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Phospholipids

5. THE EFFECT OF COD-LIVER OIL IN THE DIET ON THE COMPOSITION OF HEN'S EGG PHOSPHOLIPIDS*

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The degree of unsaturation of the lipids of the hen's egg is known to be influenced by the composition of the fats present in the diet (Henriques & Hansen, 1903; McCollum, Halkin & Drescher, 1912; Terroine & Belin, 1927). Cruickshank (1934) found that the effect of a change in the dietary fat reached its maximum after about 16 days, reflecting the time taken for the laying down of the yolk in the oviduct. Although the iodine value of the yolk lipids could be increased greatly by feeding highly unsaturated oils such as linseed or hempseed, relatively saturated fats such as mutton fat or palm oil had only a slight depressing effect, and foreign saturated acids such as lauric and myristic were not transmitted to the yolk.

Reiser (1951) added 2% of cod-liver oil to a fat-free feed and found evidence of pentaene and hexaene structures in the fatty acids of the yolk lipids by spectrophotometric analysis. On a rigorously fat-free diet hexaethenoid acids were found to disappear completely from the egg lipids, and the proportions of the other polyethenoid acids diminished; however, the total amount of unsaturated acids was not affected (Reiser, Gibson, Carr & Lamp, 1951). These workers examined either the total lipids of the yolk, or the glycerides and phospholipids as separated by precipitation with acetone.

* Part 4: Rhodes & Lea (1957).

It has recently been shown (Hanahan, 1954; Rhodes & Lea, 1956*a*) that the fatty acid in the α' position of the phosphatidylcholine (lecithin) and of the phosphatidylethanolamine of normal eggs is apparently always unsaturated, whereas that in the β position is almost entirely saturated. Moreover, the unsaturated acids of the phosphatidylethanolamine and of a small fraction of the phosphatidylcholine possessed unusually high average unsaturation for an animal lipid of non-marine origin (Rhodes & Lea, 1956*a*). The hens had been receiving a normal diet which, however, contained some fish meal.

The object of the present work was to ascertain whether the highly selective deposition of unsaturated and saturated acids in the α' and β positions of the yolk phospholipids would persist when a high proportion of fish oil, rich in polyethenoid acids, was included in the diet.

EXPERIMENTAL

Materials

Basic ration. This was a commercial laying meal containing 5% of white-fish meal.

Defatted meal. The meal (1 kg.) was refluxed with diethyl ether (2 l.) for 2 hr., filtered off and the residue re-extracted with fresh solvent. The final residue was dried and supplemented with 5 mg. of β-carotene and 22 mg. of α-tocopherol/kg.

Cod-liver oil and the methyl esters of its more unsaturated acids. The fresh medicinal cod-liver oil used had an iodine value of 167, corresponding to a mean unsaturation of approximately 6·9 m-moles of double bond/g. or 2 double bonds/mol. of its constituent fatty acids. The acids were obtained by alkaline hydrolysis and were fractionated by the method of Silk, Sephton & Hahn (1954), with precautions against atmospheric oxidation. The more unsaturated acids recovered from the acetone-soluble lithium salts represented 28% of the total, and had an average unsaturation of 12·3 m-moles of double bond/g., equivalent to approximately 4 double bonds/mol. A repetition of the lithium salt separation raised the unsaturation only to 12·9 m-moles/g. with a yield of 44%, and a single precipitation was therefore used in the main preparation. After esterification with methanol the resulting esters of the more unsaturated acids were dissolved in light petroleum (b.p. 40–60°) and passed through a column of alumina to remove any products of autoxidation present; the solvent was then removed as completely as possible.

Methods

Feeding. White Leghorn × Rhode Island Red pullets were fed with meal (basic ration) and water *ad lib.* When additions were made to the feed the oil was mixed daily with 100 g. of the meal, and when this had been consumed a further 100 g. of plain meal was given, part of which was left uneaten. Cod-liver oil in amounts of 10 and 30 g. daily, representing 14 and 34% of the total calories respectively, was readily accepted by the hens when given in this way, and had no effect on appetite or frequency of laying.

The defatted meal was not well accepted, however, and when the esters of the highly unsaturated acids were included the feed was refused almost entirely and the birds ceased to lay, probably because of the low intake of food. Methyl esters of tallow fatty acids have been fed to chicks in amounts up to 15% of the ration without evidence of toxicity (Reiser, Dieckert & Hamilton, 1956). The odour of the unsaturated esters was indistinguishable from that of the oil and no obvious explanation for their rejection could be found.

Analytical. The general analytical methods used have been described previously (Rhodes & Lea, 1957).

Examination of the yolk lipids. Eggs were collected daily and the yolks freeze-dried. The lipid of a single yolk was extracted by shaking the dried material twice with 50 ml. portions of chloroform–methanol (1:1, v/v) at 0° for 30 min. under nitrogen. The combined extracts were evaporated to dryness and the total lipid was applied as a 5% (w/v) solution in chloroform–methanol (98:2, v/v) to a column of 10 g. of silicic acid. The column was washed with the same solvent to complete the elution of the neutral fat, and the adsorbed phospholipids were recovered by elution with methanol–chloroform–water (75:20:5, by vol.).

The phosphatidylcholine and phosphatidylethanolamine were separated by chromatography on silicic acid in chloroform–methanol (70:30, v/v) as previously described (Rhodes & Lea, 1957).

The component fatty acid in the α' position and the corresponding lyso-compound were obtained from each phospholipid by the action of the phospholipase *A* of snake venom (*Agkistrodon piscivorus piscivorus*) by the technique of Hanahan, Rodbell & Turner (1954).

RESULTS

Experiment 1

One bird, laying regularly on the basic ration, was given 10 g. of cod-liver oil daily for 19 days, followed by 30 g. of oil daily for 15 days, and was then put on defatted meal for a further 19 days. Over the whole period the hen laid an average of 5·4 eggs/week. The results of the analyses of the lipids of the eggs examined (Table 1) confirmed the

Table 1. *Effect of diet on the unsaturation of hen's egg phospholipids* (*Expt.* 1)

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine.

Days on diet	Neutral fat (iodine value)	Lipid phosphorus/ yolk (mg.)	Unsaturation (double bonds/atom of P)				
			Total phospholipid	PC	PE	LysoPC	LysoPE
			(*a*) Feed included 10 g. of cod-liver oil/day				
0	73·6	60·7	2·26	2·00	3·64	0·16	0·42
1	75·0	59·8	2·28	—	—	—	—
4	76·5	60·9	2·51	—	—	—	—
8	79·0	56·2	3·05	—	—	—	—
12	79·8	56·9	2·98	—	—	—	—
19	83·7	58·4	3·24	2·75	4·77	0·18	0·35
			(*b*) Feed included 30 g. of cod-liver oil/day				
8	83·4	56·2	3·25	—	—	—	—
13	84·7	50·3	3·69	3·10	5·23	0·25	0·40
14	88·9	49·4	3·53				
			(*c*) Fed defatted meal				
10	75·8	62·3	2·91	—	—	—	—
19	74·7	66·4	2·42	—	—	—	—
			(*d*) Fed more highly unsaturated esters				
			No eggs after 5 days feeding				

findings of Cruickshank (1934) that about 2 weeks are required to bring the egg lipids into equilibrium with the dietary fat.

The addition of 10 g. of cod-liver oil daily (Table 1*a*) raised the mean unsaturation of the glycerides by 14% and that of the total phospholipids by 43%. The gross increase in the unsaturation of the phosphatidylethanolamine was greater than that of the phosphatidylcholine (1·13 and 0·75 double bonds/atom of P respectively), but the percentage increases were almost the same (31 and 37%). No change was found in the slight unsaturation of the derived lyso-compounds, that is of the component β-fatty acid of the diacylphospholipids, indicating that the increase in unsaturation had been confined to the acids in the α′ position of both phospholipids.

When the rate of feeding of the oil was raised to 30 g./day (Table 1*b*), the mean unsaturation increased further, the highest levels reached corresponding to increases of 21% for the glycerides, 56% for the total phospholipids, 44% for the phosphatidylethanolamine and 55% for the phosphatidylcholine. Even at these levels the increases in unsaturation were still confined to the α′ acids of the phospholipids.

The distribution of the polyethenoid acids in the individual phospholipids was further examined by subdivision of the peaks eluted from the silicic acid columns. The more unsaturated components appeared in the earlier fractions, as had previously been found (Rhodes & Lea, 1956*b*). The recovery of the phospholipid P of the phosphatidylcholine (eggs *b*13 and *b*14, Table 1) in fractions within given ranges of unsaturation was compared with similar figures for the phosphatidylcholine from a normal egg (Table 2). It appears from these data that the polyethenoid acids from the fish oil had been laid down in the phosphatidylcholine largely at the expense of the major proportion of diethenoid and monoethenoid acids normally present.

A similar fractionation of the more rapidly running phosphatidylethanolamine peak was not carried out, but it is clear from Table 1 that the α′ acids, which reached a mean unsaturation of over 4·8 double bonds/molecule, cannot have contained more than a small proportion of monoethenoid or diethenoid component acids.

When the hen was returned to a fat-free diet (Table 1*c*), the unsaturation of the egg lipids rapidly fell towards the normal value.

Table 2. *Fractionation of egg phosphatidylcholines, according to unsaturation, by chromatography on silicic acid columns*

Unsaturation limits (double bonds/atom of P)	Lipid phosphorus recovered (%)		
	Normal feed	Cod-liver oil supplement	Diff.
Above 5	1·1	14·4	+13·3
4–5	5·8	20·6	+14·8
3–4	14·8	19·5	+ 4·7
2–3	21·8	25·8	+ 4·0
Below 2	56·5	19·7	−36·8

Experiment 2

The inclusion of the more-highly unsaturated esters at the end of Expt. 1 (Table 1*d*) coincided with the cessation of egg production. A fresh pullet was used, therefore, to compare the effect of 10 g. of these esters in place of the whole oil. Use of the esters nearly doubled the mean unsaturation of the unsaturated acids in the dietary supplement, and increased the net weight of the highly unsaturated acids fed by a factor of four.

The feed containing the esters was only partly consumed, and after four eggs had been laid in 5 days one more was obtained on the twelfth day. After 20 days the bird was killed and the contents of the oviduct were examined. There were 19 yolks present, the largest being about 0·9 cm. in diameter. These were dried and processed together.

The analyses showed: neutral fat 300 mg., iodine value 105; phospholipid 65 mg., total P 2·60 mg., unsaturation 3·72 double bonds/atom of P. When separated, the phosphatidylethanolamine fraction comprised 59% of the lipid P, of unsaturation 4·39 double bonds/atom of P; and the phosphatidylcholine 41%, of unsaturation 2·89 double bonds/atom of P. These levels of unsaturation were close to those found in complete eggs from the hen fed the basic ration supplemented with cod-liver oil (Table 1; *a*19, *b*13, *b*14) but the proportion of phosphatidylethanolamine in these early stages of development was much higher than the figure of <20% found in the entire egg (Rhodes & Lea, 1957).

Experiment 3

In order to obtain larger amounts of the highly unsaturated phospholipids a third hen was fed with the basic ration containing 15 g. of cod-liver oil daily for 41 days. The composition of the lipids of the eggs responded to the change of feed in the same general way as in Expt. 1 (Table 3).

Isolation of the α′ acids. Phosphatidylethanolamine, prepared from the combined six eggs (Table 3) containing 28·0 mg. of lipid P (0·90 m-mole), was dissolved in 200 ml. of diethyl ether and treated with 5 mg. of venom in 5 ml. of aqueous 0·005 M-calcium chloride solution, and 0·5 m-mole of ammonia in ether (Long & Penny, 1957). A considerable precipitate formed within 2 hr., and after 4 hr. the solvent was removed by evaporation under reduced pressure. The residue was dissolved in 5 ml. of chloroform and applied to a column of

Table 3. *Effect of feeding 15 g. of cod-liver oil/day on the unsaturation of the egg lipids (Expt. 3)*

Days on diet	Neutral fat (iodine value)	Total lipid phosphorus (mg.)	Unsaturation (double bonds/atom of P)		
			Total phospholipid	PC	PE
0	72·6	61·0	2·33	—	—
7	78·5	72·2	2·87	—	—
15	84·7	68·2	3·04	2·70	4·79
27, 28, 30, 32, 34, 35	86·7	340	3·15	2·71	4·81

5 g. of silicic acid. Elution was continued with chloroform and the effluent collected. In all of these manipulations an atmosphere of nitrogen was maintained over the solutions.

After removal of the solvent 250 mg. (0·83 m-mole) of colourless liquid acids was recovered, free of phosphorus, iodine value 374, corresponding to an average unsaturation of approximately 4·4 double bonds/mole. The α' acids were recovered, therefore, in good yield and with the expected degree of unsaturation.

The α' acids were similarly obtained from phosphatidylcholines.

DISCUSSION

The cod-liver oil fed in these experiments had an iodine value of 167, equivalent to 6·58 m-moles of double bond/g. Assuming the presence of 16% of saturated acids (Hilditch, 1947) and a mean molecular weight of 300 for the unsaturated acids, the mean unsaturation of the unsaturated acids of the oil was of the order of 2·4 double bonds/molecule.

In Expt. 1 a total of 65·8 m-moles of double bond was fed in the 10 g. of oil/day, and the maximum increases in the unsaturation of the egg lipids when in equilibrium with this feed amounted to 0·40 m-mole of double bond/g. of glyceride and 0·98 m-mole/m-mole of phospholipid (Table 1*a*). The increase in the total unsaturation of an egg of average size would therefore be of the order of 4 m-moles, and, if laid daily, this represents no more than 6% of the unsaturation fed. It is not surprising, therefore, that trebling the level of oil fed (Table 1*b*), or the substitution of the esters of the more highly unsaturated acids for the whole oil, produced only a slight further increase in the average unsaturation of the yolk lipids.

The synthetic pathway from the ingested fat to the egg phospholipid appeared to be selective for the highly unsaturated acids in the feed; the average unsaturation of the α' acids of the phosphatidylethanolamine, for example, at 3·2 double bonds/mol., was considerably higher than that of the total acids of the oil (2·0 double bonds/mol.) or of the unsaturated acids in the oil (2·4 double bonds/mol.), but nevertheless rose markedly when the oil was fed. Furthermore, the polyethenoid acids of the feed were incorporated into the two phospholipids approximately in proportion to their initial unsaturation, the ratio of the unsaturation in the α' position of the phosphatidylethanolamine to that in the phosphatidylcholine being 1·75 on the normal diet, and remaining at 1·72 and 1·70 after feeding the oil (eggs *a* 0, *a* 19 and *b* 13 and *b* 14, Table 1).

Reiser *et al.* (1951) found that the laying hen on a fat-free diet is able to maintain the total level of unsaturated acids in the egg, including some 'essential' diethenoid acid, by synthesis from other carbon sources, the polyethenoid acids present on a normal diet being replaced by monoethenoid. In the present work only part of the less highly unsaturated acids was replaced by the polyethenoid acids fed, even when a large excess was present in the diet. The mono- and di-ethenoid acids were almost eliminated from the phosphatidylethanolamine, but considerable quantities remained in the phosphatidylcholine. The ease of interchangeability of the unsaturated fatty acids of the egg phospholipids is in agreement with the observations of Hanahan (1956), who concluded that the unsaturated acid in the α' position of the lecithins of rat liver was in a metabolically more reactive site than the saturated acid in the β position.

The considerable difference between the compositions of the unsaturated fatty acids in the α' positions of the two glycerophospholipids of the egg, which was maintained throughout the changes induced by the experimental diets, affords no support for a biosynthetic route to these compounds in the laying hen involving a common precursor, such as the reaction of an $\alpha\beta$-diglyceride with the cytidine diphosphate derivative of choline or ethanolamine, or an unspecific interchange of the basic alcohols of the completed molecule (Kennedy, 1956).

SUMMARY

1. The addition of 10 g. of cod-liver oil/day to the diet of a laying hen increased the unsaturation of the neutral fat and more particularly of the phospholipids of the egg yolk.

2. The increases in unsaturation of the egg phosphatidylcholine and phosphatidylethanolamine were confined entirely to the fatty acids esterified in the α' positions of the glycerophospholipids, the β-acid remaining mainly saturated.

3. The polyethenoid acids of the fish oil were selectively incorporated into the phospholipids apparently at the expense of the monoethenoid and diethenoid acids normally present. An average unsaturation of 4·8 double bonds/mol. was reached in the α' acids of phosphatidylethanolamine.

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