Phosphatidylcholine *de Novo* Synthesis and Modification Are Carried Out Sequentially in HL60 Cells: Evidence from Mass Isotopomer Distribution Analysis†

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ABSTRACT: The traditional (parallel) model of molecular species synthesis of phosphatidylcholine is based on the substrate specificity of two glycerolphosphate acyltransferases. Preformed molecular species of diacylglycerols are then converted to phosphatidylcholine. In this investigation, we used [1,2,3,4-$^{13}C_4$]-palmitate as a tracer to determine the turnover rates of diacylglycerols and phosphatidylcholines. In HL60 cells, the fractional turnover rate is 34.1 ± 16.6%/h for 1,2-dipalmitoylglycerophosphocholine (16:0,16:0-GPC), which accounts for ∼10% of total diacylglycerol turnover. The turnover rates of other phosphotidylcholines reflect the primary event of 16:0,16:0-GPC turnover. In addition, the distribution of mass isotopomers is used to study the biosynthesis of diacylglycerols and phosphatidylcholines. On the basis of precursor−product enrichments, we propose a sequential model to account for the synthesis of phosphatidylcholine molecular species. In this model, 1,2-dipalmitoylglycerol is the only molecular species used for the synthesis of phosphatidylcholine. This precursor is converted to 1,2-dipalmitoylglycerophosphocholine, which is then deacylated to provide substrates for chain elongation and/or desaturation. These modified acyl substrates are then reacylated back to form other molecular species. This sequential model is consistent with palmitate being the dominant fatty acid product derived from mammalian fatty acid synthase. It has the advantage of protecting cells from acyl modification by exogenous substrates. Furthermore, this sequence generates only inert 1,2-dipalmitoylglycerol instead of the active diacylglycerol molecular species that contain unsaturated fatty acids.

Phosphatidylcholines are major phospholipid constituents of cell membranes. Usually, these lipids account for more than 50% of total phospholipids (*1*). In addition to their function as structural elements of membranes, these compounds are also important sources for the generation of diacylglycerol and other lipid cellular signals (*2*, *3*). Diacylglycerols are activators of protein kinase C, which in turn phosphorylate proteins involved in proliferation. Therefore, the homeostasis of these lipids is important in understanding cellular proliferation and apoptosis.

Traditionally, the investigation of the metabolism of phosphatidylcholine (PC)[1] is approached using metabolic labeling of radioactive precursors, such as fatty acids, choline, phosphorus, and glycerol (*4*−*6*). In these studies, the incorporation of radioactivity into PC is used as an indicator of synthetic rate. There are several potential problems with these experimental approaches. First of all, the incorporation of radioactivity into a product depends not simply on the synthetic rate of the product; it is also affected by the labeling of the precursor pool. In previous studies, the specific activity of the labeled precursor pool is not determined. Second, the isolation of the PC product for radioactivity counting is usually done by thin-layer chromatographic separation. With the rather limited resolution power of thin-layer chormatography, the chemical identity and purity are not assessed in most of the investigations. Finally, PC is not a single chemical entity; it is comprised of many different compounds with varied fatty acid chains. Some of these compounds are derived directly from the precursor, while others are produced from secondary reactions of the primary products (*7*−*9*). The resulting radioactivity from such a heterogeneous group can be difficult to interpret.

In addition to using metabolic labeling experiments for the assessment of synthesis and degradation, *in vitro* studies using cellular homogenate or isolated enzymes are used to determine rate-limiting steps and molecular specificity in the synthesis of PC (*10*−*12*). As a result of the limited water solubility of lipid substrates, assay systems are always carried out in the presence of detergent at high concentrations. It is uncertain whether the activity obtained from these nonphysiological conditions in the presence of detergent reflects actual *in vivo* occurrence. As a consequence, consensus results have never been obtained (*12*, *13*).

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[1] Abbreviations: PC, phosphatidylcholine; DAG, diacylglycerol; MIDA, mass isotopomer distribution analysis; GC−MS, gas chromatography−mass spectrometry; 16:0,16:0-DAG, 1,2-dipalmitoylglycerol; 16:0,18:1-DAG, 1-palmitoyl-2-oleoylglycerol; 16:0,18:0-DAG, 1-palmitoyl-2-stearoylglycerol; 16:0,16:0-GPC, 1,2-dipalmitoylglycerophosphocholine; 16:0,16:1-GPC, 1-palmitoyl-2-palmitooleoylglycerophosphocholine; 16:0,18:1-GPC, 1-palmitoyl-2-oleoylglycerophosphocholine; 18:1,18:1-GPC, 1,2-dioleoylglycerophosphocholine; 18:0,18:0-GPC, 1,2-distearoylglycerophosphocholine.

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In contrast to radioactive isotopes and *in vitro* systems for enzyme analysis, stable isotope tracers coupled with mass spectrometric analysis offer a sensitive, selective, and versatile approach to determining metabolic fluxes and enzymatic reactions in cells, organs, and living systems under physiological conditions (*14*). This investigative approach can determine the specific activity (enrichment) and amounts of specific molecules contained in a mixture. In addition, the distribution of the mass isotopomers among products is a useful tool in determining the product−precursor relationship, the precursor enrichment, and the metabolic pathways involved (*15*, *16*). Mass isotopomers are forms of a chemical entity with different isotope distributions. The analysis of the distribution of mass isotopomers is often termed mass isotopomer distribution analysis (MIDA).

From the results of conventional metabolic labeling and enzymatic analyses, a general consensus about PC metabolism in mammalian cells evolves (*17*). It is known that CTP: phosphocholine cytidyltransferase, which leads to the production of CDP-choline, is the rate-limiting enzyme (*18*). The regulation of this enzyme activity is through association and dissociation with the cell membrane. A number of factors, such as fatty acids, diacylglycerols, phorbol esters, and choline, stimulate the activity of this enzyme by promoting its association with the membrane. Besides this *de novo* synthetic pathway, another significant pathway exists which modifies the fatty acid composition by deacylation and reacylation.

In this investigation, we describe the use of mass isotopomer distribution analysis to determine the metabolic pathway and fluxes of the synthesis of molecular species of phosphatidylcholine. We use HL60 cells as a model system because these cells have been widely used in lipid signaling transduction research (*19*). In addition, HL60 cells can be grown in serum-free medium that minimizes the contribution of unknown factors and variations resulting from fetal bovine serum (*19*). Our results do not support the conventional model of parallel molecular species synthesis based on differential activities of acylglycerol transferases (*10*−*12*). Instead, our data are consistent with a sequential model through a dipalmitoylglycerophosphocholine intermediate, which is subject to acyl chain modification.

EXPERIMENTAL PROCEDURES

Reagents. The regular serum medium for a cell culture was RPMI1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY) and antibiotics (400 units/L penicillin, 4 mg/L streptomycin, and 10 μg/L amphotericin). Serum-free medium was prepared from commercial RPMI1640 supplemented with HEPES (10 mM), insulin (50 μg/L), transferrin (50 μg/L), sodium selenite (50 ng/L), penicillin (400 units), streptomycin (4 mg/L), and amphotericin (10 μg/L). [1,2,3,4-$^{13}C_4$]Palmitate (>99% isotopically pure) was obtained from Isotec (Miamisburg, OH). Sphingomyelinase (*Bacillus cereus*) was purchased from Sigma. 1,3-Dipentadecanoylglycerol and 1,2-pentadecanoylglycerophosphocholine used as internal standards for diacylglycerols and phosphatidylcholines, respectively, were purchased from Sigma or Avanti Polar Lipids (Alabaster, AL).

Preparation and Analysis of a Culture Medium Containing [1,2,3,4-$^{13}C_4$]Palmitate. The medium containing [1,2,3,4-$^{13}C_4$]palmitate in regular RPMI1640 with either 10% serum or 0.3% BSA was prepared as previously described (*21*). Their palmitate content was determined by gas chromatography on a Hewlett-Packard 5980B capillary column gas chromatograph as described previously (*21*). The palmitate isotopic (M + 4) enrichment was determined by an Agilent Technologies (Wilmington, DE) 5973 MSD gas chromatograph−mass spectrometer. The peak areas of *m*/*z* 317 (M4) versus *m*/*z* 313 (M0) were determined. Since the natural abundant isotope contribution to M4 from the natural M0 peak is negligible, the isotope enrichment was calculated by the equation $E = M4/(M0 + M4)$.

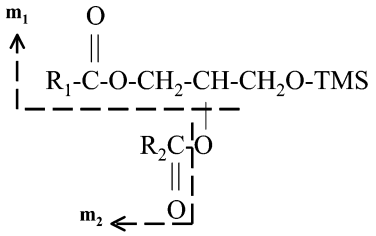
Cell Culture. HL60 human promyelocytic leukemia cells were purchased from ATCC (Manassas, VA). These cells were maintained in RPMI1640 with 10% fetal calf serum. Subculture was performed twice weekly by diluting subconfluent cells in new medium to a final concentration of 2×10^5 cells/mL. The cells were counted with a hemocytometer (Sigma). Cell viability was determined with trypan blue exclusion.

The cell treatment was performed in 25 mL of either regular 10% serum medium or serum-free medium in T-75 culture flasks at a cell density of 1×10^6 cells/mL. At the end of the experiments, the cells were collected by centrifugation. The collected cells were washed twice in PBS and brought up in 0.5 mL of Dulbecco's PBS [50 μL of this cell mix was diluted with an equal volume of 1% deoxycholate (DOC) for later protein determination] and frozen for later lipid analysis. Protein concentrations were determined using Lowry's reagent as previously described (*20*).

Lipid Analysis. PC was separated from phosphatidylethanolamine and other phospholipids (phosphatidylinositol, phosphatidylserine, and cardiolipin) using stepwise elution in a silica gel column (*20*). The latter eluting methanol fraction contains sphingomyelin, in addition to PC. These polar phospholipids were converted to the less polar diacylglycerols and ceramides via the action of sphingomyelinase (*20*). Diacylglycerols were then separated from ceramides with a second column of silica gel. Diacylglycerols were analyzed as their trimethylsilyl derivatives. In general, a cell pellet mixture (400 μL from $\sim 2 \times 10^7$ cells) with internal standards (7.4 nmol of 1,3-dipentadecanoylglycerol and 28 nmol of 1,2-pentadecanoylglycerophosphocholine) was used for lipid extraction and class separation.

Capillary Gas Chromatographic Analysis of Diacylglycerols. A Hewlett-Packard 5980 gas chromatograph was used for the analysis. The separation was achieved with a short (5 m, 0.25 mm inside diameter, 0.25 μm coating) column of methylsilicone (ZB-1 from Phenomenex, Torrance, CA). The temperature zones for the instrument were set at 290 °C for both the injector and FID detector. The initial oven temperature was set at 200 °C, and then increased at 20 °C/min to 280 °C. A splitless injection was used. The data analysis was performed with HP ChemStation software.

Gas Chromatographic−Mass Spectrometric Measurement of Diacylglycerols. An Agilent model 5973 Network Mass Selective Detector coupled to a model 6890 gas chromatographic system was used for the identification and isotope enrichment determination. The inlet system was set at 270 °C, the MSD transfer line at 280 °C, the MS source at 230 °C, and the MS quad at 150 °C. A column identical to that used for GC analysis was used. The oven temperature was



	R_1	R_2	m_1	m_2+74	M-15
16:0,16:1-	$C_{15}H_{31}$	$C_{15}H_{29}$	385	311	623
16:0,16:0-	$C_{15}H_{31}$	$C_{15}H_{31}$	385	313	625
16:0,18:1-	$C_{15}H_{31}$	$C_{17}H_{33}$	385	339	651
16:0,18:0-	$C_{15}H_{31}$	$C_{17}H_{35}$	385	341	653
18:1,18:1-	$C_{17}H_{33}$	$C_{17}H_{33}$	411	339	677
18:0,18:0-	$C_{17}H_{35}$	$C_{17}H_{35}$	413	341	679

FIGURE 1: Mass fragments, m_1 and $m_2 + 74$, are used for the determination of isotope enrichments of sn1 and sn2 acyl groups. M − 15 fragments, which contain both sn1 and sn2 carbons, are used for the determination of isotopomer distribution.

set at 200 °C, and then increased at 20 °C/min to 280 °C. The components of the sample were repetitively scanned from *m*/*z* 200 to 800 at a rate of 1.27 scans/s.

Mass Isotopomer Distribution Analysis (MIDA). Diacylglycerols are synthesized from two fatty acids (sn1 and sn2). Besides the M4-labeled palmitate precursor, other fatty acids can become labeled from this precursor through combinations of desaturation and chain elongation. Because the precursor is labeled as M4, the final diacylglycerol products should contain isotopomers of M0, M4, and M8. M0 is the isotopomer containing no label at all on sn1 or sn2. M4 is the isotopomer containing either sn1- or sn2-labeled acyl groups. M8 is the isotopomer containing both sn1- and sn2-labeled acyl groups. If the fractional enrichment of the precursor sn1 acyl pool equals *p* and that of the sn2 acyl pool equals *s*, then the mass isotopomer distribution of diacylglycerols should follow the equations M0 = (1 − *p*)-(1 − *s*), M4 = (1 − *p*)*s* + *p*(1 − *s*), and M8 = *ps*, if both sn1 and sn2 acyl groups are drawn from homogeneous pools. The parameter *p* is calculated from the (*m*/*z* 389)/(*m*/*z* 385 + *m*/*z* 389) ratio for sn1 palmitate (*21*). Similarly, the parameter *s* is calculated from the (*m*/*z* 243)/(*m*/*z* 239 + *m*/*z* 243) or (*m*/*z* 317)/(*m*/*z* 313 + *m*/*z* 317) ratio for sn2 palmitate (*21*). Other diacylglycerols are calculated as follows: (*m*/*z* 315)/(*m*/*z* 311 + *m*/*z* 315) for sn2 palmitooleate, (*m*/*z* 343)/(*m*/*z* 339 + *m*/*z* 343) for sn2 oleate, (*m*/*z* 345)/(*m*/*z* 341 + *m*/*z* 345) for sn2 stearate, (*m*/*z* 415)/(*m*/*z* 411 + *m*/*z* 415) for sn1 oleate, and (*m*/*z* 417)/(*m*/*z* 413 + *m*/*z* 417) for sn1 stearate. The mass spectral fragments that were monitored for isotope enrichments are summarized in Figure 1. There is overlap of sn2 palmitooleate mass fragments from 1-palmitoyl-2-palmitooleoylglycerol (16:0,16:1-DAG) with the sn2 palmitate from 1,2-dipalmitoylglycerol (16:0,16:0-DAG); therefore, the *m*/*z* 315 ion for this acyl group also contains some M2 isotope contribution from the *m*/*z* 313 ion of palmitate. The abundance of *m*/*z* 315 is corrected for this contribution; it is 5.8 ± 0.2% of that of *m*/*z* 313 ($n = 6$).

For a two-pool system with one unlabeled pool and one active labeled pool, the enrichments of precursor sn1 fatty acids are not known and assumed to equal p'. A fraction *f* is the fraction of the old and unlabeled PC pool. This *f* is equal to $1 - (p/p')$ since the apparent *p* is diluted from p' by the unlabeled pool. The true enrichment of the precursor sn2 fatty acids then becomes $s' = s/(1 - f)$. The mass isotopomer distribution from this system would be as follows: M0 = $f + (1 - p')(1 - s')(1 - f)$, M4 = $(1 - p')s'(1 - f) + s'(1 - p')(1 - f)$, and M8 = $p's'(1 - f)$. A simple mathematical relationship of these parameters is formulated using Excel (Microsoft). The *f* values are varied until the derived mass isotopomer distributions of M0, M4, and M8 are the closest fit to the experimentally derived distribution. In general, this close fit is within 0.03 of the experimentally derived values.

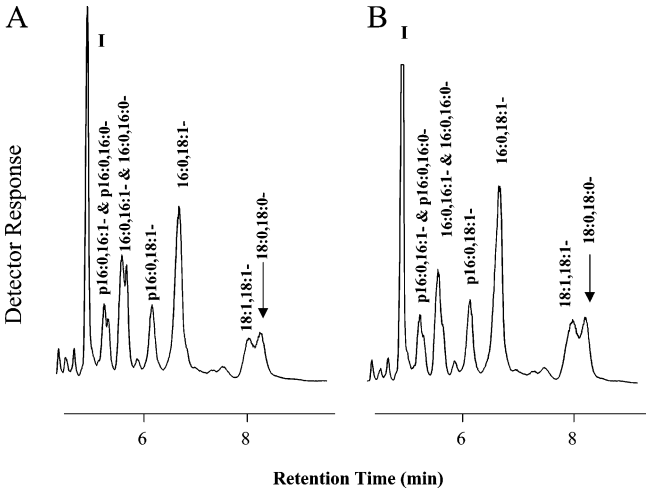


FIGURE 2: Gas chromatographic profiles of phosphatidylcholine molecular species from HL60 cells (2×10^7 cells): p16:0,16:1-, 1-hexadecyl-2-palmitooleoyl-GPC; p16:0,16:0-, 1-hexadecyl-2-palmitoyl-GPC; 16:0,16:1-, 1-palmitoyl-2-palmitooleoyl-GPC; 16:0,16:0-, 1,2-dipalmitoyl-GPC; p16:0,18:1-, 1-hexadecyl-2-oleoyl-GPC; 16:0,18:1-, 1-palmitoyl-2-oleoyl-GPC; 18:1,18:1-, 1,2-dioleoyl-GPC; 18:0,18:0-, 1,2-distearoyl-GPC. I is the internal standard, 1,2-dipentadecanoyl-GPC (28 nmol). (A) Serum-free control. (B) In the presence of palmitate (67 μM) in complex with bovine serum albumin. Both were incubated for 3 h.

The experimentally derived mass isotopomer distributions are calculated from the following ions: *m*/*z* 625 (M0), 629 (M4), and 633 (M8) for 16:0,16:0-DAG. These formulas were used: M0 = (*m*/*z* 625)/(*m*/*z* 625 + *m*/*z* 629 + *m*/*z* 633), M4 = (*m*/*z* 629)/(*m*/*z* 625 + *m*/*z* 629 + *m*/*z* 633), and M8 = (*m*/*z* 633)/(*m*/*z* 625 + *m*/*z* 629 + *m*/*z* 633). Likewise, *m*/*z* 623, 627, and 631 ions were used for 16:0,16:1-DAG. *m*/*z* 651, 655, and 659 ions were used for 1-palmitoyl-2-oleoylglycerol (16:0,18:1-DAG). *m*/*z* 653, 657, and 661 ions were used for 1-palmitoyl-2-steroylglycerol (16:0,18:0-DAG). *m*/*z* 677, 681, and 685 ions were used for 1,2-dioleoylglycerol (18:1,18:1-DAG). *m*/*z* 679, 683, and 687 ions were used for 1,2-distearoylglycerol (18:0,18:0-DAG). The mass fragments used for the determination of sn1 and sn2 acyl group enrichments are summarized in Figure 1. In addition, the M − 15 mass fragments, which contain both sn1 and sn2 groups, used for the determination of mass isotopomer distribution are denoted.

RESULTS

Phosphatidylcholine Content and Molecular Species in HL60 Cells Grown under Serum and Serum-Free Conditions. As shown in Figure 2A, 1-palmitoyl-2-oleoylglycerophosphocholine (16:0,18:1-GPC) is the major molecular species. It is followed by 1-palmitoyl-2-palmitooleoylglycerophosphocholine (16:0,16:1-GPC) and 1,2-dipalmitoylglycerophosphocholine (16:0,16:0-GPC). Other significant molecular

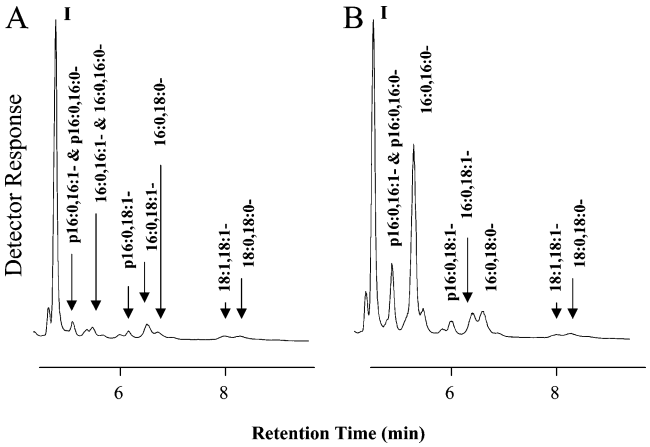


FIGURE 3: Gas chromatographic profiles of diacylglycerol molecular species from HL60 cells (2×10^7 cells): p16:0,16:1-, 1-hexadecyl-2-palmitooleoylglycerol; p16:0,16:0-, 1-hexadecyl-2-palmitoylglycerol; 16:0,16:1-, 1-palmitoyl-2-palmtiooleoylglycerol; 16:0,16:0-, 1,2-dipalmitoylglycerol; p16:0,18:1-, 1-hexadecyl-2-oleoylglycerol; 16:0,18:1-, 1-palmitoyl-2-oleoylglycerol; 16:0,18:0-, 1-palmitoyl-2-stearoylglycerol; 18:1,18:1-, 1,2-dioleoylglycerol; 18:0,18:0-, 1,2-distearoylglycerol. I is the internal standard, 1,2-dipentadecanoylglycerol (6.4 nmol). (A) Serum-free control. (B) In the presence of palmitate (67 μM) in complex with bovine serum albumin. Both were incubated for 3 h.

species include 1,2-dioleoylglycerophosphocholine (18:1,18:1-GPC) and 1,2-distearoylglycerophosphocholine (18:0,18:0-GPC). The rest of the significant lipids are ether lipids which include 1-hexadecyl-2-palmitooleoylglycerophosphocholine (p16:0,16:1-GPC), 1-hexadecyl-2-palmitoylglycerophosphocholine (p16:0,16:0-GPC), and 1-hexadecyl-2-oleoylglycerophosphocholine (p16:0,18:1-GPC). Other minor molecular species of phosphatidylcholine can be detected with GC−MS, but none are more than 1% of the total amount.

Diacylglycerol Content and Molecular Species in HL60 Cells. The molecular species distribution of diacylglcerol (Figure 3A) does not reflect that observed in phosphatidylcholine. Significant amounts of 1-hexadecyl-2-palmitooleoylglycerol (p16:0,16:1-DAG), 1-hexadecyl-2-palmitoylglycerol (p16:0,16:0-DAG), 1-palmitoyl-2-palmitooleoylglycerol (16:0,16:1-DAG), 1,2-dipalmitoylglycerol (16:0,16:0-DAG), 1-hexadecyl-2-oleoylglycerol (p16:0,18:1-DAG), 1-palmitoyl-2-oleoylglycerol (16:0,18:1-DAG), and 1-palmitoyl-2-stearoylglycerol (16:0,18:0-DAG) are found. These species are evenly distributed. In this fraction, 16:0,18:0-DAG is present in approximately the same amount as 16:0,18:1-DAG. However, the corresponding phosphatidylcholine, 16:0,18:0-GPC, exists in only trace amounts (<1% of the total), while the closely related 16:0,18:1-GPC is the predominant species. The total amount of diacylglycerol is ~1.3 nmol/10^7 cells, roughly 2% of the amount of phosphatidylcholine.

Phosphatidylcholine and Diacylglycerol in HL60 Cells Grown in Palmitate-Enriched Media. When the cells were grown in the presence of palmitate (27−68 μM) for 1−7 h, the level of total diacylglycerols is significantly elevated 4−6-fold (Figure 3B). The increase in the amount of 16:0,-16:0-DAG, which is elevated by more than 10−20-fold, accounts for most of the increase in the level of diacylglycerol. The increase in the amount of diacylglycerol is directly related to the palmitate concentration in the media (data not shown). Furthermore, use of serum-free media resulted in an increase in the amount of diacylglycerol greater than that found in corresponding cells grown in serum media.

In contrast to the diacylglycerol level, the cellular content of phosphatidylcholine (Figure 2B) is not significantly altered by the presence of palmitate in the medium. The levels of only p16:0,16:0-GPC and 16:0,16:0-GPC are slightly, but significantly, elevated when the cells are grown in serum-free media containing palmitate. The molecular species of diacylglycerols and phosphatidylcholines are summarized in Table 1.

Enrichments of Fatty Acids in sn1 and sn2 Positions of Diacylglycerols. Steady-state enrichments and concentrations of 16:0,16:0-DAG are reached between the 3 and 24 h time points of incubation (Figure 4A). The steady-state enrichments that are attained do not differ between sn1 and sn2 palmitate (sn2/sn1 ratio = 1; Table 2). Furthermore, these enrichments are the same as the medium palmitate enrichments (81 ± 6% for media vs 84 ± 4% for 16:0,16:0-DAG; $n = 6$). In comparison, the enrichments of sn1 palmitate on 16:0,18:1-DAG and 16:0,18:0-DAG are smaller than those on 16:0,16:0-DAG during early incubations, but eventually reached the same enrichments at the end of 24 h incubations. However, the sn2 oleate (from 16:0,18:1-DAG) and stearate (from 16:0,18:0-DG) enrichments are significantly smaller than those of the sn1 palmitate (Table 2). The differences in enrichments between the sn2 and sn1 acyl groups are significantly greater for 16:0,18:1-DAG than for 16:0,18:0-DAG.

During shorter incubation times (20−60 min) and with a lower initial palmitate concentration (27 μM) in the medium, near-steady-state concentrations and enrichments were reached in 20 min (Figure 4B). However, essentially the same relationship as in experiments with a higher palmitate concentration and a longer incubation time (Figure 4A) exists between sn1 and sn2 acyl group enrichments. During a prolonged incubation (24 h) as in Figure 4A but with a lower palmitate concentration (<27 μM), the enrichments decrease significantly to almost natural abundance (data not shown).

Enrichments of Fatty Acids in sn1 and sn2 Positions of Phosphatidylcholines. The enrichments in sn1 and sn2 acyl groups from phosphatidylcholines are shown in Figure 5. One major difference between sn2 and sn1 palmitate of 16:0,16:0-GPC is that significantly greater enrichments occur at the sn2 position (sn2/sn1 ratio > 1; Table 3). For 16:0,-16:1-GPC, the sn1 palmitate and sn2 palmitooleate enrichments are not significantly different from each other. Likewise, the sn2 acyl enrichments are the same as those at sn1 for 18:1,18:1-GPC and 18:0,18:0-GPC. Like the relationship of sn1 and sn2 acyl enrichments in the diacylglycerol fraction, the sn2 oleate of 16:0,18:1-GPC is significantly less enriched than the sn1 palmitate.

Mass Isotopomer Distribution of Diacylglycerols. The expected mass isotopomer distributions of diacylglycerols are calculated on the basis of compartmental analysis (Table 2). As shown in Figure 6A, the actual distributions of M0, M4, and M8 are compared to those calculated on the basis of a one-compartment model. Obviously, the actual M0 and M8 distributions are larger than those predicted from the one-compartment model, and the actual M4 distribution is smaller than predicted (Table 2). This type of discrepancy suggests a possible two-compartment model with a second completely unlabeled old pool. A perfect fit of mass isotopomer

Table 1: Molecular Species Distribution (DAG and PC) of HL60 Cells under Different Growth Conditions[a]

	p16:0,16:1-DAG and p16:0,16:0-DAG	16:0,16:1-DAG and 16:0,16:0-DAG	p16:0,18:1-DAG	16:0,18:1-DAG	16:0,18:0-DAG	total
A ($n = 10$)	0.42 ± 0.17	0.35 ± 0.13	0.20 ± 0.07	0.35 ± 0.10	0.29 ± 0.05	1.32 ± 0.45
B ($n = 4$)	0.32 ± 0.09	0.23 ± 0.10	0.25 ± 0.06	0.63 ± 0.24	0.50 ± 0.31	1.44 ± 0.38
C ($n = 6$)	2.72 ± 1.03^b	6.30 ± 2.37^b	0.57 ± 0.36^b	1.32 ± 0.43^b	0.82 ± 0.40	10.91 ± 3.53*
D ($n = 6$)	$1.64 \pm 0.42^{b,c}$	$3.65 \pm 2.22^{b,c}$	0.24 ± 0.07^c	0.58 ± 0.23^c	0.80 ± 0.11	5.99 ± 2.30*$^+$
	p16:0,16:1-PC and p16:0,16:0-PC	16:0,16:1-PC and 16:0,16:0-PC	p16:0,18:1-PC	16:0,18:1-PC	18:1,18:1-PC and 18:0,18:0-PC	total
A ($n = 10$)	7.47 ± 2.95	11.71 ± 2.84	8.8 ± 3.30	18.77 ± 4.69	7.28 ± 2.06	54.03 ± 12.79
B ($n = 4$)	6.83 ± 1.08	13.20 ± 2.36	8.63 ± 1.82	25.43 ± 5.46	11.50 ± 2.86	65.58 ± 13.23
C ($n = 6$)	14.18 ± 1.87^b	19.52 ± 5.58^b	10.28 ± 1.71	16.92 ± 2.17	5.26 ± 0.88	65.28 ± 8.30
D ($n = 6$)	7.15 ± 1.65^c	19.28 ± 5.35	6.92 ± 2.08	21.37 ± 5.26	4.55 ± 1.01^b	59.27 ± 14.63

[a] All values are expressed as nanomoles per milligram of protein. Abbreviations: p16:0,16:1-, 1-hexadecyl-2-palmitooleoylglycerol; p16:0,16:0-, 1-hexadecyl-2-palmitoylglycerol; 16:0,16:1-, 1-palmitoyl-2-palmitooleoylglycerol; 16:0,16:0-, 1,2-dipalmitoylglycerol; p16:0,18:1-, 1-hexadecyl-2-oleoylglycerol; 16:0,18:1-, 1-palmitoyl-2-oleoylglycerol; 16:0,18:0-, 1-palmitoyl-2-stearoyl; 18:1,18:1-, 1,2-dioleoylglycerol; 18:0,18:0-, 1,2-distearoylglycerol. Growth conditions: (A) serum-free medium, (B) 10% serum medium, (C) serum-free medium containing palmitate (27−68 μM) in complex with BSA, and (D) 10% serum medium containing palmitate (27−68 μM). All analyses were carried out on cells collected after incubation for 1−7 h. Statistics are from a Student's *t* test. There is no difference in profiles under conditions A and B, except 18:1,18:1-PC and 18:0,18:0-PC ($p < 0.01$). [b] Significantly different from controls (no palmitate). [c] Significantly different under conditions C and D.

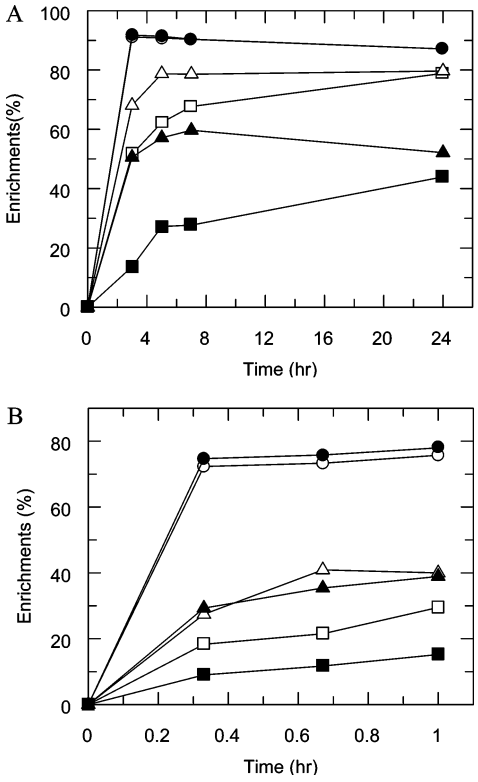


FIGURE 4: Enrichments of fatty acids in sn1 and sn2 of diacylglycerols: (○) sn1−16:0 from 16:0,16:0-DAG, (●) sn2−16:0 from 16:0,16:0-DAG, (□) sn1−16:0 from 16:0,18:1-DAG, (■) sn2−18:1 from 16:0,18:1-DAG, (△) sn1−16:0 from 16:0,18:0-DAG, and (▲) sn2−18:0 from 16:0,18:0-DAG. (A) Cells were incubated from 3 to 24 h in 68 μM [1,2,3,4-$^{13}C_4$]palmitate in complex with BSA in serum-free medium. (B) Cells were incubated from 20 to 60 min in 27 μM [1,2,3,4-$^{13}C_4$]palmitate in complex with BSA in serum-free medium. The ions used for these enrichment determinations were as follows: *m*/*z* 389 (M4) vs *m*/*z* 385 for sn1−16:0 of 16:0,16:0-DAG, 16:0,18:1-DAG, and 16:0,18:0-DAG; *m*/*z* 243 vs *m*/*z* 239 or *m*/*z* 317 vs *m*/*z* 313 for sn2−16:0 of 16:0,16:0-DAG; *m*/*z* 343 vs *m*/*z* 339 for sn2−18:1 of 16:0,18:1-DAG; and *m*/*z* 345 vs *m*/*z* 341 for sn2−18:0 of 16:0,18:0-DAG.

distribution to those of experimentally determined values is obtained when an unlabeled pool fraction is assigned to the model. In general, the unlabeled 16:0,16:0-DAG pool fraction decreases with incubation time, i.e., 0.19 ± 0.05 ($n = 6$; 20 min), 0.13 ± 0.04 ($n = 8$; 40 min), 0.10 ± 0.03 ($n = 4$; 60 min), and 0.07 ± 0.03 ($n = 4$; 3−5 h) (Table 2, *f* fraction).

While 16:0,16:0-DAG is fitted to a two-compartment model without any adjustment, the isotopomer distributions of 16:0,18:1-DAG and 16:0,18:0-DAG do not fit in either a one-compartment or two-compartment model during a short incubation (0.66 h, Table 2). The actual M8 distributions are larger, while the M4's are smaller than those calculated from a one-compartment model. When the data are fitted to a two-compartment model for the best M8 fit, the M0's become greater while the M4's become smaller than the determined values (data not shown). An adjustment of changing M0's to M4's (M4 adj in Table 2) is needed to make the fit. This indicates that labeled fatty acids exist in the unlabeled diacylglycerol compartment. The exchange of labeled fatty acids with the acyl groups in this old and unlabeled pool shifts M0 in this compartment to M4 without any change in M8. When the incubation time is prolonged, the size of the unlabeled pool decreases and the adjustment for M4 disappears. In these longer incubations, the observed isotopomer distribution fits a two-compartment model.

Mass Isotopomer Distribution of 1,2-Dipalmitoylglycerophosphocholine. A two-compartment model also accounts for the synthesis of 16:0,16:0-GPC (Table 3). These two compartments are comprised of an unlabeled pool (*f*, Table 3), which decreases in size with incubation time, and a labeled pool that increases in size with incubation time.

There is one significant difference between the label pattern of 16:0,16:0-DAG and 16:0,16:0-GPC. While the enrichments on sn1 and sn2 of 16:0,16:0-DAG are not significantly different, the sn2 enrichments of 16:0,16:0-GPC are 10−20% greater than those for sn1 during short incubations (Table 3). In addition, the M4 distributions are significantly greater than the distributions predicted from a simple two-compartment model, while the M0 enrichments are smaller. We believe that this pattern results from the replacement of unlabeled palmitoyl group with the labeled palmitate (Figure 6B). For every replacement of the acyl group in the unlabeled compartment, there is a decrease in M0 and a corresponding increase in M4 species. Therefore, acyl group modification is not specific for the generation of

Table 2: Isotope Enrichments and Isotopomer Distribution of Diacylglycerols after HL60 Cells Had Grown in [^{13}C]Palmitate Medium[a]

time (h)	*N*	*E*,sn1 (%)	*E*,sn2 (%)	isotopomers (%), determined			isotopomers (%), predicted from one-compartment model			two-compartment model			
				M0	M4	M8	M0	M4	M8	sn2/sn1 ratio	*f*	*E*,sn1,pre (%)	*E*,sn2,pre (%)	M4,adj (%)
								16:0,16:0-DAG						
0.66	4	68.8 ± 6.1	71.1 ± 5.1	18.3 ± 2.9	27.5 ± 5.6	54.3 ± 7.6	9.8 ± 4.3	42.3 ± 4.0	48.5 ± 8.6	1.03 ± 0.02	0.13 ± 0.03	78.7 ± 6.3	78.7 ± 6.3	0
3−5	4	74.8 ± 6.3	76.3 ± 6.1	11.0 ± 4.4	29.5 ± 4.5	60.0 ± 8.7	6.8 ± 3.0	37.0 ± 6.5	56.3 ± 9.4	1.02 ± 0.02	0.07 ± 0.03	79.9 ± 4.9	79.9 ± 4.9	0
24	3	60.2 ± 4.9	62.0 ± 3.8	21.3 ± 5.7	41.3 ± 0.6	37.7 ± 5.7	15.7 ± 3.2	47.3 ± 2.1	37.3 ± 5.9	1.03 ± 0.02	0.04 ± 0.02	62.5 ± 4.0	62.5 ± 4.0	0
								16:0,18:1-DAG						
0.66	4	23.3 ± 3.0	94. ± 4.0	74.5 ± 4.4	21.5 ± 2.9	4.3 ± 1.5	69.8 ± 5.6	28.0 ± 4.2	2.3 ± 1.5	0.40 ± 0.12	0.40 ± 0.11	40.1 ± 10.0	16.4 ± 7.9	16.0 ± 2.9
3−5	4	49.0 ± 4.9	20.6 ± 5.9	46.0 ± 6.4	42.8 ± 3.1	11.3 ± 3.4	40.8 ± 6.5	49.0 ± 2.8	10.3 ± 3.8	0.42 ± 0.10	0.12 ± 0.09	56.2 ± 6.7	23.1 ± 4.9	0.0 ± 4.2
24	3	56.3 ± 4.0	19.1 ± 1.7	37.7 ± 4.2	51.7 ± 2.1	11.0 ± 2.0	35.3 ± 4.0	54.0 ± 2.0	11.0 ± 2.0	0.34 ± 0.01	0.05 ± 0.02	59.3 ± 4.7	20.1 ± 2.0	0.0 ± 1.0
								16:0,18:0-DAG						
0.66	4	38.0 ± 4.3	23.8 ± 2.3	48.8 ± 5.4	32.0 ± 4.2	19.3 ± 3.8	47.3 ± 2.9	43.8 ± 2.2	9.0 ± 1.4	0.63 ± 0.11	0.53 ± 0.06	79.3 ± 6.9	53.2 ± 16.0	13.5 ± 4.1
3−5	4	56.8 ± 5.1	37.5 ± 11.4	31.5 ± 11.8	38.3 ± 1.7	30.3 ± 12.8	27.3 ± 7.8	50.8 ± 1.5	21.8 ± 8.8	0.65 ± 0.14	0.28 ± 0.05	79.0 ± 10.4	52.4 ± 17.5	7.8 ± 7.0
24	3	53.6 ± 4.0	36.4 ± 6.5	33.7 ± 6.5	45.7 ± 2.3	20.7 ± 5.0	29.7 ± 5.1	50.7 ± 0.6	19.7 ± 5.0	0.68 ± 0.09	0.08 ± 0.04	58.2 ± 3.2	39.5 ± 5.6	0.3 ± 0.6

[a] Abbreviations: 16:0,16:0-DAG, 1,2-dipalmitoylglycerol; 16:0,18:1-DAG, 1-palmitoyl-2-oleoylglycerol; 16:0,18:0-DAG, 1-palmitoyl-2-stearoylglycerol; *N*, number of experiments; *E*,sn1, M4 enrichment determined on the sn1 acyl group; *E*,sn2, M4 enrichment determined on the sn2 acyl group; *E*,sn1,pre, enrichment of the precursor acyl group for sn1 acyl groups; *E*,sn2,pre,%, enrichment of the precursor pool for sn2 acyl groups; sn2/sn1 ratio, enrichment ratio of sn2 acyl and sn1 acyl groups; *f*, unlabeled pool fraction of a two-compartment model; M4,adj, adjustment of adding the M4 isotopomer to fit the model predicted distribution; M0, isotopomer with no label; M4, isotopomer with four labels; M8, isotopomer with eight labels. HL60 cells were grown in [1,2,3,4-$^{13}C_4$]palmitate (27 μM)-enriched medium for 0.66−24 h.

FIGURE 5: Enrichments of fatty acids in the sn1 and sn2 positions of phosphatidylcholines: (A) (○) sn1−16:0 from 16:0,16:0-GPC, (●) sn1−16:0 from 16:0,16:1-GPC, (□) sn1−16:0 from 16:0,18:1-GPC, (■) sn1−18:1 from 18:1,18:1-GPC, and (△) sn1−18:0 from 18:0,18:0-GPC and (B) (○) sn2−16:0 from 16:0,16:0-GPC, (●) sn2−16:1 from 16:0,16:1-GPC, (□) sn2−18:1 from 16:0,18:1-GPC, (■) sn2−18:1 from 18:1,18:1-GPC, and (△) sn2−18:0 from 18:0,18:0-GPC. The cells were incubated from 3 to 24 h in 69 μM [1,2,3,4-$^{13}C_4$]plamitate in complex with BSA.

unsaturated acyl groups; it also occurs for a saturated acyl group. This futile cycle of palmitoyl group modification occurs in the old and unlabeled 16:0,16:0-GPC pool.

Mass Isotopomer Distribution of 1-Palmitoyl-2-palmitooleoylglycerophosphocholine. Our data (Table 3) show that the synthesis of 16:0,16:1-GPC follows a two-compartment model with the enrichments of sn1 palmitate and sn2 palmitooleate indistinguishable from each other. Furthermore, the precursor palmitate pool enrichment is significantly more diluted than the palmitate pool used for 16:0,16:0-GPC synthesis. Instead of the same enrichments as the medium palmitate, the concentration of this precursor palmitate pool varies from 14.4 to 51.5% and increases with incubation time.

Mass Isotopomer Distribution of 1-Palmitoyl-2-oleoylglycerophosphocholine. The isotopomer distribution of 16:0,18:1-GPC is consistent with a one-compartment model of synthesis (Figure 6C). Differing from those for 16:0,16:1-GPC, the enrichments on sn2 oleate are significantly smaller than those on sn1 palmitate with a ratio of ~0.4 (Table 3). Since a one-compartment model best describes the biosynthesis of 16:0,18:1-GPC, its precursor palmitate enrichments are the experimentally determined sn1 enrichments [*E*,sn1 (%) in Table 3]. The sn1 precursor enrichments of 16:0,18:1-GPC are significantly lower than those used for the synthesis of 16:0,16:1-GPC.

Mass Isotopomer Distribution of 1,2-Distearoylglycerophosphocholine and 1,2-Dioleoylglycerophosphocholine. These

Table 3: Isotope Enrichments and Isotopomer Distribution of Phosphatidylcholines after HL60 Cells Had Grown in [^{13}C]Palmitate-Enriched Medium[a]

time (h)	*N*	*E*,sn1 (%)	*E*,sn2 (%)	isotopomers (%), determined			isotopomers (%), predicted from one-compartment model				two-compartment model			
				M0	M4	M8	M0	M4	M8	sn2/s n1 ratio	*f*	*E*,sn1,pre (%)	*E*,sn2,pre (%)	M4,adj (%)
								16:0,16:0-GPC						
0.33	5	28.3 ± 6.8	44.7 ± 7.8	50.8 ± 8.8	27.8 ± 2.4	21.6 ± 6.5	47.4 ± 14.8	42.0 ± 8.3	10.6 ± 6.6	1.60 ± 0.13	0.63 ± 0.07	75.7 ± 4.8		18.4 ± 1.1
0.66	4	27.8 ± 6.4	44.2 ± 4.1	51.4 ± 9.0	26.6 ± 5.3	21.6 ± 4.2	46.6 ± 9.3	43.4 ± 5.4	10.0 ± 4.1	1.7 ± 0.15	0.66 ± 0.12	80.9 ± 9.1		18.2 ± 2.8
3−5	7	50.0 ± 5.4	58.9 ± 4.8	29.1 ± 7.9	34.1 ± 2.8	36.9 ± 6.6	20.9 ± 4.5	49.6 ± 1.0	29.6 ± 5.3	1.18 ± 0.06	0.32 ± 0.05	73.1 ± 6.6		10.1 ± 4.8
24	3	54.5 ± 3.9	60.8 ± 4.6	24.3 ± 4.7	43.7 ± 0.6	32.0 ± 4.6	19.3 ± 4.0	49.0 ± 1.0	31.3 ± 4.6	1.12 ± 0.06	0.08 ± 0.0	59.2 ± 4.2		1.0 ± 1.7
								16:0,16:1-GPC						
0.33	5	9.8 ± 3.0	11.0 ± 3.7	88.2 ± 3.3	8.0 ± 2.0	3.6 ± 1.8	80.6 ± 6.0	18.4 ± 5.3	1.0 ± 0.7	1.12 ± 0.12	0.19 ± 0.02	14.4 ± 22	16.3 ± 3.4	
0.66	4	14.2 ± 4.6	17.5 ± 5.9	81.7 ± 5.5	12.3 ± 3.5	6.0 ± 2.0	70.7 ± 8.5	26.0 ± 7.0	2.3 ± 1.5	1.22 ± 0.16	0.20 ± 0.07	20.5 ± 7.5	26.0 ± 6.8	
3−5	7	36.9 ± 5.0	41.7 ± 6.0	53.7 ± 5.5	28.8 ± 2.2	17.5 ± 4.2	37.0 ± 6.7	47.3 ± 2.2	15.7 ± 4.6	1.13 ± 0.03	0.27 ± 0.03	49.0 ± 3.9	53.5 ± 4.2	
24	3	49.9 ± 2.8	43.2 ± 2.4	37.0 ± 3.6	43.0 ± 1.0	20.3 ± 2.3	28.3 ± 2.3	50.0 ± 0.0	21.7 ± 2.1	0.87 ± 0.05	0.03 ± 0.02	51.5 ± 5.2	45.7 ± 1.8	
								16:0,18:1-GPC						
0.33	5	6.8 ± 1.8	2.8 ± 0.4	91.8 ± 1.3	7.6 ± 1.8	0.6 ± 0.5	90.6 ± 2.1	9.2 ± 2.2	0.0 ± 0.0	0.42 ± 0.07				
0.66	4	9.3 ± 3.0	3.8 ± 0.9	91.0 ± 0.0	8.7 ± 0.6	1.0 ± 0.0	87.3 ± 3.8	12.3 ± 3.2	0.3 ± 0.6	0.42 ± 0.05				
3−5	7	30.1 ± 4.4	11.1 ± 2.5	65.2 ± 5.1	29.8 ± 3.3	5.2 ± 1.8	62.2 ± 5.5	34.4 ± 4.2	3.4 ± 1.1	0.36 ± 0.03				
24	3	50.9 ± 3.2	19.0 ± 1.5	41.3 ± 4.2	48.7 ± 2.5	9.7 ± 1.5	39.7 ± 3.5	50.3 ± 2.1	9.7 ± 1.5	0.37 ± 0.01				
								18:1,18:1-GPC						
0.33	5	0.8 ± 0.2	1.3 ± 0.2	97.4 ± 0.5	2.0 ± 0.0	0.2 ± 0.4	98.0 ± 0.0	2.0 ± 0.0	0.0 ± 0.0	1.65 ± 0.23				
0.66	4	1.7 ± 0.5	3.0 ± 1.5	93.0 ± 4.0	4.3 ± 2.1	2.3 ± 2.5	95.3 ± 1.5	4.7 ± 1.5	0.0 ± 0.0	1.68 ± 0.44				
3−5	7	4.4 ± 1.2	5.7 ± 1.3	89.8 ± 2.2	9.3 ± 2.2	1.3 ± 0.5	90.3 ± 2.6	9.5 ± 2.4	0.0 ± 0.0	1.29 ± 0.10				
24	3	14.3 ± 1.0	15.1 ± 1.2	72.7 ± 1.5	24.3 ± 1.5	3.3 ± 0.6	72.7 ± 1.5	25.3 ± 1.5	2.3 ± 0.6	1.06 ± 0.02				
								18:0,18-GPC						
0.33	5	1.3 ± 0.2	1.5 ± 0.2	96.8 ± 0.4	2.8 ± 0.4	0.2 ± 0.4	97.2 ± 0.4	2.8 ± 0.4	0.0 ± 0.0	1.22 ± 1.25				
0.66	4	1.9 ± 0.9	2.5 ± 1.2	95.0 ± 2.0	3.7 ± 1.2	1.0 ± 1.0	95.7 ± 2.1	4.3 ± 2.1	0.0 ± 0.0	1.34 ± 0.53				
3−5	7	8.2 ± 2.9	8.2 ± 2.4	85.5 ± 3.7	13.0 ± 3.2	1.8 ± 1.0	84.3 ± 4.6	14.8 ± 4.6	0.5 ± 0.6	1.02 ± 0.07				
24	3	29.9 ± 5.4	21.7 ± 1.5	59.3 ± 5.1	35.7 ± 4.2	5.0 ± 1.0	54.7 ± 4.0	38.7 ± 2.5	6.3 ± 1.5	0.75 ± 0.19				

[a] Abbreviations: 16:0,16:0-GPC, 1,2-dipalmitoylglycerophosphocholine; 16:0,18:1-GPC, 1-palmitoyl-2-oleoylglycerophosphocholine; 16:0,16:1-GPC, 1-palmitoyl-2-palmitooleoylglycerophosphocholine; 18:1,18:1-GPC, 1,2-dioleoylglycerophosphocholine; 18:0,18:0-GPC, 1,2-distearoylglycerophosphocholine; *N*, number of experiments; *E*,sn1, M + 4 enrichment determined on the sn1 acyl group; *E*,sn2, M + 4 enrichment determined on the sn2 acyl group; *E*,sn1,pre, enrichment of the precursor acyl group for the sn1 acyl group; *E*,sn2,pre, enrichment of the precursor pool for the sn2 acyl group; sn2/sn1 ratio, enrichment ratio of sn2 acyl and sn1 acyl groups; *f*, unlabeled pool fraction of a two-pool model; M4,adj, adjustment of adding the M4 isotopomer to fit the model-predicted distribution; M0, isotopomer with no label; M4, isotopomer with four labels; M8, isotopomer with eight labels. HL60 cells were grown in [1,2,3,4-$^{13}C_4$]palmitate (27 μM)-enriched medium for 0.66−24 h. The phosphatidylcholine fraction was isolated through silica gel column chromatography and analyzed with gas chromatography−mass spectrometry as trimethylsilyl derivatives after enzymatic hydrolysis to liberate diacylglycerols.

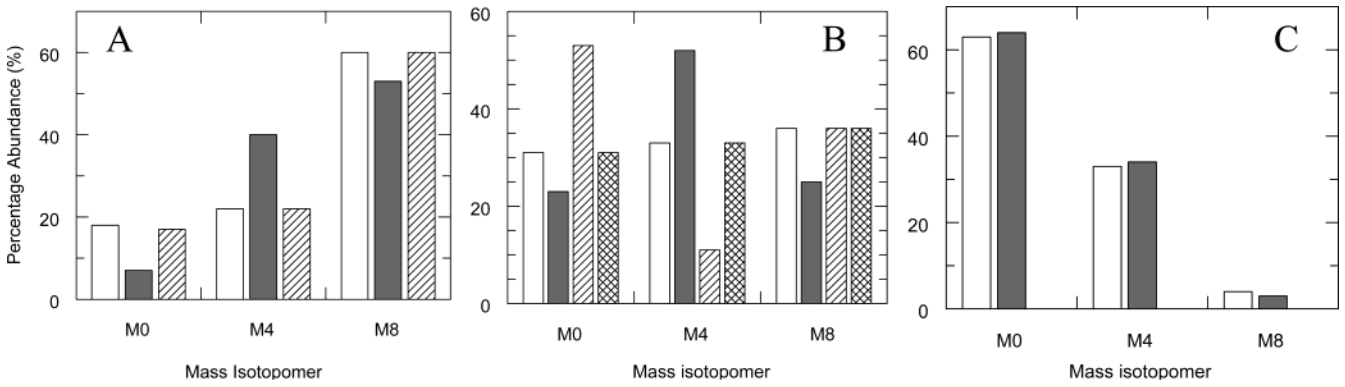


FIGURE 6: Mass isotopomer distribution analysis of phosphatidylcholines. (A) 1,2-Dipalmitoylglycerol: (empty bars) actual mass isotopomer distribution determined from the percentage composition of *m*/*z* 625 (M0), *m*/*z* 629 (M4), and *m*/*z* 633 (M8), (gray bars) expected mass isotopomer distribution calculated from the enrichments determined on sn1 (72%) and sn2 (74%) of palmitate groups based on a one-compartment model, and (hatched bars) expected mass isotopomer distribution calculated from the enrichments determined on sn1 and sn2 palmitate based on a two-compartment model with an unlabeled second pool fraction of 0.15. (B) 1,2-Dipalmitoylglycerophosphocholine: (empty bars) actual mass isotopomer distribution determined from the percentage composition of *m*/*z* 625 (M0), *m*/*z* 629 (M4), and *m*/*z* 633 (M8), (gray bars) expected mass isotopomer distribution calculated from the enrichments determined on sn1 (41.9%) and sn2 (60.1%) of palmitate groups based on a one-compartment model, (hatched bars) expected mass isotopomer distribution calculated from the enrichments determined on sn1 palmitate based on a two-compartment model with an unlabeled second pool fraction of 0.51, and (cross-hatched bars) modification of the two-compartment model by acylation of 22% of M0 with M4 palmitoyl-CoA to M4. (C) 1-Palmitoyl-2-oleoylglycerophosphocholine: (empty bars) actual mass isotopomer distribution determined from the percentage composition of *m*/*z* 651 (M0), *m*/*z* 655 (M4), and *m*/*z* 659 (M8) and (gray bars) expected mass isotopomer distribution calculated from the enrichments determined on sn1 (29.9%) palmitate and sn2 (9.4%) oleate groups based on a one-compartment model.

compounds are only minor components of the total phosphatidylcholine. The enrichments on sn2 are slightly greater than the enrichments on sn1 acyl groups in the early incubation periods for both compounds. They are not significantly different from each other after extended incubation times (Table 3). The mass isotopomer distribution of M0, M4, and M8 follows a one-compartment model. Since a one-compartment model is the best fit, the experimentally determined sn1 and sn2 acyl enrichments are their precursor enrichments. These precursor enrichments are smaller than the precursor values used for 16:0,16:1-GPC and 16:0,18:1-GPC syntheses. Furthermore, the precursor enrichments for 18:1,18:1-GPC synthesis are smaller than those for 18:0,18:0-GPC synthesis. It appears that the extent of precursor enrichments is related to the complexity of the synthesis. The more steps that are needed to yield the compound, the more diluted the precursor sn1 acyl enrichments become.

Turnover Rates of Diacylglycerols and Phosphatidylcholines. In general, the turnover rate of an endogenous compound is determined from the increase in enrichments with time at a steady state. Apparently, the turnover rates for dipalmitoylglycerol cannot be determined this way. The amount of this metabolite is elevated, and the enrichments are saturated at the precursor values after a short incubation time. Nevertheless, the rate of synthesis of 16:0,16:0-DAG is estimated from the increase in the pool size of this metabolite after a short incubation (10 min) in 7−53 μM labeled palmitate. In these experiments ($n = 4$), the level of 16:0,16:0-DAG is increased to 3.67 ± 0.18 nmol/10^7 cells from a basal value of 0.35 ± 0.13. Therefore, the rate of synthesis of 16:0,16:0-DAG is at least 3.3 nmol/10 min (3.67−0.35). In these experiments, the amounts of 16:0,18:1-DAG and 16:0,18:0-DAG are not significantly changed; therefore, their turnover rates are determined from the increase in the enrichments on sn1 palmitate. The fractional turnover rates are 16.03 ± 2.13%/10 min for 16:0,18:1-DAG and 29.88 ± 3.00%/10 min for 16:0,18:0-DAG. On the basis of the pool sizes (Table 1), the absolute turnover rates are 0.06 nmol/10 min for 16:0,18:1-DAG and 0.09 nmol/10 min for 16:0,18:0-DAG.

The fractional turnover rates of 16:0,16:0-GPC are determined from the slope of the sn1 palmitate enrichment increase in 3−7 h incubations and 10−60 min incubations. With shorter incubation times (10−60 min), the fractional turnover rates are 34.1 ± 16.6%/h ($r^2 = 0.94 \pm 0.05$; $n = 7$). From the pool size of 16:0,16:0-GPC (Table 1), the absolute turnover rate is 0.33 nmol/10 min, accounting for ~10% of the total precursor 16:0,16:0-DAG turnover. When the incubation time is longer, from 3 to 7 h, the fractional turnover rates decreased to 2.8 ± 0.4%/h ($r^2 = 0.97 \pm 0.04$; $n = 7$). The increase in sn1 and sn2 acyl group enrichments in other PC species essentially parallels that of dipalmitoylglycerophosphocholine. This relationship indicates that the turnover of 16:0,16:0-GPC is the primary event and the turnovers of other phosphatidylcholines are downstream events controlled by the enrichments of the precursor 16:0,16:0-DAG. The slopes of the enrichment changes of these lipids cannot be used to calculate their turnover rates; they only reflect the rate of the primary event, the turnover of 16:0,16:0-GPC.

Enrichments of Fatty Acids in Phosphatidylcholine and Diacylglycerol in the Presence of Oleate. When oleic acid was added in the medium to the same extent to replace palmitate, no significant change in the diacylglycerol and phosphatidylcholine pattern in cells was found after incubation for up to 24 h. Especially striking is the lack of an increase in the level of dioleoylglycerol or any other oleoyl-containing diacylglycerols. When unlabeled oleic acid was incubated together with labeled palmitate to study the kinetics of phosphatidylcholine synthesis, the near-10-fold elevation of dipalmitoylglycerol seen with palmitate alone is significantly reduced by 11−33%. Similarly, the enrichments of palmitate (both sn1 and sn2) in this diacylglycerol are decreased (Table 4). Oleate dose-dependent decreases in sn1 and sn2 enrichments of 16:0,16:0-GPC and 16:0,16:1-GPC and sn2 of 16:0,18:1-GPC are apparent. In contrast, an oleate

Table 4: Changes in sn1 and sn2 Acyl Enrichments of Phosphatidylcholines Following Co-Incubation (3 h) of Oleic Acid with Labeled Palmitate[a]

		16:0,16:0-GPC		16:0,16:1-GPC		16:0,18:1-GPC		18:1,18:1-GPC		18:0,18:0-GPC	
[PA] (μM)	[oleate] (μM)	sn1 (%)	sn2 (%)	sn1 (%)	sn2 (%)	sn1 (%)	sn2 (%)	sn1 (%)	sn2 (%)	sn1 (%)	sn2 (%)
18	0	42.91	55.75	30.58	34.19	24.48	8.11	2.71	3.79	5.42	5.59
18	17	31.27	43.81	18.91	22.85	28.76	4.64	2.82	3.12	5.2	4.98
18	9	37.46	49.85	23.03	26.72	28.46	5.67	2.87	3.48	5.65	5.65
18	5	37.51	52.14	23.38	27.12	27.57	7.29				

[a] Cells (3×10^7) were incubated in the presence of [1,2,3,4-$^{13}C_4$]palmitate (PA) and in the absence or presence of oleate. The M + 4 enrichments of sn1 and sn2 acyl groups of phosphatidylcholines were then determined. Abbreviations: 16:0,16:0-GPC, dipalmitoylglycerophosphocholine; 16:0,16:1-GPC, 1-palmitoyl-2-palmitooleoylglycerophosphocholine; 16:0,18:1-GPC, 1-palmitoyl-2-oleoylglycerophosphocholine; 18:1,18:1-GPC, 1,2-dioleoylglycerophosphocholine; 18:1,18:1-, 1,2-distearoylglycerophosphocholine.

dose-dependent increase in sn1 enrichments of 16:0,18:1-GPC is evident. There are no significant changes in the enrichments of 18:1,18:1-GPC and 18:0,18:0-GPC. These data indicate that oleate is not a substrate for the *de novo* biosynthesis of diacylglycerol and phosphatidylcholine.

DISCUSSION

The biosynthesis of phosphatidylcholine occurs from the initial formation of 1-acylglycerol 3-phosphate, which is then acylated to phosphatidic acid (*10*−*12*). Dephosphorylation of phosphatidic acid to diacylglycerol provides substrates that react with CDP-choline to yield phosphatidylcholine. In addition to conversion to phospholipids, the diacylglycerol intermediates are also used for the synthesis of triacylglycerols, which is the stored lipid form.

The acyl group at the sn1 position of PC is mostly saturated, while the sn2 acyl groups are mostly unsaturated fatty acids. The mechanism controlling PC molecular species has not been established precisely. It is thought that the molecular species are determined by the substrate preference of the two glycerolphosphate acyltransferases. These diacylglycerol molecular species are then converted to the corresponding PCs. We term this "conventional" model the parallel model of PC synthesis. In this parallel model, different molecular species of diacylglycerol are synthesized first and then converted to PC. However, *in vitro* data from a number of laboratories show contradictions with respect to the substrate specificity of the acyltransferases (*10*, *12*, *13*). In addition to the possible substrate preference of the two acyltransferases in *de novo* diacylglycerol synthesis, there is also a process of active deacylation and reacylation of the sn2 acyl group mediated by phosphatidylcholine acyltransferase. This latter enzyme is believed to be mainly responsible for the unsaturated acyl group found in the sn2 position. Our data are not completely in agreement with this general scheme.

Using ^{13}C-labeled palmitate as tracer, the sequence of acylation to form the diacylglycerol molecules can be deduced from the analysis of mass isotopomer distribution. We chose palmitate labeled at the carbon 1−4 positions (M4) as a tracer. The advantage is elimination of the background contribution from natural abundance isotopes for calculation of isotope distribution. Another advantage is elimination of possible complications from labeled fatty acids that are generated from resynthesis through labeled acetyl-CoA. The resynthesized fatty acids contain no more than two labels on the molecule.

The power of MIDA is explained by a single-compartment model in which two molecules of palmitate in the same homogeneous pool combine to form the sn1 and sn2 acyl groups of the dipalmitoylglycerol molecules. The final dipalmitoylglycerol molecules would contain eight labels (M8), in which both palmitate groups are labeled. In addition, four labels (M4) are produced from the combination of one labeled palmitate and one unlabeled palmitate. Furthermore, the unlabeled product (M0) is formed if both precursor palmitate molecules are not labeled. From the distribution of M0, M4, and M8 of dipalmitoylglycerol, the enrichment of the precursor palmitate can be derived from the binomial distribution principle. Any deviation from this predicted distribution pattern would indicate a mode of synthesis other than the single-compartment model. In addition, if the derived precursor enrichment is smaller than the determined or predicted one, this indicates dilution of the precursor label pool from endogenous *de novo* palmitate synthesis or other unlabeled sources.

Model for the Biosynthesis of Phosphatidylcholine Molecular Species. Summarizing from the MIDA data of individual phosphatidylcholine molecular species, we propose a modified model for phosphatidylcholine biosynthesis (Figure 7). In this model, 16:0,16:0-DAG is the main species and the origin of most of the phosphatidylcholines. 16:0,-16:0-DAG is converted to 16:0,16:0-GPC, which is then modified to produce other PC molecular species. This model differs from the conventional parallel model in which the diverse diacylglycerol molecular species are synthesized first and then converted into their phosphatidylcholine products. One expected and essential feature of the parallel model is that all palmitoyl groups in DG and PC should draw their precursor from the same pool with the same precursor enrichments. Our MIDA data do not support this prediction.

In our sequential model, the total pool of 16:0,16:0-GPC is not one homogeneous pool. Instead, it is a heterogeneous pool with a gradient from the newly synthesized and labeled 16:0,16:0-GPC to the old and unlabeled species. Other molecular species of PC derive their precursors from different points on the pool gradient. Each molecular species is confined to a single rapidly turning over compartment. Because of the location along the gradient, 16:0,16:0-GPC species with differing enrichment dilutions are used as the precursor. This precursor 16:0,16:0-GPC species are then deacylated. The deacylated palmitate is used for desaturation and/or elongation. Because palmitooleate has a significantly smaller pool, the dilution of the isotope enrichment is not significant. As a consequence, the amount of label on palmitooleate that is reacylated to sn2 is the same as in the original palmitoyl group. However, in the synthesis of the most abundant 16:0,18:1-GPC, the dilution on sn2 oleate is

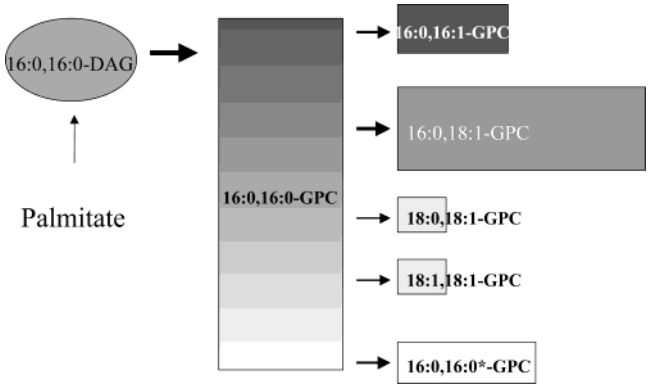


FIGURE 7: Model for the biosynthesis of phosphatidylcholine in HL60 cells. This is a sequential model. Palmitate is used to synthesize 1,2-dipalmitoylglycerol (16:0,16:0-DAG) which is then converted to 1,2-dipalmitoylphosphatidylcholine (16:0,16:0-GPC). The synthesis of 16:0,16:0-GPC follows a two-compartment model with gradient from newly synthesized and labeled 16:0,16:0-GPC to the old existing and unlabeled species. Other phosphatidylcholines derive their precursors from this 16:0,16:0-GPC gradient at different points. The palmitate used for desaturation and elongation to form palmitooleate, oleate, and stearate is derived from the acyl groups of the precursor 16:0,16:0-GPC. The elongated or desaturated fatty acids are then reacylated back to form modified phosphatidylcholine. The sn2 acyl group modification is not limited to unsaturated fatty acids. Deacylation and reacylation to sn2 of 16:0,16:0-GPC with labeled palmitate occur throughout the whole gradient to form 16:0,16:0*-GPC.

significant; this is the result of mixing with a larger unlabeled endogenous oleate pool. In addition to the synthesis of PC with different modified acyl groups, the sn2 is also reacylated with palmitate that is mostly labeled. The deacylation of the less labeled 16:0,16:0-GPC pool (in the distal end of the pool gradient) followed by reacylation with the highly labeled palmitate pool would shift the isotopomer distribution to a higher M4 composition. This process accounts for the M4 adjustment seen in Tables 2 and 3.

In our model, the synthesis of diacylglycerol is substrate specific. Both glycerolphosphate acyltransferases prefer palmitate as the substrate. Other fatty acids, such as oleate, cannot be utilized for the synthesis of the corresponding diacylglycerols at any appreciable rate. The preference for palmitate as a substrate is consistent with the fact that mammalian fatty acid synthase produces predominantly palmitate as the end product (*22*, *23*). The production of other molecular species of PC does not depend on the specificity toward unsaturated fatty acids of the acyltransferases that are involved. It depends on microcompartmentalization and delivery of the precursor substrate. In this model, changes in the microcompartment that lead to enhanced desaturation and/or elongation activities should have more impact on the molecular species profile (*24*) than exogeneously added fatty acids. This is consistent with our data showing that large amounts of oleate failed to alter the molecular species pattern in the cells.

Turnover Rates of Diacylglycerols and Phosphatidylcholine in HL60 Cells. Most studies dealing with the regulation and synthesis of phospholipids do not determine actual turnover rates. Usually, incorporation of radioactivity is used as an index of the relative change in synthetic rates. Our study is one of few that measure turnover parameters in cultured cells. Kuwae *et al*. (*25*) utilized the incoporation of ^{18}O into fatty acids following the incubation of macrophages in ^{18}O-labeled water to determine the rate of turnover of acyl groups in phospholipids. They reported a composite turnover rate of 20%/h for PC during the first hour of incubation. We obtain a fractional turnover rate of 34%/h during the first hour. The difference in turnover rates could be due to the difference in cell types. However, the ^{18}O method determines the enrichments of hydrolyzed fatty acids, which are diluted by less enriched secondary molecular species. In turn, this could lead to an artificially lower turnover rate.

The turnover rates of PC decrease with incubation time. After prolonged incubation of up to 7 h, the turnover rates decrease to 2.8%/h. This decrease could be due to a decreased fatty acid concentration as the fatty acid is used up. In addition, downregulation of the synthetic mechanism could be a contributing factor. The total turnover rates of phosphatidylcholine are governed by the turnover of 16:0,16:0-GPC.

Initially, the synthetic rate of 16:0,16:0-DAG is high, at more than 3.3 nmol/10 min per 10 million cells. This rate of synthesis is higher than its rate of disposition; therefore, it results in more than 10-fold increases in 16:0,16:0-DAG pool size. Other diacylglycerols turn over at significantly lower rates (<3%). The enrichments of these diacylglcyerols (Table 2) are higher than the precursor enrichments derived from the corresponding PCs (Table 3). Therefore, these diacylglycerols (16:0,18:1-DAG and 16:0,18:0-DAG) are not likely to be precursors for the synthesis of 16:0,18:1-GPC and 16:0,18:0-GPC. The absence of 16:0,18:0-GPC is further evidence that the molecular species of PC is not derived from preformed DG molecular species. Even though 16:0,18:0-DAG has a larger pool size and higher turnover rate than 16:0,18:1-DAG, the molecular species, 16:0,18:1-GPC, is the most dominant (35%) PC in these cells. We believe that 16:0,18:1-DAG and 16:0,18:0-DAG are synthetic precursors for triglycerides.

Conclusion. In this report, we use mass isotopomer distribution analysis and a compartmental model to determine the synthetic sequence of phosphatidylcholines. Our results show that the molecular species of phosphatidylcholines in HL60 cells are synthesized sequentially using 16:0,16:0-GPC as the synthetic intermediate. This sequence is consistent with the endogenous supply of palmitate from mammalian fatty acid synthase (*22*, *23*). In addition, the synthesis of PC molecular species is tightly controlled with a minimal influence of exogenous fatty acids. This has been observed in cultured cells. The modification of phospholipids from exogenous fatty acids requires extensive incubation over several days (*26*, *27*). In comparison, the conventional parallel model of synthesis would open the phospholipids to ready modification from exogenous sources. From an evolution sense, this sequential model serves to preserve the molecular composition of endogenous lipids. Furthermore, there is the biological advantage of a synthetic sequence going through inert dipalmitoylglycerol instead of through biologically active diacylglcyerols with unsaturated acyl groups. Free diacylglycerols are bioactive molecules. However, the active species are confined to those with shorter chain lengths or unsaturated forms (*28*−*31*). In our studies, a 20-fold increase in the level of 16:0,16:0-DAG was observed when cells were grown in palmitate-enriched medium, yet no apparent growth effect in these cells occurred.

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