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Phospholipid Metabolism in the Eggs and Embryos of the Sea Urchin *Arbacia punctulata*†

Eli Schmell and W. J. Lennarz*

ABSTRACT: The incorporation of labeled phospholipid precursors into the phospholipids of *Arbacia punctulata* eggs and of embryos prior to the first cell cleavage has been investigated. Incorporation of [^{3}H]choline into phosphatidylcholine was not detected in either eggs or embryos although fertilization resulted in a fourfold stimulation of [^{3}H]choline uptake into the cells. Both eggs and embryos, when incubated with [^{3}H]ethanolamine, incorporated radioactivity into phosphatidylethanolamine.The incorporation by unfertilized eggs was three to four times greater than that observed in embryos. However, when eggs were preincubated with [^{3}H]ethanolamine and subsequently fertilized, the resultant preloaded eggs and embryos behaved identically in the incorporation of ethanolamine into phosphatidylethanolamine. In contrast, when $^{32}P_i$ or [^{3}H]inositol was used as a lipid precursor, embryos were markedly more active than eggs in incorporation of these compounds into phospholipids. With both precursors the major reason for the marked difference in incorporation between eggs and embryos was related to an increased ability of the embryos to transport the labeled precursor. Incorporation of $^{32}P_i$ into phospholipids was highly specific in both eggs and embryos. Ninety-five per cent of the $^{32}P_i$ incorporated into phospholipid was found in the phosphoinositides: phosphatidylinositol, diphosphoinositide, and triphosphoinositide. The most highly labeled lipid was diphosphoinositide. When eggs and embryos were incubated in [^{3}H]inositol, radioactivity was incorporated into the three phosphoinositides. Although in both cases the major inositol-labeled lipid was phosphatidylinositol, when eggs were preloaded with [^{3}H]inositol and then fertilized, an increase in the proportion of label in the polyphosphoinositides was detected in the resultant embryos, when compared to the unfertilized eggs.

In recent years a wealth of information has accumulated on the many changes that occur in the eggs of the sea urchin upon fertilization (reviewed by Giudice, 1973). In general terms, these changes may be classified into two categories. One category includes changes in metabolic activities within the cytoplasm, such as an increase in respiration and a markedly enhanced rate of protein synthesis. The other category encompasses changes that are likely to be related to alterations in the properties of the plasma membrane of the egg. These changes include: (a) a marked change in the membrane potential; (b) extrusion of H^+; (c) uptake of Ca^{2+}; (d) activation of a Na^+-dependent transport system for several amino acids; (e) development of a K^+-dependent membrane potential; and (f) activation of a transport system for inorganic phosphate (reviewed by Giudice, 1973; Epel *et al.*, 1969).

A unique aspect of a number of these membrane-associated changes is that once the alteration has been triggered by fertilization it remains a permanent cell property. For example, P_i uptake is markedly enhanced in embryos and is linear throughout several cell cleavages (Brooks and Chambers, 1954). Amino acid and nucleoside transport behave similarly, at least until the first cell cleavage (Giudice, 1973). Thus, the plasma membrane of the embryo is locked in an active form, whereas the plasma membrane of the egg remains in an inactive state. This unique situation should prove convenient for studying the chemical and enzymatic differences in the membrane that may be brought about by fertilization of the egg cell.

As a prelude to such a comparative study of the two membranes, we have studied the incorporation of a variety of lipid precursors into membrane phospholipids of the egg and the embryo prior to the first cleavage. These studies revealed marked differences in the efficacy of incorporation of different lipid precursors into egg and embryo lipids. In addition, a pro-

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nounced specificity for the incorporation of inorganic phosphate into the different classes of lipids was observed.

Materials and Methods

Materials. ^{3}H-Labeled *myo*-inositol (2.83 Ci/mmol), [^{3}H]choline chloride (2.34 Ci/mmol), [^{14}C]phosphorylcholine (23 mCi/mmol), and carrier-free $^{32}P_i$ were purchased from New England Nuclear Corp. [^{3}H]Ethanolamine (320 mCi/mmol) was purchased from Amersham-Searle. [^{3}H]Leucine (35.5 Ci/mmol), [^{3}H]lysine (55.0 Ci/mmol), and [^{14}C]phenylalanine (383 mCi/mmol) were purchased from New England Nuclear. Penicillin G, streptomycin sulfate, bovine pancreatic trypsin, and *sn*-glycerol 3-phosphate were purchased from Sigma. *Escherichia coli* alkaline phosphatase was purchased from Worthington. Di- and triphosphoinositide from beef brain were generous gifts from Dr. G. Hauser of the McClean Hospital, Boston, Mass., and from Dr. David White of the University of Nottingham, Nottingham, England. All other lipid standards were purchased from Serdary Research Laboratories in London, Ontario, Canada.

Isolation of Gametes and Embryos. Arbacia punctulata were obtained from either Gulf Specimen Co., Inc., or Connecticut Valley Marine Biological Supply Co., Inc., and maintained at 18–19° in aquaria containing Instant Ocean purchased from Aquarium Supplies, Inc. Eggs and sperm were collected by electrical shock as described by Harvey (1956). When gametes were suspended in freshly prepared sea water adjusted to the same pH and density as the aquarium water, the percentage of eggs fertilized was low and variable. Consequently, all experiments and manipulations of gametes were performed in aquarium water to which penicillin G (35 μg/ml) and streptomycin sulfate (50 μg/ml) were added after the water was passed through an ultrafine glass filter.

In all experiments, a packed pellet of eggs (0.2 ml, 4–8 mg of protein), obtained using a hand centrifuge operated at 30–100*g*, was resuspended to 2 ml in fresh sea water. This, and all subsequent steps, were performed at room temperature (18–20°). The final concentration of eggs was approximately 5×10^4–5×10^5 cells per ml. Eggs were dejellied prior to fertilization by adding 15 μl of 0.2 N HCl to adjust the pH to 5.4. After 3 min the acid and jelly coat were removed by washing the cells three times with fresh sea water. Fertilization was induced by addition of 50–100 μl of undiluted sperm, and, after 10 min, the fertilized eggs were washed twice by hand centrifugation and resuspended in sea water. In all experiments involving removal of the fertilization "membranes," 0.1% trypsin was added immediately after fertilization. After 10 min excess sperm and trypsin were removed by hand centrifugation of the embryos. The resulting cells were washed twice with fresh sea water. As a control, the same number of washings and centrifugations were performed on eggs as well as embryos. Except in preloading experiments noted in the text, 15 min after fertilization isotope was added to both eggs and embryos. Throughout the incubations cell suspensions were gently swirled every 3–5 min by hand in order to prevent settling and to maintain adequate aeration.

In all experiments fertilization of at least 95% of the eggs occurred, as adjudged by elevation of the fertilization "membrane" evident upon phase microscopy. In addition, at least 95% of the embryos underwent the first cleavage 90 min after fertilization, although most experiments with embryos were terminated prior to cleavage. This observation, coupled with the enhanced protein synthesis after fertilization described in the Results section, indicated that the procedures employed for fertilization and for incubation of the embryos were suitable for normal development.

Uptake of [^{3}H]Choline into Eggs and Embryos. Eggs and embryos were incubated as described above with 20 μCi of [^{3}H]choline chloride per 2 ml of incubation mixture. At various times 400-μl aliquots of cells were transferred to a test tube containing 10 ml of fresh sea water. The cells were then washed several times by hand centrifugation until the supernatant sea water was free of radioactivity. The resulting pellet of cells was resuspended to 2 ml with fresh sea water and a small aliquot was transferred to a scintillation vial and subjected to brief sonic oscillation in scintillation fluid. The remaining cells were lysed and precipitated by adding 4 ml of 15% trichloroacetic acid (Cl_3CCOOH). The lysed cells were centrifuged for 5 min at 1000*g* and the Cl_3CCOOH-soluble material was removed. The Cl_3CCOOH pellet was washed with 1 ml of 10% Cl_3CCOOH. Both Cl_3CCOOH supernates were pooled and an aliquot was counted to determine radioactivity in the Cl_3CCOOH-soluble fraction. The Cl_3CCOOH pellet was then extracted with 2 ml of $CHCl_3$–CH_3OH (2:1) for 45 min at 37°. The cell residue was pelleted by centrifugation at 1000*g* for 10 min and washed with 2 ml of $CHCl_3$–CH_3OH (2:1). The lipid extracts were pooled and washed with 0.9% saline as described below under Isolation and Chromatography of Lipids, and radioactivity in the lipid and aqueous layers was determined. Radioactivity remaining in the cell residue was determined after drying the residue *in vacuo* to remove any residual organic solvent.

Isolation and Chromatography of Lipids. In order to isolate the lipids, 200-μl aliquots of eggs and embryos (except where noted otherwise) were precipitated by the addition of four volumes of 15% Cl_3CCOOH. The acid-insoluble material was collected by centrifugation and then extracted for 1 hr at 45° in 20 volumes of $CHCl_3$–CH_3OH (2:1) with constant stirring. After filtration through glass wool the lipid extract was washed first with 0.2 volume of 0.9% saline acidified to pH 2.0 with 6 N HCl, and then with 0.2 volumes of $CHCl_3$–CH_3OH–H_2O (3:48:47). The $CHCl_3$ layer was then evaporated to dryness and either counted in Triton-toluene scintillation fluid or stored for chromatography.

Lipids were chromatographed on SG-81 paper treated with EDTA (Steiner and Lester, 1972a,b) using the following solvent systems: (A) $CHCl_3$–CH_3OH–4 N NH_4OH (9:7:2); (B) diisobutyl ketone–acetic acid–H_2O (60:45:6); (C) $CHCl_3$–CH_3OH–H_2O (65:35:8); (D) $CHCl_3$–CH_3OH–H_2O (65:25:4). Labeled lipids were detected by exposing X-ray film to the chromatogram overnight, or longer when necessary. To quantify the individual lipids, the radioactive spots were cut from the chromatogram and counted in Triton scintillation fluid. Standards were detected with rhodamine spray for lipid or molybdate spray for lipid phosphate (Dittmer and Lester, 1964).

In all experiments using $^{32}P_i$ some residual radioactivity, probably inorganic phosphate, remained in the lipid phase even after two washes. It was therefore necessary to chromatograph the ^{32}P-labeled lipids in solvent system A to accurately quantify the extent of lipid labeling. In this solvent system $^{32}P_i$ remains at the origin.

Preparation and Characterization of Deacylated Lipids. Radioactive lipids were deacylated by the method of Lester and Steiner (1968). Authentic phosphatidylinositol, diphosphoinositide, and triphosphoinositide were deacylated by the method of Dittmer and Wells (1969). Deacylated lipids were spotted on S and S green ribbon paper, and developed in two dimensions by the method of Waechter *et al.* (1969). Radioactive glycerophosphoryl derivatives were detected by exposing X-ray film to the paper. Standards were detected with benz-

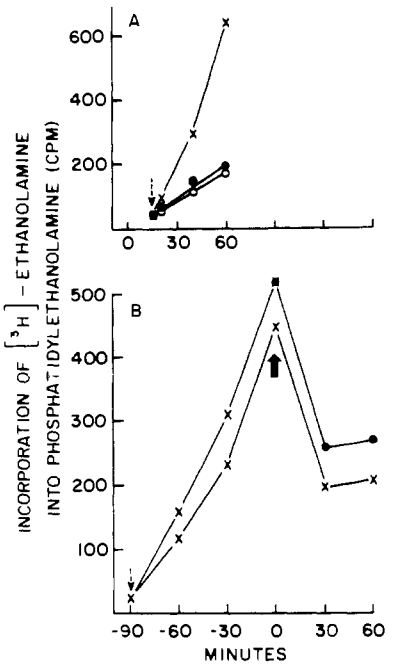

FIGURE 1: Incorporation of [^{3}H]ethanolamine into phosphatidylethanolamine. (A) Eggs and embryos (4.7 mg of protein) were incubated in 2 ml of sea water with 20 μCi of [^{3}H]ethanolamine (320 mCi/mmol), and radioactivity incorporated into phospholipids was determined as a function of time as described under Materials and Methods: (×) eggs; (●) embryos; (○) trypsin-treated embryos. (B) Two sets of eggs (5.0 mg of protein) were incubated with 40 μCi of [^{3}H]ethanolamine (320 mCi/mmol) for 90 min. After 90 min (shown by the arrow) the eggs were hand centrifuged and washed several times, and sperm was added to one tube of eggs. Incorporation of radioactivity into phospholipid was determined as a function of time as described under Materials and Methods: (×) eggs; (●) embryos.

idene-periodate (Wawszkiewicz, 1961) and P_i with Hanes–Isherwood spray (Hanes and Isherwood, 1949). The deacylated lipids were also analyzed by anion exchange column chromatography as previously published (Lester and Steiner, 1968). We are grateful to Dr. R. Lester of the University of Kentucky for performing this analysis. In order to determine if the $^{32}P_i$ incorporated into the phosphoinositides was in the phosphomonoester or phosphodiester linkage the deacylated ^{32}P-labeled lipids were treated with 70 μg of *E. coli* alkaline phosphatase in 100 μl of 5 mM Tris-HCl buffer (pH 8.3) at 37° for 24 hr.

Incorporation of Radioactive Amino Acids into Egg and Embryo Protein. Eggs and embryos were incubated with 20 μCi of [^{3}H]leucine, [^{3}H]lysine, or [^{14}C]phenylalanine. At various times 200-μl aliquots of the cell suspensions were removed and added to 400 μl of 15% Cl_3CCOOH, and the mixture was incubated at 80–90° for 15 min. The suspension was cooled to room temperature and centrifuged, and the Cl_3CCOOH-soluble material was removed. The Cl_3CCOOH-insoluble material was then successively washed with 1 ml of 10% Cl_3CCOOH, 2 ml of ethanol, and 2 ml of ethanol–ether (1:1). Each washing consisted of resuspending the insoluble material in the respective solvent, followed by brief centrifugation and removal of the soluble material. The radioactivity remaining in the pellet after the third wash was determined in Triton–toluene scintillation fluid.

Protein and Phosphate Determination. Protein was determined on sonicated eggs and embryos by the method of Lowry *et al.* (1951). Phospholipid phosphorus and sea water P_i were determined by the method of Bartlett (1959).

Results

Incorporation of Amino Acids into Protein in Eggs and Embryos. One of the most thoroughly documented changes that occurs soon after fertilization is the marked increase in protein synthesis (Epel, 1967). In order to establish that fertilization and development were normal under the conditions utilized in the present study, a series of experiments was performed in which the incorporation of labeled amino acids into protein was measured in eggs and embryos. Using [^{3}H]leucine, a 12-fold increase in the rate of protein synthesis occurred in fertilized eggs. Similar marked increases in the rate of translation were observed using [^{3}H]lysine or [^{14}C]phenylalanine (data not shown).

Incorporation of Choline, Phosphorylcholine, and Ethanolamine into Phospholipids in Eggs and Embryos. The two major lipids in the eggs of several species of sea urchins (Mohri, 1964), including *Arbacia* (E. Schmell and W. J. Lennarz, unpublished observations), are phosphatidylcholine and phosphatidylethanolamine. To determine whether or not these

TABLE I: Uptake of [^{3}H]Choline into Eggs and Embryos.

	Min after Addition of [^{3}H]Choline					
	0		15		45	
	Eggs	Embryos	Eggs	Embryos	Eggs	Embryos
Whole cells	1430[a]	2200	15,280	51,700	N.D.[b]	N.D.
Cl_3CCOOH soluble	1400	2380	17,360	60,200	20,520	63,520
Cl_3CCOOH insoluble						
Residue	0	0	0	5	0	0
Lipid	3	22	6	23	20	27
Aqueous	0	0	0	0	0	0

[a] Values expressed are total counts per minute in each fraction. [b] N. D., not determined. Eggs and embryos (8.0 mg of protein) were incubated in 2 ml of sea water containing 20 μCi of [^{3}H]choline chloride (2.34 Ci/mmol) and uptake of radioactivity into 400-μl aliquots of whole cells was determined as a function of time. At each time point the cells were precipitated with Cl_3CCOOH and the Cl_3CCOOH-soluble radioactivity was quantified. The Cl_3CCOOH-insoluble material was further fractionated as described under Materials and Methods.

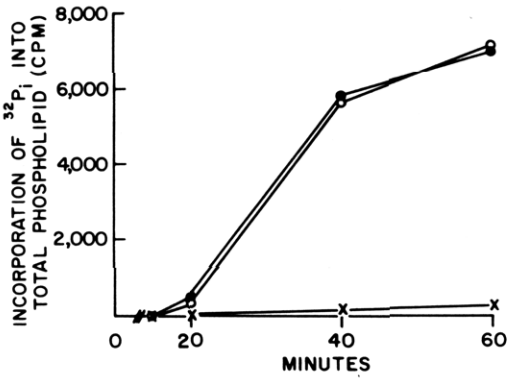

FIGURE 2: Incorporation of $^{32}P_i$ into phospholipids. Eggs and embryos (6.0 mg of protein) were incubated in 2 ml of sea water with 500 μCi of $^{32}P_i$ (effective specific activity in sea water, 2.5 Ci/mmol of P_i). Radioactivity incorporated into phospholipid in 500-μl aliquots was determined as a function of time as described under Materials and Methods: (×) eggs; (●) embryos; (○) trypsin-treated embryos.

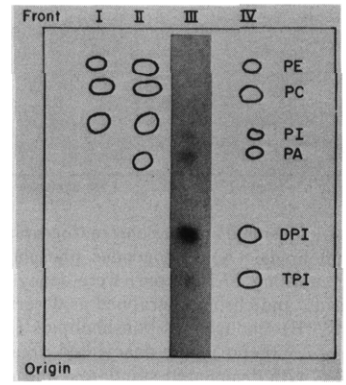

FIGURE 3: Chromatography of ^{32}P-labeled phospholipids on SG-81 paper in solvent system A. Lanes I–III: endogenous gamete lipids (50–100 nmol of lipid P containing 2500 cpm) detected with molybdate, rhodamine, and autoradiography, respectively. Lane IV: authentic lipid standards (10–15 nmol each) detected with rhodamine. Abbreviations used in the figure are: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; DPI, diphosphoinositide; TPI, triphosphoinositide.

lipids could be labeled in intact *Arbacia* eggs or embryos, experiments with labeled choline, phosphorylcholine, and ethanolamine were performed.

As shown in Table I incorporation of [^{3}H]choline into phosphatidylcholine was virtually zero in both eggs and embryos, although a significant amount of the potential precursor was accumulated by both cell types. Trypsin-treated embryos behaved identically with untreated embryos in choline uptake; both exhibited a fourfold stimulation in choline uptake when compared to unfertilized eggs. Furthermore, all of the radioactive precursor accumulated by both eggs and embryos was soluble in Cl_3CCOOH after lysis and precipitation of the cells with Cl_3CCOOH. Greater than 70% of the Cl_3CCOOH-soluble radioactivity was found in [^{3}H]choline, as identified by paper chromatography using the first dimension solvent cited by Waechter *et al.* (1969). Incorporation of [^{14}C]phosphorylcholine into phosphatidylcholine could not be detected in either eggs or embryos; however, uptake of this precursor was not studied. In contrast, when experiments using comparable amounts of labeled ethanolamine were performed, incorporation into the lipids of both the eggs and the embryos was detected. As shown in Figure 1A, the incorporation of ethanolamine was linear with time, but three–four times greater in eggs than in embryos. In both eggs and embryos all the radioactive lipid cochromatographed with authentic phosphatidylethanolamine in the four solvent systems described under Materials and Methods.

Inasmuch as this difference in incorporation in eggs and embryos could be due to a difference in the uptake of ethanolamine by the eggs and the embryos, a difference in the pool sizes of unlabeled ethanolamine in the cells, or a difference in the activity of the enzyme(s) that catalyze the incorporation into phosphatidylethanolamine, preloading experiments were undertaken. Two sets of eggs were preincubated with [^{3}H]ethanolamine for 90 min. After this time, free [^{3}H]ethanolamine was removed by washing the eggs several times with fresh sea water, and then one tube of eggs was fertilized. Incorporation of label into lipid in both eggs and embryos was followed during the preloading period as well as for 70 min after excess [^{3}H]ethanolamine had been removed. As shown in Figure 1B, no further incorporation of labeled ethanolamine into lipid was observed in the washed eggs or embryos, presumably because the free [^{3}H]ethanolamine was not retained by the eggs during the washing procedures. In fact, an identical loss of radioactivity from phosphatidylethanolamine, probably resulting from turnover, was observed with both the prelabeled eggs and embryos. In any event, this finding suggests that the threefold difference of incorporation into phosphatidylethanolamine observed between eggs and embryos (Figure 1A) is the result of more effective uptake of ethanolamine by eggs. Since trypsin treated embryos also show low incorporation of ethanolamine, the apparent barrier in embryos is not the fertilization membrane.

Incorporation of Inorganic Phosphate into Phospholipid in Eggs and Embryos. The incorporation of $^{32}P_i$ into total lipids was studied in both eggs and embryos. As shown in Figure 2, $^{32}P_i$ incorporation into the total lipids of the embryo was 20–40-fold greater than that observed in the egg. This result was not unexpected in view of the limited ability of the egg to take up P_i (Brooks and Chambers, 1954). In order to study the distribution of $^{32}P_i$ in the individual lipid classes, the labeled total lipids were separated by chromatography on silica gel paper in four solvent systems. In addition, the labeled, water-soluble backbones of the lipids were analyzed both by ion exchange chromatography and by two-dimensional paper chromatography.

Chromatography on SG-81 paper in solvent system A revealed that none of the major lipids of the embryo were labeled (Figure 3). All of the radioactivity was found in three components. The fastest moving component which, as in this experiment, was sometimes barely resolved into two spots, had an R_F of 0.58–0.59 and cochromatographed with authentic phosphatidylinositol and phosphatidic acid. A slower moving component with an R_F of 0.50 corresponded to diphosphoinositide, and the slowest moving material, R_F 0.26, cochromatographed with authentic triphosphoinositide. Similar results were obtained in solvent systems B and C, although in these systems phosphatidylinositol and phosphatidic acid were never resolved. Although the labeled egg lipids were examined only in solvent system A an identical distribution of radioactivity was observed. Despite the fact that virtually all the radioactivity was found in the polyphosphoinositides, as shown in Figure 3, these compounds, in contrast to phosphatidylinositol and phosphatidic acid, could not be detected with rhodamine or molybdate sprays; they therefore constitute a negligible percentage of the total phospholipids. Trypsin-treated embryos, which lack fertilization membranes, showed no difference in both the extent and specificity of labeling from the untreated embryos. Control experiments with sperm and activated sperm (*i.e.*, sperm ex-

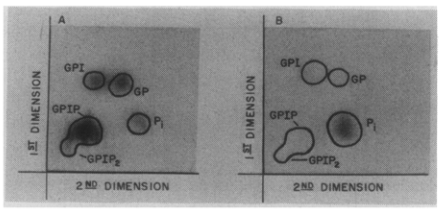

FIGURE 4: Two-dimensional paper chromatography of deacylated ^{32}P-labeled phospholipids. (A) Endogenous phospholipids (350–700 nmol of lipid P containing 17,500 cpm) were deacylated, mixed with deacylated standards, and chromatographed as described under Materials and Methods. (B) Endogenous phospholipids (350–700 nmol of lipid P containing 17,500 cpm) were deacylated, treated with alkaline phosphatase, mixed with deacylated standards, and chromatographed as described under Materials and Methods. In A and B deacylated standards were detected with benzidine–periodate, P_i was detected with Hanes–Isherwood spray, and ^{32}P was detected by autoradiography, as described under Materials and Methods. Abbreviations used in the figure are: GP, glycerol 3-phosphate; GPI, glycerophosphorylinositol; GPIP, glycerophosphorylinositol phosphate; $GPIP_2$, glycerophosphorylinositol diphosphate.

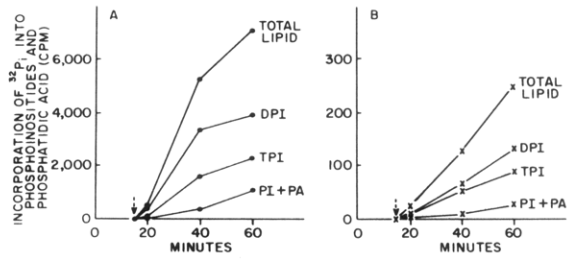

FIGURE 5: Kinetics of incorporation of $^{32}P_i$ into phosphoinositides and phosphatidic acid. Embryos (A) and eggs (B) (5.4 mg of protein) were incubated in 2 ml of sea water containing 500 μCi of $^{32}P_i$ (specific activity, 2.5 μCi/mmol). Aliquots of 500 μl were removed at various times, and the lipids isolated as described under Materials and Methods. The lipids were chromatographed on SG-81 paper in solvent system A, detected on X-ray film, and the individual phospholipids were quantified by counting the radioactive spots in Triton–toluene scintillation fluid. The abbreviations used are the same as in Figure 3.

posed to eggs) revealed that polyphosphoinositides were also synthesized by these cells, but at a rate 100–1000 times lower (per milligram of cell protein) than in embryos.

In order to further characterize the labeled embryo lipids as triphosphoinositide, diphosphoinositide, phosphatidylinositol, and phosphatidic acid, the lipids were deacylated with mild alkali. Greater than 90% of the radioactivity in the lipid was converted to a water-soluble form upon mild alkaline hydrolysis. The remaining 5–10% of the radioactive $CHCl_3$–CH_3OH soluble material, when chromatographed on SG-81 paper, contained all of the original labeled lipids plus one new lipid moving slower than triphosphoinositide, which probably was a lyso compound.

The deacylation products of the labeled lipids were subjected to two-dimensional paper chromatography. As shown in Figure 4A, the majority of the label was localized in glycerophosphorylinositol phosphate and glycerophosphorylinositol diphosphate. In order to determine if the labeled phosphate moiety was primarily in phosphomonoester linkage, rather than in phosphodiester linkage, a second aliquot of the deacylated lipids was chromatographed after alkaline phosphatase treatment. As shown in Figure 4B, greater than 95% of the radioactivity was found in free $^{32}P_i$, the remaining amount being present in the phosphodiester linkage of glycerophosphorylinositol.

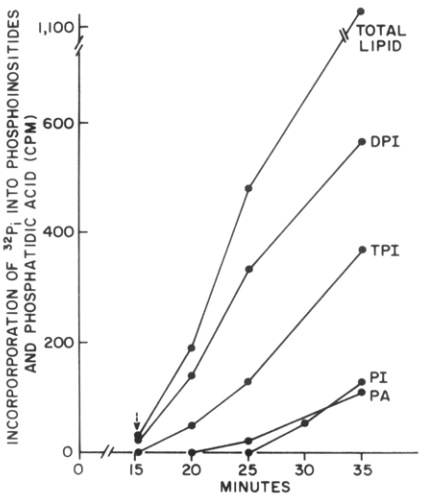

FIGURE 6: Early time course of $^{32}P_i$ incorporation into phospholipids in embryos. Embryos (7.0 mg of protein) were incubated in 2 ml of sea water with 200 μCi of $^{32}P_i$ (1 Ci/mmol) and phospholipids were isolated as described under Materials and Methods. The lipids were chromatographed on SG-81 paper in solvent system A, detected on X-ray film, and the individual phospholipids were quantified by counting the radioactive spots in Triton–toluene scintillation fluid. The abbreviations used are the same as in Figure 3.

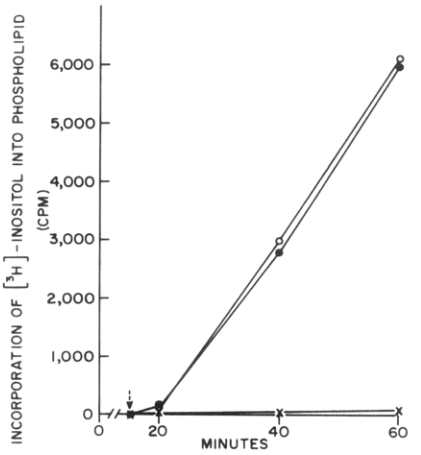

FIGURE 7: Incorporation of [^{3}H]inositol into phosphoinositides. Eggs and embryos (6.4 mg of protein) were incubated in 2 ml of sea water containing 25 μCi of [^{3}H]inositol (2.83 Ci/mmol), and radioactivity incorporated into phospholipid as a function of time was determined as described under Materials and Methods: (×) eggs; (●) embryos; (○) trypsin-treated embryos.

As further evidence of the identity of the ^{32}P-labeled lipids, labeled total lipids were mixed with unlabeled phosphatidylinositol, diphosphoinositide, and triphosphoinositide and then deacylated. The resulting water-soluble deacylation products were analyzed by ion exchange chromatography (Lester and Steiner, 1968), kindly performed by Dr. Robert Lester at the University of Kentucky. The results (not shown) confirmed the presence of the water-soluble backbones corresponding to phosphatidylinositol, phosphatidic acid, diphosphoinositide, and triphosphoinositide, and indicated that the most highly labeled lipid is diphosphoinositide.

In order to investigate the relative rates of labeling of these lipids, and to determine the proportions of each, a kinetic study of incorporation of $^{32}P_i$ into each lipid was undertaken. The results shown in Figure 5 indicate that in both eggs and embryos 53% of the $^{32}P_i$ is in diphosphoinositide, 33% in triphosphoinositide, and the remaining 14% in phosphatidylinositol and phos-

TABLE II: Per Cent Composition of [^{3}H]Phosphoinositides in Eggs and Embryos.[a]

	Phosphatidylinositol	Diphosphoinositide	Triphosphoinositide
Eggs	95.5	1.7	0.45
Embryos	92	3.28	1.94

[a] [^{3}H]Phospholipids (100 nmol of lipid phosphorus) from the experiment shown in Figure 7 were chromatographed on SG-81 paper with authentic phosphoinositide standards in solvent system A. The paper was cut into 1-cm strips and radioactivity corresponding to the various phosphoinositides was determined in Triton–toluene scintillation fluid. Approximately 97% of the recovered radioactivity was found in the three phosphoinositides.

phatidic acid. In another experiment, lipids were isolated from embryos at relatively early time points after addition of $^{32}P_i$ to establish the order of appearance of radioactivity in the lipids. The results shown in Figure 6 demonstrate that diphosphoinositide is most rapidly labeled, although significant radioactivity is detected in triphosphoinositide after incubation for 5 min with $^{32}P_i$. In contrast, label does not appear in phosphatidic acid until 10 min and in phosphatidylinositol until 15 min after addition of $^{32}P_i$.

Incorporation of Inositol into Phospholipids in Eggs and Embryos. In the hope of gaining further insight into the metabolism of phosphoinositides in the eggs and embryos, both were incubated with ^{3}H-labeled *myo*-inositol. In contrast to the results with choline and phosphorylcholine, inositol was incorporated into the lipids of both eggs and embryos. Upon chromatography of the ^{3}H-labeled lipids on SG-81 paper in solvent system A or B all radioactivity was found in phosphatidylinoitol, diphosphoinositide, and triphosphoinositide. As shown in Figure 7 the initial rate of incorporation of [^{3}H]inositol into embryo lipids is 60-fold greater than into egg lipids. Because differences in rates of transport of inositol in eggs and embryos might account for this difference in incorporation, preloading experiments were performed. Eggs were preincubated for 30 min in sea water containing [^{3}H]inositol; exogenous inositol was then removed by collecting the eggs and washing them twice with sea water. One aliquot of the washed eggs was then inseminated, and incorporation of inositol into both egg and embryo lipids was determined. As shown in Figure 8 the rate of labeling in embryos is only greater than the egg by a factor of three when the eggs are preloaded with [^{3}H]inositol rather than by the factor of 60 observed when the eggs are not preloaded. Thus, it would appear that the pronounced difference in incorporation observed in Figure 7 is due to a difference in the ability of eggs and embryos to take up inositol. It should be noted that, in contrast to the results with ethanolamine, incorporation of inositol continues in both eggs and embryos despite the fact that exogenous inositol was removed. Thus, both cell types are capable of retaining a pool of free inositol (or a modified form of it) that subsequently can be utilized for phosphatidylinositol synthesis. A comparison of the composition of the inositol-containing lipids from eggs and embryos labeled by preloading is shown in Table II. It is clear that in both cases phosphatidylinositol is the major inositol-labeled lipid. Although *quantitative* comparisons are not meaningful because of possible differences in the pool sizes of the two labeled precursors, this result taken in conjunction with the finding that some ^{32}P-labeled phosphatidylinositol is formed in $^{32}P_i$ labeling experiments suggests that both eggs and embryos contain enzymes that catalyze synthesis of phosphatidylinositol by either a *de novo* pathway or by base exchange.

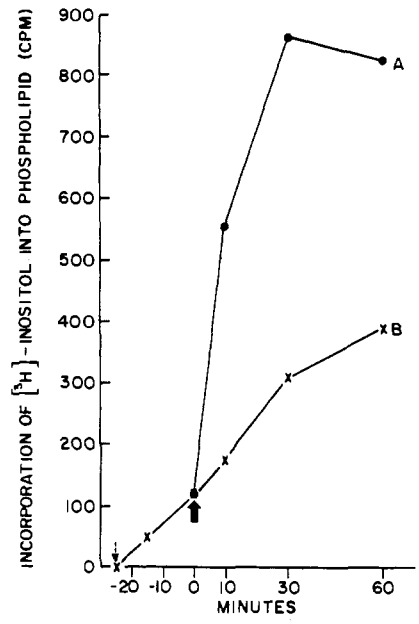

FIGURE 8: Incorporation of [^{3}H]inositol into phosphoinositides in preloaded eggs and embryos. Two sets of eggs (6.4 mg of protein) were incubated for 30 min in 2 ml of sea water containing 50 μCi of [^{3}H]inositol (2.83 mCi/mmol). After 30 min (arrow) the eggs were collected by hand centrifugation, washed several times with fresh sea water, and suspended in 2 ml of fresh sea water. To one set of eggs (A) sperm was added. The other set (B) was not fertilized. Incorporation of radioactivity into phospholipid was determined as a function of time as described under Materials and Methods: (×) eggs; (●) embryos.

Discussion

Although the amount of biochemical information about fertilization and the early stages of development of echinoderm embryos is increasing at a rapid rate, relatively little is known about the distribution and metabolism of lipids in these cells. More than 25 years ago Whitely (1949) analyzed the compounds labeled with $^{32}P_i$ upon incubation of *Arbacia punctulata* embryos with $^{32}P_i$. He found that 95% of the $^{32}P_i$ was in "small molecules" and 5% was in the acid-insoluble fraction. Of this 5%, "essentially none" was found in phospholipid. However, the techniques of lipid chemistry in 1949 precluded the detection and analysis of phosphoinositides. More recently, Mohri (1964) studied lipid metabolism during "early" development of the embryos of *Paracentrotus lividus*. Using [^{14}C]acetate and [^{14}C]glycerol to label lipids, he found no change in total lipid or phospholipid content through the mesenchyme blastula stage. Recently Pasternak (1973) studied phosphatidylcholine and phosphatidylinositol synthesis in the embryos and eggs of *Stronglyocentrotus purpuratus* and *Lytechinus*

pictus. He reported an increased uptake and phosphorylation of [^{3}H]choline immediately following fertilization, but incorporation of choline into phosphatidylcholine was only observed after several hours. Uptake of inositol was detected but only a low level of incorporation into phosphatidylinositol was reported. Using cell-free preparations, Ewing (1973) studied changes in cholinephosphotransferase activity in *Arbacia punctulata* eggs and in embryos through the pluteus stage in a particulate fraction. However, the experimental design of all of these studies was such that changes in lipid metabolism occurring between the time of fertilization and the first cell cleavage could not be detected (see below).

Because information on the incorporation of precursor into phospholipids may be of value in comparative studies of the membranes of eggs and embryos, we have studied the incorporation of choline, phosphorylcholine, ethanolamine, inositol, and P_i into the lipids of eggs and embryos. Phosphatidylcholine and phosphatidylethanolamine are known to be the major phospholipids in sea urchin eggs (Harvey, 1956; Mohri, 1964; E. Schmell and W. J. Lennarz, unpublished studies). However, using [^{3}H]choline as a lipid precursor we were unable to detect the synthesis of phosphatidylcholine in mature eggs or early embryos despite the fact that both cell types took up labeled choline. Under the experimental conditions, synthesis of as little as 0.03 pmol of ^{3}H-labeled lipid would have been readily detected.

It is interesting to compare our findings with those reported by Pasternak (1973). Using *Strongylocentrotus purpuratus* and *Lytechinus pictus*, Pasternak found only very low levels of incorporation of [^{3}H]choline into the phosphatidylcholine of eggs that were pulsed with choline and then incubated for varying times up to 40 hr. Moreover, when 1-hr embryos were pulsed with choline for 20 min no significant incorporation into lipid was observed. Only upon long term incubation, after the embryo had undergone several cell cleavages, was incorporation into phosphatidylcholine detected. Similarly, we have found that in *Arbacia punctulata* there is no significant incorporation of [^{3}H]choline or [^{14}C]phosphorylcholine into phosphatidylcholine in unfertilized eggs or in embryos up to 90 min following fertilization. Both of these reports are consistent with the idea that during early sea urchin development, when there is no apparent increase in the total surface area of the embryo plasma membrane, phosphatidylcholine synthesis is negligible. However, as new membranes are apparently synthesized later on in development, *i.e.*, during the time period studied by Pasternak, there is a marked increase in phosphatidylcholine synthesis.

In contrast to these findings with choline, both eggs and embryos incorporated [^{3}H]ethanolamine into phosphatidylethanolamine. When eggs and embryos were incubated in sea water containing [^{3}H]ethanolamine the unfertilized eggs were three–four times more active than the embryos in phosphatidylethanolamine synthesis. However, preloading experiments showed that the apparent difference between eggs and embryos in incorporating ethanolamine into phosphatidylethanolamine is due to the relatively greater ability of eggs to take up ethanolamine. This apparent lower uptake of ethanolamine into embryos, when compared to eggs, is somewhat surprising in view of the general increase in transport processes that is observed following fertilization (*cf.* introductory statement). In any case, ethanolamine incorporation apparently does not represent net synthesis, since it is evident from the pulse chase experiments (see Results section) that the ethanolamine moiety of phosphatidylethanolamine is in a rapid state of turnover. It thus appears that immediately after fertilization there is no significant net synthesis of the two major phospholipids of the embryos.

In contrast to Whitely's negative findings on $^{32}P_i$ incorporation into the lipids of *Arbacia* eggs (Whitely, 1949), we observed incorporation of $^{32}P_i$ into the phospholipids of both eggs and embryos. Embryos incorporated 20–40 times more $^{32}P_i$ into phospholipid, which is consistent with earlier observations that P_i transport is stimulated 30-fold upon fertilization of *Arbacia* eggs (Brooks and Chambers, 1954). In both cell types, 95% of the $^{32}P_i$ in lipid was found in the phosphoinositides and only 5% in phosphatidic acid; no other phospholipids were labeled. Furthermore, greater than 95% of the $^{32}P_i$ in the phosphoinositides was in the monoester linkages of di- and triphosphoinositides, the remaining 5% being found in the phosphodiester linkage of the phosphoinositides. This result, coupled with the kinetic data in Figure 6 showing the time course of appearance of label in the phosphoinositides of embryos, suggests that $^{32}P_i$ is rapidly incorporated into the very small pool of polyphosphoinositides, perhaps by a process involving a phosphorylation–dephosphorylation cycle. *De novo* synthesis of phosphatidylinositol, which is present in much greater amounts, occurs at a much slower rate.

Preloading experiments with [^{3}H]inositol showed a threefold stimulation of incorporation of label into the phosphoinositides following fertilization. The major labeled lipid found both before and after fertilization was phosphatidylinositol, although after fertilization the proportion of di- and triphosphoinositide increased slightly. In Pasternak's study (1973) with two other species of sea urchin, incorporation of inositol into lipid was also observed, although the lipids were not characterized in detail.

Recently, Palmer (1973) has shown that in the protozoan *Crithidia fasciculata* the level of polyphosphoinositides is higher in logarithmic phase cells than in stationary phase cells. Talwalker and Lester (1973) have shown a direct relationship between the levels of polyphosphoinositides and the adenylate energy charge in yeast. All of these results suggest an association between increased levels of polyphosphoinositides and enhanced metabolic activity of cells. Evidence has been reported in several systems that di- and triphosphoinositides are synthesized by successive phosphorylations of phosphatidylinositol with ATP as the phosphate donor (Kai and Hawthorne, 1969). Moreover, phosphatases have been shown to convert the polyphosphoinositides to phosphatidylinositol (Kai and Hawthorne, 1969). It is thought that the combined action of phosphatases and kinases accounts for the rapid turnover of the phosphomonoester groups which has been observed in a variety of biological systems (LeBaron *et al.*, 1960; Wagner *et al.*, 1961; Brockerhoff and Ballou, 1962; Sheltaway and Dawson, 1969; Santiago-Calvo *et al.*, 1964; Andrade and Huggins, 1964; Schneider and Kirschner, 1970; Steiner and Lester, 1972a,b). Thus, the level of polyphosphoinositides in any cell type may be controlled by the relative activity of specific kinases and phosphatases. The effects of cyclic AMP on kinases and phosphatases have been studied in detail in mammalian cells. In sea urchin embryos the level of cyclic AMP is known to increase at the time of cell cleavage (Yasumasi *et al.*, 1973), but the precise role of these compounds in the developmental process is not yet known.

In any case, the present study has established the presence of low levels of phosphoinositides in *Arbacia* eggs and embryos, and shown that following fertilization there is preferential incorporation of $^{32}P_i$ into the polyphosphoinositides until at least the first cell cleavage. However, as in other cell types, the biological role of these phospholipids remains unknown.

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