Phospholipid composition of chick brain during development

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SUMMARY The phospholipid composition of chick embryo brain was examined over the interval 4–18 days of incubation. Phosphatidyl ethanolamine accounted for about 25% of the total lipid phosphorus during this interval, while phosphatidyl choline decreased from 70 to 50%. Sphingomyelin formed a relatively low fraction of the total phospholipid. The sum of inositol phosphatides and phosphatidyl serine was about 5%, with slight variation during the interval studied. A phospholipid resembling cardiolipin was detected. Its content increased to 4% during this incubation period.

KEY WORDS brain · phospholipids · chick embryo · phosphatidyl choline · phosphatidyl ethanolamine · phosphatidyl serine · sphingomyelin · phosphatidyl inositol · cardiolipin

STUDIES OF PHOSPHOLIPIDS in incubated hens' eggs were first made in 1909, when Plimmer and Scott (1) reported the gross changes in amounts of various classes of compounds containing phosphorus. Although this early work has been greatly extended (2–13), our knowledge of the phospholipid composition of individual organs is as yet incomplete. In addition, the origin of individual phospholipids occurring in particular organs is still somewhat obscure. For this reason the studies reported in this paper and the accompanying one (14) were instigated.

MATERIALS AND METHODS

The embryos were obtained from Hy-line 950-A eggs purchased from Jenk's Hatchery, Tangent, Ore. The eggs were incubated in a Jamesway incubator Model 252-B (James Co., Los Angeles, Calif.) at 37° and 90% humidity.

Abbreviation: TLC, thin-layer chromatography.

Chemicals

J. T. Baker "analyzed" reagent grade chloroform and methanol were used without distillation. Phosphatidyl serine and phosphatidyl ethanolamine were obtained from Nutritional Biochemical Corp., Cleveland, Ohio; phosphatidyl choline from British Drug Houses Ltd., Cambridge, Mass.; sphingomyelin from Sylvan Chemical Co., Edgewood Cliffs, N.J.; phosphatidyl inositol from Calbiochem, Los Angeles, Calif.; and cardiolipin and lysolecithin from Sigma Chemical Co., St. Louis, Mo. Commercial phospholipid products further purified by column chromatography were used as standards. These standards were shown by TLC to be 90–98% pure. A small quantity of phosphatidic acid was kindly supplied by Dr. H. L. Hokin of the University of Wisconsin.

Extraction of Lipids

Lipids were extracted from embryo tissue and yolk by the method of Bieber, Cheldelin, and Newburgh (12), with the following modifications: 12–16 volumes of chloroform–methanol 2:1 (v/v) per g wet weight of material was used for the initial extraction of lipids; the organic layer was washed twice with 0.2 volume of 0.88% aqueous KCl; after the first washing the organic layer was made to its original volume by adding methanol. This procedure was considered adequate since further extraction of tissue yielded less than 0.4% additional lipid P. The water washings contained less than 0.4% of the total lipid P, and analysis of these phospholipids indicated a composition similar to that of the main fraction. The amount of lipid P in brain varied from 0.3 to 17 μmoles/brain (8–25 μmoles/g wet weight) during the incubation period of 5–18 days. At least 25 brains were used in each experiment. The entire brain was removed by severing the spinal cord at the junction of the myelencephalon.

TABLE 1 CHROMATOGRAPHY OF EMBRYO LIPID EXTRACT ON SILICIC ACID–HYFLO*

Eluent	Volume of eluate	Peak Number	Tubes	Phospholipids in Fraction
	ml			
$CHCl_3$	400	1	1–70	None (neutral)
C–M 12:1	100	2	80–85	PE+, PS, CA-like
C–M 9:1	100	3	86–98	PE, PS
C–M 4:1	250	4	100–120	PE, PS, PC, PI
C–M 3:2	300	5	123–160	PC, PI
C–M 3:2		6	163–195	PC, PI
Methanol	200	7	201–212	SPM, PC

Column: 1.4 cm diam, containing 12 g of Mallinkrodt 100 mesh silicic acid and 6 g of Johns-Manville Hyflo Supercel. Elution of 420 μmoles of lipid-P applied to the column was carried out under nitrogen pressure at a flow rate of 1–3 ml/min. Each tube contained 400 drops. The recovery of lipid P was 98–100%. The peaks were located by phosphorus analysis.

* Abbreviations: C–M, chloroform–methanol; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; CA, cardiolipin; PC, phosphatidyl choline; PI, phosphatidyl inositol; SPM, sphingomyelin.

Column Chromatography

Chromatography on silicic acid–Hyflo columns was carried out by the method of Bieber et al. (12). DEAE-cellulose acetate, silicic acid–silicate–water, and silicic acid–NH_3 columns also were used as described by Rouser and co-workers (15, 16).

The results of a typical batch elution on silicic acid are shown in Table 1. The mixed phospholipid peaks were further purified by column chromatography as shown in Table 2. Cardiolipin, found in peak A from the original peak 2, could be separated from phosphatidyl ethanolamine by further chromatography on silicic acid. Sphingomyelin was not obtained free from phosphatidyl choline.

TABLE 2 RECHROMATOGRAPHY OF PHOSPHOLIPID PEAKS FROM SILICIC ACID CHROMATOGRAPHY

Peak Number from Table 1	Column Used	Phospholipid	Isolated
		Peak A	Peak B
2	Silicic acid–NH_3 (150 ml of C–M 4:1, methanol)	PE (CA)*	PS
3	Silicic acid–NH_3 (eluted like peak 2)	PE (PS)*	PS
4	Silicic acid–NH_3 (eluted like peak 2)	PE (PS)* (PC)*	PS
5	DEAE-cellulose (250 ml of C–M 7:1, 180 ml of C–M 4:1)	PC	PI†
6	DEAE-cellulose (eluted like peak 5)	PC	PI†
7	Silicic acid (100 ml of $CHCL_3$, 200 ml of C–M 1:1, methanol)	PC	SPM, PC

Abbreviation, column sizes, and conditions as in Table 1. Recoveries were 90–100%.

* Parenthesis indicates a minor contamination by the phospholipid indicated.

† PI was eluted by adding 2% concd NH_4OH to C–M 1:1.

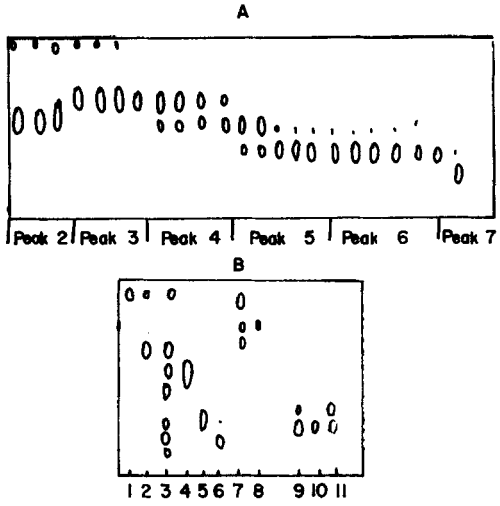

FIG. 1. *A*. Representative thin-layer chromatograms of fractions taken from a silicic acid–Hyflo column of a brain lipid extract. The manner of combining material from several tubes into "peaks" is shown. Peak *2* contains PS and CA-like phospholipid (for abbreviations see Table 1); peak *3*, PE and CA-like phospholipid; peak *4*, PS and PE; peak *5*, PC and PI (PC has the lower R_F value); peaks *6* and *7*, PC and SPM.
B. Thin-layer patterns of *1*, rechromatographed CA-like phospholipid (PS has been removed); *2*, rechromatographed PE (PS has been removed but a trace of CA-like phospholipid remains); *3*, phospholipid standards which are (from the bottom) LL, SPM, PC, PI, PS, PE, and CA; *4*, rechromatographed PS; *5*, rechromatographed PC; and *6*, rechromatographed SPM which still contains some PC. *7* contains standard PE, CA, and phosphatidic acid; *8*, the front-running phospholipid (shown in the top TLC plates), behaves like authentic CA and not like phosphatidic acid. *9* shows a mixed SPM–PC fraction; *10*, the same fraction after treatment with base; and *11*, standards of SPM and PC. Separation was on Silica Gel G and the solvent used was chloroform–methanol–7 N NH_3 70:30:4.

The purity of the individual peaks was determined by TLC (17–19). The spots were detected by spraying with either Zinzadse reagent (20), ninhydrin (19), Dragendorff reagent (19), silver nitrate (19), or a dichromate spray (21). Typical thin-layer plates are shown in Fig. 1.

Portions of the isolated phospholipids (1–200 μmoles of lipid P) were dried in test tubes in vacuo. Approximately 4 ml of 3–6 N HCl in methanol–water 1:1 was then added to each tube. The lipids were hydrolyzed in a boiling water bath for 12 hr (48 hr for samples of phosphatidyl inositol). Rapid evaporation was prevented by placing a glass marble on the tube. After hydrolysis and ether extraction the HCl solution was evaporated and the residue taken up in a small amount of water. This material was then chromatographed on washed Whatman No. 1 paper using the ethanol–NH_3 solvent of Artom, Lofland, and Oates (22) for the separa-

TABLE 3 CHARACTERIZATION OF PHOSPHOLIPIDS ISOLATED FROM CHICK EMBRYO BRAIN

Phospholipid	Test on TLC Plates*			Ester: Phosphorus	Nitrogen: Phosphorus	Remarks
	Z	N	D			
CA	+	−	−	1.9	—	No amines in acid hydrolysate
PS	+	+	−	—	—	Serine in acid hydrolysate
PE	+	+	−	2.0	1.0	Ethanolamine in acid hydrolysate
PC	+	−	+	2.1	1.0	Choline in acid hydrolysate
SPM	+	−	+	0.9	—	Choline in acid hydrolysate
PI	+	−	−			No amines in acid hydrolysate. Positive inositol test, *cis*-hydroxyls test on TLC.

* Abbreviations used in these columns: Z, Zinzadse reagent for phospholipids; N, ninhydrin reagent; and D, Dragendorff reagent.

TABLE 4 PHOSPHOLIPID COMPOSITION IN CHICK EMBRYO BRAIN DURING DEVELOPMENT

Age	Percentage of Each Phospholipid*						Total Recovery
	CA-like	PE	PS	PC	PI	SPM	
hr							%
118	—	26	—	70	3	6	105
144	1.1	25	1.5	56 (?)†	—	4	88
159	0.4	23	1.5	70	3	3	101
244	1.6	28	3	62	3	3	101
290	0.7	26	7	51	4	2	91
307	1.9	24	6	58	—	3	93
378	2.1	27	—	59	3	3	94
390	4.0	28	6	49	2	3	92
427	—	24	3	55	—	3	85

* $\frac{\text{Lipid P in fraction}}{\text{Total lipid P chromatographed}} \times 100$.

† The low total (88%) for the 144 hr fraction may account for the apparently low PC value.

tion of the nitrogen bases. After development they were sprayed first with ninhydrin solution, then Dragendorff reagent.

Another aliquot of the isolated phospholipid was used for quantitative ester (23), phosphorus (24), and nitrogen (25) determinations. Free *myo*-inositol was determined by the spot test of Feigl and Gentil (26).

RESULTS

The results of the analysis and characterization of various fractions given in Table 3 are typical of results obtained at several ages of embryos. The sphingomyelin peak obtained on silicic acid–Hyflo columns was contaminated with phosphatidyl choline. Assay of nonsaponifiable phosphorus in this peak indicated that it contained 53% sphingomyelin; the ester:phosphorus ratio indicated 57% sphingomyelin. Figure 1 shows that the least polar phospholipid of chick embryo brain behaved on TLC like commercial cardiolipin and not like phosphatidic acid.

Quantitative changes in the relative amounts of major phospholipids in the whole embryo during development have been reported by Kugler (7) and more recently by Bieber et al. (12). The phospholipid composition of brain during development is shown in Table 4. The plasmalogen content was not determined; hence the percentages given include plasmalogens. Several Zinzadse-positive and some Zinzadse- and ninhydrin-positive components comprising about 1.5% of the total lipid P appeared in the chloroform–methanol 2:1 and methanol eluates from DEAE-cellulose columns. These minor components were not identified further.

An unidentified phospholipid was detected by TLC in some brain fractions. This particular component moved on thin-layer chromatograms slightly ahead of phosphatidyl choline and was always mixed with it. For this reason the "phosphatidyl choline" in Table 4 represents phosphatidyl choline plus a small amount (less than 5%) of this compound.

The increase in phospholipid content during development is shown in Table 5. Data on the DNA content, obtained from other batches of chick brains, have been published (27). Between 96 and 254 hr a rapid increase in phospholipid per cellular unit (i.e., per milligram of DNA) is evident. After 254 hr the amount of phospholipid per cell remains relatively constant, while brain phospholipid as a whole increases.

TABLE 5 PHOSPHOLIPID INCREASE IN BRAIN DURING INCUBATION DEVELOPMENT

Age of Brain	Phospholipid Per Brain	Phospholipid:DNA
hr	*μmoles*	*μmoles/mg*
96	0.14	5.8
168	1.62	7.8
254	4.90	15.2
336	9.10	15.1
360	11.3	17.1
432	18.8	21.0
1-day hatched chick	ca. 24	ca. 16

DISCUSSION

From these studies it is evident that the relative proportions of phosphatidyl ethanolamine and phosphatidyl inositol remain relatively constant during development (see Table 4). Phosphatidyl serine increases slightly while sphingomyelin remains constant after 159 hr, but the small amounts of these two phospholipids make it difficult to draw any definitive conclusions. It is of interest to note that the cardiolipid-like phospholipid(s) increase gradually over the period studied.

In relation to the processes of development it is of interest to compare the rate of increase of phospholipid per whole brain with that per cellular unit (per milligram of DNA). Between 96 and 168 hr a very rapid increase of phospholipid per brain occurs, in contrast to a much lower increase per cellular unit. This indicates a rapid cellular proliferation without a similar increase in brain mass. After 254 hr, it appears that each new brain cell accumulates a typical amount of phospholipid, since the ratio phospholipid: DNA becomes constant by 254 hr.

The low concentration of sphingomyelin in chick brain indicates that if a direct relationship exists between sphingomyelin and myelination, then myelination in the chick embryo up to 18 days (2–3 days before hatching) is not complete. The sphingomyelin content increases from 2.5% of total phospholipids at 3 days to 11% at 5 months (28), suggesting a relationship between sphingomyelin and brain development. Garrigan and Chargaff noted (29) a marked increase in concentration of sphingosine found in brain glycolipid from 10-day chick embryo to that found 2 days after hatching. The sphingomyelin content of animal adult brain tissue generally is greater than 10% (30). In the adult brain of man, ox, cat, rabbit, rat, pigeon, guinea pig, and fish, cephalin predominates over lecithin by about 1.5:1 (30). In contrast, lecithin is more prominent than cephalin in chick during the embryonic period.

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