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Phospholipid Patterns of the Developing Chick Embryo*

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The phospholipids of unincubated egg yolks and of chick embryos of eggs incubated 4, 6, 12, 14, and 18 days have been separated by silicic acid chromatography. The major components have been identified as lipids containing ethanolamine, choline, sphingosine, and inositol in the egg and the embryos. Serine-containing lipids were found in the embryo but not in the egg yolk. The phospholipid pattern in the developing embryo was similar throughout the period studied, although changes in the relative amounts of the individual phosphatides were apparent as development progressed. Serine lipids appeared to increase, and lecithin decreased. The most prominent change occurred in the sphingomyelins, which at least doubled in relative amount between 4 and 18 days of incubation. It has been suggested that the change in the sphingomyelins is a reflection of nerve myelination in the chick embryo. The lipid phosphorus content was found to remain relatively constant throughout the period studied when determined from DNA content. When determined on the basis of fat-free Kjeldahl nitrogen or fat-free dry weight, lipid phosphorus declined steadily.

Although there have been reports in the literature on the changes of lipid phosphorus in the egg yolk and the chick embryo during incubation, little information on the separation of the phospholipids has been reported. Egg yolk phospholipids have been extensively studied by Rhodes and Lea (1957). Studies of ether-soluble lipid phosphorus in the developing chick embryo and the residual yolk have been carried out by Plimmer *et al.* (1909), Masia and Fukutomi (1923), Kugler (1936), and Tsuji *et al.* (1955). In the above studies of the embryo phospholipids, separation of the individual phospholipids was not attempted, and the results, reported in terms of cephalin, lecithin, and sphingomyelin, were based primarily on analysis of nitrogen bases.

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The purpose of the present paper is to present the results of studies on the quantitative separation and identification of the major phospholipid components of unincubated egg yolk and chick embryos at 4, 6, 12, 14, and 18 days of incubation. It is hoped that this information can be used to correlate changes in phospholipid patterns with morphologic changes during embryogenesis.

Materials and Methods

Fertilized eggs from White Leghorn chickens were obtained from Hanson's Hatchery, Corvallis, Ore. The eggs were incubated in electric incubators as specified by the manufacturer.

The lipid isolation technique used was the same for all samples. The wet tissue was homogenized for 2 minutes in a Waring Blendor with a solution of chloroform–methanol (2:1 v/v). The total volume of solvent used was ten times the wet weight of the tissue. One half of the solvent was used in the first extraction, with an extraction time of fifteen minutes. After centrifugation the precipitate was extracted with the second portion of the solvent for 30 minutes. After centrifugation of this mixture the first and second extracts were combined and washed with solutions of KCl as described by Folch *et al.* (1957). In the first wash, a volume of 0.88% KCl equal to 0.2 of the volume of the lipid extract was thoroughly mixed

with the lipid extract. The mixture was centrifuged and the aqueous layer removed. Three further washings were done with a second solution, consisting of chloroform, methanol, and 0.74% KCl (3:48:47).

The residual material remaining after the first lipid extraction was saved for DNA and protein determinations. In order to check the completeness of the extraction this residue was allowed to stand in an equivalent volume of chloroform–methanol (2:1) solution for an additional 12 hours. Phosphorus determinations were made on the resulting extract. In like manner the dried aqueous washings were extracted and total phosphorus determined to ascertain the loss due to the washing procedure. Since less than 0.5% of the total phosphorus was found in either the residue or the aqueous washings, the extraction procedure was considered adequate.

The washed lipid extracts were dried and dissolved in a small volume of chloroform. This lipid solution was applied to the silicic acid columns, usually within 4 hours of the start of its preparation. Mallinckrodt reagent silicic acid, 200–400 mesh, was used in all column chromatography, with the addition of Johns-Manville Hyflo Super-Gel as an aid in speeding solvent flow. All solvents used were reagent grade. Columns of 10 g silicic acid and 5 g Hyflo Super-Gel, 1.4 mm in diameter, were washed with chloroform, which was allowed to flow through them overnight. Lipid (0.6–1.0 mg lipid P per gram of silicic acid) was applied to the column and the neutral lipids were removed with chloroform. The phospholipids were eluted with increasing concentrations of methanol in chloroform. Nitrogen pressure was used to maintain a flow rate of 1.0–1.5 ml/minute. The positions of the phospholipid peaks were determined by phosphorus analysis by the method of Bartlett (1959). The tubes of each peak were pooled and diluted to a constant volume to be used for quantitative determinations. Further separation of pooled peaks II and III of the embryos were carried out by a small-scale modification of the method of Rouser *et al.* (1961).

Nitrogen determinations were carried out by the micro-Kjeldahl procedure of Lang (1958). Microbiological assays were used for choline (Horowitz and Beadle, 1943) and for inositol (Williams *et al.*, 1941). Sphingosine nitrogen was estimated by the modified procedure of McKibbin and Taylor (1949). Aliquots of the peaks to be analyzed for sphingosine were dried and hydrolyzed with 4 ml of saturated barium hydroxide at 100°. After 5 hours 0.4 ml of concentrated hydrochloric acid was added and the hydrolysis continued for an additional 2 hours. The hydrolysates were extracted with three portions of chloroform, the first consisting of 5 ml and the final two of 3 ml each. The combined extracts were dried and dissolved in a known volume. Aliquots were used for total nitrogen determination by a micro-Kjeldahl method. DNA was determined by the diphenylamine method of Burton (1956).

Lipids were hydrolyzed in sealed tubes at 100–110° with 6 N HCl for 12 hours and the hydrolysates were chromatographed with Whatman #1 paper by an ascending technique. The solvent systems of Artom *et al.* (1958) and Magee *et al.* (1960) were used. Choline was detected by Dragendorf's reagent (Block *et al.* 1958), amino compounds by ninhydrin, inositol by a spray described by Nagai and Kimura (1958), and sphingosine with fluorescein (Saito, 1960).

Further qualitative identification of unhydrolyzed phospholipid peaks was made with a paper chromatographic procedure similar to that of Horhammer *et al.* (1959). The unhydrolyzed lipid samples were chromatographed in their ether solvent system at 0–4° by ascending technique. Lipids were detected with malachite green and ninhydrin.

Results

The results of the separation of the egg yolk phospholipids are shown in Figure 1. Figure 2

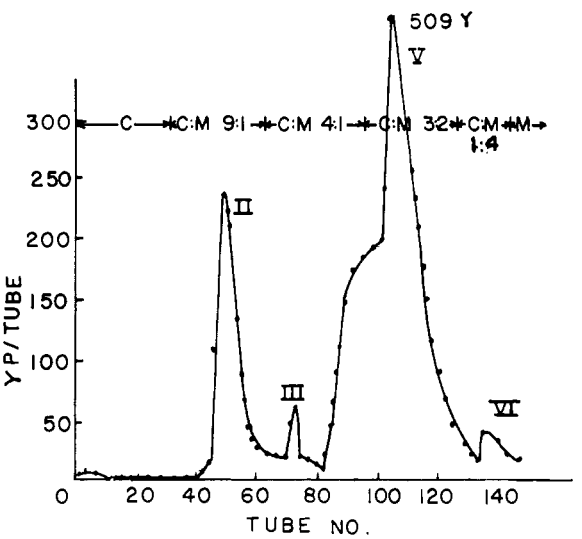

FIG. 1.—Chromatography of egg yolk phospholipids. C = chloroform, M = methanol. Numerals refer to peaks described in text.

shows a representative profile of the separations obtained with the embryo phospholipids. Figures 3 and 4 show the further separation peaks II and III. Similar patterns were observed with all embryonic ages studied. The percentages of the recovered lipid phosphorus in individual peaks, and the major constituents present in the peaks, are shown in Table I. The pattern observed in the egg yolk is clearly quite different from those obtained with the embryos in that the yolk contains no detectable serine phosphatides, which are present in all stages of the embryo studied. The embryo profiles remain quite similar throughout development, but changes in the relative amounts of lipid phosphorus in the individual

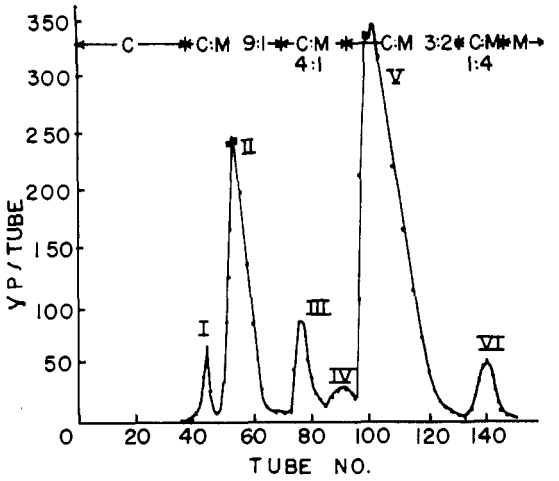

FIG. 2.—Chromatography of phospholipids of 6-day embryo. C = chloroform, M = methanol. Numerals refer to peaks described in text.

peaks are evident. Important among these changes is the increase in the sphingomyelin content from 6% to 14% during embryogenesis. There also appears to be an increase in peak IIb (a serine phosphatide) as development progresses. It is interesting to note that the total lipid phosphorus in the non-choline-containing peaks (peaks I, II, III, and IV) remains at approximately 35% throughout development. Similarly the choline-containing peaks (peaks V and VI) represent approximately 63% of the total lipid

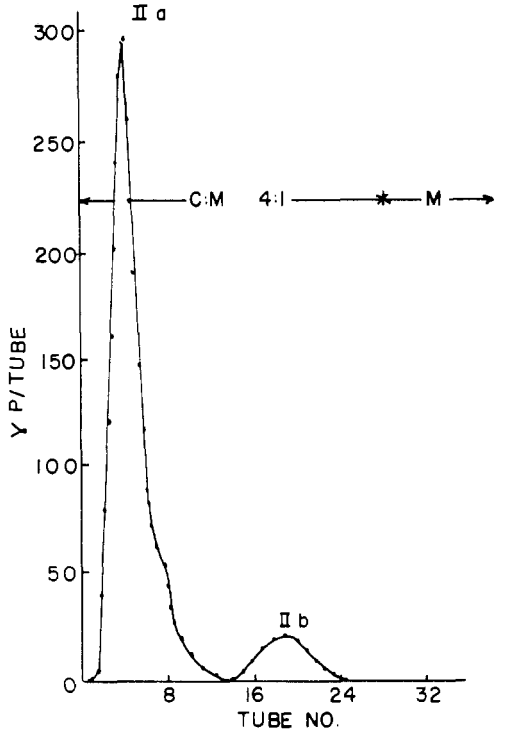

FIG. 3.—Separation of peak II into constituent phospholipids. C = chloroform, M = methanol.

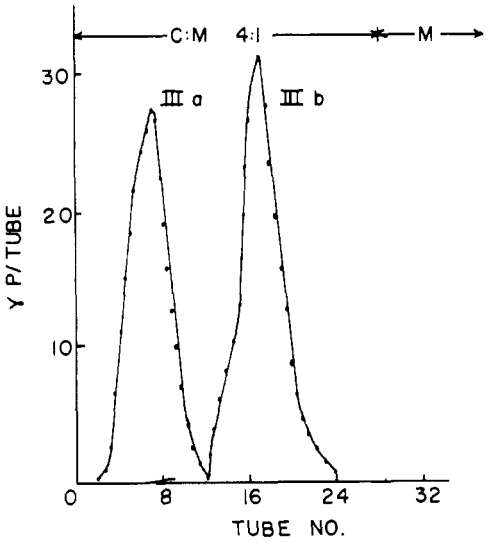

FIG. 4.—Separation of peak III into constituent phospholipids. C = chloroform, M = methanol.

phosphorus at all the stages of embryonic development studied.

Paper chromatography of the individual unhydrolyzed peaks indicated that each peak from the silicic acid column was relatively homogeneous. In addition their identity was established by comparison with the movement of commercial samples of lecithin, sphingomyelin, and the phosphatidyl derivatives of inositol, serine, and ethanolamine.

Table II gives the range of ratios of nitrogen to phosphorus obtained for the individual peaks from different experiments. Table III reports the data relating the lipid phosphorus content to DNA, fat-free nitrogen, and fat-free dry weight. The ratio of lipid phosphorus to DNA remains relatively constant throughout development, while the ratios of lipid phosphorus to nitrogen and dry weight decrease throughout the period studied.

DISCUSSION

Very little information on separation and identification of the phospholipids of developing embryos is recorded in the literature. The chick would seem to be an ideal source of material for such a study owing to the ease of identifying stages of development, as well as the relative abundance and availability of material. The major drawback is the large number of embryos required in the early stages of development in order to obtain sufficient material. The dissection of the many embryos required in early stages of development becomes a limiting factor, since the phospholipids are notorious for their autooxidation and autolysis during extraction and purification. It is quite possible that such lengths of time could result in considerable alteration of the phospholipids and thus introduce artifacts.

Table I shows the percentage of lipid constit-

TABLE I
AVERAGE PERCENTAGE OF ELUTED LIPID PHOSPHORUS PRESENT IN INDIVIDUAL LIPIDS

Peak	Constituent[a]	Egg	Age of Embryo (Days) 4	6	12	14	18
I	Serine	0	2.8	3.1 (2.9–4.0)[c]	3.1 (2.9–3.3)	2.7 (2.4–3.2)	2.8 (2.2–3.4)
IIb	Serine	0	1.5	1.2 (1.1–1.3)	4.4 (4.1–4.6)	3.1 (3.0–3.4)	6.9 (5.9–7.2)
a	Ethanolamine	18.9	17.6	21 (20–22)	20 (19–21)	19 (19–22)	14 (12–15)
IIIb	Serine	0	4.0	4.2 (2.5–4.3)	3.9 (2.7–4.4)	3.9 (2.4–4.0)	3.7 (2.8–5.5)
a	Ethanolamine	3.9	6.6	6.6 (4.1–6.7)	2.7 (1.8–3.2)	4.2 (2.6–4.3)	3.9 (2.8–6.0)
IV	Inositol[b]						
	Serine / Ethanolamine	0	3.2	4.9 (3.9–6.5)	3.0 (2.1–4.7)	4.6 (2.8–5.8)	6.3 (3.7–7.3)
V	Choline	72.0	58.2	55 (52–58)	54 (51–56)	51 (50–53)	49 (45–50)
	Inositol	0	3.2	3.7	1.9	2.6	4.1
VI	Sphingomyelin	5.3	6.2	6.6 (4.5–8.9)	10 (9.1–11)	11 (11–12)	14 (13–15)
% Recovery of Phosphorus Added to Column		100	101	103	100	98	99

[a] In addition to glycerol, phosphorus, and fatty acids. [b] Not separated further. [c] Range of values in different experiments.

TABLE II
MOLAR NITROGEN TO PHOSPHORUS RATIOS IN INDIVIDUAL PEAKS

Age of Embryos	Peak I	II	III	IV	V	VI
Egg		1.1	1.1		1.1	1.6–2.1
4-day	0.9	1.0–1.2	1.1–1.5	0.9–1.0	1.0–1.1	1.5–1.9
6-day	1.0–1.2	1.0–1.1	1.1–1.3	0.8–1.0	1.0–1.1	1.9–2.1
12-day	0.7–1.0	1.1–1.3	1.0–1.2	0.7–0.9	1.0–1.1	1.5–2.0
14-day	1.0–1.5	1.0–1.1	1.0–1.1	0.7–0.9	0.9–1.2	1.5–2.1
18-day	1.2–2.3	1.0–1.3	1.1–2.5	0.8–1.3	1.0–1.5	1.9–2.0

TABLE III
ESTIMATION OF LIPID PHOSPHORUS PRESENT ON THE BASIS OF DNA, PROTEIN NITROGEN, AND DRY WEIGHT

Age of Embryos	mg Lipid P / mg DNA	mg Lipid P / mg Protein[a]	mg Lipid P / g Dry Weight[a]
Egg	3.2	0.18	
4-day	0.10	0.057	5.6
6-day	0.095	0.042	4.7
12-day	0.11	0.045	4.8
14-day	0.11	0.032	3.7
18-day	0.11	0.019	2.5

[a] Fat-free.

uents found in the individual lipid peaks at each stage of development studied. The values for the egg and 4-day embroyo are from single experiments, while all others are average values obtained in three or four individual experiments. Slight variations were observed among individual experiments with embryos of the same age, but considering the methods and the biological material used the agreement was remarkably good. The extractions and isolation of the phospholipids were accomplished rapidly and where feasible under nitrogen to avoid artifacts as much as possible.

As shown in Table II there is some variation in the ratio of nitrogen to phosphorus obtained in individual experiments. Some unidentified ninhydrin-reactive components were observed on chromatography of the lipid hydrolysates. These may have been amino acid contaminants which were dissolved in the lipid fractions, and the amount in any given separation may be responsible for the observed variation of the ratio of nitrogen to phosphorus. Such contamination is generally recognized as one of the problems of lipid separation and purification. The wide range of values in peaks I and IV may be in part due to the small amount of material, usually less than 5% of the total lipid phosphorus, present. Thus these nitrogen determinations are subject to greater error than those of the larger peaks.

The only constituent identified in peak I of the embryo phospholipids was serine. Similar results were obtained (Hanahan *et al.*, 1957) in a study of rat liver phosphatides.

Mixtures of serine and ethanolamine phosphatides were found in peaks II and III, but further separation of these peaks permitted calculation of the amounts of phosphatidyl serine and phosphatidyl ethanolamine. An increase in phosphatidyl serine is observed during development of the embryo, particularly between 14 and 18 days of age. Correlation of this change with morpho-

logic or functional changes at the corresponding time can only be speculative owing to lack of information on the distribution and function of the serine phospholipids. The amount of phosphatidyl ethanolamine remains relatively constant.

The inositol-containing phospholipids are found in peaks IV and V in the embryo lipids. It is evident from Table I that the inositides constitute less than 5% of the total lipid phosphorus, assuming an intramolecular ratio of unity for inositol to phosphorus. There appears to be little change in the relative amount of inositol-containing lipids throughout the period studied.

As is evident from Table I, peak V is the major peak in the egg yolk and embryo phospholipids. The major constituent appears to be lecithin, but a significant amount of inositol-containing lipids is also present. These lipids are probably responsible for the low N:P ratio (less than one) in this peak. There appears to be a decrease in the relative percentage of lecithin phosphorus as development proceeds.

The most striking change in relative amounts of lipid phosphorus occurs in the sphingomyelin peak, VI. The percentage of lipid phosphorus in this peak at least doubles between 4 and 18 days of incubation. A large increase in the sphingomyelin content of the chick embryo brain has also been reported between 13 and 16 days of incubation by Mandel *et al.* (1949). The period of active myelination and the development of well-defined electrocardiograms in the chick embryo appears to be between 10 and 19 days of incubation (Heald, 1960). The period recorded in Table I (4 to 18 days of incubation) spans the period of active myelination, and the increase in sphingomyelin content from 6 to 14% may be due mainly to the myelination phenomenon. The evidence presented is only suggestive, and it must be recognized that any attempt to equate quantitative changes in sphingomyelin content with myelin sheath formation suffers from lack of information on the sheath composition. Nevertheless a correlation between the biochemical and the morphologic changes seems reasonable.

Consideration of Table III shows the ratio of lipid phosphorus to DNA to remain relatively constant throughout development. Since DNA can be used as a measure of cell number, these data might be taken as an indication of a constant amount of lipid phosphorus per cell regardless of cell type. Such a conclusion seems untenable, however, in view of the data on the increase in phospholipid content of chick brain between 13 and 16 days (Mandel *et al.*, 1949) and the discussion of brain formation presented by McIlwain (1955). A more likely alternative is that the constant ratio observed is a fortuitous coincidence resulting from the variations among the many individual developing tissues. The decrease in the ratio of lipid phosphorus to either protein nitrogen or dry weight after 12 days is most likely a reflection of the formation of muscle or other nonlipid constituents after this stage of development.

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