25687, and National Institutes of Health General Clinical Research Center Grant RR-00334.

Manuscript received 23 June 1988 and in revised form 29 August 1988.

REFERENCES

1. Goodnight, S. H., W. S. Harris, W. E. Connor, and D. R. Illingworth. 1982. Polyunsaturated fatty acids, hyperlipidemia and thrombosis. *Arteriosclerosis.* **2:** 87-113.
2. Leaf, A, and P. C. Weber. 1988. Cardiovascular effects of n-3 fatty acids. *N. Engl. J. Med.* **318:** 549-557.

3. Herold, P. M., and J. E. Kinsella. 1986. Fish oil comsumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. *Am. J. Clin. Nutr.* **43:** 566–598.
4. Dyerberg, J. 1986. Linolenate-derived polyunsaturated fatty acids and prevention of atherosclerosis. *Nutr. Rev.* **44:** 125–134.
5. Crawford, M. A., M. N. Casperd, and A. J. Sinclair. 1976. The long chain metabolites of linoleic and linolenic acids in liver and brain in herbivores and carnivores. *Comp. Biochem. Physiol.* **54B:** 395–401.
6. Salem, N., H-Y. Kim, and J. A. Yergey. 1986. Docosahexaenoic acid: membrane function and metabolism. *In* Health Effects of Polyunsaturated Fatty Acids in Seafoods. Academic Press, New York. 263–317.
7. Fliesler, S. J., and R. E. Anderson. 1983. Chemistry and metabolism of lipids in the vertebrate retina. *Prog. Lipid Res.* **22:** 79–131.
8. Bernsohn, J., and F. J. Spitz. 1974. Linoleic and linolenic acid dependency on some brain membrane-bound enzymes after lipid deprivation in rats. *Biochem. Biophys. Res. Commun.* **57:** 293–298.
9. Neuringer, M., and W. E. Connor. 1986. N-3 fatty acids in the brain and retina: evidence for their essentiality. *Nutr. Rev.* **44:** 285–294.
10. Neuringer, M., G. J. Anderson, and W. E. Connor. 1988. The essentiality of n-3 fatty acids for the development and function of the retina and brain. *Annu. Rev. Nutr.* **8:** 517–541.
11. Neuringer, M., W. E. Connor, C. V. Petten, and L. Barstad. 1984. Dietary omega-3 fatty acid deficiency and visual loss in infant rhesus monkeys. *J. Clin. Invest.* **73:** 272–276.
12. Neuringer, M., W. E. Connor, D. S. Lin, and L. Barstad. 1986. Biochemical evidence of n-3 fatty acid deficiency in brain and retina of perinatal and juvenile rhesus monkeys. *Proc. Natl. Acad. Sci. USA.* **83:** 4021–4025.
13. Bourre, J. M., G. Pascal, G. Durand, M. Masson, O. Dumont, and M. Picinotti. 1984. Alterations in the fatty acid compositon of rat brain cells (neurons, astrocytes, and oligodendrocytes) and of subcellular fractions (myelin and synaptosomes) induced by a diet devoid of n-3 fatty acids. *J. Neurochem.* **43:** 342–348.
14. Mohrhauer, H., and R. T. Holman. 1963. Alteration of the fatty acid composition of brain lipids by varying levels of dietary essential fatty acids. *J. Neurochem.* **10:** 523–530.
15. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride–methanol. *J. Lipid Res.* **5:** 600–608.
16. Tinoco, J., R. Babcock, I. Hincenbergs, B. Medwadowski, and P. Miljanich. 1978. Linolenic acid deficiency: changes in fatty acid patterns in female and male rats raised on a linolenic acid-deficient diet for two generations. *Lipids.* **13:** 6–17.
17. Miller, R. G. 1966. Simultaneous Statistical Inference. McGraw-Hill, New York. 8.
18. Hartree, E. F. 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48:** 422–427.
19. Bosman, H. B., and G. Z. Pike. 1971. Membrane marker enzymes: isolation, purification and properties of 5'-nucleotidase from rat cerebellum. *Biochim. Biophys. Acta.* **227:** 402–412.
20. Fiske, C. H., and Y. SubbaRow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:** 375–400.
21. Sec, Y. P., and P. S. Fitt. 1972. Determination of inorganic phosphate in the presence of Triton X-100. *Anal. Biochem.* **49:** 430–435.
22. Miller, D., J. H. Soares, S. Cupett, and V. White. 1971. Comparative interaction and deposition of ω3 and ω6 fatty acids in chick brain. *Nutr. Rep. Int.* **4:** 19–30.
23. Galli, C., H. I. Trzeciak, and R. Paoletti. 1971. Effects of dietary fatty acids on the fatty acid composition of the brain ethanolamine phosphoglyceride: reciprocal replacement of n-6 and n-3 polyunsaturated fatty acids. *Biochim. Biophys. Acta.* **248:** 499–454.
24. Marco, G. J., L. J. Machlin, E. Emery, and R. S. Gordon. 1961. Dietary effects of fats upon fatty acid composition of the mitochondria. *Arch. Biochem. Biophys.* **94:** 115–120.
25. Century, B., L. A. Witting, C. C. Harvey, and M. K. Horwitt. 1963. Interrelationships of dietary lipids upon fatty acid composition of brain mitochondria, erythrocytes and heart tissue in chicks. *Am. J. Clin. Nutr.* **13:** 362–368.
26. Edwards, H. M., and J. E. Marion. 1963. Influence of dietary menhaden oil on growth rate and tissue fatty acids of the chick. *J. Nutr.* **81:** 123–130.
27. Feingnenbaum, A. S., and H. Fisher. 1959. The influence of dietary fat on the incorporation of fatty acids into body and egg fat of the hen. *Arch. Biochem. Biophys.* **79:** 302–306.
28. Cruickshank, E. M. 1939. The effect of cod-liver oil and fishmeal on the flavor of poultry products. *Proc. World's Poultry Congr. (Cleveland)* **7:** 539–542.
29. Cruickshank, E. M. 1934. Studies in fat metabolism in the fowl. The comparison of the egg fat and depot fat of the fowl as affected by the ingestion of large amounts of different fats. *Biochem. J.* **28:** 965–977.
30. Menge, H., C. C. Calvert, and C. A. Denton. 1965. Influence of dietary oils on reproduction in the hen. *J. Nutr.* **87:** 365–370.
31. Menge, H., C. C. Calvert, and C. A. Denton. 1965. Further studies of the effect of linoleic acid on reproduction in the hen. *J. Nutr.* **86:** 115–119.
32. Balnave, D. 1970. Essential fatty acids in poultry nutrition. *World's Poult. Sci. J.* **26:** 442–459.
33. Tinoco, J. 1982. Dietary requirements and functions of α-linolenic acid in animals. *Prog. Lipid Res.* **21:** 1–45.
34. Lamptey, M. S., and B. L. Walker. 1976. A possible essential role for dietary linolenic acid in the development of the young rat. *J. Nutr.* **106:** 86–93.
35. Anderson, G. J., and W. E. Connor. 1988. Uptake of fatty acids by the developing rat brain. *Lipids.* **23:** 286–290.
36. Adam, O., G. Wolfram, and N. Zoellner. 1984. The effect of linoleic and linolenic acids on prostaglandin formation and kidney function in man. *Fette Seifen Anstrichm.* **86:** 180–183.
37. Sanders, T. A. B., and F. Roshanai. 1983. The influence of different types of ω3 polyunsaturated fatty acids on blood lipids and platelet function in healthy volunteers. *Clin. Sci.* **64:** 91–99.
38. Dyerberg, J., H. O. Bang, and O. Aagaard. 1980. α-Linolenic acid and eicosapentaenoic acid. *Lancet.* **i:** 199.
39. Singer, P., W. Jaeger, S. Voigt, and I. H. Thiel. 1984. Defective desaturation and elongation of n-6 and n-3 fatty acids in hypertensive patients. *Prostaglandins Leukotrienes Med.* **15:** 159–165.
40. Budowski, P., and M. A. Crawford. 1985. α-Linolenic acid as a regulator of the metabolism of arachidonic acid: dietary implications of the ratio n-6:n-3 fatty acids. *Proc. Nutr. Soc.* **44:** 221–229.
41. Hansen, H., and B. Jensen. 1983. Urinary prostaglandin E2 and vasopressin excretion in essential fatty acid-deficient rats: effect of linolenic acid supplementation. *Lipids.* **18:** 682–690.
42. Budowski, P., C. M. Hawkey, and M. A. Crawford. 1980. α-Linolenic acid protects against encephalomalacia in the chick. *Ann. Nutr. Aliment.* **34:** 369–400.

43. Marion, J. E., and H. M. Edwards. 1964. The response of laying hens to dietary oil and purified fatty acids. *Poultry Sci.* **43:** 911–918.
44. Miller, D., and V. White. 1975. Chick tissues fatty acid composition as affected by selenium, DL-α-tocopherol acetate, ethoxyquin and polyunsaturated oils. *Nutr. Rep. Int.* **12:** 245–255.
45. Flier, J., B. R. Lokesh, and J. E. Kinsella. 1985. Increased 5′-nucleotidase activity in plasma membranes from rat liver following ingestion of fish oil. *Nutr. Res.* **5:** 277–283.