Origin of phospholipids in the chick embryo during development

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SUMMARY Inorganic-^{32}P, injected into yolks of eggs incubated to produce embryos of different ages, was incorporated into all phospholipid fractions in both whole chick embryo and embryo brain. Specific activity values compared between individual phospholipids of the same incubation age and in eggs injected at the same time did not vary more than twofold between one another.

Biologically prepared phosphatidyl-^{32}P choline and phosphatidyl-^{32}P ethanolamine, when injected into yolks of eggs, gave a very different pattern of incorporation into embryo brain from that given by inorganic ^{32}P. When the labeled choline phosphatide was injected, a phosphatidyl choline fraction was isolated whose specific activity was 30–40 times greater than those of other phospholipid fractions. Phosphatidyl-^{32}P ethanolamine injection gave a qualitatively similar result.

Glycerol-1,3-^{14}C and acetate-1-^{14}C were incorporated to a much lesser extent than inorganic-^{32}P.

The hypothesis is advanced that as the embryo develops, de novo synthesis from inorganic phosphate decreases and intact phospholipid is transferred from the yolk to the embryo and its organs.

KEY WORDS chick · embryo · brain · ^{32}P incorporation into · phosphatidyl choline · phosphatidyl ethanolamine · phosphatidyl serine · sphingomyelin · cardiolipin · phosphatidyl inositol · ^{32}P-labeled phospholipids

SEVERAL GROUPS (1–6) have studied the fate of yolk lipids during the development of the chick embryo. The findings of these groups may be summarized as follows: (*a*) yolk contains a rich supply of phospholipid which decreases while embryo phospholipid increases; (*b*) hydrolysis of lipid-phosphorus to form inorganic phosphorus occurs in the yolk, (*c*) substances labeled with ^{32}P and ^{14}C are incorporated into embryo phospholipids, but ^{32}P is not significantly incorporated into yolk phospholipids; (*d*) some cholesterol and triglycerides are transported into embryo tissue without degradation and resynthesis. The questions of how much embryonic phospholipid may be synthesized de novo and whether variation occurs between individual compounds remain obscure. In addition, except for the work of Budowski, Bottini, and Reiser (5), little is known concerning the variations that might occur in phospholipid synthesis at different sites (such as different organs or the circulatory systems of the embryo).

In the work reported here, parameters have been measured which provide information concerning the time, morphological site, and quantity of phospholipids synthesized de novo in the developing chick embryo. The results are consistent with a mechanism in which part of the embryo phospholipid originates from intact yolk phospholipid. The transfer of intact yolk phospholipid apparently occurs more readily at later stages of development.

Abbreviation: TLC, thin-layer chromatography.

MATERIALS AND METHODS

The sources of fertile eggs and chemicals are indicated in the preceding paper (7). In addition, carrier-free sodium phosphate-^{32}P was purchased from Nuclear Consultants Corp., Glendale, Calif. Glycerol-1,3-^{14}C (2 mc/mmole) and sodium acetate-1-^{14}C (50 mc/mmole) were obtained from New England Nuclear Corp., Boston, Mass. The extraction, aqueous KCl washing, separation, and characterization of phospholipids were performed as described in the preceding paper (7). The isolated fractions were rechromatographed where necessary in order to obtain the purest fractions possible, as tested by TLC. Tubes corresponding to the leading and trailing edges of a column peak were not included in the combined fraction.

Phosphorus Assay in the Aqueous KCl Layer

Total phosphorus and inorganic phosphorus (P_i) in the presence of phosphate ester were determined by the method of Bartlett (8). The nonlipid P occurring in the KCl washings in this procedure was separated into an organic and an inorganic fraction and ^{32}P was determined in each fraction. The aqueous KCl solution was evaporated at 40° to half or less of its original volume and then diluted to a known volume for analysis of P_i and total P. Duplicate aliquots of this solution were 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 (9). The precipitate was collected, washed with dilute base, and dissolved in dilute HCl for determination of radioactivity.

Assay for ^{32}P

The radioactivity of phosphorus compounds which were isolated in either aqueous or organic solvents was determined as follows: to a 1 ml aliquot of the ^{32}P fraction to be assayed was added 1 ml of a solvent blank (which was H_2O if the ^{32}P was added in chloroform–methanol 2:1 (v/v), or chloroform–methanol 2:1 when the ^{32}P sample was in H_2O). This procedure eliminates the necessity of corrections for variations in counting due to differences in the final composition of the scintillator solution. To this 2 ml of solution was added 10 ml of a modified (40 g of naphthalene per liter instead of 60 g/liter) Bray's solution (10) that was less than 10 days old. The vials were counted in a Packard Tri-Carb scintillation counter. The counting efficiency, which was approximately 50%, was independent of the chemical bonding of phosphorus or concentration of the ^{32}P compound in the range 0–5 μmoles of lipid P per vial.

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

In these experiments, carrier-free sodium phosphate-^{32}P was diluted with sufficient sterile chick Ringers solution to permit the injection of 2–16 μc of $^{32}P_i$ per egg. Within each group of eggs of the same age which were injected on the same day, the same dose of $^{32}P_i$ was administered to each egg via a 50 μl syringe (Hamilton Co., Whittier, Calif.). The $^{32}P_i$ was injected into the yolk in such a manner as to avoid injection directly into the embryo. The injection of 100 μc of $^{32}P_i$ into a single egg had no visible effect on the development of 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 and after all the eggs were injected they were reincubated.

The injection solution was diluted 1000-fold and a 1 ml aliquot was counted at the same time as the isolated experimental samples. Normalized specific activities

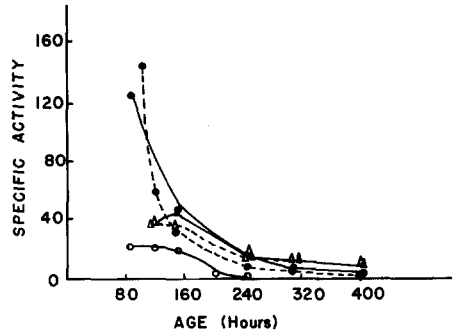

FIG. 1. Normalized specific activities (see definition in text) of yolk, embryo, and brain P_i and embryo and brain phospholipid (PL) with age of incubation. The first point for P_i of the whole embryo was estimated from specific activities of P_i from 96- and 110-hr embryos. The embryos were injected after 43 hr of incubation. Solid line, P_i; dashed line, PL. Symbols are: ● whole embryo, △ brain, and ○ yolk. Whole embryo PL values are corrected so as to exclude brain PL.

(used in Tables 1, 2, 4, 5, 6, 10, and 11 and in Fig. 1) of individual phosphorus compounds were determined by using the following formula:

Normalized Specific Activity =

$$\frac{\text{cpm of fraction}/\mu\text{moles P in fraction}}{\text{cpm of injected solution}/1000} \times 100.$$

Allowing for all the errors in counting and phosphorus determination, the normalized specific activity values fall within 5% of the most accurate value which could be determined for each sample.

Preparation of Labeled Phospholipids for Injection

$^{32}P_i$ (48×10^4 dpm) and glycerol-1,3-^{14}C (7.0×10^4 dpm) were injected into 30 eggs (145-hr old) and the eggs were incubated for an additional 99 hr. The embryos were pooled and a ^{32}P-labeled phosphatidyl choline fraction was isolated by column chromatography on DEAE-cellulose and rechromatography on silicic acid–Hyflo (7). No ^{14}C was found in this phosphatidyl-^{32}P choline fraction.

In another attempt to prepare doubly-labeled phospholipid, $^{32}P_i$ (3.3×10^9 dpm) and acetate-1-^{14}C (1.3×10^9 dpm) were injected into 34 eggs (120-hr old) and the phospholipids were isolated from whole embryos after reincubation for an additional 118 hr. A ^{32}P-labeled phosphatidyl ethanolamine was prepared by column chromatography on silicic acid–Hyflo and rechromatography on silicic acid–ammonia (7). This phosphatidyl ethanolamine fraction (as well as other phospholipid fractions) contained negligible amounts of ^{14}C. About 0.6% of the added ^{14}C was found in the neutral lipid fraction. These low levels of ^{14}C incorporation prevented the biological preparation of doubly-labeled phospholipid by this means.

TABLE 1 RATIOS OF THE SPECIFIC ACTIVITY OF PHOSPHORUS FRACTIONS ISOLATED FROM CHICK EMBRYOS

$^{32}P_i$ was injected into 43-hr old embryos.

Time after Injection	Brain PL* / Embryo PL	Brain Pi / Embryo P_i†	Brain PL / Brain P_i	Embryo PL / Embryo P_i†
hr				
75	0.51	—	1.4	—
101	1.1	1.0	1.1	0.94
116	1.3	1.1	1.5	—
165	1.4	0.87	1.5	0.96
203	1.3	0.89	1.5	1.1
265	1.5	1.5	1.9	1.8
360	2.1	1.8	1.6	1.5
384	2.3	2.9	1.2	1.5

* PL = phospholipids.
† Embryo P_i includes brain P_i in this column.

TABLE 2 RELATIVE SPECIFIC ACTIVITIES OF PHOSPHORUS FRACTIONS IN CHICK EMBRYO

Age of Embryo	Specific Activity of Phosphorus Fraction Relative to Yolk P_i: Yolk P_i*	Embryo P_i	Embryo PL†	Brain P_i	Brain PL
hr					
88	1.0	4.6	5.3	—	—
144	1.0	2.4	2.3	2.5	2.7
208	1.0	230.	220.	200.	310.
244	1.0	72.	78.	65.	100.
378	1.0	20.	77.	77.	165.

* Although the specific activity of yolk P_i declines with age, its value is set at 1.0 at each age to permit a comparison of activities of different ^{32}P pools at the designated ages.
† PL = phospholipid.

RESULTS

Injection of $^{32}P_i$ into 43-hr old Embryos

In these experiments $^{32}P_i$ was injected into the yolk of eggs which had been previously incubated for 43 hr. At various subsequent times of incubation, the phospholipids were extracted from yolk, whole embryo, and brain and analyzed. Specific activities of the individual phospholipid fractions, of the total water-soluble phosphorus, and P_i were determined.

The ratios of the specific activity of various phosphorus-containing fractions isolated from the embryo or brain are given in Table 1. The specific activity of yolk P_i relative to other phosphorus fractions is shown in Table 2. The total incorporation of $^{32}P_i$ into embryo and brain is given in Table 3. The percentage incorporation for different batches of eggs over comparable time intervals varied by about 10% in the latter half of incubation and up to 20% in the first half. The change in the specific activity of P_i and phospholipids during development is shown for yolk, whole embryo, and brain in Fig. 1. If the specific activity is plotted for individual phospholipids, the relation of the brain curve to the embryo curve is very similar and the interpretation of the data would be qualitatively the same for individual phospholipids as for the whole extract.

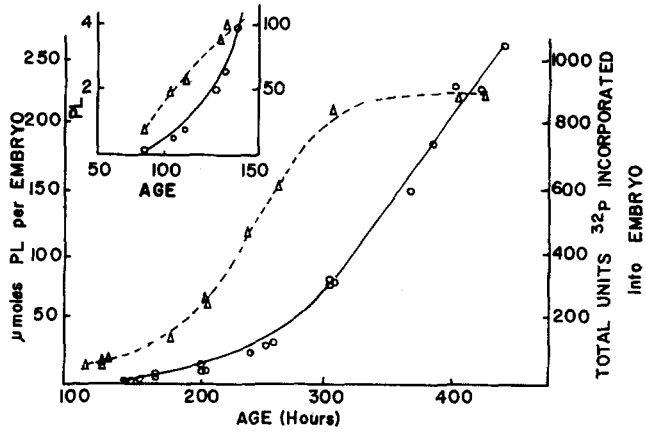

FIG. 2. Total quantity (in micromoles) of phospholipid (PL) and total "units" of ^{32}P per embryo appearing during the incubation period when $^{32}P_i$ was injected at 43 hr. "Units" are the normalized specific activity of PL multiplied by number of µmoles of lipid P, i.e., "normalized cpm." O—O, PL; △---△, ^{32}P.

The increase of phospholipid per embryo and total incorporation of ^{32}P per embryo have been plotted against time in Fig. 2. The same information is shown for brain in Fig. 3.

The specific activities of different phospholipids isolated from the whole embryo are given in Table 4 and those of the brain phospholipids in Table 5. Specific activities, when determined in individual tubes of a column peak, were about the same as for a pooled peak. Variation in specific activity with fatty acid composition was not apparent. It is apparent that in the whole embryo the specific activities of phosphatidyl choline and sphingomyelin were consistently lower than the specific activity of the other phospholipids. A measure of bio-

TABLE 3 PERCENTAGE OF INJECTED ^{32}P INCORPORATED INTO PHOSPHOLIPID OF INCUBATED CHICK EMBRYOS*

Whole Embryo: Age	Whole Embryo: % Radioactivity Incorporated	Brain: Age	Brain: % Radioactivity Incorporated
hr		*hr*	
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

* Injection time was 43 hr.

TABLE 4 SPECIFIC ACTIVITY OF PHOSPHOLIPID FRACTIONS OBTAINED FROM WHOLE CHICK EMBRYOS INJECTED AT 43 HR WITH $^{32}P_i$

Age of Embryo†	Phospholipid Fraction*							
	PE-2	PE-3	PS-2	PS-3	PI-5	PC-5	PC-6	SPM-7
hr								
209	16	16	17	17	18	14	14	11
259	13	14	14	6.1	12	—	11	8.4
309	7.6	8.4	8.1	4.5	—	—	5.6	3.4
386	3.9	3.4	2.8	—	—	2.2	—	1.7
451	2.2	2.2	2.5	1.7	1.1	0.84	2.2	—

* The designation of the fraction is by peak number on silicic acid–Hyflo columns. Specific activities were measured after phospholipids were separated by column chromatographic methods which have been described (7). Abbreviations: PE, phosphatidyl ethanolamine; PS, phosphatidyl serine: PI, phosphatidyl inositol: PC, phosphatidyl choline; and SPM, sphingomyelin.
† Time of hatching was about 500 hr.

TABLE 5 SPECIFIC ACTIVITIES OF PHOSPHOLIPID FRACTIONS ISOLATED FROM CHICK EMBRYO BRAIN INJECTED AT 43 HR WITH $^{32}P_i$

Age of Embryo	Phospholipid Fraction*									
	CA-1	PE-1	PE-2	PE-3	PS-2	PI-4	PI-5	PC-5	PC-6	SPM
hr										
Experiment† 1										
118				45		41			39	35
144	64			49		53			42	44
244	23			26		23			24	21
308	20			16		14			13	15
403									8	
Experiment 2										
159	57	47	44	55	48	39	40		36	32
378	7.3	9.2	10			8.1	8.7	8.0	8.0	7.9
Experiment 3										
208		36				7		24		30
427		8.2				7.9		6.3	8.5	7.6

* Designation of the phospholipid fractions and abbreviations are as in Table 4; in addition CA = cardiolipin.
† An experiment means a set of eggs which were injected at the same time and incubated together initially.

logical variation from experiment to experiment is afforded in Table 5 by comparison of specific activity values of the same phospholipid fractions at nearly the same ages. The specific activity of yolk phospholipid was about 1/10,000 of embryo phospholipid except toward the latter part of the incubation when this value rose to about 1/5000 of the specific activity of the embryo phospholipids. Hevesy, Levy, and Rebbe (1) noted a similar trend.

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

In another series of experiments, $^{32}P_i$ was injected into separate batches of eggs which had been previously incubated for either 0, 215, 288, or 400 hr. The phospholipids of embryo tissue were then analyzed on the second, third, or fourth day after the injection. The specific activity of various phosphorus compounds was determined and the results are given in Table 6. Yolk phospholipid did not incorporate a significant amount of activity in these experiments.

Studies with Glycerol-1,3-^{14}C

Into each of 100 eggs which had been incubated for 42 hr was injected 50 μl of a chick Ringers solution containing glycerol-1,3-^{14}C (6.8×10^4 cpm/egg). The eggs were reincubated and from some of the eggs, after 47 and 124 hr of total incubation time, various fractions were isolated from the yolk (less albumen and extraembryonic membranes). In addition, brain tissue was removed from the remaining eggs after a total of 210 and 354 hr incubation and analyzed. The results summarized in Table 7 indicate that glycerol is metabolized in yolk, but is not extensively incorporated into lipid fractions of the brain.

Injection of Labeled Phospholipids

The ^{32}P-labeled phosphatidyl choline (see Methods) was dissolved in chloroform–methanol 2:1. A known portion of this solution was evaporated to 5 ml and added to 10 ml of water containing 2% Tween 20. A nearly clear emulsion of phosphatidyl choline was obtained after re-

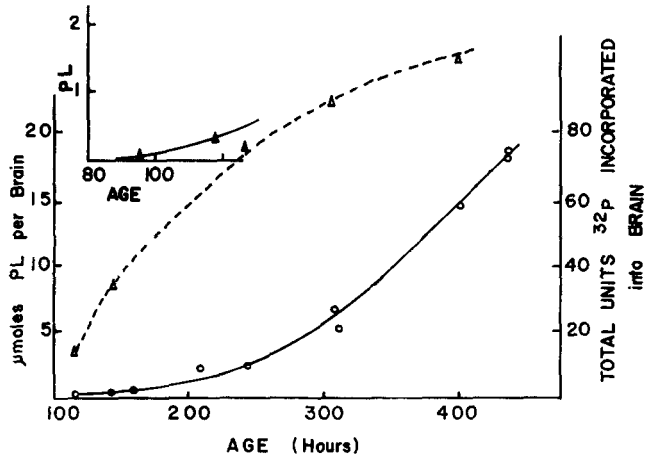

FIG. 3. Total amount (in micromoles) of phospholipid (PL) and total units of ^{32}P per brain appearing during the incubation period when $^{32}P_i$ was injected at 43 hr. For definitions of symbols see Fig. 2. Insert, magnification of early time period.

moving nearly all organic solvent by gentle heating under nitrogen. This emulsion was then injected into 47 eggs which had been previously incubated for 280 hr. Twenty-two micromoles of the ^{32}P-labeled lipid (ca. 13,000 cpm/µmole P) were added per yolk (this is approximately 2% of the total phosphatidyl choline in yolk at this age). After 144 hr of further incubation, phospholipids were extracted from brains and from embryo tissue less brain. Results of this experiment are given in Table 8. The phosphatidyl choline (PC-1) isolated by the same procedures as the phosphatidyl choline that was injected had a much higher specific activity than did other phospholipids or water-soluble phosphorus compounds.[1] This particular fraction represented approximately 9% of the total phosphatidyl choline. The major fraction of phosphatidyl choline (PC-2) was eluted from the silicic acid–Hyflo column after the fraction with the high specific activity and had a much lower specific activity. Analysis by TLC indicated that the injected phosphatidyl choline and PC-1 were less polar than PC-2. Of the added ^{32}P radioactivity, 0.4% was incorporated into brain phospholipid.

In another experiment purified phosphatidyl-^{32}P ethanolamine (102,000 cpm/µmole P) was emulsified in 10 ml of 1% Tween 20 (as above) and injected into 19 336-hr old eggs. After 425 hr of total incubation, phospholipid fractions were isolated from brain. The results are shown in Table 9. The phosphatidyl ethanolamine fraction with the highest specific activity did not emerge on the silicic acid–NH_3 column in the same place as the phosphatidyl ethanolamine which was injected, but in the second peak from this column. This result would be expected if the phosphatidyl ethanolamine had become oxidized during handling. This peak also contained minor amounts of phosphatidyl serine (2–4%). Since the phosphatidyl serine occurs in such a low amount, and since all the fractions containing phosphatidyl ethanolamine had a relatively high specific activity (Table 10), it is unlikely that the high specific activity of the phosphatidyl ethanolamine was due to this minor contaminant. Since the fatty acid constituents were not determined, the possibility of an exchange of these constituents cannot be eliminated. In this experiment, 1.4% of added ^{32}P radioactivity was incorporated into brain phospholipid.

[1] The techniques of extraction, washing and column chromatography eliminate nonlipid phosphorus. Thin-layer chromatograms of the high specific activity fraction showed only phosphatidyl choline.

TABLE 6 ANALYSIS OF VARIOUS FRACTIONS AFTER INJECTION OF $^{32}P_i$ INTO EMBRYOS AT DIFFERENT AGES AND FURTHER INCUBATION

Injection Time	Incubation Time Following Injection	Phosphorus Fraction Contains:*	Source	Specific Activity
hr	*hr*			
0	96	PE (ca. 90%), PS, CA	Brain	31
		PC (ca. 90%), PI	Brain	21
		SPM (ca. 60%), PC	Brain	14
		Inorganic P	Brain	28
		All H_2O Sol. P	Brain	33
215	92	CA (ca. 80%), PS	Brain	9.0
		PE (ca. 90%), PS	Brain	9.9
		PE (ca. 90%), PS	Brain	10
		PC (ca. 90%), PI, PS	Brain	9.7
		SPM (ca. 60%), PC	Brain	9.6
		Phospholipid extract	Embryo	7.4
		Inorganic P	Brain	17
		Inorganic P	Embryo	5.3
288	48	Phospholipid extract	Brain	1.2
		Inorganic P	Brain	3.2
		All H_2O sol. P	Brain	2.1
		All H_2O sol. P	Blood	14
		Inorganic P	Blood	10
	92	Phospholipid extract	Embryo	1.0
		Inorganic P	Embryo	1.5
		Phospholipid extract	Brain	1.3
		Inorganic P	Brain	1.6
		All H_2O sol. P	Brain	1.6
400	70	Phospholipid extract	Brain	1.9

* Phospholipids were not completely separated in these experiments; the phospholipid extract was either chromatographed on silicic acid–Hyflo once or not chromatographed at all. The percentage composition of individual phospholipids at different incubation ages is given in the previous paper (p. 552). Approximate amounts of individual phospholipids in this table were estimated by spot size on thin-layer chromatograms.

DISCUSSION

The work of Hevesy et al. (1) and of Branson, Brooks, and Piper (2) showed that P_i present in yolk is a precursor of embryonic lipid P. The work reported extends

TABLE 7 DISTRIBUTION OF LABEL FROM GLYCEROL-1,3-^{14}C INTO YOLK AND CHICK EMBRYO BRAIN.

Injection was at 42 hr. Incubation.

Fraction	Extraction Time	% of Added Counts Found in Fraction
Yolk	*hr*	
Phospholipid	47	1.5*
Neutral lipid	47	1.5
Water-soluble acids†	47	2
Water-soluble amines†	47	4
Glycerol‡	47	92
Lipid (neutral and phospholipid)	124	1.3
Water-soluble acids†	124	3
Water-soluble amines§	124	39
Glycerol‡	124	49
Brain		
Lipid extract	210	0.7
Fatty acid	210	0.5
Deacylated lipid moieties	210	0.2
Water soluble acids and amines	210	0.01
Glycerol‡	210	0.1
Lipid extract	354	0.9
Water-soluble compounds	354	1.4

* This value may be partially due to impurities in this fraction.

† Obtained by ether extraction of the acidified aqueous KCl wash of the lipid extract.

‡ Non-ionic, water-soluble fraction which chromatographs on paper like glycerol.

§ Fraction retained on cation-exchange column.

TABLE 8 INCORPORATION OF INJECTED ^{32}P-LABELED PC INTO EMBRYO AND BRAIN PHOSPHORUS FRACTIONS*

Injection at 280 hr and extraction at 424 hr

Phosphorus Fraction	Specific Activity†
Water-soluble P from brain	10
Embryo phospholipid extract	3
Embryo P_i	43
Brain P_i	15
PE (plus small amount of PS)	38
CA	59
PE-1 (first PE peak from the column)	6
PE-2 (second PE peak from the column)	225
PC-1	1600
PC-2, SPM	38

* See Tables 4 and 5 for abbreviations.

† Specific activity is $\frac{\text{cpm}/\mu\text{mole P}}{\text{cpm of aliquot (0.1) of injected PC}}$.

this to individual phospholipids. In addition, the results of our experiments permit us to approach the important question of how much of the synthesis is de novo and how much is due to direct transfer of yolk phospholipid to the embryo. Making certain assumptions, the extent of de novo synthesis from P_i may be estimated from the net amount of phospholipid synthesized, the specific activity of the phosphorus pool from which the phospholipid is made, and the total incorporation into phospholipid.

The data shown in Tables 10 and 11 were obtained from the curves in Figs. 1, 2, and 3. The method of calculation is indicated in the footnotes to the tables. The interpretation of the data given here is facilitated by the fact that the curves in Figs. 1–3 are quite regular, with no sharp fluctuations.

The values in the last column of Tables 10 and 11 are assumed to represent the fraction of phospholipid which is synthesized de novo in a given incubation interval in whole embryo and brain respectively. Although the number is calculated on the basis of the average specific activity of extracted P_i in the time interval designated (assuming that this P_i represents the P_i which is incorporated into phospholipids), other water-soluble ^{32}P-phosphates would be part of the phosphorus pool available for phospholipid synthesis. Experimentally, it was found that the P_i and water-soluble organic phosphorus in whole embryo (which would include nucleotides, sugar phosphates, and the common intermediates of phospholipid synthesis) had nearly the same specific activities. The water-soluble organic phosphorus in brain usually had a specific activity slightly greater than that of P_i. The molar ratio of P_i to brain lipid P is about 1:6 and the P_i :ATP ratio in brain is about 2:1 (reference 11, page 7). An intermediate such as glycerophosphoryl choline would represent a small fraction (on a molar basis) compared to the lipid P in brain. In rat, for example, glycerophosphoryl choline represents only 0.8% of the brain fresh weight (12). Phospholipid in chick brain represents about 10% of the fresh weight. Since the size of the water-soluble phosphorus pool is quite small, it is unlikely that there would be enough label in the water-soluble phosphorus pool (excluding

TABLE 9 INCORPORATION OF INJECTED ^{32}P-LABELED PE INTO BRAIN PHOSPHORUS FRACTIONS*

Injection at 336 hr and extraction at 425 hr.

Phosphorus Fraction	Specific Activity†
CA, PE (first peak from a silicic acid column)	5.2
PE (isolated like donor PE; silicic acid–Hyflo, then silicic acid–NH_3)	2.1
PE, PS (minor)—highest specific activity peak	32
PC, PI (minor)—fourth peak from a silicic acid column	0.83
PC peak from silicic acid (major fraction)	0.16
SPM, PC (minor)—last eluted peak from silicic acid	1.7
Water-soluble P (excludes P_i)	0.22
P_i only	1.1

* See Tables 4 and 5 for abbreviations.

† Specific activity here is $\frac{\text{cpm}/\mu\text{mole P}}{\text{cpm of aliquot (0.1) of injected PE}}$.

P_i) to account for the label that did become incorporated into phospholipid.

There is considerable evidence that certain small ions do not pass rapidly through the "blood–brain barrier." Table 1, column 3, provides some evidence that brain P_i does not mix with embryo P_i. In mouse brain, Heald reports (reference 11, page 17) that the equilibrium between water-soluble phosphorus in blood and brain requires 7 days. Webster (13) found that in normal chicken after 3 days, acid-soluble phosphorus in plasma and brain had not equilibrated. Changus, Chaikoff, and Ruben (14) have shown that young rat brain incorporated $^{32}P_i$ more rapidly than at older ages. The data in Table 6, for injection into 288-hr old embryos, show that specific activities of water-soluble phosphorus in blood and brain differ 7-fold after 48 hr. Table 6 also shows that $^{32}P_i$ injected into yolk at early and late ages is assimilated by the embryo and embryo brain. Specific activity values in Table 6 are of the same order of magnitude as when $^{32}P_i$ was injected at 43 hr, indicating that there are no barriers to P_i passage from yolk to embryo at later ages.

Summarizing at this point, the figures in the last column of Tables 10 and 11 represent the fraction of ^{32}P in phospholipid from whole embryo and brain respectively that arises from P_i and water-soluble phosphorus of embryo and brain. In view of the approximations made, these values must be regarded more as showing a trend rather than defining rigidly the fraction of de novo synthesis.

TABLE 10. RELATIONSHIP BETWEEN INCREASE OF PHOSPHOLIPIDS AND INCORPORATION OF $^{32}P_i$ INTO EMBRYO PHOSPHOLIPIDS DURING INCUBATION

Incubation Interval	Δ μmoles of PL per embryo	Δ Units of ^{32}P in PL	Specific Activity of Embryo P_i	μmoles of ^{32}P incorporated / Δ μmoles of PL per embryo
hr				
0–100	0.25	31	ca. 160	ca. 0.8
100–150	2.4	74	75	0.4
150–200	6.8	115	38	0.4
200–250	21	245	24	0.5
250–300	41	265	12	0.5
300–350	64	145	5	0.5
350–400	72	30	2.5	0.2
400–450	65	20	2.0	0.2

The columns are: 1, the absolute incubation time interval for the eggs; 2, the net increase in μmoles of phospholipid (PL) in the interval designated; 3, the net increase in total ^{32}P units per embryo (see Fig. 2) in the interval designated; 4, the average specific activity of the P_i over the incubation interval designated as determined from the midpoint of the specific activity curve for P_i (Fig. 1); and 5, the apparent fraction of PL which has been synthesized from P_i and water-soluble phosphates.

TABLE 11 RELATIONSHIP BETWEEN INCREASE OF PHOSPHOLIPIDS AND INCORPORATION OF $^{32}P_i$ INTO BRAIN PHOSPHOLIPIDS DURING INCUBATION

Incubation Interval	Δ μmoles of PL per brain	Δ Units ^{32}P in PL	Specific Activity P_i Brain	μmole ^{32}P incorporated / Δ μmole PL/brain
hr				
100–150	0.8	34	40	ca. 1.
150–200	0.8	25	36	0.9
200–250	1.3	15	21	0.5
250–300	2.2	11	12	0.4
300–350	4.0	8	8	0.3
350–400	4.7	8	6	0.3
400–450	4.8	6	4	0.3

The columns have the same meaning as those in Table 10, except that the data pertain to the brain (see Figs. 1 and 3).

Since it was not possible to obtain substantial incorporation from glycerol-1,3-^{14}C or acetate-1-^{14}C, determination of the extent of de novo synthesis from other precursors of phospholipids is precluded. The experiments with labeled glycerol and acetate do suggest that much of the incorporation of glycerides from yolk occurs without substantial degradation.

The possibility of incorporation of intact phospholipid from the yolk into embryo phospholipids is suggested by the experiments with ^{32}P-labeled phosphatidyl choline and phosphatidyl ethanolamine. When phosphatidyl-^{32}P choline was injected into yolks of eggs which had been incubated for 280 hr and the phospholipids were isolated by the same chromatographic procedure used to prepare the donor phosphatide, the specific activity of this fraction was 40 times greater than that of brain P_i or of other phospholipid fractions, except for one phosphatidyl ethanolamine fraction which had an intermediate value. Similar results (albeit less dramatic) were obtained with ^{32}P-labeled phosphatidyl ethanolamine.

Two schemes to explain the source of brain phospholipids may now be considered. The numbers indicate, in micromoles of phosphorus, pool sizes at the time that phosphatidyl-^{32}P choline (PC-^{32}P) was injected. Data on pool size are taken from published work (7, 15–17).

Scheme I

PC-^{32}P injected
22
↙
PC-yolk → P_i-yolk → P_i-embryo
1180 63 570
P_i-embryo → P_i-brain[2] → PC-brain
570 22 10

Scheme II

PC-^{32}P injected
22
↙
PC-yolk → PC-embryo → PC-brain
1180 150 10

[2] From unpublished results.

TABLE 12 Pool Sizes (in Micromoles) of Phosphorus Compounds in the Incubated Egg during the Incubation Period

Age	Yolk				Embryo	
	Wet Wt.*	Lipid P†	P_i*	Water-Soluble P‡	P_i*	Lipid P§
days	*g*					
0	17.5	2100	Trace	61	0	0
5	37.2	2040‖	107	61	6	1.5
7	27.6	1800	97	48	19	4.8
9	19.9		96	77	31	17
11	16.6		106	60	140	46
13	7.7	1700	32	23	330	100
15	8.4		16	16	615	183
16		1600				200
17	11.6		16	16	955	230
19	6.3	1210	16	9.6	1310	270
20	4.2			9.6		250
21		300	9.7		1320	

* Data from Kugler (16, 15).
† This column was determined from data of Kugler (16).
‡ This is water-soluble P exclusive of P_i, as determined by Kugler (15).
§ Our data.
‖ Our value.

If Scheme I operates exclusively, the added phosphatidyl-^{32}P choline would be hydrolyzed and the results would be similar to those in experiments when P_i was added to yolk (Tables 4–6). Since the same phosphatidyl choline fraction that was added to yolk had the highest specific activity in brain, an obvious conclusion is that scheme II is at least partially active.

Another observation of interest is that donor phosphatidyl-^{32}P choline was diluted only about 8-fold after injection (as determined from the specific activities of the donor phosphatide and that obtained from the injected embryos); phosphatidyl-^{32}P ethanolamine was diluted 30-fold. Thus, as may be seen from the pool size of phosphatidyl choline in yolk, there was apparently little mixing of injected phospholipid with yolk phospholipid, and thus incorporation of injected phospholipid was probably rapid.

When $^{32}P_i$ was added to yolk, the specific activity of P_i isolated from yolk declined slowly from 80 to 160 hr incubation, then dropped to a very low level and did not fall appreciably after that. This decline does not correspond either to the measured amount of P_i in yolk during incubation (15) or to the specific activity of embryo phospholipid. Although the situation is complicated by changing pool sizes, if all yolk phospholipid were converted to hydrolytic products and the P_i thus derived were used to make embryo phospholipid, the specific activity of yolk P_i should have been nearer to (and parallel in rate of decrease to) the specific activity in embryo phospholipid. Actually, the specific activity in embryo phospholipid was close to that of P_i extracted from embryo and far removed from that of yolk P_i in the latter three-quarters of the incubation period (Table 2). Table 12 shows specifically that transfer of phosphorus from yolk to embryo does not depend on the build-up of yolk P_i. Since the yolk P_i pool is quite small in later embryonic stages (Table 12) it is difficult to account for the large build-up of embryonic P_i without organic phosphorus getting into the embryo. A good deal of yolk phospholipid might be hydrolyzed in the liver or blood and the P_i used for various purposes such as bone formation in the embryo.

From the data reported here, it seems reasonable to conclude that yolk phospholipid is to some extent transported intact, or at least without breaking phosphodiester bonds, to embryo and brain tissue. There is also evidence from Budowski, Bottino, and Reiser (5) which is consistent with the hypothesis that triglycerides are transported from yolk to the brain without intermediate breakdown.

Finally, it seems plausible to suppose that as the embryo develops and occupies more space in the egg, and as the membranes with a substantial circulatory system engulf the yolk, more phospholipid would become accessible to uptake by the blood. Prior to hatching, the yolk sac becomes continuous with the gut and is drawn inside the embryo (note column 3 of Table 12). Davison, Dobbing, Morgan, and Wright (4) have pointed out that histologically it has been demonstrated that the chick liver parenchymal cells become heavily laden with lipid in the latter embryonic stages. The fact that turnover of phospholipid in liver is rapid compared to that in brain and other organs has led Davison to suppose that phospholipids are synthesized in the liver and transported via the blood to other organs.

A somewhat oversimplified scheme to summarize these relations is presented in Fig. 4. From the available evidence, several reasonable conclusions may be drawn

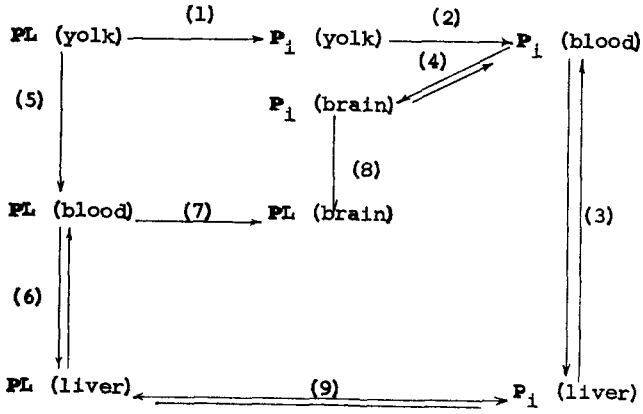

FIG. 4. Synthesis of phospholipid (PL) or transport of P_i and PL within the developing chick embryo. Steps are numbered 1–9 for easy reference in the text.

concerning the several reactions: namely, reaction 1 is irreversible and in the initial part of the incubation period produces a relatively small pool of P_i in the yolk; reaction 2 does not appear to account for the major amount of embryo phosphorus after 5–6 days of incubation; reactions 3 and 6 are fast in comparison to the other reactions. The sequence 1, 2, 4, 8 seems to account for most of the brain phospholipid up to about 200 hr of incubation, after which time the sequence 5–7 becomes increasingly important.

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