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The major phospholipids of the chick embryo were studied between 4 and 19 days of incubation. Inorganic ^{32}P was added to yolks of the developing embryos at various ages as a method to study the morphological site and time of synthesis of individual phospholipids. At different ages of the incubation (after $^{32}P_i$ injection), phospholipids were extracted from the brain and whole embryos with chloroform: methanol (2:1, v/v) and were chromatographed on either DEAE-cellulose of silicic acid-ammonia columns, depending on the nature of the peak. Lipid-phosphorus was determined in the separated peaks; by this analysis the relative composition of the phospholipids was calculated in brain. Lecithin declined from about 70% to 50% of the total lipid-phosphorus while relative amounts of the other phospholipids changed little; phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and sphingomyelin were

approximately 26%, 5%, 3%, and 3%, respectively, over the incubation period studied.

A non-nitrogen phospholipid from brain, representing about 2% of the total lipid-phosphorus, was found to behave on thin layer plates like commercial cardiolipin and unlike phosphatidic acid. After base hydrolysis of this sample, its chromatographic behavior was unlike α-glycerol phosphate, the product expected by base hydrolysis of phosphatidic acid.

Specific activities were of the same order of magnitude for all phospholipids in brain and whole embryo at all ages studied. Roughly, the order of specific activities was: cephalin > lecithin > sphingomyelin. Water soluble phosphates isolated from the tissue during the extraction had specific activities within a 2- or 3-fold range of cephalin.

A high specific activity phosphatidyl choline-^{32}P was prepared biologically by the chick by injection of about 100 μc of $^{32}P_i$ per egg. This ^{32}P-phospholipid fraction was injected into 11-day old embryos. A phosphatidyl choline fraction from chick brain was isolated whose specific activity was approximately 40 times greater than all other phosphorus-containing fractions. Similar results were obtained by using labeled phosphatidyl ethanolamine.

Glycerol-1,3-^{14}C and acetate-1-^{14}C, when added to yolks of growing embryos, when compared to $^{32}P_i$ was not

significantly incorporated into the phospholipids.

The implications of the results are considered; a mechanism by which phospholipid would be at least partially transported from yolk to the brain without breaking phosphodiester bonds is suggested. The results of both kinds of experiments, i.e., those in which $^{32}P_i$ was added and those in which ^{32}P-phospholipid was added, indicated that part of the brain phospholipid was synthesized de novo, but a considerable portion of the total phospholipid in brain might arise from undegraded yolk phospholipid, more so in the latter stages of incubation than the earlier ones.

ORIGIN AND COMPOSITION OF PHOSPHOLIPIDS IN CHICK EMBRYO AND CHICK EMBRYO BRAIN DURING DEVELOPMENT

by

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Typed by Judy Saunders

To my parents

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ORIGIN AND COMPOSITION OF PHOSPHOLIPIDS IN CHICK EMBRYO AND CHICK EMBRYO BRAIN DURING DEVELOPMENT

INTRODUCTION

Until recent times embryological studies have included principally observations of morphological and cytological changes occuring during growth and differentiation. More recently investigators have sought to explain these changes in chemical and physical terms. Molecular changes which occur during development are a step removed from observations seen cytologically in that the changes studied occur with reference to other molecular changes, not necessarily with reference to other cells. The progress achieved in being able to physically measure life on an ever decreasing scale of size has made possible ever increasing knowledge about the sequence of events that are basic to the growth and development of multicellular forms of life. That changes in phospholipid composition and concentration in chick embryos are revelant to underlying cause and effect relationships is hence anticipated, but it may turn out that such changes, in providing the physical environment necessary for biological function, require a less rigorous chemical definition than say enzymes.

As a branch of chemistry, organic chemistry is more advanced than biochemistry. Methods used by organic chemists to understand a reaction include the study of starting

material, the kinetics of reactions, catalytic effects, and examination of product. In the work reported here, products and overall kinetics are studied within the dimensions of a developing living system. On the basis of what has been achieved by product and kinetic study in organic chemistry, it is hoped that the findings reported here will be a contribution to the field which is called chemical embryology. The chick embryo has received considerable attention for some time as a system for studies of chemical embryology. The morphology of the chick is well documented by Lillie (34). The relation of chemical changes to physiological changes which occur in the developing chick has been treated in the classic work of Needham (38).

Studies of phospholipids in incubated hens' eggs date back to 1909 when Plimmer, Aders, and Scott (39) reported changes in relative amounts of various classes of phosphorus compounds. Extensions of this work have been published by Cohn and Bonot (13); Jost and Sorg (28); Sereno, Montezemolo and Balboni (43); Masia, Yasura, and Fukutomi (37); Kugler (30, 31); Szepsenwol, Mason, and Shontz (49); Tsuji, Brin, and Williams (50); Rhodes and Lea (40); Hooghwinkel and van Niekerk (27); and Bieber, Cheldelin, and Newburgh (4).

Since phospholipids contain phosphorus, carbon, and hydrogen, different workers have used the radioisotopes ^{32}P and ^{14}C to elucidate sequences and morphological site of lipid forming reactions which occur in the incubated egg.

This approach was pioneered in the chick embryo by Hevesey, Levy, and Rebbe in 1938 (25); other studies were by Branson, Brooks, and Piper (7); Stokes, Fish, and Hickey (48); Davison, Dobbing, Morgan and Wright (15); Budowski, Bottino, and Reiser (10); and Camerino and Wright (11). The data obtained by these groups established that during the incubation of hen's eggs: (a) egg yolk contains a rich supply of phospholipid which progressively decreases while embryo phospholipid increases; (b) hydrolysis of lipid-P to form inorganic P occurs in yolk; (c) substrates labeled with ^{32}P and ^{14}C become incorporated into embryo phospholipids, but inorganic ^{32}P in particular is not significantly incorporated into yolk phospholipids; (d) some lipids (cholesterol and triglyceride) seem to be transported to some extent into embryo tissue without being degraded and re-synthesized in the process; and (e) the composition of individual phospholipids of the embryo does not strictly reflect the composition of yolk phospholipids.

Branson _et al._, Hevesey _et al._, Stokes _et al._, and Budowski _et al._ concentrated on establishing the site of lipid and phospholipid synthesis in the incubated egg. Hevesey and Branson groups obtained data consistent with a mechanism by which yolk phospholipids are hydrolyzed to produce inorganic P and other products and the inorganic P is then made available for synthesis of embryo phospholipids. Budowski and co-workers presented evidence for the breakdown

and re-synthesis of yolk phospholipid during the time that the embryo develops in the fertilized egg. The chief shortcoming of the ^{32}P work was that all phospholipids were combined into one class, and quantitative aspects of their synthesis were not considered. The question of how much embryonic phospholipid was synthesized de novo (from inorganic P, diglyceride, and base) was not answered. The work of Budowski et al. is more comprehensive since it includes data on phospholipids and triglycerides in yolk and several organs of the chick embryo; this data must be taken into account in proposing any overall mechanism regarding the fate of yolk lipids and phospholipids.

In the work reported in this thesis, phospholipids of the incubated egg were studied not only as a class, but as individual subclasses, i.e., phospholipids differing in base or alcohol side chain. Parameters have been measured which provide information concerning the time, morphological site, and quantity of phospholipids synthesized in the developing chick embryo. The phospholipid composition of chick embryo brain with age of incubation and the characterization of a previously undefined minor phospholipid from chick embryo brain are reported.

METHODS AND MATERIALS

Fertile Eggs

The embryos were obtained from Hy-line 950-A eggs purchased from Jenk's Hatchery, Tangent, Oregon. The eggs were incubated in a Jamesway incubator, Model 252-B (James Co., Los Angeles, Cal.), under conditions of controlled temperature and humidity (98° F dry bulb, 86° F wet bulb).

Chemicals

J. T. Baker 'analyzed' reagent grade chloroform ($CHCl_3$) and methanol (MeOH) were used without distillation. Commercial phospholipid (PL) products were obtained for primary chromatographic standards. Phosphatidyl serine (PS) and phosphatidyl ethanolamine (PE) were obtained from Nutritional Biochemical Corp. Phosphatidyl choline (PC) was obtained from British Drug Houses Ltd. Sphingomyelin (SPM) was purchased from Sylvana Chemical Co. The phosphatidyl inositol (PI) used was a product of Cal Biochem. Cardiolipin (CA) and lyso-lecithin (LL) were from Sigma Chemical Company. The silicic acid for chromatography was Mallinckrodt 100 mesh $SiO_2 \cdot xH_2O$. DEAE-cellulose used was the Bio-Rad Cellex-D brand. Hyflo Super-Cel was obtained from Fisher Scientific Co. A small quantity of somewhat impure phosphatidic acid (PA) was kindly supplied by Dr. H. L. Hokin of the University of Wisconsin. A few mgs of the

barium salt of α-glycerol phosphate was a gift of Dr. A. Baich. Carrier free radioactive sodium phosphate ^{32}P ($^{32}P_i$) was purchased from Nuclear Consultants Corp., Glendale, Cal. Glycerol-1,3-^{14}C (2 mc/mmole) and sodium acetate-1-^{14}C (50 mc/mmole) were obtained from New England Nuclear, Boston, Mass.

Extraction of Lipids

The method of obtaining lipids from embryo tissue and yolk was that used by Bieber *et al.* (4), with the following modifications: 12 to 16 volumes of 2 parts $CHCl_3$ to 1 part MeOH (C:M, 2:1, v/v) per g wet weight of material was used for the extraction of lipids, the organic layer was washed twice with 0.88% aqueous KCl, and after the first washing the organic layer was made to its original volume by adding MeOH. This method was shown to be satisfactory since overnight extraction of tissue yielded only 0.4% more of total lipid-P than extraction for 15 minutes. Further, lipid-P lost in the water layer was only 0.4% of total lipid-P and was not of a composition drastically different than that of the main lipid fraction; i.e., the more polar phospholipids (PL's) were not preferentially removed from the organic layer by water washing. Therefore, the total lipid-P not experimentally included beyond the extraction and washing steps amounted to only 1%.

To obtain brain PL, the entire brain was removed. At ages of incubation less than five days, the part of the head containing brain was used as an approximation of the brain.

Column Chromatography

All columns used were 1.4 cm in diameter (the few exceptions are noted) and were operated under N_2 pressure such that the flow rate was from 1-3 ml/minute. A Gilson Model 15 fraction collector (Madison, Wis.) was employed for all column work and 400 drops/tube were collected. Chromatography on silicic acid-Hyflo columns was carried out by the method of Bieber *et al*. (4). DEAE-cellulose acetate, silicic acid-silicate-water, and silicic acid-NH_3 columns were also used and are described by Rouser *et al*. (41, 42).

Most of the commercial PL's required purification. Commercial PC (3 g) was first dissolved into diethylether, the insoluble material filtered off, and the ether solution diluted with cold acetone to precipitate PC. The PC precipitate PC. The PC precipitate was dissolved in C:M (7:1) and chromatographed on DEAE-cellulose acetate (41). The eluate volume from 20-60 ml contained PC. This fraction was chromatographed on 15 g of silicic acid which had been treated with water (41). PC emerged in C:M:H_2O (80:20:1.5, v/v/v).

Commercial SPM (0.52 g) was dissolved in $CHCl_3$ and chromatographed on 15 g of activated silicic acid. Elution was with 200 ml of $CHCl_3$ (elutes neutral lipid) followed by

C:M (1:1). The SPM fraction was dissolved in C:M (7:1) and passed through a DEAE-cellulose acetate column. A white material was obtained of which 90% of the lipid-P was SPM (PC was the only PL impurity).

Commercial PI (0.32 g) was dissolved in 100 ml of C:M (2:1). The organic layer was washed with 0.2 volumes of 0.88% of aqueous KCl (as above) and chromatographed on 15 g of activated silicic acid. The fractions of PI obtained by eluting with 200 ml of $CHCl_3$, 100 ml of C:M (2:1), and MeOH produced several streaked spots on thin layer (TL) chromatographs. It was generally found difficult to obtain chromatographically pure PI from either the commercial sample or from embryo tissue.

Commercial PE (0.24 g) was chromatographed on DEAE-cellulose; the column was eluted with 120 ml of $CHCl_3$ and 200 ml of C:M (2:1). PE was obtained in 180-280 ml of eluate.

Commercial PS (0.09 g) was purified on 10 g of a silicic acid-NH_3 column (42). PS was eluted in MeOH after elution with 150 ml of C:M (4:1). This sample appeared to be highly oxidized (yellow color) and contained oxidized PE as well as PS. CA and LL did not require purification. Chromatographic standards used in later experiments were purified PL's obtained from the chick.

A typical extraction and chromatographic separation of lipids will now be described. Six dozen fertile eggs

obtained one afternoon were placed in the incubator on the next morning. After incubating for 173 hours, 58 embryos, weighing 47 g wet weight, were removed and homogenized in approximately 300 ml of C:M (2:1). The homogenate was filtered through glass wool (filter paper adsorbs lipids), and the solid material homogenized again in approximately 300 ml of C:M (2:1). The filtered extract (approximately 700 ml) was shaken gently with 140 ml of 0.88% aqueous KCl. After the mixture stood several hours in the cold, aqueous and organic layers were separated and the organic layer made to its original volume by adding MeOH (considerable MeOH is extracted into the water layer). A second identical washing was performed. The combined water washings were saved for further analysis. The organic layer was evaporated at 35° C in a Büchi "Rotovapor" in vacuo using a water aspirator. The residue was dissolved in C:M (2:1) and made to a volume of 250 ml. The solvent was removed again by evaporation. This process of evaporation and re-dissolving breaks proteo-lipid bonds (19). The residue (420 μmoles of lipid-P) was taken up in a small volume of $CHCl_3$ and chromatographed on silicic acid-Hyflo prepared as described above (12 g of silicic acid, 6 g of Hyflo). All solvents used in the extraction process were purged with purified nitrogen gas, but an absolute nitrogen atmosphere was not adhered to.

"Batch" elution of the extract on silicic acid was carried out with 400 ml of $CHCl_3$, 100 ml of C:M (12:1),

200 ml of C:M (9:1), 250 ml of C:M (4:1), 300 ml of C:M (3:2), and 200 ml of MeOH. This entire elution under nitrogen pressure required a night and a day. The peaks were located by P analysis (3) and the results are given in Table II-A.

TABLE II-A

Chromatography of Embryo Lipid Extract on Silicic Acid

Eluate	Peak No.	Tubes	Phospholipids
$CHCl_3$	1	1-70	none (neutral)
C:M (12:1)	2	80-85	PE, PS, CA-like
C:M (9:1)	3	86-98	PE, PS
C:M (4:1)	4	100-120	PE, PS, PC, PI
C:M (3:2)	5	123-160	PC, PI
C:M (3:2)	6	163-195	PC, PI
MeOH	7	201-212	SPM, PC

These peaks, which were mixtures of PL, were re-chromatographed to obtain fractions which were 80-98% pure on the basis of P and base or alcohol group. The fatty acid composition of the peaks was not measured.

Peaks 2 and 3 (Table II-A) were re-chromatographed on silicic acid-NH_3 columns eluting routinely with 150 ml of C:M (4:1), then with MeOH. Complete separation of PE and PS was not generally achieved, although contamination of PS in PE was usually minimal and not significant. PE contamination

of PS was more noticeable due to the large amount of PE compared to PS.

Peak 6, 152 μmoles of primarily PC, was dissolved in a small volume of C:M (7:1) and re-chromatographed on a tightly packed DEAE-cellulose acetate column 1.2 x 15 cm (5 g of resin). Elution was with 250 ml of C:M (7:1), 180 ml of C:M (4:1) followed by MeOH:NH_3 (9:1). The first peak eluted was nearly 100% PC and the peak eluted in MeOH:NH_3 contained principally inositides.

Peak 7 was re-chromatographed on 4 g of silicic acid by eluting with 100 ml of $CHCl_3$ and 200 ml of C:M (1:1) and MeOH. The main fraction was eluted in C:M (1:1) and was PI with some PC contamination. Peak 8 was re-chromatographed on 9 g of the silicic acid-Hyflo mixture, eluting with 100 ml $CHCl_3$, 200 ml C:M (1:1), and MeOH. Two SPM peaks were obtained, one from tubes 30-41 and a second (eluted entirely in MeOH) from tubes 48-55. The results of this entire procedure are given in Table II-B.

Near 100% recoveries of lipid-P added to the silicic acid-Hyflo columns could be attained. In a typical column separation, recovery of total P and ^{32}P were 98% and 100%, respectively. The PC peak (eluted in C:M, 3:2, on silicic acid-Hyflo) when chromatographed on DEAE-cellulose, was usually recovered in 65-90% yield; the losses were probably oxidized PL. Complete recoveries on the DEAE-cellulose column could be attained by eluting with 150 ml of C:M (7:1),

TABLE II-B

Re-chromatography of PL Peaks from Silicic Acid Chromatography

Peak No. from Sil. Acid[a]	Second Col. Used	PL Peaks Resolved on Second Col.		
		Peak I	Peak II	Peak III
2	Silicic acid-NH_3	PE	PS	
3	Silicic acid-NH_3	PE (PS)[b]	PS	
4	Silicic acid-NH_3	PE (PS)	PS (?)[c]	
5	Silicic acid-NH_3	PC, PE	PC, PE	PC
6	DEAE-cellulose	PC	PI	
7	Silicic acid	PC, PI		
8	Silicic acid-Hyflo	SPM	SPM	

[a] See Table II-A.

[b] Parenthesis indicate contamination with the PL in the parenthesis.

[c] Too little PL was present in this peak to identify it.

150 ml of C:M (2:1), 100 ml of MeOH, 200 ml of C:M:conc. NH_3 (1:1:0.1), and finally with MeOH:conc. HCl (95:5). On silicic acid-NH_3 columns 90-100% recoveries were routinely achieved if the fractions had not been exposed to air and heat for long periods of time. The usual procedure in collecting fractions was to take the tubes comprising the symmetrical part of the PL peaks in order to avoid overlapping parts of adjacent peaks.

Rouser (41) has eliminated the problem of oxidation of lipids in air by maintaining lipids in an atmosphere of purified nitrogen gas. Such precautions are mandatory if one wishes to obtain good separations of completely unoxidized lipids and 100% recoveries on DEAE columns. In experiments reported here, nitrogen conditions were employed during the extraction and silicic acid chromatography of the PL. The losses of PL on DEAE (by not using rigorous nitrogen conditions throughout) were not of particular disadvantage in view of the parameters to be measured and in view of the overall aim of this research.

These procedures were used routinely to separate PL's by column chromatography. Exact duplication of the elution procedure for different batches of tissue was not specifically sought, since PL mixtures of different compositions would not produce the same chromatographic pattern. Variations from the usual chromatographic procedure are sufficiently covered in the Results section.

Identification of Lipid Fractions

The first clue as to the identity of PL fractions was their position of elution from silicic acid columns according to Bieber *et al*. (4) and from the other columns according to Rouser *et al*. (41, 42). Known chemical and chromatographic methods were used to show that a given PL fraction contained a specific base or alcohol group (ethanolamine, serine, choline, or inositol). The methods used for identification are summarized below.

Paper Chromatography of Intact PL

The method of Kennedy and Collier (29) was not found to be of general value because thin layer chromatography (TLC) proved more versatile and was faster and the dyes mentioned in this paper were not readily available.

Paper Chromatography of Acid Hydrolysis Products

PL samples (1 to 200 μmoles of lipid-P) in 2 x 15 cm pyrex tubes were dried and about 4 ml of 3-6 N HCl was added. The lipids were hydrolyzed in a boiling water bath for about 12 hours (or 48 hours for PI samples). MeOH was added from time to time in order to improve solubility in the aqueous HCl solution. A large marble on top of the tube prevented rapid evaporation. After hydrolysis and ether extraction, the HCl solution was evaporated and the residue

was taken up in a small amount of water. Ethanolamine, serine, and choline were identified by chromatography on washed Whatman No. 1 paper using ethanol-NH_3 solvent of Artrom *et al*. (1) and spraying first with ninhydrin solution, then with Dragendorff reagent (44) which is specific for choline.

Chemical Analysis

To obtain molar ester to phosphorus ratios (E/P), the method of Snyder and Stevens (46) was used to measure ester bonds. Phosphorus was determined by the Bartlett (3) method.

Nitrogen was determined by the Lang modification (32) of the micro Kjeldahl assay to obtain molar nitrogen to phosphorus (N/P) ratios.

Free *myo*-inositol was identified by the spot test of Feigel and Gentil (18) which is specific for only the *myo* geometrical isomer of inositol.

Thin Layer Chromatography (TLC)

Materials.

1. Silica gel G (according to Stahl).
2. Silica gel H, Brinkman Instrument Co. (contained no $CaSO_4$ binder (45)).
3. Spreader by C. Desaga (Heidelberg, Germany).
4. Glass plates: 20 x 20 cm, 20 x 10 cm, and 20 x 5 cm.

5. Solvent tanks, 33 cm wide and 24 cm high with a central groove so two plates 20 x 20 could be chromatographed simultaneously (Brinkmann, Great Neck, N. Y.).

Spray solutions.

1. Fifty percent H_2SO_4 (36). This is a non-specific spray for C-H compounds.
2. Eighty percent H_2SO_4 saturated with $Na_2Cr_2O_7$. This solution was found to be more sensitive than 50% H_2SO_4.
3. Zinzadse reagent (26). This spray will detect as little as 0.005 μmole of PE or PC. It was found to be specific for PL's only among phosphate esters (sugar phosphates did not produce the characteristic blue color).
4. Ninhydrin (44). This spray was used to detect PE, PS, and SPM or free amino acids.
5. Dragendorff reagent (44). Choline containing lipids were detected with this reagent (the same reagent used to detect free choline on paper chromatograms).
6. Silver-NH_3 (44). This reagent will react with adjacent cis OH groups or adjacent cis OH-NH_2 groups. PI shows a dark spot after treatment. PS and PE react more mildly.

Procedure. A 30 g portion of silica gel G mixed with 65 ml of water covered five 20 x 20 cm plates when the thickness of the gel was 0.25 mm. The same coverage (or a little more) was attained with 45 g of silica gel H in 0.001 M Na_2CO_3 (45). The plates were activated at 110° to 120° for about 30 minutes prior to application of the lipid solutions which were usually concentrated before applying in spots 10 to 40 mm^2 in area, set about 2.5 cm apart. Application was made by capillary tubes and the amount of lipid-P applied was from 0.1 to 0.01 of a μmole. The developing tank was lined laterally on all sides with a sheet of Whatman No. 1 filter paper which was wetted with solvent prior to chromatography. The solvent front was allowed to proceed to about 0.6 to 0.9 of the plate height. The plates were removed, air dried, and treated with one of the reagents listed above. Usually a single specific reagent was sprayed onto each plate, followed by dichromate spray after the initial reaction. Ninhydrin or Dragendorff reagent followed by Zinzadse spray could be used on a single plate. To clearly locate PL, one should spray with Zinzadse, then lightly with 50% H_2SO_4. The background will slowly colorize after blue PL spots appear.

When lipid was to be recovered from the plate, the spreader was set at 0.50 mm. After chromatographing, the area of the plate containing the lipid was transferred to a small sintered glass funnel and washed with a solvent which

would elute the lipid.

Base Hydrolysis

Lipids were saponified under conditions described by Garbus (21). The samples (0.1 to 10 μmole of lipid-P) were dried in 2 x 15 cm test tubes and 0.2 ml of $CHCl_3$, 0.4 ml of C:M (2:1), and 0.5 ml of 0.5 N NaOH was added. After mixing, the solutions were allowed to stand 10 to 30 minutes at room temperature or 10 minutes at 35° C. The pH was adjusted to the acid side with 1 N HCl, and 3 ml of water was added. Fatty acids and unsaponifiable lipid were extracted from the aqueous solution by several 3 ml portions of diethyl ether. The aqueous layer was evaporated to dryness and saved for further analysis (as will be described).

Phosphorus Assay in the Aqueous KCl Layer

Total phosphorus and P_i in the presence of phosphate ester in the aqueous KCl wash were determined by the Bartlett (3) method. P_i was separated from organic phosphate (esters and anhydrides) so that when ^{32}P was used, radioactivity could be determined for P_i, organic P, and total P. To accomplish this, the aqueous KCl solution, separated from the organic lipid containing layer (the original extraction), was evaporated at low heat (40° C) to half or less of its original volume, then made to a convenient volume for P_i and total P analysis. Duplicate (usually 2 x 0.2

volumes) aliquots of the solution were withdrawn and mixed with an appropriate amount of activated carbon. After filtering, 3 ml of carrier P_i (10 mg/ml) was added and P_i was precipitated as $MgNH_4PO_4$ as described by Lehninger (33). The precipitate was collected on fine-pore analytical filter paper, washed with dilute base, and then dissolved in dilute aqueous HCl, allowing the HCl solution to pass from the filter paper directly into a 10 ml volumetric flask. From this solution ^{32}P was assayed and the specific activities of P_i, organic P, and total P were calculated.

Assay for ^{32}P

^{32}P was measured by liquid scintillation counting from aqueous and organic solutions. One ml of water and 1 ml of C:M (2:1) were added to each scintillation vial (20 ml size from Packard Instrument Co.). Ten ml of Bray's solution (9)[a] was then added to samples and standards. In this way ^{32}P from an aqueous or a C:M (2:1) solution may be added to the vials and the assay may be carried out without correcting for efficiencies due to differences in make-up of scintillator solution. The vials (total volume in each is 12 ml) were cooled and counted in a Packard scintillation counter, Model 314-DC, at 1250 volts (tap 9). Cpm from the green channel were recorded with the discriminators set from

[a] Bray's solution was modified to 40 g of naphthalene per liter of solution.

20-∞. The efficiency was about 50%. Standards were always counted with samples since the counting efficiency varied ±3% from day to day. Bray's solution should be made fresh about every two weeks, or counts become unreliable. The efficiency of this method was independent of the chemical bonding of P or concentration of the ^{32}P compound. Large increases in lipid concentration (up to 10 mg/vial) in the vials did not alter the efficiency. This is perhaps due to the fact that $CHCl_3$ and H_2O quench the solution maximally. Toluene scintillator solution was used to monitor column separations when radioactive lipids were being chromatographed.

Injection of $^{32}P_i$ into Fertile Eggs

In the experiments reported here, 1 or 2 ml of carrier free sodium phosphate ^{32}P ($^{32}P_i$) was diluted with sufficient sterile chick ringers solution (10 to 100 ml) such that 2 to 16 μc of ^{32}P were injected per egg. Within a given batch of eggs (i.e., in a group of eggs the same age which were injected on the same day), the same dose of ^{32}P was administered to each egg via a 50 μL syringe (Hamilton Co., Whittier, Calif.). The eggs containing 0- to 8-day old embryos were placed for 10 to 15 minutes at an angle of 45° (blunt end up) prior to injection. They were then swabbed with 70% ethanol and a small hole was made with a pointed probe near the midpoint of the egg. The needle was inserted

one-third to one-half of the distance into the egg's center, the same distance each time with a given batch of eggs. This permits injection into the yolk and misses the embryo, since at early ages the embryo floats on the yolk surface. At later ages, 8 to 15 days, the position of the embryo was determined by candling the eggs before injection. After 15 days, injection was made a few cm deep into the pointed end of the egg; this was the only way one could avoid piercing the embryo. After injection, the holes were sealed with a small piece of plastic tape. An effort was made to complete the injection within a few hours time and the eggs were reincubated immediately afterwards. Incubation time, as recorded in the data, did not include the period during which eggs were out of the incubator.

A 1 ml aliquot of the injection solution was diluted to 1000 ml and this solution was used as the standard in calculating specific activities and percentages of incorporation. The amount of ^{32}P added to eggs was not visibly harmful. Two eggs which received about 100 μc of ^{32}P at five days of incubation grew normally to ten days (time of harvest).

RESULTS

Separation of Lipids by Column Chromatography

Lipid extracts from yolk, whole embryo, and brain were separated on the columns as described under Methods. Figures 1 to 5 are representative examples of the separations achieved by the columns used. The isolated PL fractions were made to a concentration of 0.05 to 5 μmoles of lipid-P/ml in C:M (2:1). Assays of these solutions and results of the assays are presented in the remaining part of this section. The separations achieved were primarily on the basis of the group linked by an ester bond to P in the intact lipid; fatty acid composition was not measured. Since PL's containing more unsaturated fatty acids are eluted earlier on silicic acid (23), some degree of fractionation of the PL's by the fatty acid composition was presumably achieved when a particular PL appeared in several distinct peaks (PE is an example).

Characterization of Brain PL Fractions

The quantitative and qualitative analysis of PL fractions consisted of determining E/P and N/P ratios, identifying the acid hydrolysis products, chromatographing intact PL's by thin layer chromatography (TLC), and chromatographing products of base hydrolysis by TLC; the methods have been described in the previous section.

Figures 1 to 3

Chromatographic separation of PL from brain isolated from embryos at 159, 244, and 378 hours incubation age. In all figures showing column separations, P concentration (in relative units) is plotted against tube number. In Figure 1, cpm per tube are given on one vertical scale and the actual P concentration on the other vertical scale.

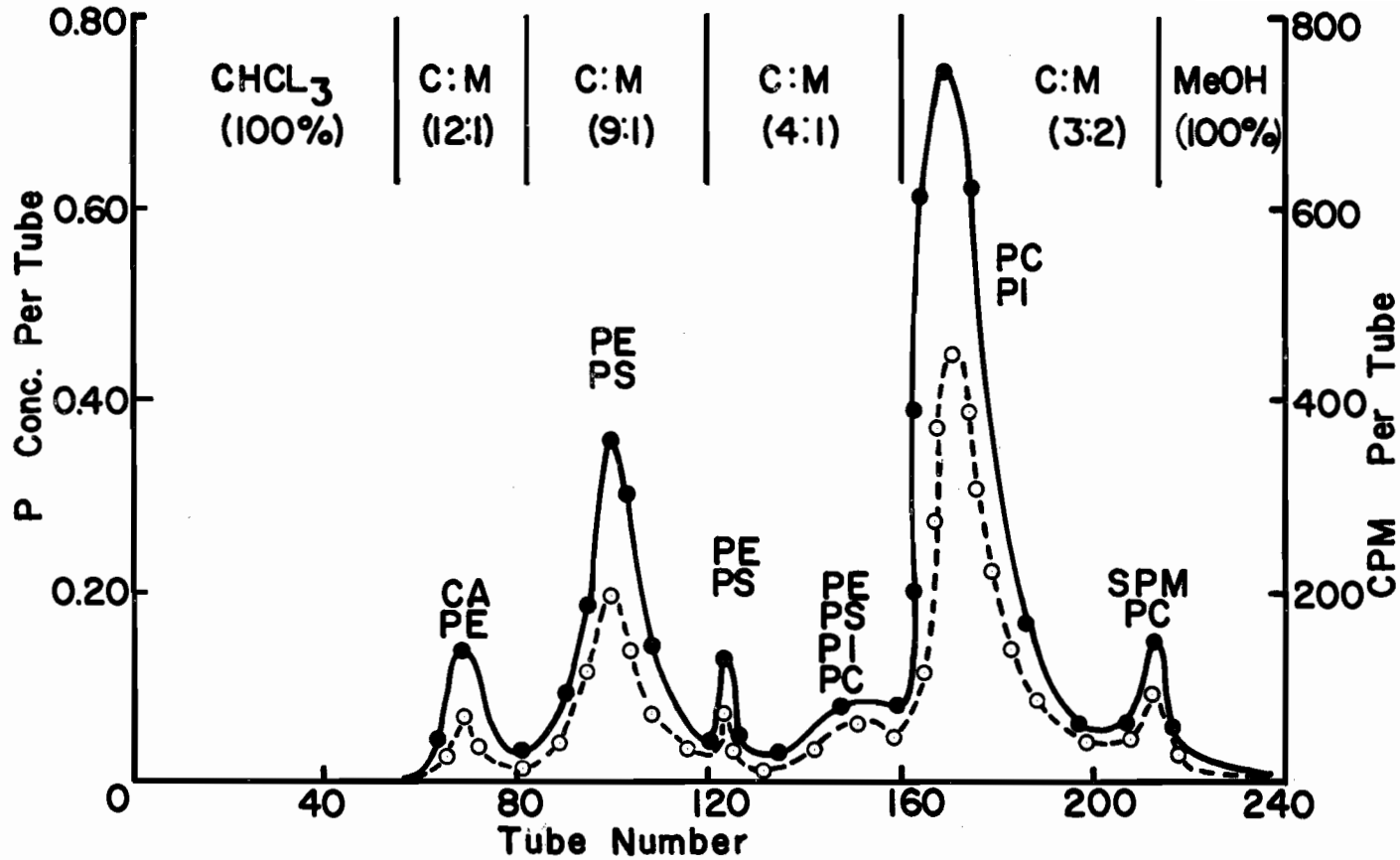


Figure 1
159-Hour Brain

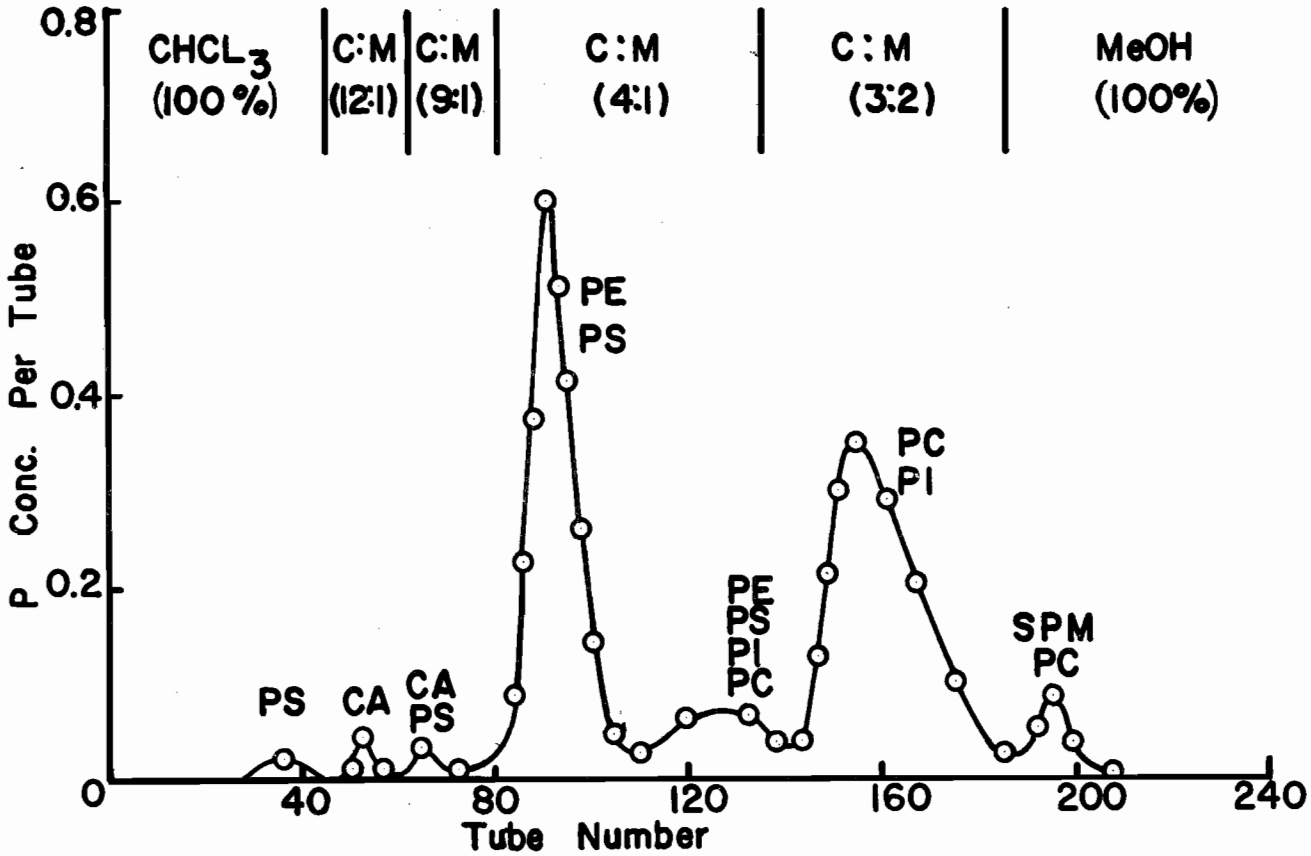


Figure 2

244-Hour Brain

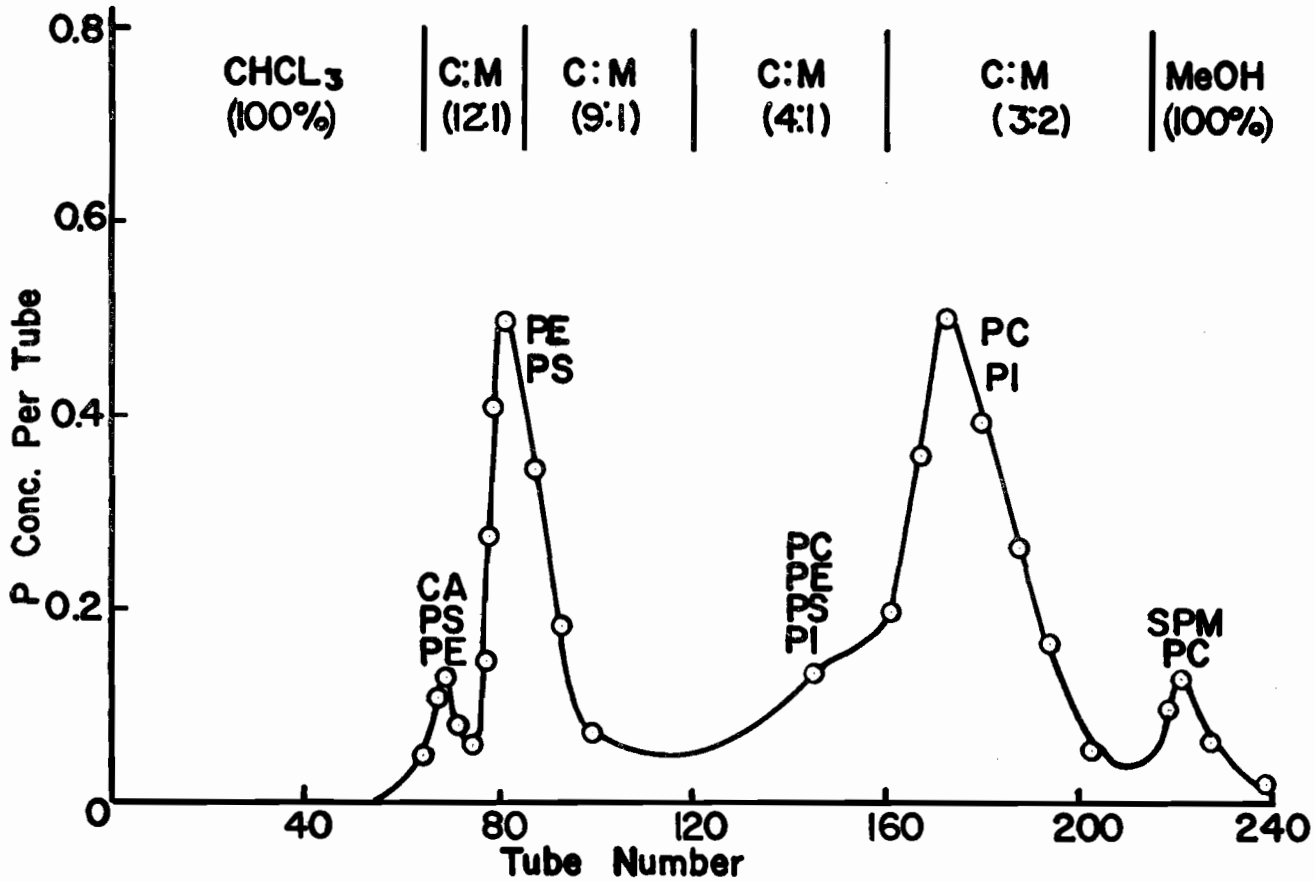


Figure 3

378-Hour Brain

Figure 4

Chromatographic separation of the C:M (3:2) peak (tubes 163-190) from silicic acid-Hyflo on DEAE-cellulose acetate. PL was from brain of chick incubated for 378 hours.

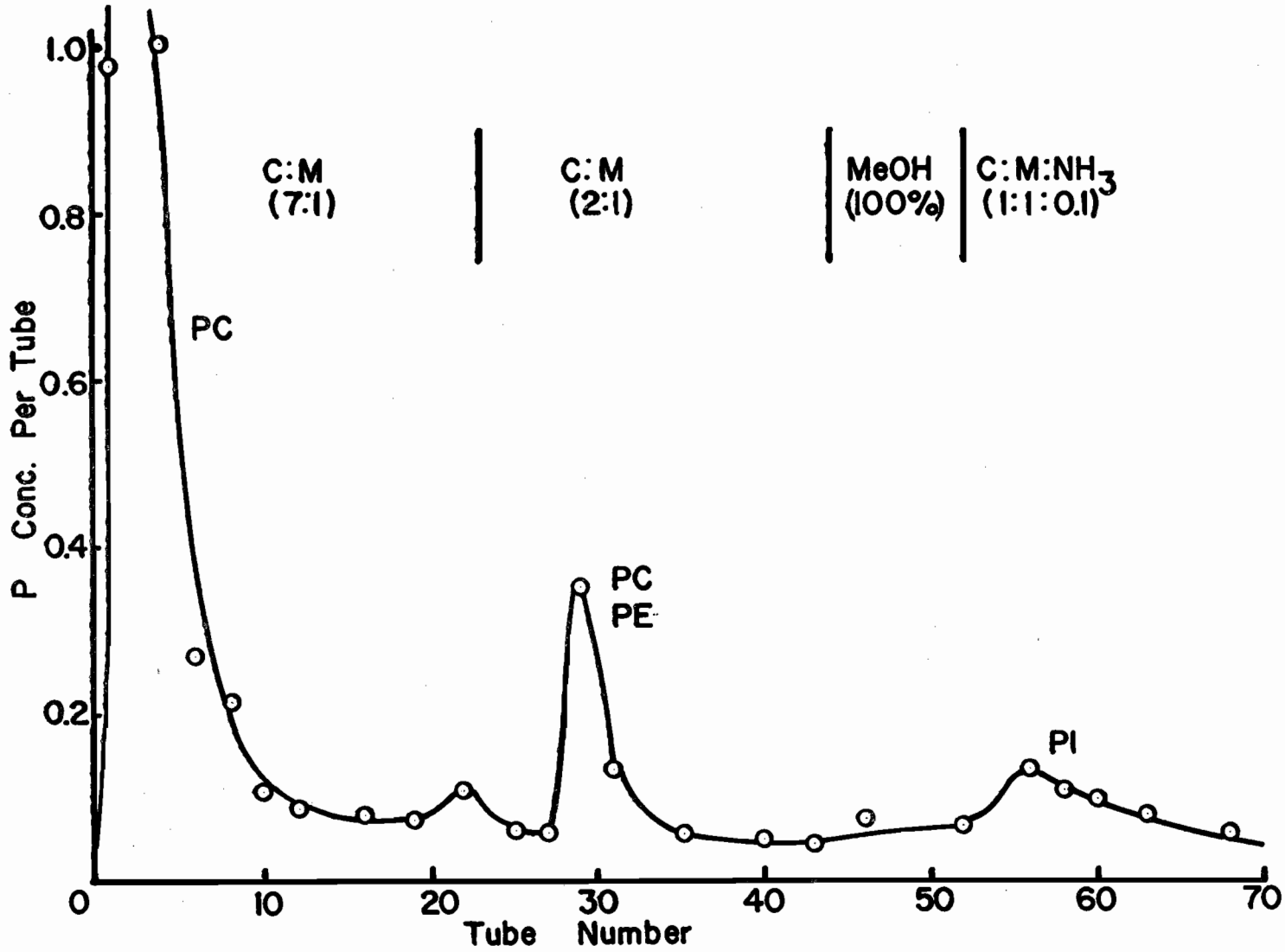

1.0
0.8
0.6
0.4
0.2
P Conc. Per Tube
C:M
(7:1)
C:M
(2:1)
MeOH
(100%)
C:M:NH3
(1:1:0.1)
PC
PC
PE
PI
0
10
20
30
40
50
60
70
Tube Number

Figure 4

Figure 5

Chromatographic separation of the C:M (9:1) peak from the silicic acid-Hyflo column on a silicic acid-NH_3 column. In this instance the PL extract was from 173-hour embryo.

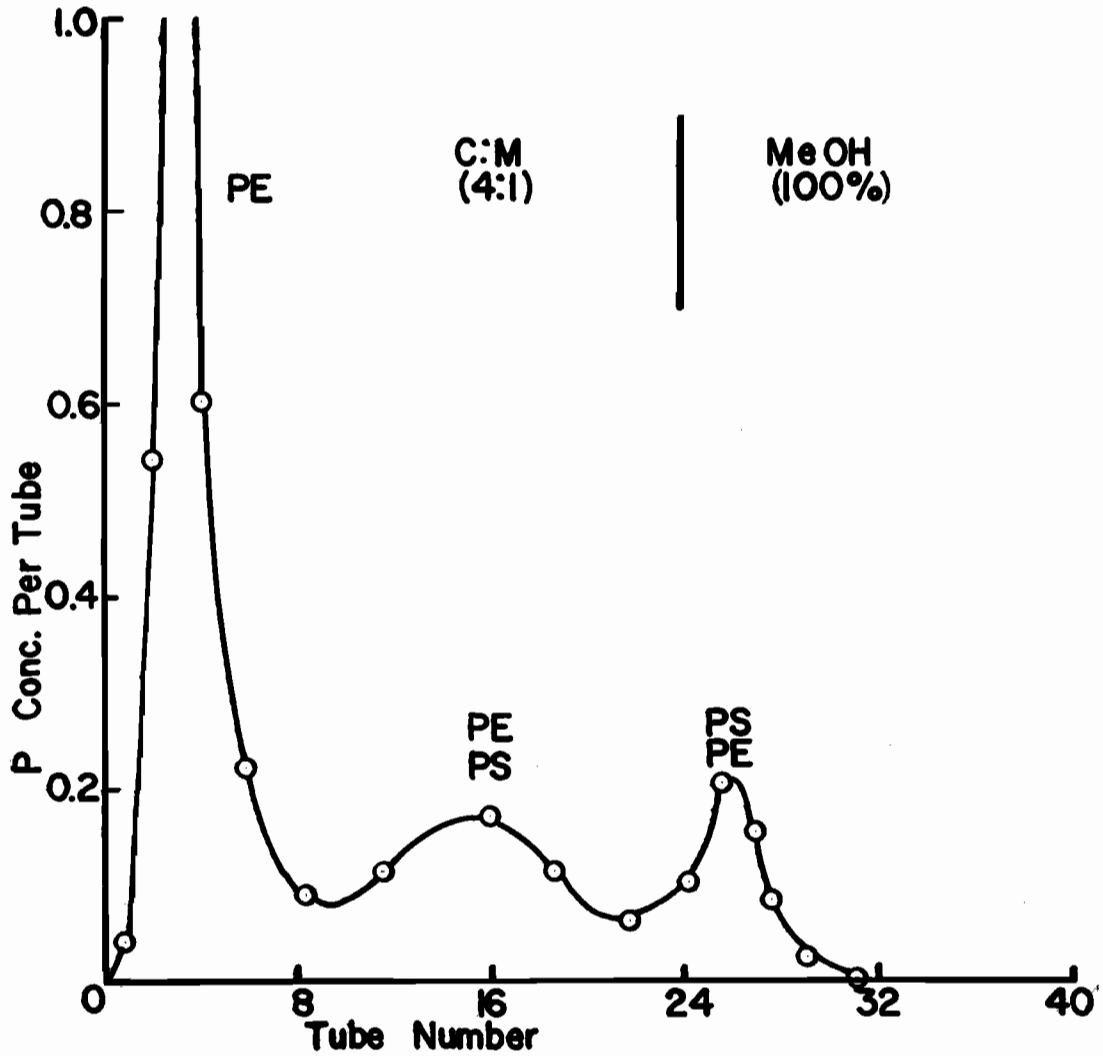

1.0
0.8
0.6
0.4
0.2
0
P Conc. Per Tube
PE
C:M
(4:1)
MeOH
(100%)
PE
PS
PS
PE
8
16
24
32
40
Tube Number

Figure 5

Acid Hydrolysis Products

Table III-B refers to brain PL fractions which were found to contain choline, serine, ethanolamine, and inositol in their acid hydrolysates. These hydrolytic products were found in the PL peaks expected, based on the experience of Bieber *et al*. (4) and Rouser *et al*. (41, 42); that is, specific PL's were eluted on chromatographic columns in the order given by these two groups.

E/P and N/P Ratios

Ester and phosphorus were measured at the same time to obtain the molar E/P and N/P ratios (considerable error would be introduced by measuring the same PL solution at two temperatures differing by 7 or 8 degrees since the thermal expansion of $CHCl_3$ is large). Representative samples of these ratios are recorded in Table III-A. E/P ratios were sometimes greater than 2.0, indicating some glyceride contamination. SPM fractions were always contaminated with PC, even when the fraction was chromatographed on several columns. The E/P ratio in SPM fractions is a measure of PC or LL contamination in SPM, as the amide bond of SPM does not react readily under the conditions of the ester assay (6).

TABLE III-A

E/P and N/P Ratios in
Typical Brain Phospholipid Fractions

Chromatographic Fraction and Age[a]		Behaves on TLC like:	E/P[c] ±5%	N/P[c] ±8%
SA-12:1, SA-15:1	288 hr.	CA	-	no N
SA-12:1, SA-15:1	396 hr.	CA	1.9	no N
SA-9:1	208 hr.	PE	1.8	1.0
SA-9:1, SA-4:1	208 hr.	PE	2.1	0.8
SA-4:1	396 hr.	PE	2.1	-
SA-3:2	288 hr.	PC	2.0	1.0
SA-3:2	208 hr.	PC	2.1	0.8
SA-3:2	240 hr.	PC	2.3	-
Commercial SPM[b]		SPM	0.3	-
SA-3:2 to MeOH	208 hr.	SPM	0.8	-
SA-3:2 to MeOH	208 hr.	SPM	1.3	1.1
SA-3:2 to MeOH	320 hr.	SPM	0.9	-
SA-3:2 to MeOH	396 hr.	SPM	0.9	-

[a] Abbreviations used above are: SA = silicic acid and hr. = hours of incubation. Designation of the fractions is by their chromatographic behavior (i.e., SA-12:1, 288 hr. means a silicic acid-Hyflo column fraction obtained when the solvent was C:M (12:1) and the source was from brain of a 288-hour old embryo; where two columns are indicated, the information in the table was obtained after the particular fraction had been chromatographed the second time).

[b] SPM fractions were contaminated with PC. The E/P ratio here is a measure of PC and LL contamination. Since polar N contaminates emerge towards the end of the column, N/P ratios were not routinely measured for SPM fractions.

[c] Average errors of these determinations are given.

TLC of Intact Phospholipids

Figures 6-10 are typical reproductions of TLC plates of lipid extracts and fractions which were obtained from the columns. One can conveniently judge the completeness of column separations from these TLC plates. Table III-B, given in seven parts, summarizes the results obtained on TLC plates by giving the fraction and its reaction with different spray reagents (see Methods on TLC). Blank spaces in the table mean the particular spray was not definative.

Figure 7 shows the monitoring of a PL fraction chromatographed on silicic acid-Hyflo; the spots are given consecutively by tube number. Neutral lipid (which moves to the solvent front on these TLC plates) is seen in Tubes 4, 8, and 74. Tubes 75-92 contain PE and a CA-like PL while the middle tubes, 124-170, contain mixtures of PE, PS, PI, and PC (PC shows strongly on Plate 3); SPM can be seen in the latter tubes (175-204).

Plate 1 of Figure 9 suggests that one PL component is present in brain (B) that is not present in embryo (E) without the brain (the fourth spot from the bottom in brain PL on Plate 1). This PL was not identified on this plate but is likely an inositide. The fraction "P2" on Plate 2 of the same figure contains PC and an unknown which has been designated "PX"; the 7:1 fraction in Figure 10 also contains this component. On most TLC plates, the PC spot all but hid PX,

making it difficult to judge the relative amounts of PC and PX. PX reacted slightly with Dragendorff reagent and ninhydrin. In the acid hydrolysate of a fraction in which PX was noted by TLC, there was one Dragendorff positive spot and no ninhydrin positive spots. These results and the mobility of PX in the systems used would suggest that PX is perhaps a dimethaminoethanol phosphatide. Another possibility is that the X in PX might be methylcholine or carnitine. Further purification and characterization of this unknown component which migrates with the PC fraction remains to be done.

In Figure 10, the fractions eluted from DEAE-cellulose in C:M (2:1) and in MeOH (the second and third columns from the left) comprised about 1.5% of total lipid-P. These spots were not identified except by their reactions with spray reagents (Table III-B, Part 6). Inositol was found in acid hydrolysates of the C:M:NH_3 fraction.

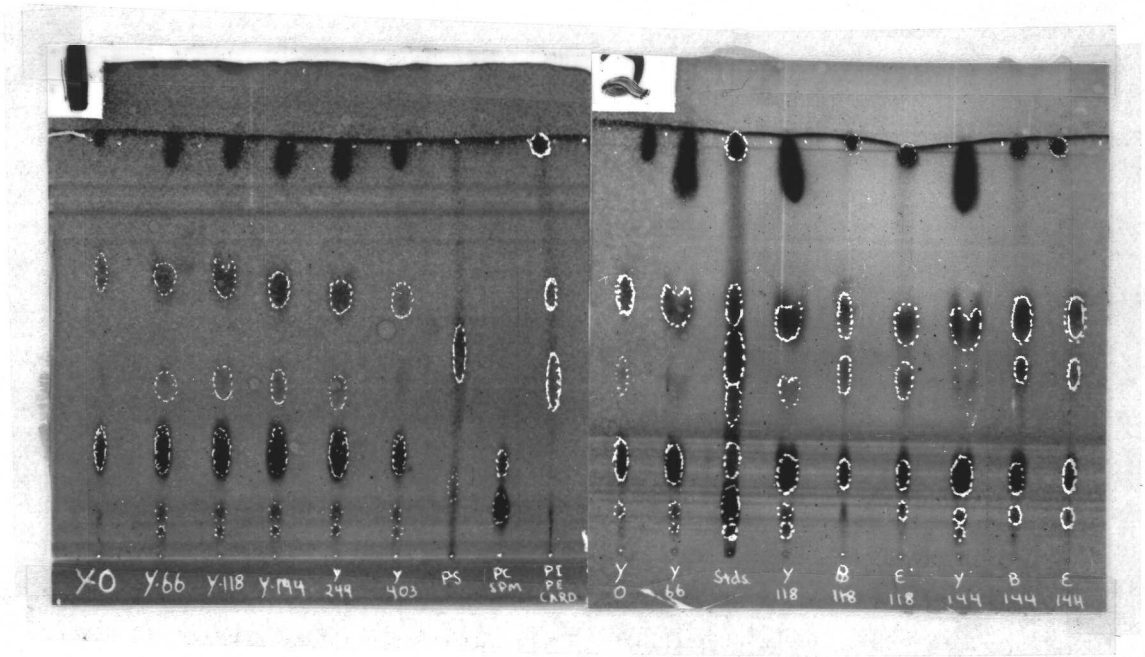
Sphingomyelin

Since the molar percent of lipid-P that was found to be brain SPM by column chromatography was less than that of the whole embryo (4) or from that reported for other brain tissue (35, p. 644), investigations were conducted to check this result. A lipid extract from seven-day (170 hours) embryo brain was chromatographed on silicic acid-Hyflo. The last apparent peak and all the P beyond this peak was pooled and this combined fraction was chromatographed on paper

Figure 6

TLC plates of PL fractions from yolk (Y), brain (B), and whole embryo (E) at 0 hours, 66 hours, 118 hours, 144 hours, 244 hours, 308 hours, and 403 hours incubation (fractions are labeled on the plates). Silica gel H was the adsorbent and the plates were developed with C:M:acetic acid: water (50:25:7:3) according to Skipski (45). The plates were sprayed first with Zinzadse reagent (to detect PL), then with chromic acid (to detect all organic components). PL spots are denoted by the dotted circle. From these plates it is evident that the CA-like PL is not present in yolk, and PI and PE are present in smaller quantities than in the brain or whole embryo. LL could be detected with Zinzadse reagent only in yolk, although faint spots sometimes appeared with chromic acid reagent in brain and embryo (see Plate 3, B-244 and E-244).

Figure 6


1
Y-O
Y-66
Y-118
Y-144
Y 244
Y 403
PS
PC SPM
PI PE CARD
2
Y O
Y 66
Stds
Y 118
B 118
E 118
Y 144
B 144
E 144

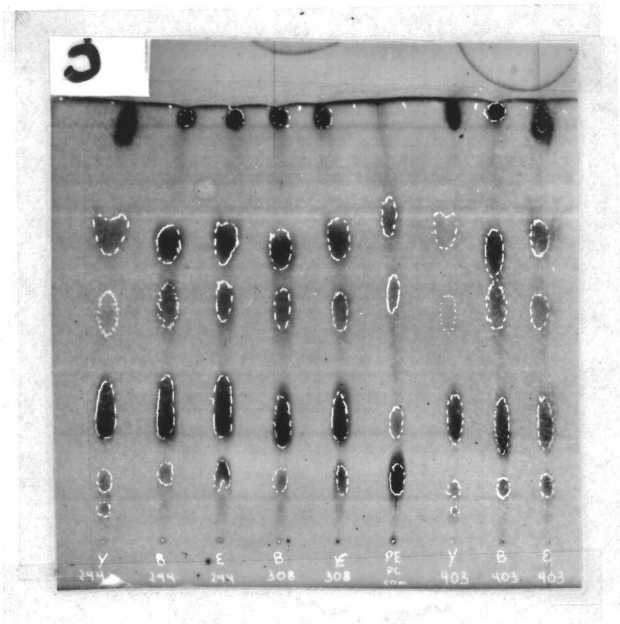

3
Y 244
B 244
E 244
B 308
E 308
PE PC
Y 403
B 403
E 403

Figure 7

TLC plates of a PL extract from a 288-hour chick brain. The numbers refer to tube fractions from a silicic acid-Hyflo column. Silica gel G was the adsorbent and chromatograms were developed with C:M:NH_3 (60:35:5) and sprayed as in Figure 6. Starting from the top of the plates, the spots are respectively: neutral lipid and CA-like lipid (dotted circles indicate PL), PE, PS, and PI (PS and PI move closely together), PC, SPM, and LL (seen faintly in Tube 204).

Figure 7

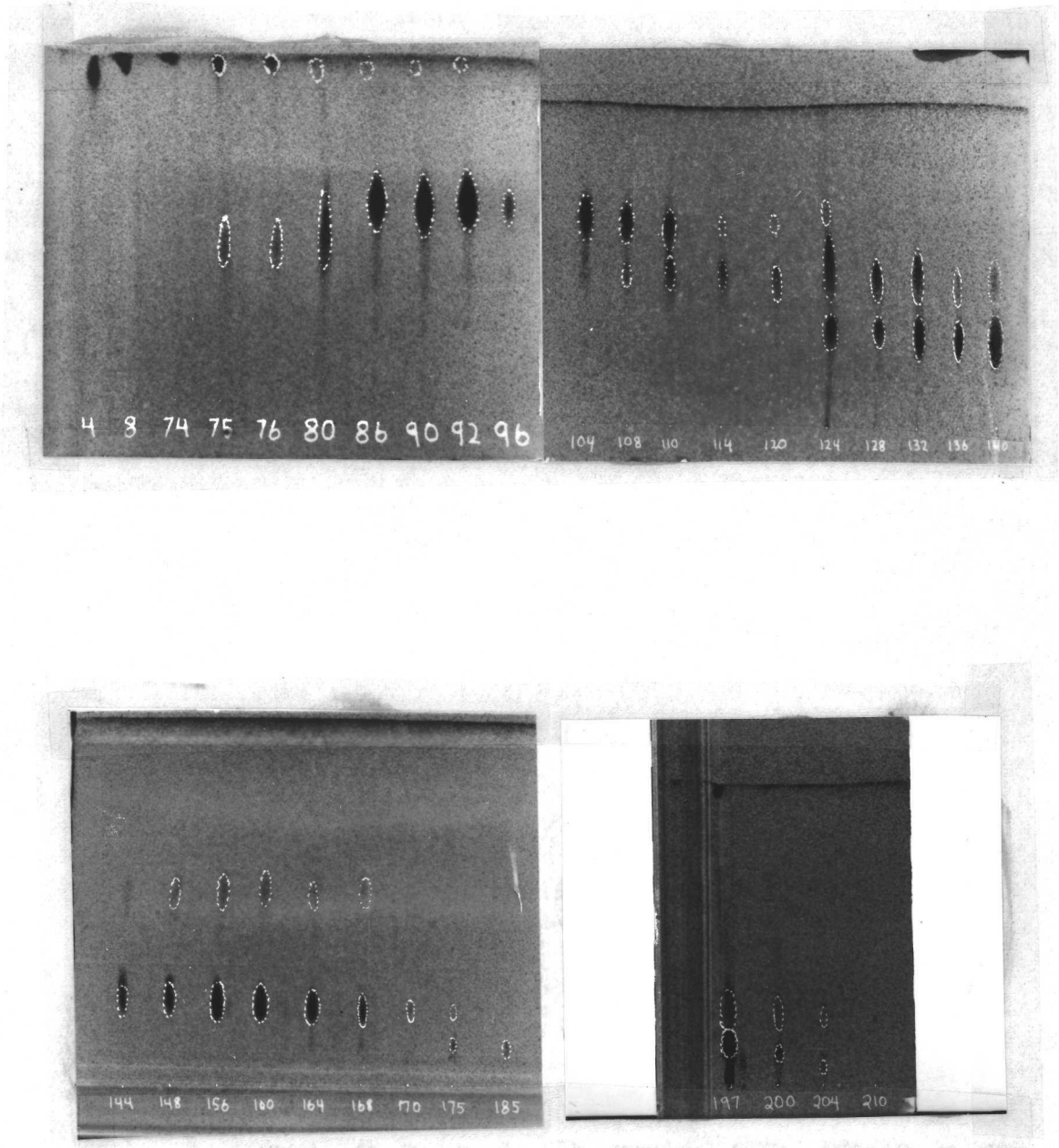

4 8 74 75 76 80 86 90 92 96
104 108 110 114 120 124 128 132 136 140
144 148 156 160 164 168 170 175 185
197 200 204 210

Figure 8

PL fractions from 118-, 144-, and 308-hour chick brain on Plates 1, 2, and 3, respectively. PL's are designated by the dotted circle. "P" denotes a peak on the silicic acid-Hyflo column, the number being the number of the peak. Silica gel G was the adsorbent and the plates were developed with C:M:NH_3:H_2O (75:25:1:3.8). The lefthand column on Plate 1 is a PL extract from 118-hour brain and the right-hand column is a 118-hour PL extract from whole embryo, showing SPM, PC, PI, PE, and CA-like PL, reading from bottom to top. PS moves with PC in this system.

Figure 8

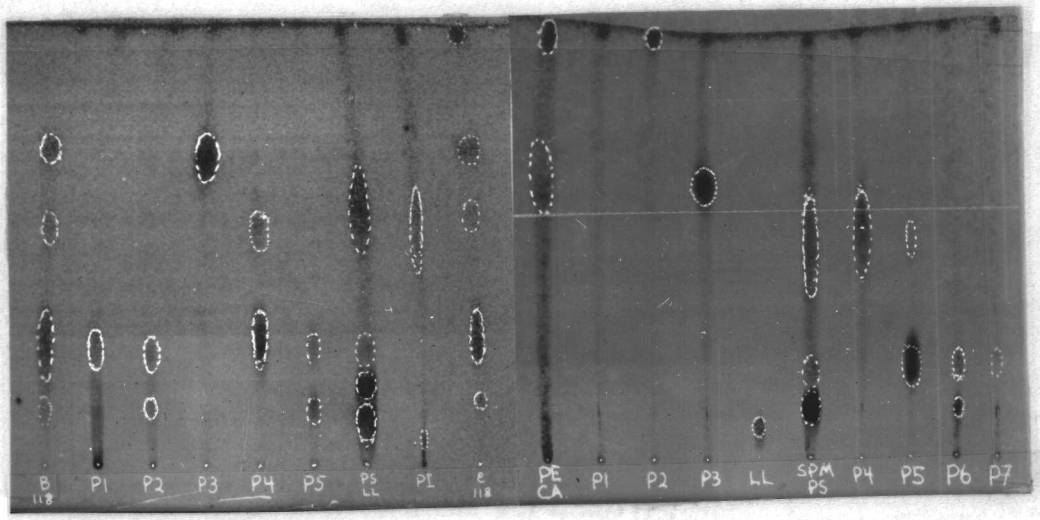


Plate 1 Plate 2

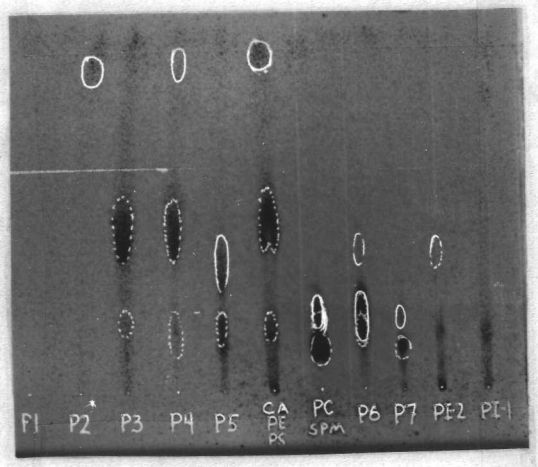


Plate 3

Figure 9

Plate 1: TLC of a PL extract from a 15-day embryo (E) minus the brain and a 15-day brain (B). Standards are also shown. Plate 2: Chromatographed peaks (P) from silicic acid columns (the number after the peak is peak number). S = a second silicic acid column, Z = positive Zinzadse test, N = positive ninhydrin test, and D = positive Dragendorff test. The lefthand spot is an inositide fraction which was eluted in C:M:NH_3 (1:1:0.1) from DEAE-cellulose. The PE standard (righthand column) also shows some PS.

Figure 9

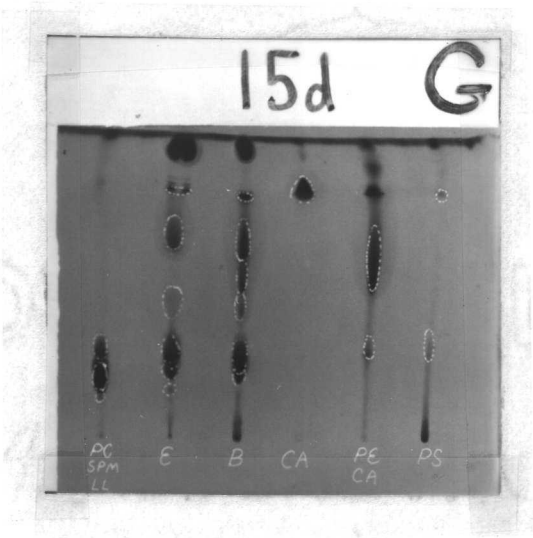


Plate 1

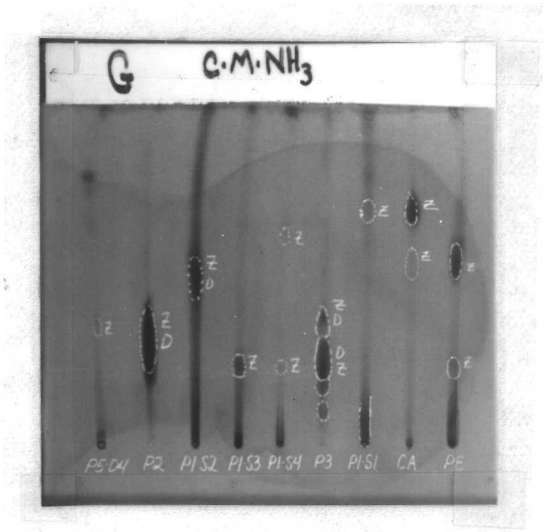


Plate 2

Figure 10

TLC of fractions chromatographed initially on silicic acid-Hyflo and then on DEAE-cellulose or silicic acid-NH_3. From left to right the fractions are designated by the eluate (C:M) in which they emerged from the DEAE column. The 2/1 and M (for MeOH) fractions represented about 1.5% of total lipid-P. Inositides were generally eluted in C:M:NH_3 (fourth column). The fifth and sixth columns are the first and second peaks, respectively, emerging from a silicic acid-NH_3 column. The seventh column is the MeOH:conc. HCl (95:5) eluate from DEAE; it contained no phosphatides. The last column was not sprayed with chromic acid.

Figure 10

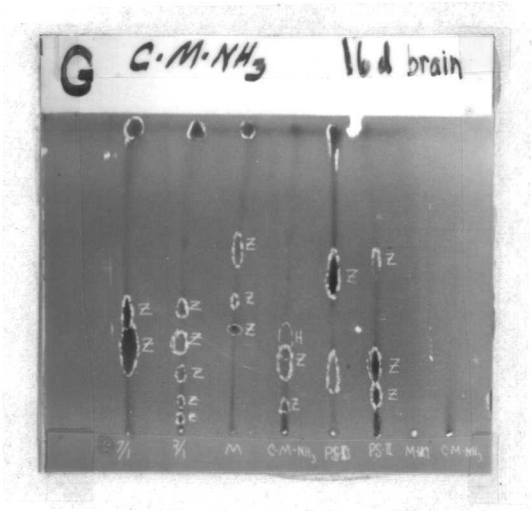


TABLE III-B

Identification of Brain PL Fractions
by TLC and Chemical Analysis

Part 1
Fractions which Migrate on TLC Plates Like CA

Fraction[a] and Hours of Incubation		Test Rgt. on TLC Plates				Other Assays
		Z	N	D	Ag	
SA-9:1	118	+	-	-		
SA-12:1, R-4:1	159	+	-	-	-	No amines in acid hydrolysate
SA-12:1, SA-15:1	288	+	-	-		
SA-12:1	396	+	-	-	-	No amines in acid hydrolysate
SA-12:1, SA-15:1	396	+	-	-		E/P was 1.9

[a] Abbreviations used in designating fractions are: SA = silicic acid-Hyflo column, DEAE = DEAE-cellulose acetate column, SS = silicic acid-silicate water column, R = silicic acid-NH_3 column, Z = Zinzadse reagent, N = ninhydrin reagent, D = Dragendorff reagent, and Ag = silver ion for cis OH groups. The fractions are designated by type of column in order of use and eluting solvent in C:M.

TABLE III-B

Part 2
Fractions which Migrate on TLC Plates Like PS

Fraction[a] and Hours of Incubation		Test Rgt. on TLC Plates				Other Assays
		Z	N	D	Ag	
SA-4:1 to 3:2	144	+	+	-		Serine in acid hydrolysate
SA-9:1, R-MeOH	159	+	+	-		
SA-12:1, R-4:1 to MeOH	288	+	+	-	faint	
SA-4:1 to 3:2	320	+	+	-		
SA-12:1, R-MeOH	396	+	+	-	faint	Serine in acid hydrolysate

[a] Fractions are designated as on the previous page.

TABLE III-B

Part 3
Fractions which Migrate on TLC Plates Like PE

Fraction[a] and Hours of Incubation		Test Rgt. on TLC Plates				Other Assays
		Z	N	D	Ag	
SA-12:1, R-4:1 to MeOH	159	+	+			Ethanolamine in acid hydrolysate
SA-9:1, R-4:1	159	+	+			Ethanolamine in acid hydrolysate
SA-4:1, DEAE-7:1	159	+	+	-		
DEAE-7:1, SS-4:1	210	+	+	-		Ethanolamine in acid hydrolysate
DEAE-2:1	210	+	+	-		
SA-12:1, SA-9:1	288	+	+			E/P was 2.0, N/P was 1.0
SA-4:1	320	+	+			
SA-12:1, SA-9:1	396	+	+	-	-	Ethanolamine in acid hydrolysate

[a] Fractions are designated as on the previous page.

TABLE III-B

Part 4
Fractions which Migrate on TLC Plates Like PC

Fraction[a] and Hours of Incubation		Test Rgt. on TLC Plates				Other Assays
		Z	N	D	Ag	
SA-3:2	144	+		+		Choline in acid hydrolysate
DEAE-7:1, SS-1.5% H_2O in C:M (4:1)	210	+	faint	+		
SA-3:2 to MeOH	244	+		+		E/P was 2.3
SA-3:2, DEAE-7:1	288	+		+		
SA-4:1, DEAE-7:1	360	+		+		
SA-4:1 to 3:2	396	+	-	+	-	Choline in acid hydrolysate

[a] Fractions are designated as on the previous page.

TABLE III-B

Part 5
Fractions which Migrate on TLC Plates Like SPM

Fraction[a] and Hours of Incubation		Test Rgt. on TLC Plates				Other Assays
		Z	N	D	Ag	
SA-3:2 to MeOH	118	+		+		Choline in acid hydrolysate
SA-MeOH	144	+		+		
DEAE-7:1, SS-2% H_2O in C:M (4:1)	159	+		+		
SA-MeOH	320	+		+		E/P was 1.2
SA-3:2 to MeOH, SA-1:1	396	+		+	-	E/P was 0.9

[a] Fractions are designated as on the previous page.

TABLE III-B

Part 6
Fractions which Migrate on TLC Plates Like PI

Fraction[a] and Hours of Incubation		Test Rgt. on TLC Plates				Other Assays
		Z	N	D	Ag	
SA-3:2, DEAE-($C:M:NH_3$)	118	+	-	-		No amines in acid hydrolysate
SA-4:1 to 3:2	144	+	-	-	+	
SA-3:2, DEAE-($C:M:NH_3$)	159	+	-			No amines in acid hydrolysate
SA-4:1 to 3:2	244	+	-	-	+	
SA-3:2 to MeOH, DEAE-($C:M:NH_3$); 2 spots[b]	288	+	-			
SA-3:2	320	+	-	-	+	
DEAE-($C:M:NH_3$)	396	+	-	-	-	Inositol test positive

[a] Fractions are designated as on the previous page.

[b] See Figure 10.

TABLE III-B

Part 7
Fractions which Migrate on TLC Plates
Unlike Any Standard PL

Fraction[a] and Hours of Incubation		Test Rgt. on TLC Plates				Other Assays
		Z	N	D	Ag	
SA-3:2, DEAE-2:1[b]	118	+				Migrates just above PC
SA-4:1, DEAE-7:1[b]	288	+	faint	-	-	
DEAE-7:1[b]	396	+	-	faint	-	No amino acid base
SA-3:2, DEAE-7:1	396	+	faint			
DEAE-2:1[c]	396	+	+	-		Migrates just below PC
DEAE-2:1[c]	396	+	+	-		
DEAE-2:1[c]	396	+	-	-		
DEAE-MeOH (R_f like PE)	396	+	-	-	-	

[a] Fractions are designated as on the previous page.

[b] This component, PX, moved with part of the PC spot on TLC plates and was difficult to estimate (refer to text for discussion).

[c] Several Z positive spots appeared in the DEAE-2:1, MeOH and C:M:NH_3 fractions (see Figure 10) which were not identified except for the fact that ethanolamine, serine, and one other ninhydrin positive substance were present in the acid hydrolysates of two of these fractions, C:M (2:1) and MeOH; these two fractions were about 1-2% of total lipid-P.

according to the method of Kennedy and Collier (29). The major component of this fraction migrated like authentic SPM, thus substantiating the TLC results. E/P ratios in the SPM fraction (which as previously noted was contaminated with PC) from three different experiments were 0.8, 0.9, and 1.3 (Table III-A). The N/P ratio is not useful in this case since trace N-containing compounds tend to emerge from the silicic acid-Hyflo column in more polar eluates and such contaminates were noted in several instances on TLC plates and on paper chromatograms.

On one separation of a 16-day brain extract, the E/P ratio in the SPM fraction was 0.88. Calculating on the basis that all ester bonds are from PC, this would mean that this particular fraction was 57±5% SPM. An aliquot of this fraction was saponified by the method of Garbus (21), and the unsaponifiable material was extracted several times with diethyl ether. The ether layer contained only one P compound, SPM, as seen on Plate 2 of Figure 11. Analysis of this ether layer for P showed that SPM content in the original fraction was 53±2%. Thus, the two methods of analysis were in agreement within experimental error.

CA-like Lipid

The PL fraction eluted first on silicic acid-Hyflo columns that appeared in both brain and whole embryo minus brain (Figure 9, Plate 1) was partially characterized from

Figure 11

TLC plates of a fraction containing SPM. Plate 1: A C:M (3:2 to MeOH) fraction from silicic acid-Hyflo which was chromatographed on a second silicic acid column. The left-hand column shows the presence of N impurities in this fraction. Plate 2: The same fraction after base hydrolysis (hydrolysis was performed on three identical samples); base hydrolysis saponifies PC but SPM is resistant to mild hydrolysis (as described in the text).

Figure 11

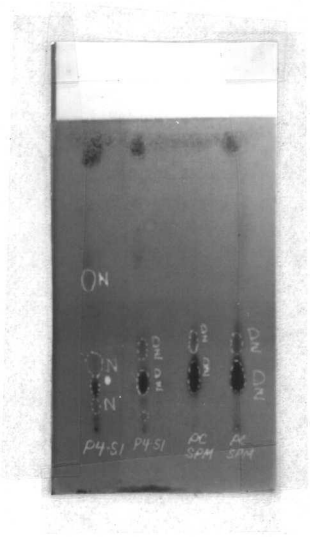


Plate 1

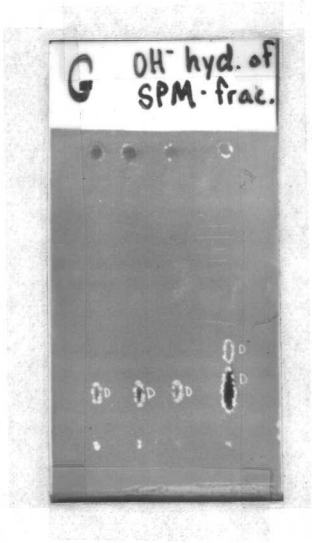


Plate 2

brain (see Table III-A and Table III-B, Part 1). For this purpose, 120 brains from 288-hour old chick embryos were collected. After extracting and chromatographing on silicic acid in the usual manner, the tubes containing the front-running PL peak were pooled. Analysis on TLC plates indicated the presence of PI, PS, and a component which migrated like CA (N/P = 0.9). This CA-like material was ninhydrin negative. The whole peak was re-chromatographed on silicic acid-Hyflo (6 g to 3 g) by elution with 200 ml of $CHCl_3$, 100 ml of C:M (15:1), 100 ml of C:M (9:1), and 200 ml of C:M (4:1). The first PL fraction emerged in C:M (15:1) and separated on TLC plates like commercial CA; 4 μmoles of lipid-P were recovered from the pooled tubes. PE and PS were eluted with C:M (9:1). Figure 12, Plates 1 and 2, show that the chromatographic behavior of this lipid is like that of authentic CA. After base hydrolysis the CA-like substance did not chromatograph with α-glycerolphosphate, the product which would be expected from base hydrolysis of PA. Therefore, this CA-like component is tentatively identified as CA.

Phospholipid Composition in the Incubated Egg

Quantitative changes in the relative amounts of major PL's during development of the chick embryo have been reported by Kugler *et al.* (30) and more recently by Bieber *et al.* (4). The results by Bieber *et al.* with whole embryos

Figure 12

TLC of CA-like PL from chick embryo brain. Plate 1: Comparison of unknown (Pl-Sl-12d) with authentic CA, PA, and PE on silica gel H. Plate 2: Comparison of unknown with authentic CA, PA, and PE on cilica gel H. In the third column the unknown and CA were co-chromatographed. Plate 3: TLC of base hydrolyzed samples. Pl-Sl-12d is the CA-like sample, Z = Zinzadse positive, N = ninhydrin positive, and α-Gly-P = authentic α-glycerolphosphate.

Figure 12

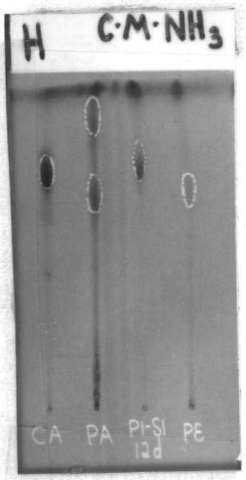


Plate 1

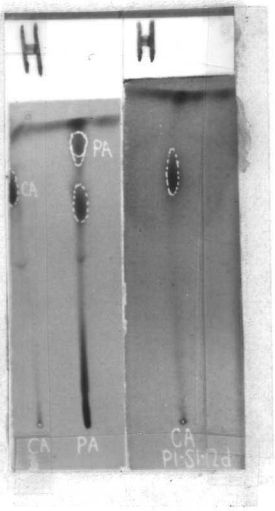
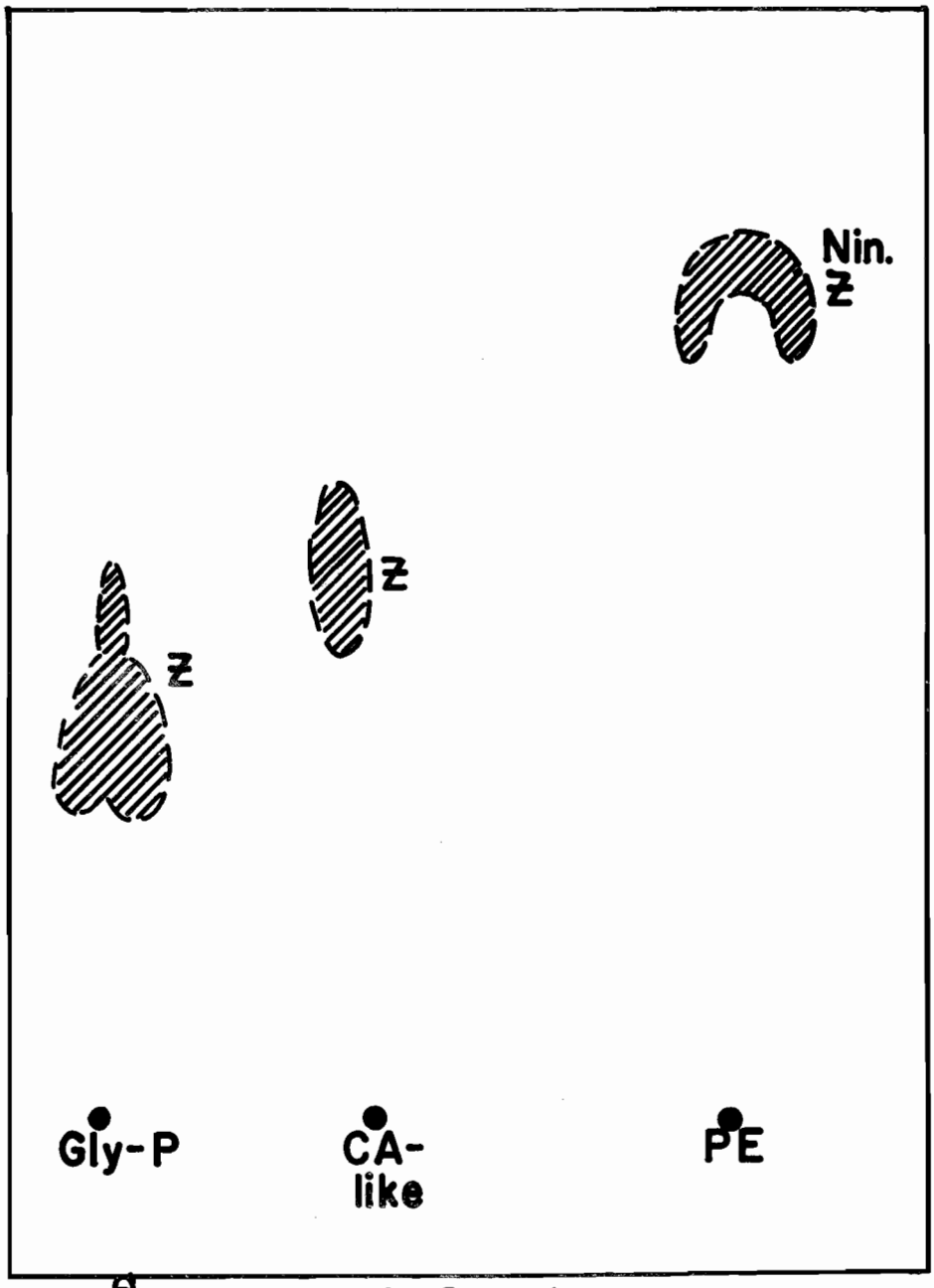


Plate 2

Figure 12



$OH^{\ominus}$ Hydrolyzed Samples

Plate 3

are compared to the present results (determined by P analysis in the pooled, separated peaks) for brain in Table III-C. The results shown in Table III-C indicate that brain SPM increases less than SPM in whole embryos. In Table III-D are given the complete PL analysis of embryo brain of different ages; the results in this table are considered to be semi-quantitative. The values of PE plus PS are probably within 3% of their true value. The actual ratio of PE to PS was only estimated, since PS was not determined by serine analysis in the presence of ethanolamine, but rather calculated from the amount of lipid-P that was present in the second and third peaks of the silicic acid-NH_3 column and by estimating the amount of ethanolamine in the acid hydrolysate by ninhydrin intensity. Although such peaks chromatographed on TL plates like PS, it was shown that oxidized PE was present to some extent in this fraction. It was evident that PS was less than 8% of total lipid-P in the period studied. PI was estimated by the amount of inositol positive lipid-P that was eluted by C:M:NH_3 (1:1:0.1) on DEAE-cellulose. Several Zinzadse positive and some Zinzadse and ninhydrin positive components comprising about 1.5% of the total lipid-P appeared in the C:M (2:1) and MeOH eluates from DEAE-cellulose columns (see Figure 10). These minor PL's were not further identified.

The unidentified PL designated PX was detected in some brain fractions (see previous discussion on PX). This

TABLE III-C

Phospholipid Composition in Whole Embryo and Embryo Brain during Development

Hours of Incubation	Phospholipid and Tissue:					
	PC-brain	PC-emb.	PE-brain	PE-emb.	SPM-brain	SPM-emb.
110	70	58	26	24	6	6.2
153	70	55	23	28	3	6.6
300	58	54	24	23	3	10
350	59	51	27	23	3	11
445	55	49	24	18	3	13

TABLE III-D

Phospholipid Composition in Brain during Development[a]

Age in Hours	Percent of Individual Phospholipid:						Total
	CA-like	PE	PS	PC[b]	PI	SPM	
118	-	26	-	70	3	6	105
144	1.1	25	1.5	56(?)	-	4	87
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	28	6	49	2	3	92
427	-	24	3	55	-	3	85

[a] $\frac{\text{Lipid-P in fraction}}{\text{Total lipid-P recovered}} \times 100$. The blank spaces mean that the individual PL was not accounted for quantitatively.

[b] The values for PC include an unknown amount of a minor PL which has been designated PX (see text for details). The 144-hour PC value appears to be low.

particular component, as seen in Figure 10, moved just ahead of PC and was always mixed with it. For this reason the percent PX was difficult to judge. In Table III-D, the PC column represents PC plus an unknown quantity of PX, probably about 5% at most.

Table III-E gives the μmoles of PL in brain and gives this data on the basis of DNA. The mgs of DNA per brain for chick embryo have been reported by Baker and Newburgh (2). Total phospholipid in the C:M (2:1) extract from brain is represented here and the data are as expected for nerve cells.

TABLE III-E

Phospholipid Increase in Brain during Incubation Development

Age in Days	μMoles of PL / brain	μMoles of PL / mg of DNA
4	0.14	5.8
7	1.62	7.8
11	4.90	15.2
14	9.10	15.1
15	11.3	17.1
18	18.8	21.0
1 day hatch	ca. 24	ca. 16

Incorporation of Added Radioactivity into Tissue of Chick Embryo

Injection of $^{32}P_i$ after 43 Hours Incubation

These experiments were conducted by incubating fertile eggs for 43 hours, then injecting 50 μL of a carrier free solution of $^{32}P_i$ into the egg yolk as described under Methods. At various subsequent times of incubation, PL was extracted from homogenized yolk, whole embryo, and brain and analyzed. Specific activities of individual PL fractions and the specific activity of the total P and P_i in the aqueous fraction were determined. Specific activity is defined in equation 1.

(Eq. 1) Sp. Act. =

$$100 \frac{\text{cpm of phosphorus fraction}}{\text{cpm of injected soln.}/1000(\mu\text{mole P in fraction})}$$

The cpm in the PL fractions and in an aliquot of the solution injected (diluted 1000 fold before counting) were measured on the same day so that corrections for ^{32}P decay were not required. Statistical analysis of the counting data from standard solutions and samples gave an average deviation of ±4%. In ten identically prepared ^{32}P solutions, the standard deviation between samples (±1.5%) was greater than the standard deviation between repeated countings of the same sample which means that the pipeting error was greater than the counting error. After these ten standards were

allowed to stand for three weeks at room temperature, the standard deviation between samples was ±10%; Bray's solution is affected by fluorescent light. Two or three identical samples were prepared for counting of each fraction; thus specific activities reported in the tables which follow, accounting for average counting errors and P determination errors, would be expected to fall within 5% of the most accurate value which could be determined for each sample.

Tables III-F to III-H give an adequate representation of the data from which interpretations and conclusions are drawn. The fractions in these tables were chromatographed on two columns (see Methods), but are designated by their position of elution from silicic acid-Hyflo (the first column).

The relation of changes in specific activity of phosphorus in yolk, whole embryo, and brain was determined in three separate batches of eggs (injected at 43 hours incubation) covering the interval from about 3 to 17 days of incubation. The specific activity of brain PL fractions are given in Table III-I. The data from three different experiments is included, thus giving one an idea of the "biological error," i.e., the variation of specific activity in different lots of eggs. Stearman (47) has made a study of the use of radioisotopes in chick embryo experiments, and his conclusion is that the variation in radiochemical data depends largely on biological variation (thus the biological

TABLE III-F

Specific Activity of Chromatographed PL Fractions from One Experiment[a]

Age of Embryo	Phospholipid Fraction:[b]			
	PE-1 C:M (12:1)	PE-2 C:M (9:1)	PE-3 C:M (4:1)	PE-4 C:M (3:2)
209 (hr)	5.8	5.8	6.4	3.3
259	4.7	5.1	4.6	-
309	2.7	3.0	4.0	-
386	1.4	1.2	-	-
451	0.8	0.8	0.3	1.2

[a] One experiment means one batch of eggs which were injected at the same time and analyzed after varying times of incubation.

[b] The designation of fraction is by peak number on silicic acid-Hyflo columns; the eluting solvent for the peak is given. The specific activities were measured after rechromatography of the peaks on silicic acid-NH_3 columns.

TABLE III-G

Specific Activities of Chromatographed PL Fractions from One Experiment

Age of Embryo	Phospholipid Fraction:[a]			
	PS-1 C:M (12:1)	PS-2 C:M (9:1)	PI-1 C:M (4:1)	PI-2 C:M (3:2)
209	5.9[b]	6.1	6.6	6.0
259	4.9	2.2[b]	4.2	-
309	2.9	1.6	-	2.8
386	1.0	-	-	-
451	0.9	0.6	0.4	3.6 (?)

[a] See note under Table III-F for explanation of abbreviations.

[b] Contaminated with PE.

TABLE III-H

Specific Activities of Chromatographed PL Fractions from One Experiment

Age of Embryo	Phospholipid Fraction:[a]		
	PC-1 C:M (4:1)	PC-2 C:M (3:2)	SPM-1[b] MeOH
209	5.0	5.1	4.0
259	3.9	-	3.0
309	-	2.0	1.2
386	0.8	-	0.6
451	0.3	0.8	0.7

[a] See Table III-F for abbreviations used here.

[b] The SPM peaks shown in this table were not re-chromatographed.

TABLE III-I

Incorporation of $^{32}P_i$ into Brain Phospholipid

		Specific Activities of Comparable Phospholipid Fractions:										
Eluate (C:M) →		1:0	12:1		9:1		4:1			3:2		MeOH
Phospholipid →		CA	CA	PE	PE	PS	PE	PI	PC	PI	PC	SPM[a]
Age	Exp. No.											
118	1						45	41			39	35
144	1	15[b]	64				49	53			42	44
244	1	11[b]	23				26	23			24	21
308	1		20		16[c]		16[c]	14			13[d]	15
403	1										8[e]	
159	2		57	47	44	48	55[c]	39[c]		40	36	32
378	2		7.3	9.2	10			8.1[c]	8.0	8.7	8.0	7.9
208	3			36				7	24			30
427	3		9.2[f]	8.2				7.9	6.3		8.5	7.6

[a] SPM nearly always was contaminated with 5-30% PC even after chromatography on two columns.
[b] Amount of lipid too small to identify.
[c] Contaminated with PS.
[d] Contaminated with PI.
[e] An estimate based on the specific activity of the whole extract (unfractionated).
[f] Contaminated with PE.

error overshadows the counting errors in data reported here). The measured specific activity of yolk PL was about 0.0001 of embryo PL except towards the latter part of the incubation when the yolk specific activity rose perceptibly to about 0.002 of the embryo specific activity. Hevesey et al. (25) noted a similar trend.

Table III-J illustrates the finding that lecithin (PC) and SPM at all ages had lower specific activities than cephalin (PE and PS combined; PS and PE usually had similar specific activities). Table III-K gives the specific activities of various P sources within the incubated egg. In Table III-L, yolk P_i is given the value of 1 at each age and specific activities of other P fractions are expressed relative to this value. The total incorporation of ^{32}P into embryo and brain is given in Table III-M.

Specific activities of P_i (isolated during the extraction of PL) is shown for yolk, whole embryo, and brain in Figure 13. Figure 14 shows the specific activity decline for the PL extract from brain and whole embryo minus brain. If one plotted the same information for individual PL's, the relation of the brain curve to the embryo curve would be very similar and the interpretation of data would be qualitatively the same for individual PL's as for the whole extract.

The net molar increase of PL per embryo and total incorporation of ^{32}P per embryo have been plotted with time

TABLE III-J

Ratios of Specific Activities of PL Fractions from Whole Embryos as a Function of Age

Age in Hours of Embryos	Specific Activity Ratios:		
	Lec/Ceph[a]	Inositide/Ceph	SPM/Ceph
110	0.9	-	-
153	0.9	1.0	0.8
209	0.8	1.1	0.7
210	0.7	-	0.5
259	0.9	0.9	0.6
309	0.7	1.0	0.4
451	1.0	-	0.8

[a] Lec/Ceph means the specific activity of a lecithin fraction divided by the specific activity of a cephalin fraction, the same fraction being used at each age; for example, lec. was the PC eluted in C:M (3:2) and chromatographed on DEAE while ceph. was PE eluted in C:M (12:1). "Cephalin" includes PE and PS. "Lecithin" denotes PC.

TABLE III-K

Specific Activity Ratios of P Fractions in Chick Embryos

Age in Hours	$\frac{\text{PL Brain}}{\text{PL Embryo}}$	$\frac{P_i \text{ Brain}}{P_i \text{ Embryo}}$	$\frac{\text{PL Brain}}{P_i \text{ Brain}}$	$\frac{\text{PL Embryo}}{P_i \text{ Embryo}}$
118	0.51	-	1.4	-
144	1.1	1.0	1.1	0.94
159	1.3	1.1	1.5	-
208	1.4	0.87	1.5	0.96
244	1.3	0.89	1.5	1.1
308	1.5	1.5	1.9	1.8
403	2.1	1.8	1.6	1.5
427	2.3	2.9	1.2	1.5

TABLE III-L

Relative Specific Activities of P Fractions in Chick Embryo

Age in Hours	Specific Activity of P Fraction Relative to Yolk P_i			
	P_i Emb.	PL Emb.	P_i Brain	PL Brain
88	4.6	5.3	-	-
144	2.4	2.3	2.5	2.7
208	230	220	200	310
244	72	78	65	100
378	20	77	77	165

TABLE III-M

Percent of Added ^{32}P Incorporated into PL of Incubated Chick Embryos

Whole Embryo		Brain	
Age in Hours	% Incorp.	Age in Hours	% Incorp.
88	1.5	118	0.25
150	2.2	144	0.42
159	2.3	159	0.80
208	5.2	208	1.3
210	5.5	308	1.5
244	7.9		
378	11	378	2.4
386	10		
427	13	427	2.5

Figure 13

Specific activities of yolk, embryo, and brain P_i with age of incubation. The first point for P_i of the whole embryo was estimated from specific activities of P_i from 96- and 110-hour embryo. ● = specific activity of P_i of whole embryo; Δ = specific activity of brain P_i; o = specific activity of P_i from yolk.

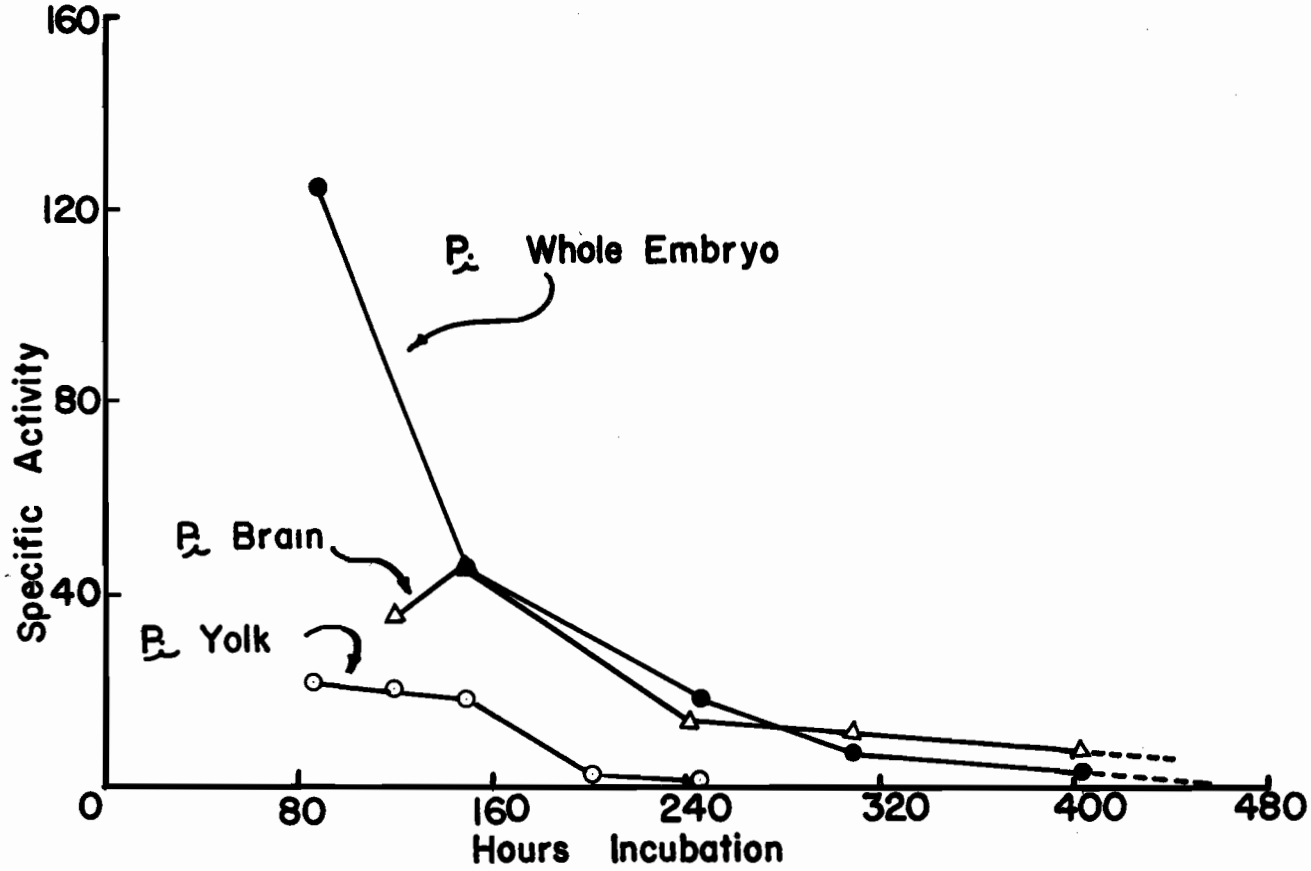

160
120
80
40
0
Specific Activity
P. Whole Embryo
P. Brain
P. Yolk
80
160
240
320
400
480
Hours Incubation

Figure 13

Figure 14

Specific activity of the PL extract from whole embryo minus brain and brain during the incubation interval studied.

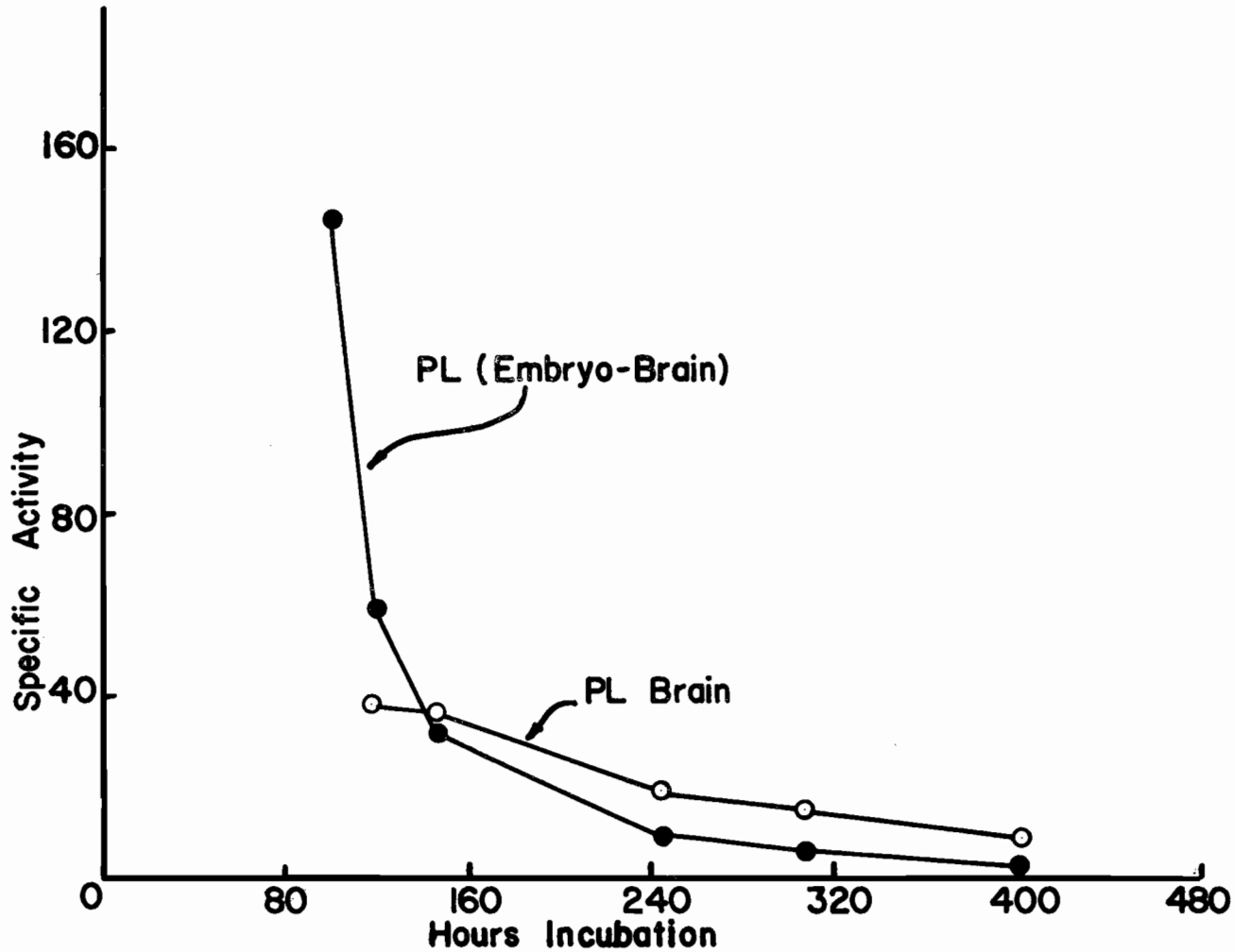

160
120
80
40
0
Specific Activity
PL (Embryo-Brain)
PL Brain
80
160
240
320
400
480
Hours Incubation

Figure 14

in Figures 15 and 16. The same information is shown for brain in Figures 17 and 18.

Injection of $^{32}P_i$ at Different Ages of Incubation

In another series of experiments, $^{32}P_i$ was injected into separate batches of eggs which were previously incubated for 0, 9, 12, and 16 days. The PL was then analyzed 4 days after injection (in one case 2 and 4 days after injection). Total ^{32}P incorporation was measured in the PL extract (brain and whole embryo tissue separately) and the specific activities were determined for individual PL fractions from brain. The radioactivity in P_i and total P were measured in the aqueous layer. The results are given in Table III-N.

When $^{32}P_i$ was injected at 16 days, the PL fractions from brain isolated at 19 days had incorporated 1.0% of added ^{32}P and the specific activity of lipid-P was 1.9.

Glycerol-^{14}C Injection

In order to gain further insight concerning the process of synthesis of PL's in the chick embryo, glycerol-1,3-^{14}C was utilized. One hundred ten eggs which had been incubated for 42 hours were each injected with 50 μL of a chick ringer solution containing ^{14}C-glycerol (6.8×10^4 cpm/egg - 0.2 mc/mmole of glycerol). After further incubation of 5 hours and 124 hours, groups of eggs were removed from the incubator

Figure 15

Total μmoles of PL/embryo appearing during the incubation period.

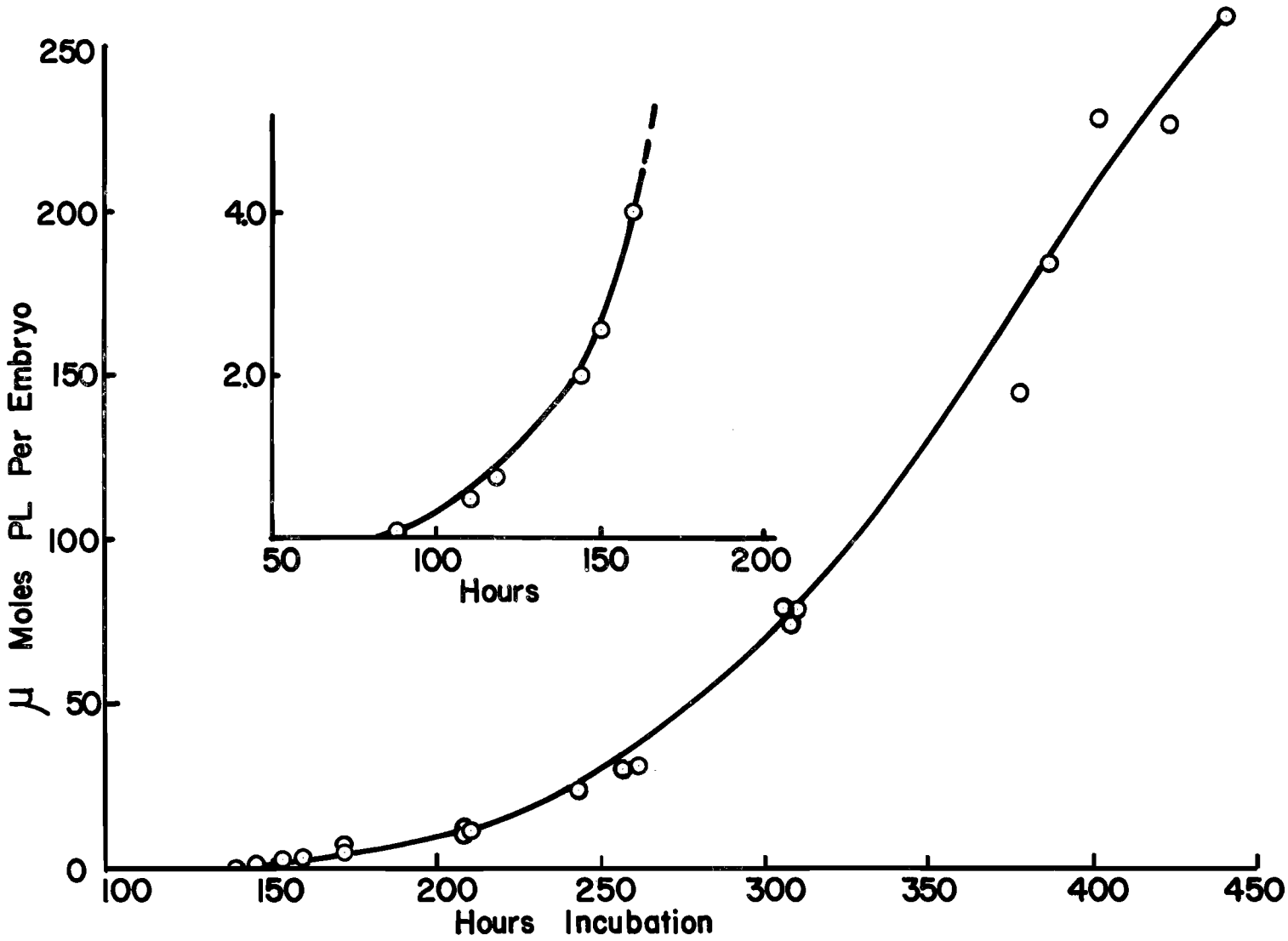

μ Moles PL Per Embryo
250
200
150
100
50
0
100
150
200
250
300
350
400
450
Hours Incubation
4.0
2.0
50
100
150
200
Hours

Figure 15

Figure 16

Total units of ^{32}P radioactivity appearing in PL during the incubation period. Different experiments are indicated by different symbols. Units are cpm (μmole lipid-P in PL)/constant.

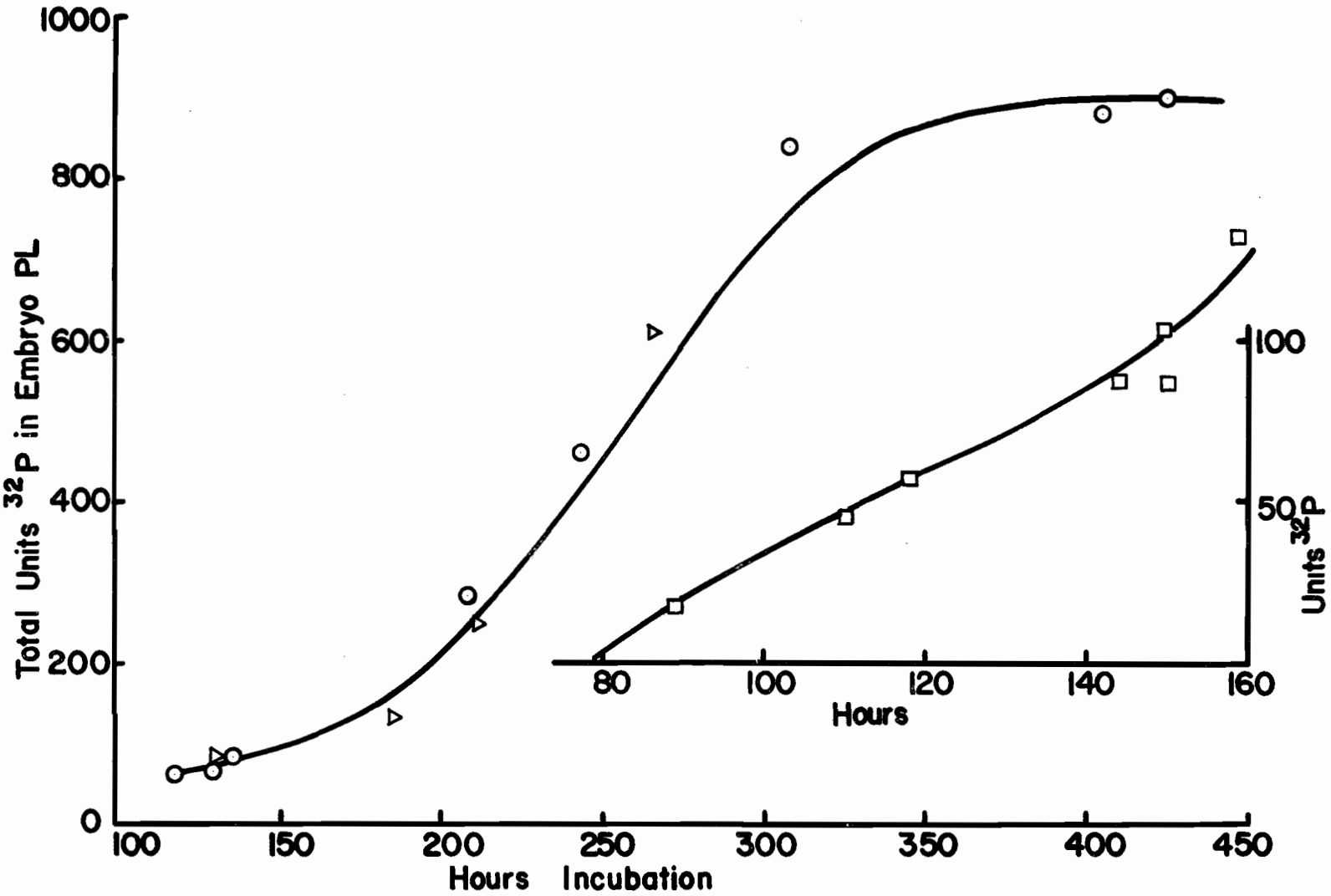

1000
800
600
400
200
0
Total Units ^{32}P in Embryo PL
100
150
200
250
300
350
400
450
Hours Incubation
80
100
120
140
160
Hours
100
50
Units ^{32}P

Figure 16

Figure 17

Total μmoles of PL/brain appearing during the incubation period.

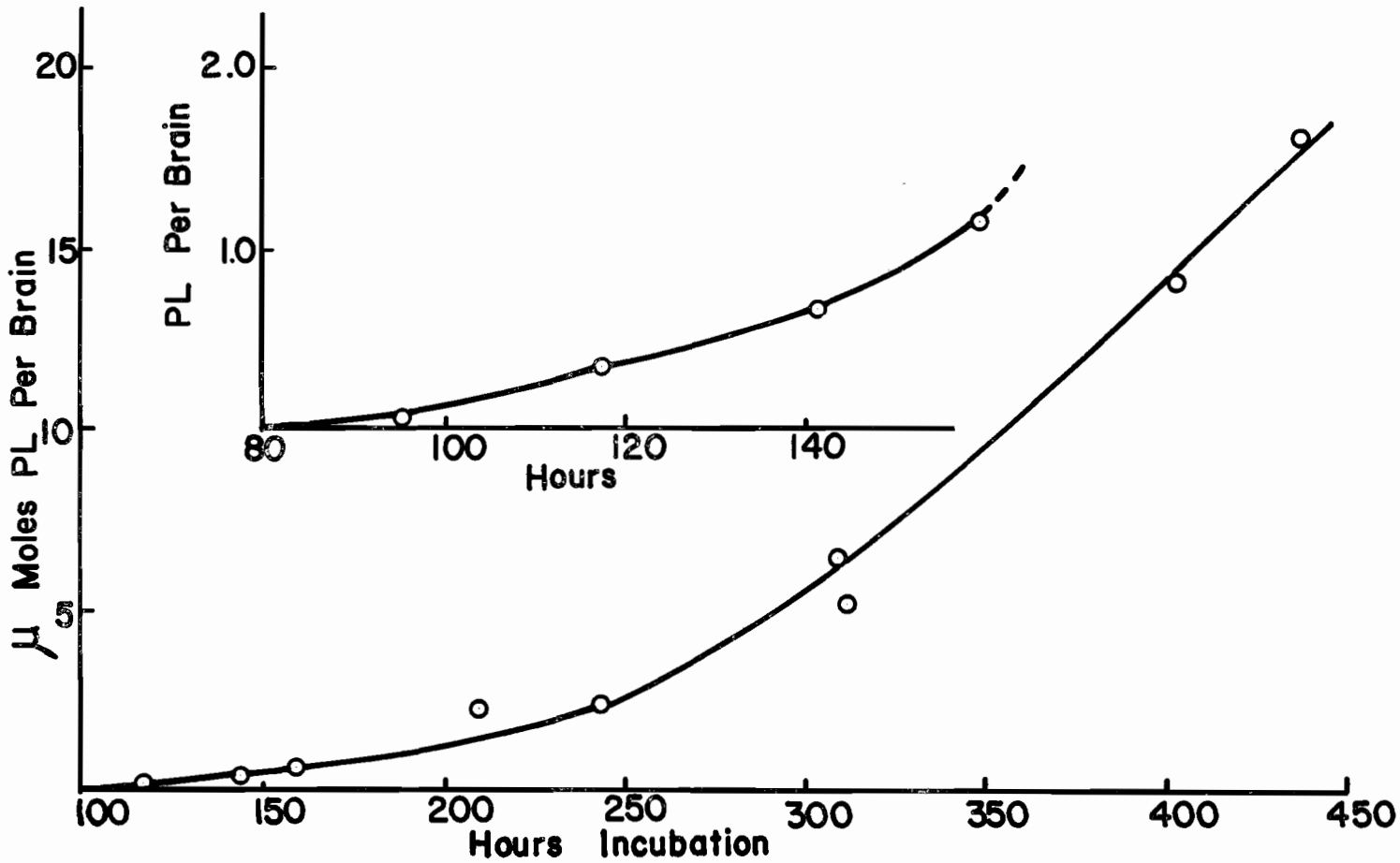

μ Moles PL Per Brain
20
15
10
5
100
150
200
250
300
350
400
450
Hours Incubation
PL Per Brain
2.0
1.0
80
100
120
140
Hours

Figure 17

Figure 18

Total units of ^{32}P radioactivity appearing in brain PL during the incubation period. One experiment is plotted here. Units are defined in Figure 16.

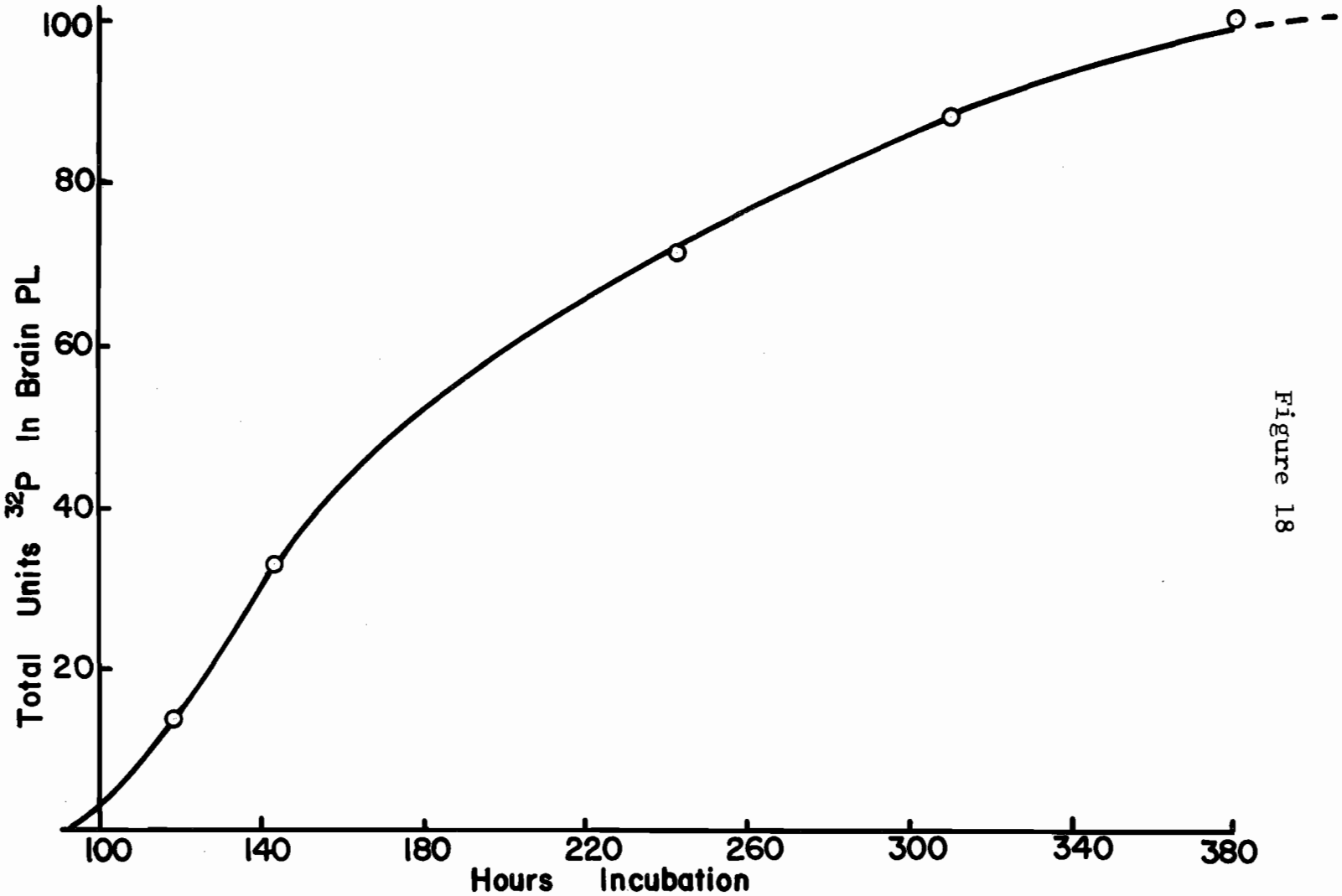

Total Units ^{32}P In Brain PL
100
80
60
40
20
100
140
180
220
260
300
340
380
Hours Incubation

Figure 18

TABLE III-N

Incorporation of $^{32}P_i$ into PL of Brain

Part 1
Injection of $^{32}P_i$ at 0 Days
and Analysis of Fractions at 4 Days

PL Fraction[a]	Sp. Act. of PL at 4 Days	P Compounds Present[b]	Percent of Total P
Peak 1	31	cephalins	37
Peak 2	21	lecithins	60
Peak 3	14	SPM and lecithin	2
P_i brain	28	Inorganic P	-
P_t brain	33	H_2O solu. P	-

[a] Peak refers to peak number on a silicic acid-Hyflo column (fractions were not re-chromatographed).

[b] As detected by TLC.

TABLE III-N

Part 2
Injection of $^{32}P_i$ at 9 Days
and Analysis of Fractions at 13 Days

PL Fraction[a]	Sp. Act. of PL at 13 Days	P Compounds Present	Percent of Total P
Peak 1	9.0	CA, PS	1.9
Peak 2	9.9	PE, PS	20
Peak 3	10	PE, PS, PY[b]	11
Peak 4	9.7	PI, PS	5.4
Peak 5	9.6	PC, SPM	5.6
PL embryo	7.4	all PL's	100
P_i brain	17	Inorganic P	-
P_i embryo	5.3	Inorganic P	-

[a] The designation is as it is in Part 1 of this table.

[b] A small quantity of unidentified PL was noted by TLC.

TABLE III-N

Part 3
Injection of $^{32}P_i$ at 12 Days and
Analysis of Fractions at 14 or 16 Days

PL Fraction	Sp. Act. of PL at: 14 days	16 days	P Compounds Present
PL brain	1.2	1.3	all PL's
PL embryo	-	1.0[b]	all PL's
P_i brain	3.2	1.6	Inorganic P
P_t brain	2.1	1.6	H_2O sol. P cpds.
P_i embryo	-	1.5	Inorganic P
P_t blood[a]	14	-	H_2O sol. P cpds.
P_i blood	10	-	Inorganic P
P_o blood	1.1	-	P_t-P_o in H_2O sol.

[a] The water soluble P in blood of embryos. Since P_i was 10 and P_o 1.1, this value is apparently too high.

[b] 4.2% of the injected P_i was incorporated into PL of whole embryos.

and aliquots of yolk (less albumin and extraembryonic membranes) were fractionated according to the scheme in Figure 19. In addition, brain tissue was removed after a total of 210 and 354 hours incubation and analyzed. The results summarized in Table III-O indicate that glycerol is metabolized in yolk, but is not extensively incorporated into lipid fractions of the brain; at least five times as much $^{32}P_i$ was incorporated as ^{14}C-glycerol in the same time interval (compare Table III-M and III-O).

Injection of Labeled Phospholipids

For these studies it would have been advantageous to use PL's labeled with ^{14}C and ^{32}P. This would have permitted the estimation of incorporation of undegraded PL in the yolk into the embryo. However, the low level of ^{14}C incorporation (both as ^{14}C-glycerol and, as will be seen, ^{14}C-acetate) as compared to ^{32}P incorporation precluded the possibility of doing such an experiment by the methods utilized here. The information necessary to count ^{32}P and ^{14}C simultaneously is given in Table III-P.

A solution containing carrier free orthophosphate ^{32}P and glycerol-1,3-^{14}C was prepared in chick ringer solution such that the $^{32}P/^{14}C$ ratio was 6.8 (477,000 dpm/70,400 dpm). This solution was injected into 30 eggs which had been incubated for 145 hours. After 244 hours total incubation, labeled PL was extracted from whole embryos and chromatographed

Figure 19

The fractionation of egg yolk in the experiment in which ^{14}C-glycerol was added to yolks of growing embryos. The percentages of ^{14}C incorporation in these fractions (designated by letter) are given in Table III-O. TLC of fraction A (PL and neutral lipid) was by the method of Dobiasova (17) with hexane:diethylether:ethylacetate (40:10:15). Glycerol in fraction F was identified by paper chromatography on Whatman No. 1 with n-butanol:ethanol:water (4:1:1:1.9).

Figure 19

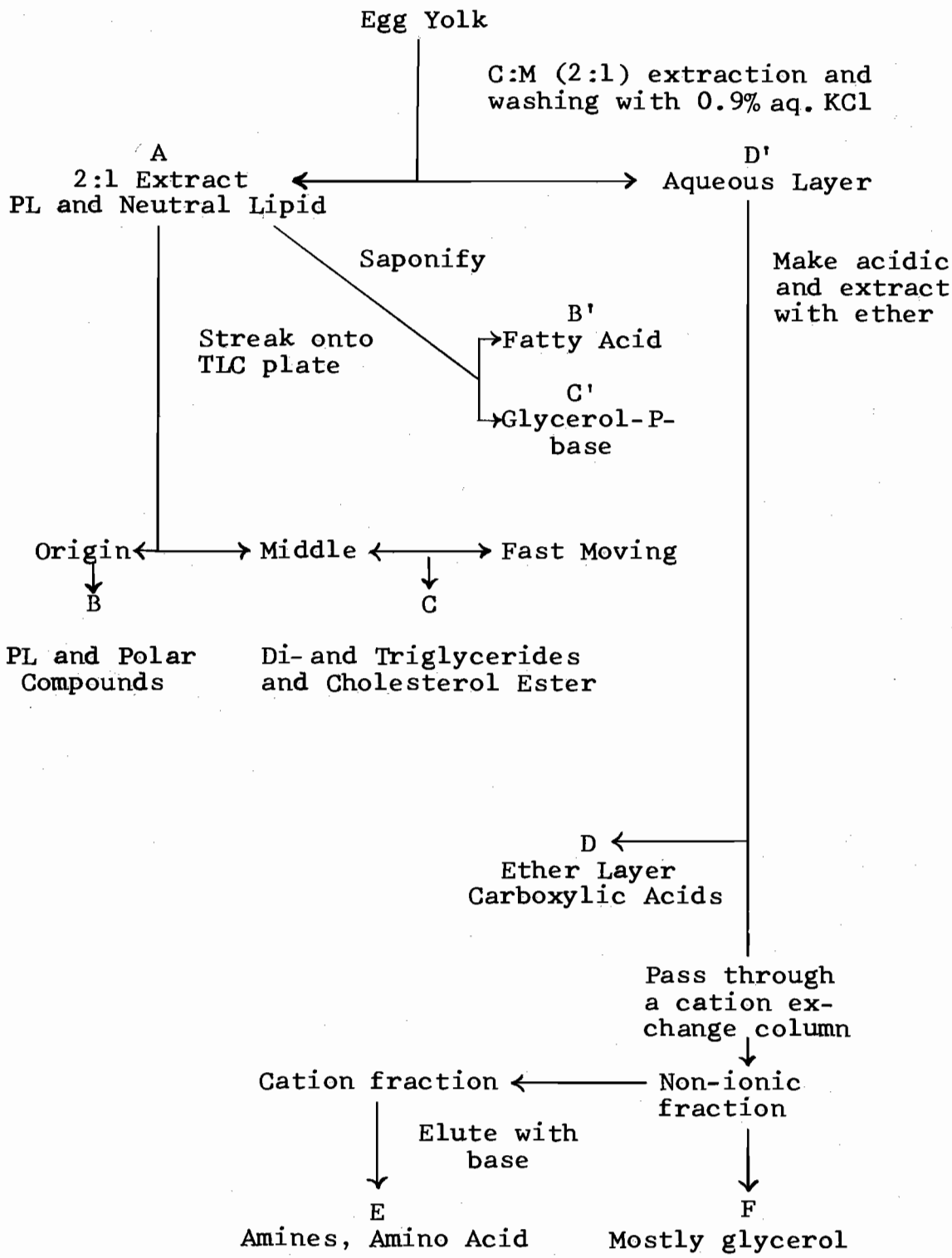

Egg Yolk
C:M (2:1) extraction and washing with 0.9% aq. KCl
A
2:1 Extract
PL and Neutral Lipid
D'
Aqueous Layer
Saponify
Make acidic and extract with ether
B'
Fatty Acid
Streak onto TLC plate
C'
Glycerol-P-base
Origin
Middle
Fast Moving
B
C
PL and Polar Compounds
Di- and Triglycerides and Cholesterol Ester
D
Ether Layer
Carboxylic Acids
Pass through a cation exchange column
Cation fraction
Non-ionic fraction
Elute with base
E
Amines, Amino Acid
F
Mostly glycerol

TABLE III-O

^{14}C Distribution in the Incubated Egg after Adding ^{14}C-glycerol to Eggs after 42 Hours of Incubation

Incubation Age in Hours	Part of Egg	Percent of Total ^{14}C Recovered in Fraction Designated in Figure 19					
		A (PL & neutral lipid)	B (PL) or B' (fatty acid)	C (di & tri-glycerides & CE[a]) or C'[b]	D (carboxylic acids) or D' (H_2O solu.)	E (amines)	F (glycerol)
47	yolk	3	1.5 (B)	1.5 (B)	2 (D)	4	92
124	yolk	1.3	-	-	3 (D)	39	49
210	brain[c]	0.7	0.5 (B')	0.2 (C')[d]	0.01 (D)	0.006	0.12
354	brain	0.9	-	-	1.4 (D')	-	-

[a] CE = cholesterol ester.

[b] C' represents the H_2O soluble fraction of saponified lipids.

[c] Brain fractions were obtained in the same manner as yolk fractions.

[d] For the same experiment, $^{32}P_i$ incorporation was 1.0% at 210 hours and 2.2% at 354 hours (i.e., when $^{32}P_i$ was injected at 43 hours and fractions isolated at these times). Further, since most of the label in brain PL and neutral lipid is in the fatty acid, it appears that at least five times as much $^{32}P_i$ gets into the phosphodiester unit (fatty acids excluded) as into ^{14}C-glycerol in the same length of time over comparable incubation intervals.

TABLE III-P

Simultaneous Analysis of ^{32}P and ^{14}C by Liquid Scintillation Counting

Isotope	Applied Volts	Discriminator Settings for Recording cpm	Efficiency of Counting
^{14}C	1190	Green channel, 60-∞	0%
^{32}P	1190	Green channel, 60-∞	30.4%
^{14}C	1330	Red channel, 10-100	7.4%
^{32}P	1330	Red channel, 10-100	32%

on a DEAE-cellulose column. Tubes 3-16, which were eluted by C:M (7:1), were pooled. This fraction was re-chromatographed on silicic acid-Hyflo (6 g to 3 g), eluting with 400 ml of $CHCl_3$, 200 ml of C:M (9:1), 200 ml of C:M (4:1), and 300 ml of C:M (2:1). Analysis of tubes 95-120 showed the presence of only PC which contained no ^{14}C despite the addition of glycerol-^{14}C. Other PL fractions were likewise devoid of measurable ^{14}C label.

An aliquot of this PC (made up in C:M (2:1)) was evaporated to approximately 5 ml and added to 10 ml of water containing 2% Tween 20; a nearly clear emulsion of PC resulted. This PC-^{32}P was then injected into 47 eggs which had been previously incubated 280 hours. From 0.2 to 0.4 ml/egg of the emulsion was injected. After 144 hours (six days) further incubation, PL fractions were recovered from brains; lipid was also extracted from embryo tissue less

brain. Of the 47 embryos injected with PC-^{32}P, 16 died in the six day interval. This might have been due to MeOH or Tween 20, but probably not due to the radioactivity or excess PC present; 22 μmoles of PC were added per yolk, which is about 2% above the normal amounts. The results of this experiment are given in Table III-Q. The PC isolated in the same manner as that injected had a specific activity much higher than other PL's or water soluble P compounds. In addition, this particular PC fraction represented a rather small percent (approximately 9%) of the total PC (the major PC coming after the high specific activity PC on the silicic acid-Hyflo column).

Table III-R shows a comparison of the percent incorporation of $^{32}P_i$ versus PC-^{32}P. In both the embryo and brain, twice as much incorporation of $^{32}P_i$ occurs as of PC-^{32}P.

Another similar experiment was performed using $^{32}P_i$ and acetate-1-^{14}C. A total of 10^9 $^{32}P_i$ cpm and 10^8 ^{14}C cpm were injected into 34 eggs. The injections were done at 120 hours and after 238 hours total incubation, a PE fraction was isolated from whole embryos by previously described methods utilizing a silicic acid-Hyflo and a silicic acid-NH_3 column. The PL in tubes 77-87 from the first column was pooled and chromatographed on the second column from which the PL in tubes 5-12 was taken for further injection. This fraction as well as the other PL fractions contained no ^{14}C. Some ^{14}C, amounting to 0.6% of the total added ^{14}C, was

TABLE III-Q

Specific Activity of PL Fractions Isolated after 427 Hours Incubation when PC-^{32}P was Injected at 280 Hours

Phosphorus Fraction[b]	Specific Activity	^{32}P Compounds Present
PL-embryo	3	All PL's
P_t-embryo	10	H_2O solu. P from wash
P_i-embryo	43	Inorganic P from wash
P_t-brain	10[a]	H_2O solu. P from wash
P_i-brain	15[a]	Inorganic P from wash
DEAE-2:1 brain	38	PE, PS (minor)
DEAE-MeOH brain	59[a]	CA, PE (very minor)
DEAE-C:M:NH_3 brain	38	Oxidized PL
DEAE-7:1, SA-12:1 brain	6	PE
DEAE-7:1, SA-9:1-4:1 brain	225	PE
DEAE-7:1, SA-4:1-3:2 brain	1600	PC
DEAE-7:1, SA-3:2-MeOH	38	SPM, PC

[a] For these fractions, cpm minus background were less than 25; the standard deviation for ^{32}P counts was about ±20%.

[b] These fractions are denoted by the way they were eluted on chromatographic columns.

TABLE III-R

Comparison of Incorporation Levels Between $^{32}P_i$ and PC-^{32}P

PL Source	Percent Incorporation when Label Added to Yolk is:	
	Ortho $^{32}P_i$	PC-^{32}P
Brain	0.8%	0.4%
Whole Embryo	5%	2.8%

found in the first neutral fraction from the silicic acid column. This fraction probably contained triglycerides and cholesterol esters. The specific activities of the PL fractions are given in Table III-S. The PE (second fraction in Table III-S) was emulsified in 1% Tween 20 and essentially all $CHCl_3$ and MeOH were removed from the emulsion by gentle heating. About 0.2 ml/egg of the emulsified PE (170,000 cpm/3 μmoles) was injected into 19 eggs of 336 hours age. After 425 hours of total incubation, the PL fractions were isolated from brain and the results are shown in Table III-T. The PE fraction which had the highest specific activity did not emerge on the silicic acid-NH_3 column in the same tubes in which the donor PE had emerged, but in the peak which had mainly PE and some PS. Specific activities of PE and PS were not determined independently in this fraction, but since the amount of PS was low and other PE fractions (like SA-12:1 in Table III-T) had relatively high specific activities, it is assumed that PE was responsible for the high

specific activity. If the PE which was added to yolks at 14 days became partially oxidized during the chromatographic procedure (the same PE would have gone through three columns prior to the last one), one might expect its chromatographic behavior to be altered. In this experiment, 1.4% of added ^{32}P counts were incorporated into brain PL.

TABLE III-S

Specific Activity of PL Fractions when Eggs were Injected at 120 Hours and PL Isolated at 238 Hours

Fraction	$\frac{^{32}P \text{ cpm}^a}{\mu\text{Moles P} \times 10^3}$	PL's Present
PL extract	98	All PL's
SA-9:1, R-4:1 (I)[b]	102	PE
SA-9:1, R-4:1 (II)[b]	99	PE and PS
SA-9:1, R-MeOH (III)[b]	93	PS and oxd. PE
SA-4:1	109	PE, PS, PI, and PC
SA-3:2 to MeOH	103	PC, PI, and SPM

[a] The specific activities given here are different than those given in previous data (calculated by equation 1) in that the term is not divided by cpm of a standard.

[b] Abbreviations are: SA = silicic acid-Hyflo, R = silicic acid-NH_3, and (I), (II), and (III) for the first, second, and third peaks, respectively.

TABLE III-T

Incorporation of ^{32}P-Labeled PE into Brain PL when Label Added to Yolk at 336 Hours and PL Extracted at 425 Hours

P Fraction[a]	Relative Specific Activity[b]	P Compounds Present
SA-12:1	5.2×10^{-3}	CA, PE
SA-9:1, R-4:1	2.1×10^{-3}	PE
SA-9:1, R-4:1 to MeOH	32×10^{-3}	PE, PS (minor)
SA-4:1	0.52×10^{-3}	PE, PS, PC
SA-4:1 to 3:2	0.83×10^{-3}	PC, PI
SA-3:2	0.16×10^{-3}	PC, PI
SA-3:2 to MeOH	1.69×10^{-3}	PC, SPM
H_2O solu. P	0.22×10^{-3}	Water sol. P
P_i only	1.14×10^{-3}	Inorganic P

[a] PL fractions are designated by position of elution on silicic acid-Hyflo and silicic acid-NH_3 columns.

[b] Relative specific activity here is

$$\frac{\text{cpm}/\mu\text{mole P}}{\text{specific activity of donor PE}}$$

DISCUSSION

Brain Lipid Composition

What may be derived from the PL composition of brain about the biological role of this molecular class is indeed meager. The present study and studies like it reveal that the composition does change during growth and differentiation and this change does parallel myelin formation; but the quality of the change dependent on the PL's, collectively and individually, may only be suggested at this time. Indeed, we are in the embryonic stage of even stating the problem.

Consider that first of all we are considering an entire organ; more than one kind of cell is represented and growth is superimposed on cell differentiation which presumably is not synchronous. The number of common chemically different species within the class "phospholipids" is about 200 not counting the less prevalent fatty acid side chain types, *cis-trans* isomers, or ironically different species. The varied fatty acid and base groups represented render PL molecules covering a wide range of melting points, acidity constants, and water solubility.

Some insight may be gained by asking the question, how does the PL composition of chick brain compare with brain of other species. Table IV-A shows data from four different brain sources. Rat brain data were reported by Freysz *et al.*

TABLE IV-A

Phospholipid Composition from Different Brain Sources

PL Fraction	Percent Composition of PL in Brain of:			
	Chick (12 day)	Rat (3 day)	Rat (5 mo.)	Beef
Cephalin	30	57	30	42
Lecithin	58	44	42	36
SPM	3	2.5	8	17
CA-like	2	-	-	1.6
Inositide	3	-	-	4.5

(20), and beef brain data by Rouser (41). The low level of SPM in chick would indicate that if a direct relationship ensues between SPM and myelination, then myelination in the chick embryo up to 18 days (2-3 days before hatch) is not complete. A relation between SPM content in human brain and myelination has been reported by Brante (8). In addition, the SPM content of three-day versus five-month rat brain (Table IV-A) suggests a relation between SPM and brain development. Another interpretation is that SPM in chick brain is not important in myelination, but sphingosine is. Garrigan and Chargaff (22) noted a marked increase in mucolipid-bound sphingosine in chick embryo brain from about ten days to two days after hatching. The SPM content of adult brain tissue generally is greater than 10% (35, p. 645). In

the brain of man, ox, cat, rabbit, rat, pigeon, guinea pig, beef, and fish, cephalin predominates over lecithin by about 1.5/1 (35, p. 644). In contrast, lecithin is more prominent than cephalin in chick at early embryonic ages.

The physical chemical properties peculiar to lecithin but not to cephalin are only vaguely known. Some of the physical properties of PL's are discussed by Bingham (5). He notes that lecithin forms micells or smectic lamellae at very low lipid concentrations--10^{-5} g/ml. At pH 7.5, PC is within its isoelectric range, while PS and PE would carry net negative charges. Bingham also discusses the physical state of PL's in the classical Danelli unit membrane. He suggests that lipids at the borderline of a solid-liquid phase might be influenced by slight chemical stimuli and this in turn would determine the membrane permeability to charged water soluble molecules.

Incorporation of ^{32}P into Embryo and Brain Phospholipids

The work of Hevesey *et al.* (25) and Branson *et al.* (7) showed that inorganic P present in yolk is a precursor of embryonic lipid-P. The work reported here shows this to be true for individual PL's.

Making certain assumptions, the extent of *de novo* synthesis from P_i, base, and diglyceride may be ascertained by knowing the net amount of PL synthesized, the specific

activity of the inorganic phosphate pool from which the PL is made, and the total incorporation into PL. These results are presented in Figures 13, 15, 16, 17, and 18 for the incubation intervals studied. The data shown in Table IV-B and Table IV-C was obtained from these curves. The method of calculation of the righthand column in Tables IV-B and IV-C is shown below:

1) μmoles of $^{32}P_i$ incorporated =

$$\frac{\text{Column 3 of Table IV-B and C}}{\text{Column 4 of Table IV-B and C}}$$

2) Column 5 of Table IV-B and C =

$$\frac{\mu\text{moles of } ^{32}P_i \text{ incorporated}}{\text{Column 2 of Table IV-B and C}}$$

Column 3 is the net increase in PL per embryo (or brain as the case may be) over the interval designated in the lefthand column of the table, and column 4 is the average specific activity of P_i as determined from the midpoint of the specific activity curve for P_i in the respective tissue (Figure 13). The major assumption is that the P_i extracted when PL is extracted from the tissue is equilibrated with the water soluble P_i pool which is incorporated into PL. The righthand figure in Table IV-B and IV-C is assumed to represent the fraction of PL which is synthesized de novo in a given incubation interval. Although the number is calculated on the basis of the specific activity of extracted P_i, other water soluble ^{32}P phosphates would be part of the

P pool available for PL synthesis. The calculation is facilitated by the fact that the curves in Figures 13, 15, 16, 17, and 18 are quite regular with no sharp fluctuations.

Experimentally, it was found that P_i and water soluble organic P (which would include nucleotides, sugar phosphates and the common intermediates of PL synthesis) had nearly the same specific activities. The water soluble organic P in brain usually had a specific activity slightly greater than P_i. Thus if lipid-P arose from ^{32}P labeled compounds other than P_i such as ATP, phosphorylcholine, CDP-choline, etc., about the same fraction or more of PL would be calculated to arise de novo. Further, the molar ratio of ortho P_i to brain lipid-P is about 1/6 and the P_i/ATP ratio in brain is about 2/1 (24, p. 7). An intermediate like phosphorylcholine would represent a small fraction molewise of the lipid-P in brain. In rat brain, for example, phosphorylcholine represents 8% of water soluble P (16).

There is considerable evidence that small ions do not pass rapidly through the "blood-brain barrier." In mouse brain, Heald reports (24, p. 17) that the equilibrium between water soluble P in blood and brain required seven days. Webster (51) found that in normal chicken brain the specific activity of brain acid soluble P was only one-fourth as great as plasma acid (water) soluble P after three days. The data in Table III-M, Part 3, show that specific activities of water soluble P in blood and brain differ by

3- or 4-fold. The specific activity decrease of the P_i in brain (and hence water soluble P) is not rapid as seen in Figure 13. Thus the rate of entry of water soluble PL precursors (like phosphorylcholine) would not appear to be fast enough to account for the amount of label that does become incorporated into PL. Further, the size of the water soluble P pool is quite small (previous paragraph) when P_i is excluded; in our experiments there would not appear to be enough label in the water soluble P pool (excluding P_i) to account for the label that did become incorporated into PL. Thus a mechanism by which lipid-P would arise in the brain from ^{32}P phosphates other than $^{32}P_i$ does not seem plausible on the basis of what is known about the biochemistry of the brain.

It seems likely that in order to account for all the ^{32}P label that appears in brain PL in a given incubation interval, water soluble P compounds (which are PL precursors) found in the brain all contribute to the ^{32}P supply for PL synthesis. If, for example, during the 250-300 hour interval (Table IV-C) half of the PL synthesized is from brain water soluble phosphates and this accounts for nearly all radioactivity, then half of the P must come from another source which is not labeled or has a comparatively low specific activity.

The experiments in which $^{32}P_i$ was added to eggs at different incubation intervals substantiates further the

conclusions drawn thus far. Table IV-D shows that less ^{32}P is incorporated per μmole of PL synthesized in brain at later ages than early ages. One interpretation of the data in Table IV-D would be that P_i and water soluble P is being rapidly diluted by cold P. The data presented in the Results section show this is not the case. An alternative explanation is that lipid-P is diluted by non-polar P.

Summarizing at this point, the figures in the right-hand column of Tables III-B and C represent the fraction of ^{32}P in brain PL that arises from P_i and water soluble P. The origin of the remaining fraction of brain PL is considered in the next section.

Injection of ^{32}P-Labeled PL

Two similar experiments were carried out to test the proposition that PL's may be transported from yolk to brain without intermittent breakdown and resynthesis. In the Results section an experiment is described in which PC-^{32}P was prepared (donor PC). This PC was injected into yolks of eggs which had been incubated 11 days. Six days later, PC-^{32}P (recipient PC) was isolated by the same chromatographic procedure used to prepare donor PC. The specific activity of this fraction was 40 times greater than brain P_i or other PL fractions except for one PE fraction which had an intermediate value (Table III-Q). We now might consider two schemes to explain the source of brain PL's, as shown below.

TABLE IV-B

Relation of Phospholipid Increase to Incorporation of $^{32}P_i$ into Embryo Phospholipids during Incubation

A Incubation Interval	B $\frac{\Delta\mu\text{mole PL}}{\text{embryo}}$	C Δ Units ^{32}P in PL	D Sp. Act. P_i	E $\frac{\mu\text{mole } ^{32}P \text{ incorp.}}{\Delta\mu\text{mole PL/emb.}}$
(Hrs.)				
0-100	0.25	31	ca. 160[a]	ca. 0.8-1.0
100-150	2.4	74	75	0.4
150-200	7.4	130	38	0.5
200-250	18	260	24	0.6
250-300	41	235	12	0.5
300-350	64	115	5	0.5
350-400	72	50	2.5	0.3
400-450	54	25	2.0	0.2

The columns are: A, the time of injection of $^{32}P_i$ and the time of extraction of PL from the tissue; B, the net increase in moles of PL in the interval designated; C, the net increase in total ^{32}P units (cpm/constant) in the interval designated; D, the average specific activity of P_i over the incubation interval designated (see Figure 13), and E, the apparent fraction of PL which has been synthesized from P_i and water soluble phosphates (see text).

[a] Since label was added at 43 hours, this figure may only be approximated.

TABLE IV-C

Relation of Phospholipid Increase to Incorporation of $^{32}P_i$ into Brain Phospholipids during Incubation

A Incubation Interval	B $\frac{\Delta\mu mole\ PL}{brain}$	C ΔUnits ^{32}P in PL	D Sp. Act. P_i	E $\frac{\mu mole\ ^{32}P\ incorp.}{\Delta\mu mole\ PL/brain}$
100-150	0.8	29	44	ca. 0.8
150-200	1.1	22	36	0.6
200-250	1.3	17	21	0.6
250-300	2.0	13	12	0.5
300-350	3.7	9	8	0.3
350-400	5.1	8	6	0.3
400-450	5.2	6	4	0.3

The columns are designated as in Table IV-B.

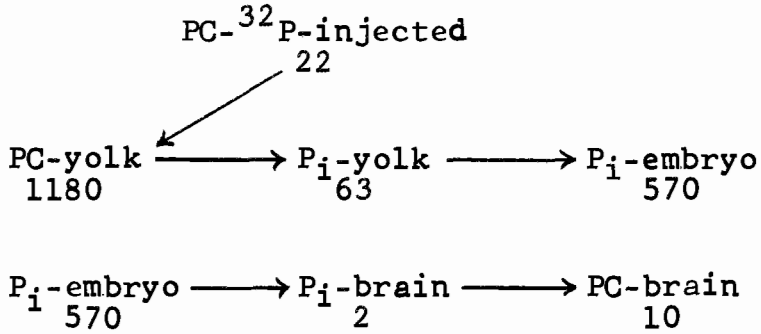
TABLE IV-D

Incorporation of $^{32}P_i$ into PL at Various Age Intervals

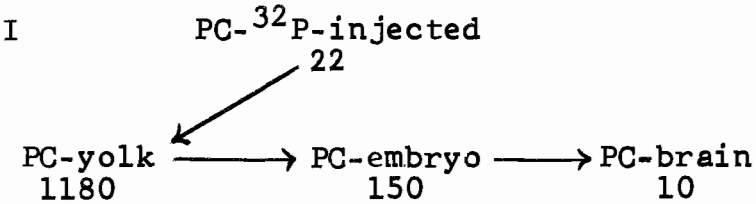
Time $^{32}P_i$ Injected	Time ^{32}P Extracted	$\Delta\mu$moles PL/brain	% of ^{32}P Incorp.	% of ^{32}P Incorp. / $\Delta\mu$moles PL/brain
	(Hrs.)			
0	96	0.16	0.13	5.0
215	307	3.4	1.2	3.5
288	336	3.1	0.23	0.7
288	380	3.7	0.36	0.5
400	470	13.4	1.0	0.7

The numbers indicate the relative pool sizes (in μmoles) estimated from the present data and that of other groups. Arrows indicate a transport or flow.

Scheme I



Scheme II



If Scheme I operates exclusively, the added PC-^{32}P would be hydrolyzed and the results would be similar to those in experiments when P_i was added to yolk. Since the same PC fraction that was added to yolk turned out to have the highest specific activity in brain, the most obvious conclusion is that Scheme II is partially active.

The second radioactive PL experiment, in which PE-^{32}P was added to yolks at 14 days (336 hours) and PL isolated at 17 days (425 hours), gave a result very similar to the one in which PC-^{32}P was injected in that the PL with the highest specific activity found in brain was the same type as donor PL (see Table III-T). It seems significant that one

fraction had a specific activity 28 times greater than that of P_i found in the brain. The argument for some direct transfer of PL would again seem to hold since if PL (in this case PE) were degraded completely in the yolk before being used by the embryo, the specific activities found in brain PL would more nearly resemble those found when $^{32}P_i$ (not PE-^{32}P) was added to the yolk. The most radioactive PE fraction obtained from labeled PE did not emerge on the silicic acid-NH_3 column exactly as the material used for injection. This fraction was shown to contain mostly PE. The fact that this recipient PE hangs onto the column longer might indicate that during the three column procedures to which this fraction was subjected (before being injected), it became partially oxidized and was eluted in more polar solvents. In the PC-^{32}P experiment, the most radioactive PC was not eluted with the major PC peak but before it. If donor PL were oxidized prior to injection into yolks of growing embryos and the chromatographic behavior of the recipient PL were slightly altered, this might mean that no transesterification of the diglyceride portion occurred. An alternative explanation is that transesterification had occurred and the new fatty acids accounted for the changed chromatographic behavior.

Another observation of interest is that donor PC-^{32}P was diluted only about 8-fold after injection (as determined from the specific activities of donor and recipient PC);

PE-^{32}P was diluted 30-fold. Thus as may be seen from the pool size of PC in yolk, there would not appear to have been very much mixing of injected PL with yolk PL and incorporation of injected PL must have been rapid.

Although many details of the incorporation process are at present unclear, it is quite evident that the behavior of injected PL-^{32}P is greatly different than $^{32}P_i$. The fact that about 1.4% of ^{32}P (added as PL) was incorporated into brain is consistent with Tables IV-B and C. Identical experiments with both $^{32}P_i$ and PL-^{32}P labeling were not carried out, but by extrapolation of the data, one would find that over the interval of 14 to 17 days approximately 0.9% of $^{32}P_i$ added would be incorporated into brain PL. Since in the case of PE-^{32}P injection, the amount of incorporation was 1.4% over this same interval, the fraction of de novo synthesis (0.9/1.4 + 0.9) appears to be about 0.4. In Table IV-C, the fraction is 0.3. Considering the experimental error and the assumptions made, the $^{32}P_i$ and PE-^{32}P experiments are in agreement in calculating the fraction de novo synthesis.

Thus from the data reported here, it seems reasonable to conclude that yolk PL is to some extent transported intact, or at least without breaking P-ester bonds, to embryo and brain tissue. The experimental findings of other groups (below) and some indirect evidence reported here substantiates this interpretation.

When $^{32}P_i$ was added to yolk, the specific activity of P_i isolated from yolk declined slowly up to 160 hours incubation, then dropped to a very low level and did not fall appreciably after that. This decline does not correspond with either the measured amount of P_i in yolk during incubation (31) or with the specific activity of embryo PL. Although the situation is complicated by changing pool sizes, if all yolk PL were converted to hydrolytic products and the P_i thus derived were used by the embryo to make PL, the specific activity of yolk P_i should have been nearer to and parallel in rate of decrease to the specific activity in embryo PL. This was not true. Actually, the specific activity in embryo PL was close to that of embryo P_i and far removed from that of yolk P_i (Table III-L). The amount of P_i present in yolk compared to embryo is small at any given age of the incubation (except very early ages). Since yolk P_i and embryo P_i do not equilibrate rapidly, it would seem difficult to account for the large build-up of embryonic P_i without organic P getting into the embryo. A good deal of yolk PL might be hydrolyzed in the liver or blood and the P_i used for various sources such as bone formation in the embryo.

There is good experimental evidence that lipids are transported intact from yolk to embryo organs. Davison *et al*. (15) showed that cholesterol-4-^{14}C when injected into yolk was taken up by brain in the later embryonic stages.

The label isolated in the brain cholesterol was found exclusively in the 4 position, showing that there was no apparent breakdown of cholesterol in the process. Budowski et al. (10) presented evidence which was consistent with the hypothesis that triglycerides were transported from yolk to the brain without intermittent breakdown. Cammerino and Wright (11) noted a decrease in the specific activity of labeled cholesterol of embryo in the latter part of the incubation, indicating a transfer of unlabeled sterol from yolk. Changus et al. (12) have shown that young rat brain incorporates $^{32}P_i$ more rapidly at young ages than at older ages. Heald (24, p. 41) in reviewing data along these lines, suggests that the transport of intact PL from blood to brain might be a possible explanation of experimental results obtained by several groups (some of them mentioned here).

Finally, it seems plausible to suppose that as the embryo develops and consumes more space in the egg, and as the membranes with a substantial blood circulatory system engulf the yolk, more PL would become accessible to uptake by the blood. Another morphological aspect of the situation is that the yolk sac becomes continuous with the gut; in fact, at the end of the incubation period, it is drawn inside the embryo. In this respect, yolk is functioning in a classical manner as a source of immediate nutrient and material for the developing embryo. Davison et al. (14) have pointed out that histologically it has been demonstrated that the chick

liver parenchymal cells become heavily laden with lipid. The fact that turnover of PL in liver is rapid compared to brain and other organs has led Davison to suppose that PL's are synthesized in the liver and transported via the blood to other organs (14).

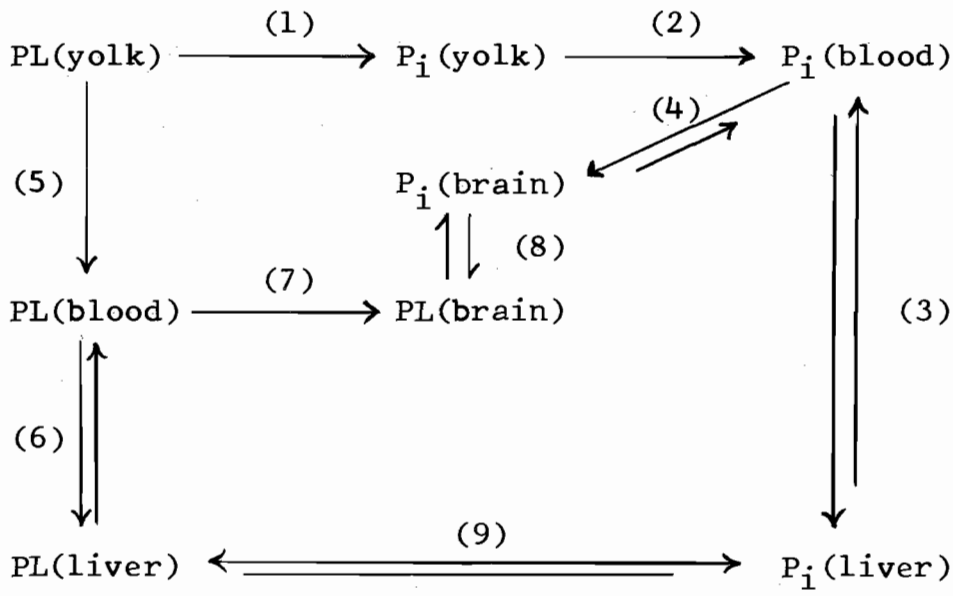
In summary an overall somewhat oversimplified mechanistic scheme is presented in Figure 20.

From the available evidence, some things may be said about the several reactions: Reaction (1) is irreversible and especially rapid in the initial part of the incubation period; reaction (2) must be slow and almost irreversible; reaction (3) is fast by comparison; reaction (4) is slow and becomes slower during embryonic development; reaction (5) is probably about as fast as (2) and is nearly irreversible; reaction (6) is comparatively rapid; reaction (7) is slower than (6) but faster than (4); reaction (8) is probably about as fast as (7) but during growth is not reversible; finally, reaction (9) is probably rapid.

Figure 20

A simplified diagram showing the routes of transport of PL and P_i and the synthesis of PL in the incubated egg. The arrows indicate either transport or synthesis.

Figure 20


PL(yolk)
(1)
P_i(yolk)
(2)
P_i(blood)
(4)
(5)
P_i(brain)
(8)
PL(blood)
(7)
PL(brain)
(3)
(6)
PL(liver)
(9)
P_i(liver)

SUMMARY

1. The relative amounts of phospholipids of chick embryo brain have been measured at intervals between 4 and 19 days of the incubation of the egg. The measurement was made by phosphorus analysis of individual phospholipids separated by column chromatography. Phosphatidyl ethanolamine and phosphatidyl choline together account for approximately 84% of total lipid phosphorus.
2. A previously uncharacterized phospholipid from chick brain has been tentatively identified as cardiolipin.
3. The sphingomyelin content of chick brain was found to be approximately 3% throughout the incubation interval studied, even in the latter stages of the incubation when myelination has been reported to be an important process.
4. Inorganic ^{32}P, when injected into the egg yolk at different ages, is incorporated into all phospholipid fractions in both whole embryo and brain. Specific activity values compared between individual phospholipid fractions of the same age did not vary more than two-fold between one another.
5. Glycerol-1,3-^{14}C, when added to yolk at the same time as inorganic ^{32}P, is incorporated into brain less than one-fifth as much as inorganic ^{32}P (by percentage of added label incorporated into the entire phospholipid extract).

Acetate-1-^{14}C is not measurably incorporated into the brain phospholipid extract compared to the amount of ^{32}P incorporated.

6. Highly labeled ^{32}P-phosphatidyl choline and ^{32}P-phosphatidyl ethanolamine were prepared biologically by injection of inorganic ^{32}P into growing embryonated eggs. In separate experiments, each of these phospholipids were injected into separate groups of yolks of developing embryos. In each experiment, brain phospholipids were extracted and fractionated several days after injection of the labeled compound. The highest specific activity phospholipid fraction was the same type as that injected. Recovered phosphatidyl choline was 30 to 40 times greater in specific activity than the other phospholipid fractions; in like manner, phosphatidyl ethanolamine was 10 to 30 times greater.

7. The significance of the widely differing results when ^{32}P-phospholipid was injected into yolks rather than inorganic ^{32}P is discussed.

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