 Preferential Incorporation of Phospholipids into Plasma Membranes during Cell Aggregation of Dictyostelium discoideum*

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Changes in phospholipids of plasma membranes and total particulate membranes of the cellular slime mold Dictyostelium discoideum were analyzed during development. The predominant classes of phospholipids in the plasma membrane were phosphatidylethanolamine and phosphatidylcholine, constituting approximately 50 and 25 molar per cent of phospholipid phosphorus, respectively. The relative amount of plasma membrane phospholipid increased by almost 30% and the relative proportion of the major phospholipid classes remained constant during the early phase of development.

An induction in phospholipid synthesis occurred during cell aggregation, resulting in a 12% increase in the cellular phospholipid content. The relative rate of synthesis increased rapidly between 3 and 9 h of development and began to drop at the postaggregation stage. Plasma membranes isolated from cells at different developmental stages showed that plasma membrane phospholipids were preferentially labeled at 6 h, giving rise to a 20-fold increase in specific activity. The specific activity of plasma membrane phospholipids was 8- to 10-fold higher than that of total particulate membranes at 6 h. The data suggest that the newly synthesized phospholipids inside the cell were preferentially incorporated into the plasma membrane at the onset of cell aggregation. Phospholipid synthesis was also examined in an aggregateless mutant strain WL3. An approximate 2-fold increase in the synthesis of phospholipids was observed, but there was no preferential transport of labeled phospholipids to the plasma membrane. These results suggest that the rapid incorporation of phospholipids into plasma membrane is coupled with chemotactic migration during differentiation. The possible role of phospholipids in cell-cell adhesion is also discussed.

Dictyostelium discoideum is a good model for investigating the role of plasma membrane in cell differentiation. It has a well-defined and well-characterized life cycle (1) and cells can be initiated to develop synchronously. The free living amoeba responds to the depletion of food by undergoing chemotactic migration to form multicellular aggregates which eventually culminate to form the fruiting bodies. After about 5 h of development, cells begin to undergo chemotactic migration toward areas of higher adenosine 3′-5′-monophosphate concentration (2, 3). This process is accompanied by a series of changes in the cell. New antigenic determinants appear on the cell surface during this time (4–6). The induction of several plasma membrane enzymes, such as adenyl cyclase, has been associated with the signal transmission system of chemotaxis, while some membrane proteins and glycoproteins have been implicated in cell-cell adhesion (7–9). Total cellular phospholipids have also been analyzed in both vegetative and developing amoebae (10). Changes in phospholipid composition were observed at the aggregation and culmination stages (11). However, the phospholipid composition of the plasma membrane has not been examined.

Since lipids constitute the major components of the plasma membrane, changes in membrane lipid composition may regulate cell migration and cell-cell interactions. Slime mold cells exhibit increased motility during the aggregation period and it is likely that the plasma membrane is turning over rapidly at the same time. Alterations in membrane lipid composition may also affect membrane fluidity and functions of other membrane components (12). During early development, cells acquire an increase in intercellular cohesiveness which allows them to transit from the solitary state to the multicellular state. In addition to proteins and glycoproteins, lipid components in the plasma membrane may affect proper cell-cell adhesion.

In this report, we characterized the changes in the composition of plasma membrane phospholipids during development. Both ethanolamine phosphoglycerides and choline phosphoglycerides were found to be the predominant species of phosphoglycerides in the plasma membrane at all stages. A rapid increase in the synthesis of phospholipids occurred at the aggregation stage and is developmentally regulated. The newly synthesized phospholipids were preferentially incorporated into the plasma membrane at the initial stage of chemotactic migration.

**EXPERIMENTAL PROCEDURES**

Materials—[14C]Orthophosphoric acid and [3H]Glycerol were purchased from New England Nuclear, Boston, Mass. Phosphatidylethanolamine (Escherichia coli, type V), phosphatidylcholine (bovine brain, type III-B), phosphatidylserine (bovine brain), phosphatidylinositol (soybean), phosphatidylglycerol (egg yolk lecithin), phosphatic acid (egg yolk), and cardiolipin (bovine heart) were purchased from Sigma. Lyso phosphatidylethanolamine and lysophosphatidylcholine were prepared by treating their respective diacyl forms with phospholipase A2 from Crotalus adamanteus (Sigma). Precoated thin layer chromatography plates (250- and 500-μ silica gel G) were obtained from Analtech Inc., Newark, N. J. Lipid chromatography grade silicic acid (mesh 325) was purchased from Sigma. Reagent grade organic solvents were used for all lipid extractions. All lipid extracts were dried down in an inert atmosphere of nitrogen.
Cell Strain and Growth Conditions—D. discoideum strain NC4 was used in all experiments except as otherwise noted. Amoebae were cultured on nutrient agar plates in association with Klebsiella aerogenes (13). Cells were collected when the bacterial lawn was partially cleared, giving a yield of approximately 1 × 10^6 cells/cm diameter Petri dish. Bacteria were removed by repeated differential centrifugation as described in Whatman's Directions for 50 filters. Amoebae were homogenized in 1 mm MgCl₂ in 10 ml of 0.05 M tris-HCl buffer, pH 7.5, and then disrupted with about 30 strokes from a motor-driven Teflon pestle at 4°C. A small sample of the homogenate was stored for protein estimation and the rest was centrifuged at 17,000 × g for 15 min. The pellet was suspended in the two-phase polymer system of Brunette and Till (14) and centrifuged at 5,000 × g for 10 min to obtain a plasma membrane fraction at the interphase. This fraction was used until the end of the experiment. The homogenate was washed twice in cold distilled water, pelleted, and stored frozen at -70°C. Plasma membranes were also isolated by the method of Green and Newell (15) with some modifications. Cells were suspended in 10% sucrose in 10 mM Tris-HCl (pH 7.5) and then lysed by nitrogen decavitation in a Parr cell disruptor. The aqueous phase was centrifuged at 5000 × g for 10 min at 25°C. The supernatant was removed, evaporated to dryness, and then resuspended in water. The pellet was resuspended in water and aliquots of 10 to 300 µg of proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Aliquots were also used for neutral hexose (22) and sialic acid determination (21).

Silicic Acid Column Chromatography—Fractions of total lipids into phospholipid and neutral glycolipids was achieved by silicic acid chromatography (23) or thin layer chromatography (24). In several experiments, the total lipid extract in chloroform was subjected to acid hydrolysis by 2 ml of 0.1 N hydrochloric acid and 0.2 ml of internal standard sodium methoxide in methanol for 24 h at 37°C (25). The hydrolysate was dia lyzed against water and then used to isolate the alkalai-stable glycolipids. Silicic acid powder (325 mesh, Sigma) was activated at 90°C for 24 h before column chromatography. The activated silic acid was made into slurry with diethyl ether and phosphate buffer (0.6 g/10 ml ether) fitted into a glass column (30 cm) fitted with a Teflon stopcock. After ether was eluted out, the column was washed with 3 bed volumes of chloroform. A sample of lipid mixture was loaded onto the column, followed by elution with 100 ml of chloroform. Subsequently, the column was washed with 50 ml of acetone followed by 30 ml of acetic acid/methanol (9/1). The eluates were pooled to obtain the neutral glycolipid fraction. Finally, the column was washed with 100 ml of methanol to elute out the polar phosphoglycerides. All fractions were concentrated under nitrogen at 35°C and redissolved in a fixed volume of chloroform.

Lipid Extraction—Separation of glycoproteins, glycolipids, and phospholipids from whole cell or plasma membranes was accomplished by a modified tetrahydrofuran (H₂Furan) extraction procedure (20, 21). Approximately 8 × 10⁷ cells or 1 to 2 mg of membranes were initially treated with boiling 2-propanol and homogenized with a motor-driven Teflon pestle. The homogenate was centrifuged at 10,000 × g for 15 min. The supernatant was removed, evaporated to dryness under nitrogen, and then dissolved in 10 ml of phosphate buffer, pH 7.5. The aqueous phase was separated carefully. The organic phase containing lipids was washed thoroughly to break the emulsion so that only 32P- or 3H-labeled lipids were extracted. This aqueous phase mixture, 1 ml of a mixture of 2 N Na₂SO₄ and 5% aqueous ammonium molybdate (1/1) and 2 ml of isobutanol was added. The mixture was blended on a Vortex mixer vigorously and then centrifuged at 3000 × g for 5 min. The aqueous phase contained the inorganic phosphates, while the organic phosphates were in the isobutanol phase. Aliquots were removed from the two phases and counted in a liquid scintillation counter.

Metabolic Labeling—Carrier-free [³²P]orthophosphate and [¹⁴C]glycerol were used to study the biosynthesis of phospholipids. Cells were developed on filter paper saturated with PD solution buffered with 2-N-morpholinoethanesulfonic acid instead of Na₂K phosphate. At different developmental stages, cells were labeled with 100 to 300 µCi of radioactive precursor/10⁸ cells for an interval of 2 h. Cells were collected and washed repeatedly with distilled water or 17% cold trichloroacetic acid. The cells were extracted in methanol/concentrated ammonia/water (70/25/3.5/2, v/v). The insoluble residue obtained from the H₂Furan extraction was shaken for 1 h with 1 ml of 13% trichloroacetic acid. After centrifugation at 3000 × g for 5 min, the clear supernatant was combined with aqueous phase obtained by the H₂Furan procedure. The soluble phase was washed again and the washing was added to the aqueous phase and then subjected to thin layer chromatography. Metabolic labeling—Carrier-free [³²P]orthophosphate and [¹⁴C]glycerol were used to study the biosynthesis of phospholipids. Cells were developed on filter paper saturated with PD solution buffered with 2-N-morpholinoethanesulfonic acid instead of Na₂K phosphate.
labeled lipid spots, the scraped material was eluted with a mixture of chloroform/methanol (2/1). The solvents were removed carefully under a stream of nitrogen and lipid-P was determined for each spot by the Bartlett procedure (29). Recovery of each phospholipid by this method was 95% or better. All experiments were repeated two or more times. The values reported represent the average of two or more determinations and the standard deviations are less than 15%.

RESULTS

Purification of Plasma Membranes—Plasma membranes were purified from D. discoideum cells at different developmental stages, using either the two-phase aqueous polymer system (8, 14) or sucrose density gradient centrifugation (see "Experimental Procedures"). The yield of membranes was slightly higher in case of the two-phase system and it was used in most biosynthesis studies, while sucrose gradient centrifugation was used to prepare large preparations of plasma membranes. Alkaline phosphatase and 5'-nucleotidase were used as plasma membrane enzyme markers. Membranes obtained by the two-phase method had an approximate 12-fold increase in the specific activity of alkaline phosphatase, but only a 3- to 5-fold increase in the specific activity of 5'-nucleotidase. Plasma membranes prepared by sucrose gradients showed an approximate 15-fold increase in alkaline phosphatase activity. Therefore, membranes prepared by both methods showed a similar degree of enrichment in plasma membrane. These results confirm the previous observations by Green and Newell (15) and by Siu et al. (31). There was also no detectable difference in the lipid composition of membranes prepared by these two methods.

Alterations in Plasma Membrane Phospholipids during Development—In the isolation of phospholipids, care was taken to inactivate the endogenous phospholipases by treating cells directly with hot 2-propanol before lipid extraction (24). Preliminary experiments were conducted to determine the total lipid-P and neutral hexose content of lipid extracts, and the tetrahydrofuran procedure was found to be more efficient than the Bligh and Dyer method (32) in the extraction of phospholipids and glycolipids without concurrent solubilization of glycoproteins. It was a relatively mild procedure and degradation of glycolipids was minimized. The sensitive Bartlett (29) procedure was used for the quantitation of phospholipid.

The amount of total phospholipids present in the cell at the 3-h stage was approximately 3.9 pg/cell (Fig. 1a). The cellular content decreased almost linearly during the first 15 h of cell differentiation. However, the amount of phospholipid per cell remained more or less constant up to 9 h. This was followed by a rapid decrease in phospholipid content during the next 6 h, resulting in an approximately 50% reduction of the cellular phospholipid content.

When data were expressed in terms of the amount of phospholipids per mg of protein, the phospholipid:protein ratio was increased from 0.06 at 3 h to 0.083 at 9 h for the whole cell homogenate (Fig. 1b). Such an increase of almost 40% reflected both the net synthesis of phospholipids and the steady turnover of cellular proteins during this period (Fig. 1a). When phospholipids were extracted from plasma membranes and quantitated in the same manner, the phospholipid:protein ratio showed a pattern of change similar to that of the whole cell homogenate. Between 3 and 9 h, the phospholipid:protein ratio of the plasma membrane increased by almost 30% and this was followed by a decrease of about 17% between 9 and 12 h. The increase in the phospholipid:protein ratio took place during the period when cells were undergoing active chemotactic migration, while the decrease corresponded to the time when cells were in aggregates, forming pseudoplasmodia. The data thus suggest that drastic changes in phospholipid metabolism occurred during the first 12 h of cell differentiation.

Membrane lipids were also fractionated by thin layer chromatography and quantitated by lipid-P determination. The major phospholipids present in the cell during development were phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), lyso-PC, lyso-PE, and trace amounts of cardiolipin. The neutral lipids included triglycerides, diglycerides, free fatty acids, sterola, sterol esters, and some unidentified pigments.

Both the particulate membranes and the plasma membranes had similar phospholipid composition. The predominant species of phospholipids in plasma membranes and total particulate membranes were PE and PC (Fig. 2). PE and PC constituted, respectively, about 50 and 25 molar per cent of the phospholipid phosphorus in the plasma membrane. The relative abundance of the various phospholipid classes did not exhibit much difference during the first 12 h of development, a small increase in the relative amount of lyso-PE was observed between 3 and 6 h. On the other hand, there

![Fig. 1. Changes in phospholipid content of plasma membrane and whole cell homogenate during development. Plasma membranes were isolated from NC4 cells at different developmental stages using the two-phase polymer system (14) and lipids were extracted as described under "Experimental Procedures." Lipid-phosphorus was estimated by the Bartlett procedure (29) and the amount of phospholipid was estimated from the lipid-P data by assuming that the average molecular weight of phospholipid is 800. Protein concentrations were determined by the method of Lowry et al. (19). A, Amount of total cellular phospholipids (△) and proteins (○) at different developmental stages; b, relative amount of phospholipids in the plasma membrane (●) and whole cell homogenate (□) at different developmental stages.](image)
Phospholipids of Dictyostelium Plasma Membrane

Fig. 2. Changes in the phospholipid composition in the plasma membrane and the total particulate membranes during development. Plasma membranes were isolated as described under Fig. 1 and particulate membranes were obtained as a pellet after centrifugation of the cell lysate at 100,000 × g for 1 h. Total lipid was extracted from 1 × 10^8 cells and then separated into different phospholipid classes by thin layer chromatography. The samples were developed consecutively by two different solvent systems: 1) chloroform/methanol/concentrated ammonia/water (70:25:3.5:2, v/v) and 2) chloroform/methanol/acetic acid/water (80:10:1.5:0.7, v/v) (49). Spots were detected by a brief exposure to I_2 vapor and their R_f values were compared with those of known standards. Spots were scraped, extracted twice with chloroform/methanol (2:1), and the solvents were removed under a stream of nitrogen. Lipid-P of the dry residue was determined and the amount of each phospholipid class is expressed as per cent of the total phospholipid content of the plasma membrane (a) or particulate membranes (b) for each developmental time point. The different phospholipid classes are (a) PE, (b) lyso-PE, (c) PC, (d) lyso-PC, (e) PI, and (f) PS.

was also a gradual decrease in the relative amount of lyso-PC in the plasma membrane between 3 and 9 h. In addition, the relative amount of PS showed a greater than 2-fold reduction in both plasma membrane and particulate membranes between 3 and 6 h.

Incorporation of ^32^P into Phosphoglycerides—To study the metabolic events leading to the initial accumulation and subsequent decrease in cellular phospholipid content, synthesis of phospholipid during development was monitored by the incorporation of ^32^P into total phosphoglycerides. The results are shown in Fig. 3a. A rapid incorporation of ^32^P into phosphoglycerides occurred between 4 and 6 h of development, reaching a maximum at 9 h. This incorporation of ^32^P decreased by almost 50% at 12 h of development. During the same period, a gradual decrease in the ^32^P-labeled organic phosphate pool became evident. The rapid synthesis of phosphoglycerides during this period might contribute to the depletion of the organic phosphate pool.

Since changes in the specific activity of the inorganic phosphate pool may affect the interpretation of the data, experiments were carried out to determine the pool size of inorganic phosphate during development (Table I). The cellular concentration of inorganic phosphate was about 8 to 9 mM during the first 9 h of development. However, an almost 2-fold increase in inorganic phosphate concentration occurred between 9 and 12 h. The uptake of [32P]phosphate from the medium also decreased by about 35% after 7 h of development. The specific activity of the inorganic phosphate pool de-

Fig. 3. Synthesis of phospholipids during development. a, Changes in ^32^P incorporation into organic phosphates and total cellular phospholipids during development. Developing cells were labeled with [32P]orthophosphoric acid at 200 μCi/10^8 cells for 2 h prior to the isolation of the particulate membrane fraction. Phospholipids and organic phosphates were extracted separately and quantitated as described under "Experimental Procedures" and the amount of radioactivity recovered from 10^8 cells in each fraction was estimated. b, Total cellular phospholipids; c, organic phosphate pool. b, Relative rate of synthesis of phospholipids during development. Developing cells were labeled with [32P]orthophosphate and phospholipids were extracted and quantitated. The relative rate of synthesis (a) was calculated by dividing the ^32^P specific activity of phospholipids with the specific activity ratio for the inorganic phosphate pool shown in Table I.

| Table I |
| Changes in specific activity of inorganic phosphate pool during development |

NC4 cells were labeled with [32P]orthophosphoric acid at 200 μCi/10^8 cells for 2 h prior to the time of collecting for analysis. Inorganic phosphate was extracted from 10^8 cells and quantitated as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Total P_i</th>
<th>F. concentration (μmol)</th>
<th>Radioactivity in P_i (dpm)</th>
<th>F. specific activity (dpm/μmol × 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>141</td>
<td>7.7</td>
<td>6.9</td>
<td>423 (1)^a</td>
</tr>
<tr>
<td>3</td>
<td>163</td>
<td>9.3</td>
<td>6.7</td>
<td>456 (1.08)</td>
</tr>
<tr>
<td>6</td>
<td>147</td>
<td>8.1</td>
<td>6.7</td>
<td>456 (1.08)</td>
</tr>
<tr>
<td>9</td>
<td>156</td>
<td>8.6</td>
<td>4.2</td>
<td>269 (0.64)</td>
</tr>
<tr>
<td>12</td>
<td>267</td>
<td>15.5</td>
<td>4.5</td>
<td>169 (0.40)</td>
</tr>
</tbody>
</table>

* Values in parentheses represent the ratios of specific activities of inorganic phosphate at different developmental stages relative to that of the 3-h stage.
creased accordingly between 7 and 12 h and there was an approximate 2.5-fold difference between 12- and 3-h cells.

In calculating the relative rate of phospholipid synthesis, the pool effect was taken into account by dividing the specific activity of the total membrane phospholipid with the relative specific activities of the inorganic phosphate pool shown in Table I. The results showed that there was an approximate 6-fold increase in the relative rate of synthesis between 3 and 9 h of development (Fig. 3b). The rate of synthesis began to drop at 12 h when cells moved into the postaggregation stages.

The pattern of \(^{32}P\) incorporation into the major phosphoglyceride classes was also examined after fractionation using thin layer chromatography. In Fig. 4, all six major phospholipids showed a similar pattern of synthesis. Their relative rates of synthesis showed an average 6-fold increase between 3 and 9 h of development, reaching a peak at 9 h, which was followed by a 30 to 50% reduction in their synthesis at 12 h. Phosphatidylethanolamine, phosphatidylethanolamine, and phosphatidylinositol were the predominant species synthesized during this period. Phosphatidylethanolamine and phosphatidylethanolamine appeared to be almost identical in their rate of synthesis although phosphatidylcholine was present in approximately one-half the amount of phosphatidylethanolamine. On the other hand, synthesis of lyso-PC was lowest even though it was only next to PC in relative abundance (Fig. 2).

**Preferential Incorporation of Phospholipids into Plasma Membrane**—To examine the labeling pattern of phospholipids in the plasma membrane, cells were pulsed with \(^{32}P\)orthophosphoric acid for 2 h before membrane purification. Contrary to the pattern obtained for the particulate membranes, plasma membrane phospholipids were labeled maximally at

![Fig. 4. Synthesis of individual classes of phospholipid during development. Developing cells were labeled with \(^{32}P\)orthophosphoric acid at 200 \(\mu\)Ci/10^8 cells for 2 h prior to isolation of the particulate membrane fraction. Phospholipid classes were scraped after separation by thin layer chromatography and their radioactivity was determined. The relative rate of synthesis was expressed in terms of the radioactivity incorporated into an individual phospholipid class per milligram of total cellular phospholipid and then normalized with the specific activity ratios of the P pool as in Fig. 3b. a, PE (○), PC (○), PI (●); b, PS (□), lyso-PE (△), and lyso-PC (△).](image)

### Table II

**Incorporation of \(^{32}P\)orthophosphoric acid into plasma membrane and total particulate membrane phospholipids during development**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Specific activity of PL (dpm/μg PL)</th>
<th>Specific activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>103 (1)</td>
<td>106 (1)</td>
</tr>
<tr>
<td>6</td>
<td>2449 (23.8)</td>
<td>240 (2.3)</td>
</tr>
<tr>
<td>9</td>
<td>364 (3.5)</td>
<td>439 (4.2)</td>
</tr>
<tr>
<td>12</td>
<td>156 (1.5)</td>
<td>242 (2.3)</td>
</tr>
</tbody>
</table>

\(<\text{Values in parentheses represent the change in the relative specific activities of PL at different developmental stages.}\>

6 h of development (Table II). The specific activity of plasma membrane phospholipids at this stage was about 24-fold higher than that of 3-h cells, while the total particulate membranes showed only a 2.3-fold increase. However, the specific activity of 9-h membranes dropped to a much lower value and was only 3.5-fold higher than that of 3-h cells.

When the specific activity of plasma membrane phospholipids was compared with that of total cellular phospholipids, \(^{32}P\) radioactivity in the plasma membrane showed a 10-fold enrichment at 6 h of development (Table II). This experiment was repeated three more times and the enrichment factor was found to vary between 8 and 10. It has been determined that an upper limit of about 3% of the total cellular protein in the vegetative cell was present in the plasma membrane (15). If this value remained unchanged during the early stages of development, it can be estimated from the data in Fig. 1 that approximately 34 \(\mu\)g out of a total of 440 \(\mu\)g of cellular phospholipids were present in the plasma membrane of 10^6 cells at 6 h, i.e. the amount of total cellular phospholipids was about 13-fold higher than that of the plasma membrane. Therefore, an estimated 50% to 80% of the newly synthesized phospholipid had to be incorporated into the plasma membrane to give rise to an enrichment factor of 8 to 10 at 6 h of development. In contrast, cells at the preaggregation (3 h) and postaggregation (12 h) stages did not exhibit any preferential incorporation of phospholipids into the plasma membrane (Table II).

Rapid labeling of plasma membrane phospholipids also became evident when the specific activities of phosphatidylethanolamine and phosphatidylethanolamine, the two predominant phospholipids, were examined (Table III). Changes in these two plasma membrane phospholipids corresponded to changes in the specific activity of total plasma membrane phospholipids (Table II). At 6 h, phosphatidylethanolamine and phosphatidylethanolamine in the plasma membrane showed 34- and 40-fold increase in specific activity, respectively, while they both showed only a 2- to 3-fold increase in the particulate fraction when compared with values obtained for 3-h cells. When compared with the corresponding phosphoglycerides of total particulate membranes, the \(^{32}P\) radioactivity in phosphatidylethanolamine and phosphatidylethanolamine of the plasma membrane showed 13-fold and 9-fold enrichment, respectively, at 6 h, the initial stage of cell aggregation. At all other stages, this ratio for either phospholipid class was close to one.

**Incorporation of \(^{3}H\)Glycerol into Phospholipids**—The synthesis of phospholipids in slime mold cells during the
aggregation period was also examined using $[^3H]$glycerol as the precursor. The pattern of changes in the relative rate of phospholipid synthesis was qualitatively similar to that observed when $[^2F]$phosphate was used to label the cells. The incorporation of $[^3H]$glycerol into the total particulate membrane phospholipids almost doubled between 3 and 9 h of development (Fig. 5). However, the incorporation of $[^3H]$glycerol returned to the 3-h level at 12 h.

When purified plasma membrane was compared with total particulate membranes, an 8-fold increase in the specific activity of phospholipid was evident, suggesting that there was a rapid accumulation of newly synthesized phospholipids in the plasma membrane between 4 and 6 h of development. The specific activity of plasma membrane phospholipids dropped by about 10% when cells were labeled beyond 7 and 9 h. However, at 12 h, the specific activity of plasma membrane phospholipids returned to the 3-h level. These results again confirmed the notion that preferential incorporation of phospholipids into the plasma membrane took place only during cell aggregation but not at the preaggregation or the postaggregation stages.

Since the synthesis of phospholipids and their preferential incorporation into the plasma membranes appeared to be a developmentally regulated event at the aggregation stage, an aggregateless mutant strain WL3 (8, 31) was also examined for its ability to synthesize membrane phospholipids. The pattern of phospholipid synthesis in WL3 was significantly different from that of the wild type strain NC4 (Fig. 6). A 2-fold increase in the incorporation of $[^3H]$glycerol into total membrane phospholipids was observed between 3 and 6 h of development and the incorporation leveled off after 6 h. Plasma membranes were also isolated from WL3 cells and the pattern of labeling by $[^3H]$glycerol was very different from that of NC4. The specific activity of plasma membrane phospholipids was significantly reduced and much lower than that of cellular phospholipids and did not exhibit the rapid labeling pattern of wild type cells (NC4) between 3 and 6 h. At 6 h, the specific activity of plasma membrane phospholipids was about 4-fold lower than that of total particulate membranes. The results thus suggest that although phospholipids were synthesized in WL3 cells, the preferential enrichment and transport of phospholipids from the cytoplasm to the plasma membrane were probably blocked.

### Table III

<table>
<thead>
<tr>
<th>Phospholipid class</th>
<th>Specific activity $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td>I. PE</td>
<td></td>
</tr>
<tr>
<td>Total particulate membrane</td>
<td>166.7 (1)</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>148.8 (0.89)</td>
</tr>
<tr>
<td>II. PC</td>
<td></td>
</tr>
<tr>
<td>Total particulate membrane</td>
<td>255.3 (1)</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>148.2 (0.58)</td>
</tr>
</tbody>
</table>

$^a$ Specific activity is expressed in terms of disintegrations per minute per pg of PE or PC.

$^1$ Values in parentheses represent the relative specific activities between total particulate membranes and plasma membranes.

DISCUSSION

Plasma membranes purified by either the two-phase aqueous polymer system or sucrose gradient centrifugation show consistently 12- to 15-fold enrichment in alkaline phosphatase, which is predominantly associated with the plasma membrane in *D. discoideum* (15, 31). Other biochemical criteria and electron microscopy have also been used to assess the purity of these preparations and were reported in a pre-
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vious paper (31). Plasma membranes prepared from cells at different developmental stages also achieve a similar degree of purity although the yield becomes lower for cells at the pseudoplasmodium stage. 5'-Nucleotidase which is a plasma membrane marker for many mammalian cells does not seem to be enriched to a similar extent in our plasma membrane fractions. A similar observation has also been made by Green and Newell (15). This could be due to the inactivation of the enzyme during the isolation procedure or its more general distribution in the slime mold cell.

Although the phosphoglycerides of plasma membranes undergo quantitative changes during the aggregation stage of cell development, their composition does not exhibit major changes and the relative ratio of the phospholipid classes generally reflects that of total membranes. An interesting feature of the plasma membrane is the presence of high concentrations of phosphatidylethanolamine and phosphatidylcholine. The abundance of these two classes of phosphoglycerides in slime mold cells has also been observed when total cellular phospholipids were examined (10, 11). On the other hand, phosphatidylinositol and phosphatidylserine are present in relatively minute amounts. This variation in phospholipid head group specificity is in agreement with other eukaryotic plasma membranes (30, 33).

Changes in the phospholipid content of plasma membranes at the aggregation stage suggest that lipid metabolism inside the cell is changing. Metabolic studies using both [32P]phosphate and [3H]glycerol as precursors to label phospholipids show that a significant increase in the rate of phospholipid synthesis takes place at the beginning of the aggregation stage. Phospholipid synthesis begins to drop when aggregates enter the slug stage. The increase in the phospholipid content of both the particulate membranes and the plasma membrane is probably the result of a net accumulation of the newly synthesized products in the cell. An 8- to 10-fold higher phospholipid specific activity was obtained in the plasma membrane fraction when either [32P]phosphate or [3H]glycerol was used as the precursor. This is a clear indication that phospholipids synthesized during the aggregation period are preferentially incorporated into the plasma membrane. The newly synthesized phospholipids probably provide the membrane precursors for the rapid turnover of plasma membranes when cell motility is greatly enhanced during aggregation. Ferber et al. (34) have reported the presence of high levels of phospholipase A and lysophospholipase in the membranes of migrating cells. These enzymes may also be partly responsible for the active turnover of membrane phospholipids.

The aggregateless mutant strain WL3 exhibits an increase in phospholipid synthesis between 3 and 6 h but fails to transport the newly synthesized phospholipid to the plasma membrane. The phospholipid transport system in WL3 cells may be blocked as a result of the pleiotropic effect of the mutation. Nevertheless, it becomes evident that the rapid increase in phospholipid synthesis and the transport of phospholipids to the plasma membrane are regulated by different mechanisms during development. This is consistent with the idea that the preferential incorporation of phospholipids into the plasma membrane is coupled with the aggregation process.

Phospholipids are known to be synthesized primarily in the endoplasmic reticulum (35) and then transported to membranes of other organelles either by the phospholipid exchange proteins in the cytosol (36, 37) or by a multistep assembly process of membrane flow from the Golgi apparatus to endoplasmic reticulum (38-40). Although the in vivo action of phospholipid exchange proteins is still not clear, it is possible that they are involved in the transfer of phospholipids to the plasma membrane. However, it seems more likely that the membrane flow mechanism may be responsible for the rapid transport of phospholipids to the plasma membrane. Such a view is consistent with the observation of Maeda and Eguchi (41) who have shown by electron microscopy the presence of intracellular vesicles and vacuoles which are characteristic of aggregating amoebae. These organelles are absent in preaggregation and postaggregation cells. Some of these vesicles and vacuoles may be involved in the secretion of cellular products such as cAMP, one class of vacuoles is filled with membranous material. Although a direct relationship between the newly synthesized phospholipids and these vacuoles has not been demonstrated, it is likely that some of them are responsible for the transport of the newly assembled membrane to the plasma membrane, while others are used for storage of membranes for the formation of prespore vacuoles at the slug stage.

Changes in total lipid composition during development have been reported (42) and they can be the result of the active membrane turnover as borne out by our present study. Cell differentiation has been shown to be affected when cells are fed with polyenoic fatty acids (43). Although changes in the fluidity of bulk lipids do not seem to occur during differentiation in normal cells (44), certain changes in the lipid composition may still affect the plasma membrane fluidity and provide the necessary microenvironment for the modulation of membrane proteins and glycoconjugates during cell adhesion.

The rapid turnover of phospholipids may give rise to asymmetry in phospholipid distribution in the plasma membrane, which has been demonstrated in both eukaryotic and prokaryotic membranes (45). It may also lead to the reorganization of various membrane components. Certain reorganization of plasma membrane proteins under the influence of cAMP and Ca**⁺** has already been shown by freeze-fracture studies (46). The lipid environment may also play an important role in modulating the activity of certain membrane-associated ligands or receptors, thus facilitating the cell cohesion process.

Recently, Kolber and Haynes (47) have demonstrated that phosphatidylethanolamine is effective in producing membrane aggregation. Studies on the aggregation of chromaffin granule membranes have also confirmed this notion (48). The high molar fraction of phosphatidylethanolamine in membranes of slime mold cells and its rapid incorporation into the plasma membrane during cell aggregation suggest that it may contribute significantly to the cohesive force between cells. The role of lipid-lipid and lipid-protein interactions in the aggregation process during development thus warrants further investigation.

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Phospholipids of Dictyostelium Plasma Membrane