Transacylase and Phospholipases in the Synthesis of Bis(monoacylglycero)phosphate†

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ABSTRACT: Bis(monoacylglycero)phosphate (monoacyl-*sn*-glycero-1-phospho-1′-monoacyl-*sn*-glycerol) is a unique lipid that represents greater than 15% of the total phospholipid of the resident alveolar macrophage. Bis(monoacylglycero)phosphate is not synthesized *de novo* but rather is derived from phosphatidylglycerol of the lung surfactant. There are two enantiomers of bis(monoacylglycero)phosphate synthesized, but only the *sn*-1, and not the *sn*-3, enantiomer accumulates *in vivo*. We recently published a scheme in which the *sn*-3 enantiomer was an intermediate in the synthesis of the final *sn*-1 bis(monoacylglycero)-phosphate. Here we further expand the understanding of the biosynthesis of bis(monoacylglycero)phosphate by examining the proposed first two steps of the pathway. A phospholipase A and a transacylase activity are partially separated by gel permeation chromatography. Both are optimally active in the acid pH range that supports the concept that they function in the lysosome−endosome compartment of the cell. Independently, these two enzyme systems are incapable of converting phosphatidylglycerol into *sn*-3 bis-(monoacylglycero)phosphate. However, combination of the two partially purified enzymes reestablishes the synthesis of *sn*-3 bis(monoacylglycero)phosphate from phosphatidylglycerol. The results presented here support our hypothesis that the phospholipase and transacylase are separate enzymes essential to the synthesis of bis(monoacylglycero)phosphate.

Bis(monoacylglycero)phosphate (BMP,[1] monoacyl-*sn*-glycero-1-phospho-1′-monoacyl-*sn*-glycerol) is a unique lipid found in most human tissues with the highest concentrations found in the pulmonary alveolar macrophage (PAM) (Rouser et al., 1969a,b; Mason et al., 1972; Siakotos & Fleischer, 1969; Baxter et al., 1969). We reported evidence suggesting that the alveolar macrophage synthesizes BMP from phosphatidylglycerol (PG) derived from lung surfactant (Cochran et al., 1985, 1987; Waite et al., 1987). The bis(glycero)-phosphate backbone remains intact during synthesis and is long-lived in the cell while the acyl groups rapidly turn over (Cochran et al., 1987). BMP has been shown to be an important component of PAM arachidonic acid metabolism even though BMP is resistant to degradation by most classical PLAs *in vitro* (Cochran et al., 1985, 1987; Waite et al., 1987). The location of the acyl groups has not been demonstrated conclusively. The high unsaturated acyl composition of *sn*-1:*sn*-1′-BMP does suggest that the acyl chains are esterified at the secondary positions. Conflicting results have been reported on this issue; NMR spectroscopic studies suggested that the acyl groups are at the primary positions (Wherret & Huterer, 1972), whereas Mason et al. (1972) reported that both acyl esters are located at secondary positions. Hydrolysis of BMP with esterified radiolabeled C20:4 by the lipase of *Rhizopus delemar* yields a combination of radiolabeled products such as lysophosphatidylglycerol (LPG, monoacyl-*sn*-glycero-1-phospho-1′-*sn*-glycerol) and free fatty acid. Since the *R. delemar* lipase cleaves primary ester bonds nonstereospecifically, the majority of the fatty esters appear to be located at secondary positions (Slotboom et al., 1970). Our laboratory, as well as those of Hostetler (Hostetler et al., 1982, 1992; Matsuzawa et al., 1978; Matsuzawa & Hostetler, 1980a,b), Wherret (Huterer & Wherret, 1982, 1989, 1990; Huterer et al., 1993; Joutti & Renkonen, 1979; Wherret & Huterer, 1972), DeBuch (Frentzen-Bertrams & Dubuch, 1981), and Renkonen (Joutti & Renkonen, 1979; Somerharju & Renkonen, 1980), has investigated the mechanism of BMP synthesis. We recently published a scheme that accounts for most of the observations on BMP synthesis made by these groups (Amidon et al., 1995). These reactions have been shown to occur in the lysosomal/endosomal organelles of the cell (Mason et al., 1972; Huterer et al., 1993; Huterer & Wherret, 1982; Waite et al., 1990, 1992; Joutti & Renkonen, 1979; Thornburg et al., 1991) (Scheme 1).

In this proposed scheme, a PLA_2 deacylates PG to form acyl-LPG that is subsequently acylated to form BMP that has the *sn*-3 rather than the *sn*-1 stereoconfiguration. This

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[1] Abbreviations: BAL, brocheal alveolar lavage; BMP, bis(monoacylglycero)phosphate (monoacyl-*sn*-glycero-1-phospho-1′-monoacyl-*sn*-glycerol); PAM, pulmonary alveolar macrophage; C20:4, arachidonic acid; C16:0, palmitic acid; C18:1, oleic acid; C18:2, linoleic acid; LPG, lysophosphatidylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; P(1,2)PD, 1,2-diacyl-3-*sn*-glycero-1,2-propanediol; P(1,3)PD, 1,2-diacyl-3-*sn*-glycero-1,3-propanediol; PEG, 1,2-diacyl-3-*sn*-glyceroethylene glycol; PLA, phospholipase A.

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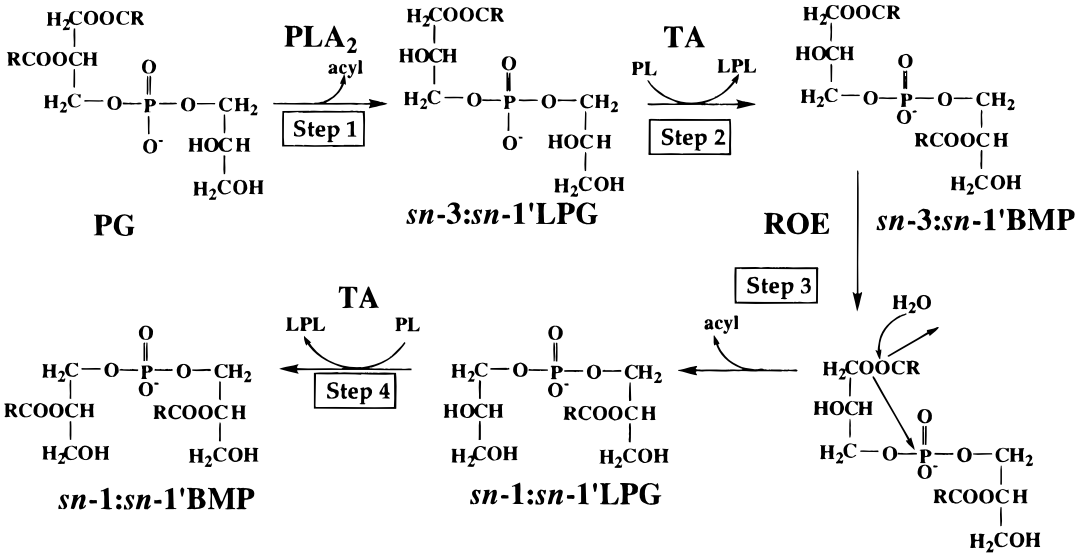
Scheme 1



sn-3-BMP then undergoes a stereorearrangement in which the phosphate moiety is transferred to the *sn*-1 carbon with the concomitant release of the 1-acyl group. We previously demonstrated that removal of the 1-acyl group is required for the conversion of PG to *sn*-1-BMP (Waite et al., 1987). The *sn*-1-LPG that results from the proposed stereoreorientation reaction is acylated to yield the final product, *sn*-1-BMP. We previously demonstrated that macrophage and liver contain lysosomal PLA_2 (Franson & Waite, 1973; Waite et al., 1992), and other laboratories have shown that LPG can be acylated in a CoA- and ATP-independent reaction (Matsuzawa et al., 1978; Frentzen-Bertrams & Dubuch, 1981; Huterer et al., 1993). Although little is known about this acylation, it appears to be a transacylation in which the acyl group of a phospholipid is transferred to the head group glycerol of LPG.

In this paper, we describe some properties of the first two steps in this proposed scheme. The PLAs and the transacylase are solubilized and separated by gel permeation chromatography. Separately, these partially purified enzyme preparations are not capable of converting PG to *sn*-3-BMP. Together, however, they are capable of this conversion that implicates them in the overall synthetic sequence proposed in Scheme 1.

MATERIALS AND METHODS

Materials

RAW 264.7 (murine monocytic, macrophage-like, Abelson leukemia virus transformed BALB/c) cells were obtained from American Type Tissue Culture. Tissue culture medium was from GIBCO, the fetal bovine serum from Flow Laboratories, and the gentamicin sulfate from Hazelton. Ampicillin, 1,2-dioleoyl-*sn*-glycerol, pyridinium dichromate, and phospholipase A_2 (EC 3.1.1.4) of Ophiophagus hannah and porcine pancreas were from Sigma. [1,2,3-^{3}H]-Glycerol and NaB^3H_4 were purchased from American Radiolabeled Chemicals. Silica gel G and H TLC plates were from Analtech. Authentic phospholipid standards were purchased from Serdary. *Escherichia coli* BB26−36 strain was generously provided by Dr. D. Leuking, Michigan Technological University.

Methods

System A: chloroform/methanol/glacial acetic acid/water (75:48:12.5:2, v/v/v/v) to 14 cm then hexanes/diethyl ether/formic acid (90:60:4, v/v/v) to 17 cm; R_f values, LPC, 0.15; LPE, 0.25; PC, 0.35; PE, 0.6; and FFA, 0.9.

System B: chloroform/methanol/glacial acetic acid (65:35:8, v/v/v); R_f values, PE, 0.5; and PG, 0.7.

System C: chloroform/methanol/ammonium hydroxide (130:70:10, v/v/v); R_f values, LPG, 0.27; PG, 0.40; BMP, 0.53; and APG, 0.75.

System D: chloroform/methanol/ammonium hydroxide (150:50:10, v/v/v); R_f values, PG, 0.23; P(1,3)PD and P(1,2)-PD, 0.487; BMP, 0.39; propanediol BMP analog, 0.55; and propanediol APG analog, 0.65.

Synthesis of Radiolabeled Substrates

Radiolabeled phospholipids were synthesized as follows. The vinyl ether bond of 5 mmol of plasmalogen PC (PE), dried as a film on a 50 mL conical tube, was cleaved by exposure to HCl fumes for 5 min to form 2-acyl-lysoPC (PE). The lipid film was neutralized by the addition of 5 mL of 100 mM Tris (pH 8.55) to minimize acyl migration from the secondary position to the *sn*-1 position that can occur at an acidic pH value (Albright et al., 1973). Routinely, 250 μCi of [^{14}C]palmitic acid (approximately 50 mCi/mmol) dissolved in ethanol was aliquoted into the reaction vessel directly before the addition of 1 mL of 10 mM $MgCl_2$ containing 5 mg of coenzyme A and 100 mg of ATP. This mixture was sonically dispersed in a water bath sonicator for 5 min. Freshly prepared rat liver microsomes were added (Waite & Van Deenen, 1967), and after 60 min in a 37 °C shaking water bath, the reaction was quenched by extracting the phospholipids by the method of Bligh and Dyer (1959). Radiolabeled products were resolved by system A_1 to 14 cm on silica gel H plates (Analtech) and then resolved in the same dimension by system A_2 to 17 cm. The positional specificity of the radiolabeled fatty acid was assayed by the formation of radiolabeled lysophospholipid and fatty acid after exhaustive hydrolysis by snake venom PLA_2 (EC 3.1.1.4). After 90 min, no diacylphospholipid remained as detected by TLC. Approximately 85% of the radiolabel was routinely found in the *sn*-1 position as determined by the

relative amounts of radiolabeled fatty acid and lysophospholipid formed. Phospholipids with the radiolabel in position 2 were synthesized by the same method except 1-acyl lysolipids (by PLA_2 hydrolysis) were used.

The glycerol-labeled substrates were synthesized as previously described (Thornburg et al., 1991; Amidon et al., 1995).

Savoy cabbage PLD was isolated by the method of Davidson and Long (1958). Transphosphatidylation reactions were carried out in a buffer containing 100 mM $CaCl_2$, 100 mM sodium acetate (pH 5.6), and the appropriate alcohol according to Comfurious et al. (1977). After incubation for 90 min, reactions were quenched with methanol/chloroform (2:1) and phospholipids were extracted according to Bligh and Dyer (1959).

A transphosphatidylation reaction was used to convert PE to 1,2-diacyl-*sn*-glycero-3-phospho-*rac*-glycerol (*sn*-3:*rac*-PG). 1-Acyl-2-lyso-*sn*-glycero-3-phospho-*rac*-glycerol (*sn*-3:*rac*-LPG) was formed by PLA_2 (porcine pancreatic) hydrolysis of PG, while 1-lyso-2-acyl-*sn*-glycero-3-phospho-*rac*-glycerol was formed by hydrolysis with *R. delemar* lipase (Cochran et al., 1987). The synthesis of PG analogs with a differing polar head group was also accomplished using a transphosphatidylation reaction, but ethylene glycol, 1,3-propanediol, (*R*)-1,2-propanediol, or (*S*)-1,2-propanediol was used in the place of glycerol. Each of these compounds was isolated by thin layer chromatography, and routinely, there was an 80−90% efficiency of the transphosphatidylation reaction.

The metabolism of substrates by intact RAW 264.7 cells was carried out as follows. RAW 264.7 cells ($3-4 \times 10^6$) were grown to confluency in 35 mm × 10 mm culture dishes. The substrate was prepared by evaporating solvent under a nitrogen stream followed by sonic dispersion in the cell culture medium (sonifier at 20% for 3 × 15 s before addition to cells, 1.5 mL/culture dish). After incubation, the medium was removed and the cells were washed twice with ice-cold saline followed by scraping cells from the dish into methanol. The phospholipids were extracted by the method of Bligh and Dyer (1959) modified by the use of 10% acetic acid in the place of water and resolved by TLC. The chloroform extractable products were isolated on silica gel H plates using system C. The products were located by scanning radioactivity and then scraped and extracted from the silica and either quantitated for radioactivity by liquid scintillation or analyzed for stereoconfiguration as described earlier (Amidon et al., 1995; Thornburg et al., 1991). Analysis of water/methanol soluble products by TLC revealed that LPG was the major tritiated metabolite (>80%) and that this LPG had the same stereoconfiguration as that recovered in the $CHCl_3$ phase of the modified Bligh and Dyer extraction system. Therefore, the amount of LPG formed was calculated as a combination of water soluble products and LPG isolated by TLC. The metabolites of P(1,2)PD, P(1,3)PD, and PEG were further studied by acylation with caproyl chloride of their BMP analog products isolated from the thin layer plate. In each case, P(1,3)PD, P(1,2)PD, and PEG yielded a product upon acylation that had the same R_f value as their acylated BMP analog (system D.) This is predicted since each compound should have a single hydroxyl group free for acylation.

The cell homogenization and fractionation was carried out as previously described (Waite et al., 1990). Briefly, RAW 264.7 cells were grown to confluency in Dulbecco's MEM with high glucose, 10 mM Hepes, 10% heat-inactivated fetal bovine serum, and 50 mg/mL gentamicin sulfate in a 245 × 245 mm sterile culture plate. The cells were washed two times in ice-cold isotonic saline and resuspended in 5 mL of 1 mM $NaHCO_3$. After homogenization 50 times by a Dounce homogenizing apparatus, the crude homogenate was centrifuged at 1000*g*. The supernatant was adjusted to 45% sucrose and fractionated using a discontinuous flotation sucrose gradient. Over 11 mL of the adjusted supernatant were pipetted 9 mL of 40%, 7 mL of 35%, 7 mL of 30%, and 2 mL of 9% sucrose, all in 1 mM Tris-HCl (pH 7.4). These tubes were centrifuged at 64000*g* for 290 min in a Beckman L5−50 instrument in a Beckman SW-27 rotor. Fractions 1, 3, and 5 were isolated by hand using a Pasteur pipet from the 9/30%, 30/35%, and 35/40% sucrose interfaces, respectively.

BMP Synthesis by Subcellular Fraction. Fraction 1 isolated from RAW 264.7 cells was used to synthesize *sn*-3:*sn*-1′-BMP. Radiolabeled PG ($5-10 \times 10^4$, 0.1−1 mM) or LPG ($5-10 \times 10^4$, 0.1−1 mM) was sonically dispersed in a buffer containing 100 mM sodium acetate (pH 4.5) and 10 mM 2-mercaptoethanol and incubated at 37 °C for 60−90 min with 50−100 μg of protein. The incubation mixture was extracted by the modified method of Bligh and Dyer, and chloroform soluble products were resolved using system C.

In order to separate the PLA and transacylase, cell homogenates or subcellular fractions were delipidated by the addition of an equal volume of ice-cold, water-saturated butanol (Huterer & Wherret, 1989; Matsuzawa et al., 1978). After gentle mixing of the two phases for 2 min, the mixture was centrifuged at 13000*g* for 15 min and the aqueous lower layer removed and filtered through a Sephacryl S-300 column (1.5 × 84 cm) equilibrated at pH 7.4 with 10 mM Tris. Eighty fractions of 1.1 mL each were collected. This Sephacryl column (1.5 × 84 cm) had a void volume of 33 mL, and a total volume of 148 mL was used to partially separate the transacylase and the phospholipase enzymes. The transacylase and the phospholipase preparations were stored in 35% glycerol (v/v) at −20 °C. Greater than 75% of the activity was recovered after 6 weeks.

The transacylase and PLA activities were assayed as follows. The conversion of LPG to BMP was used to describe transacylase activity. Solvent was evaporated under a stream of N_2 from a mixture of [1,2,3-^{3}H]-*sn*-3:*rac*-1-acyl-LPG (1×10^5 dpm, 100 pmol) and 30 nmol of RAW lipid prior to sonic dispersion in buffer A. PLA activity was determined by the hydrolysis of PG to LPG. Solvent was evaporated under a stream of N_2 from [1,2,3-^{3}H]-*sn*-3:*sn*-1′-PG (1×10^5 dpm, 100 pmol), with or without 30 nmol of RAW lipid, before sonic dispersion in buffer A. All reactions were run at 37 °C at pH 4.5 for 45−60 min and stopped by extracting the phospholipids using the modified method of Bligh and Dyer. Radiolabeled products were resolved by TLC as before. All studies were carried out two to five times in duplicate, and the results among experiments varied by no more than 10%.

Analytical Methods. The protein content was determined by the method of Peterson (1977), and the lipid phosphorus was quantitated by the method of Chalvardjian (Chalvardjian & Rudnicki, 1970).

Table 1: Effect of Ca^{2+}, ATP, and Coenzyme A on BMP Synthesis by Fraction 1[a]

additions	distribution of radiolabeled products from PG (% total radioactivity)			
	PG	LPG	BMP	APG
none	28	18	44	10
1 mM Ca^{2+}	31	16	45	8
5 mM Ca^{2+}	54	10	33	3
10 mM Ca^{2+}	73	7	19	1
10 mM Mg^{2+}	78	5	14	3
2-mercaptoethanol	23	20	46	9
CoA	30	19	44	6
ATP	41	24	30	5
ATP + Mg^{2+}	62	9	26	3
ATP + Mg^{2+} + CoA	56	9	25	10

[a] The effect of various cofactors on the conversion of [1,2,3-^{3}H]-*sn*-3:*sn*-1′-PG to [1,2,3-^{3}H]-*sn*-3:*sn*-1′-BMP by fraction 1 was examined. The standard incubation mixture was buffered at pH 4.5 with 100 mM sodium acetate with 10 mM EDTA and contained 50 μg of protein from fraction 1. Calcium, Mg^{2+}, ATP (13 μM), and coenzyme A (1 μM) were added minus EDTA as indicated to the reaction mixture. The reactions were quenched after 60 min by extracting phospholipid. Results are described as the percentage of the total radioactivity recovered for each compound.

RESULTS

The conversion of *sn*-3:*sn*-1′-PG to *sn*-3:*sn*-1′-BMP is postulated to be a two-step process beginning with the hydrolysis of *sn*-3:*sn*-1′-PG to form *sn*-3:*sn*-1′-LPG that is subsequently transacylated to form *sn*-3:*sn*-1′-BMP. A lipid-enriched, low-density subcellular fraction (fraction 1) from RAW 264.7 cells was shown to convert *sn*-3:*sn*-1′-PG to *sn*-3:*sn*-1′-BMP, but not *sn*-1:*sn*-1′-BMP (data not shown). The formation of *sn*-3-BMP was inhibited by ATP and the divalent cations Ca^{2+} and Mg^{2+} but was not influenced by CoA, EDTA, or 2−mercaptoethanol (Table 1). This fraction contains 10% of lysosomal marker enzyme yet is devoid of all other marker enzymes (Waite et al., 1987). This subcellular fraction appeared to be suitable for assaying the first two steps of the pathway proposed in Scheme 1.

The butanol-delipidated fraction 1 did not have transacylase activity in the absence of other added lipids, but the activity could be reconstituted by the addition of endogenous lipid extracted from the cells to act as the acyl donor. Without the addition of exogenous acyl donor, the *sn*-3:*sn*-1′-PG was hydrolyzed to form *sn*-3:*sn*-1′-LPG that demonstrates the presence of PLA activity (Figure 1). The addition of 10 nmol of RAW lipid yielded BMP with a concomitant decrease in the LPG recovered. In this study, the PLA(s) deacylated approximately 17% of the PG and addition of the acyl donor led to 7% acylation of the LPG to form BMP.

In order to study the phospholipase and transacylase postulated to be responsible for the conversion of *sn*-3:*sn*-1′-PG to *sn*-3:*sn*-1′-BMP, it was necessary to separate the two enzymes using Sephacryl S-300 chromatography. In most cases, butanol-extracted cell homogenates were used rather than that of fraction 1 in order to obtain sufficient material. The transacylase activity was determined by the conversion of *sn*-3:*sn*-1′-LPG to *sn*-3:*sn*-1′-BMP, while the PLA activity was measured by the hydrolysis of *sn*-3:*sn*-1′-PG to *sn*-3:*sn*-1′-LPG (Figure 2). The transacylase activity eluted before the PLA even though some overlap of the two was found. The apparent molecular weights of the transacylase and the phospholipase as determined by comparison with the elution volumes of globular proteins with known

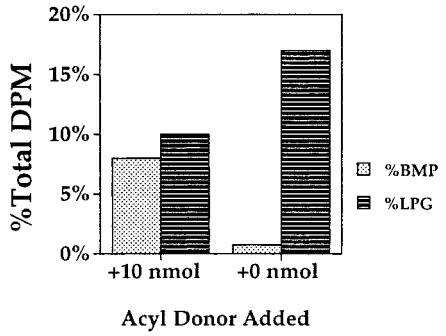


FIGURE 1: Reconstitution of BMP synthetic activity from butanol-delipidated extract. A RAW 264.7 cell homogenate was centrifuged at 1000*g*, and the supernatant was delipidated with ice-cold water-saturated butanol as described in Materials and Methods. The delipidated protein was dialyzed against 10 mM Tris (pH 7.4) for 3 h to remove any residual butanol. Lipids from the whole cell were used as the source of acyl donor in the reconstitution assay and were collected by extracting a portion of the whole-cell homogenate by the method of Bligh and Dyer (1959). Assays were run with 20 μg of delipidated protein in 100 mM sodium acetate at pH 4.5 and either 1×10^5 dpm [1,2,3-^{3}H]PG or a combination of 0.1 nmol of 1×10^5 dpm [1,2,3-^{3}H]PG and 10 nmol of RAW 264.7 lipid. Reactions were quenched after 45 min of incubation at 37 °C.

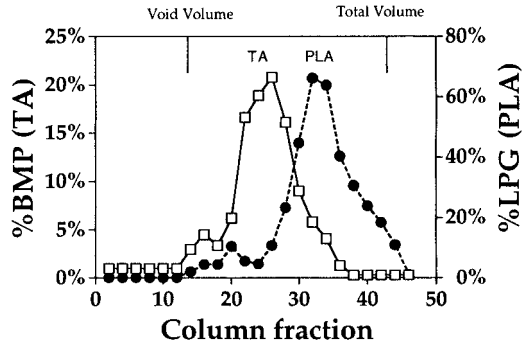


FIGURE 2: Partial separation of transacylase and PLA enzyme systems. RAW 264.7 cells were homogenized and delipidated by the addition of an equal volume of ice-cold water-saturated butanol. After centrifugation (15000*g*, 15 min), the aqueous lower phase was filtered using a Sephacryl S-300 column equilibrated with 10 mM Tris (pH 7.4). Fractions (2.8 mL, total volume) were assayed for transacylase activity by monitoring the conversion of [1,2,3-^{3}H]LPG to [1,2,3-^{3}H]-*sn*-3:*sn*-1′-BMP and PLA activity by the hydrolysis of [1,2,3-^{3}H]PG to [1,2,3-^{3}H]LPG (see Materials and Methods). RAW 264.7 lipid (25 nmol from the whole cell) was added to each assay: PLA activity (PLA):LPG formed by the hydrolysis of PG (●) and transacylase activity (TA):BMP formed by the transacylation of LPG (□).

molecular weight standards on this Sephacryl S-300 column are 190 000 for the transacylase and 36 000 for the phospholipase (data not shown). It is possible that aggregation with other proteins can occur during gel filtration which would result in an overestimation of the molecular weight of each enzyme.

A pool of transacylase activity was collected that did not contain appreciable levels of PLA activity as shown by a failure to hydrolyze a pool of PG (data not shown). Likewise, the PLA pool synthesized little BMP even though considerable LPG was formed from PG (Figure 3). The PLA degrades *sn*-3:*sn*-1′-PG to *sn*-3:*sn*-1′-LPG, and this hydrolysis steadily increases over the entire experiment. Only after 90 min was any BMP detected, and it amounted to no more than 5% of the isolated radiolabeled metabolites found. When the PLA pool was assayed with LPG as the acyl acceptor and RAW lipid as the acyl donor, a low level of transacylase

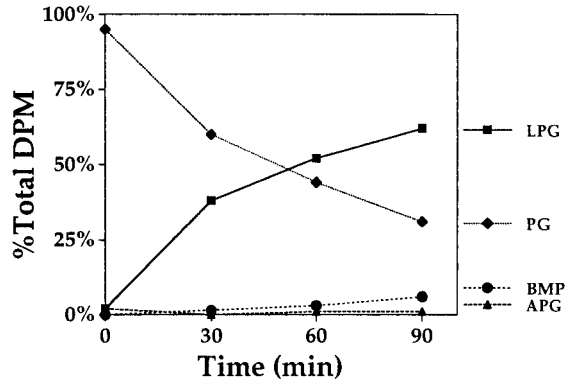


FIGURE 3: Metabolism of PG by the PLA. Fractions (32−38) from the s-300 gel filtration column containing PLA activity were pooled. This pool of PLA was defined as the partially purified PLA, and assays were conducted at pH 4.5 and 37 °C with [1,2,3-^{3}H]PG (1×10^5 dpm, 100 pmol) using 1 μg of the PLA pool protein. Results are described as a percentage of total radioactivity.

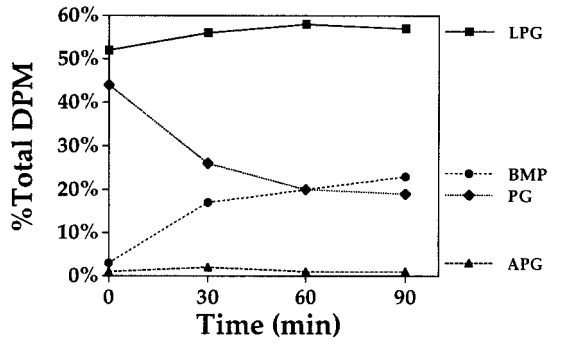


FIGURE 4: Recombination of the transacylase and PLA activities. [1,2,3-^{3}H]PG (1×10^5 dpm, 100 pmol) was first incubated with the PLA (1 μg) for 60 min, after which 5 μg of the transacylase and 30 nmol of RAW 264.7 lipid were added. At various times, the reactions were stopped by extracting the phospholipids. The time scale represents the length of time after the addition of the transacylase pool protein.

activity was detected (data not shown). While the small amount of transacylase activity found in the phospholipase pool is most likely contamination, some PLAs possess transacylase activity which may be the case here. These experiments verify that there is adequate separation of the PLA and the transacylase, since there is very low synthesis of *sn*-3:*sn*-1′-BMP when the two activities are examined independently.

Two sets of experiments were conducted to demonstrate that the PLA produced the *sn*-3:*sn*-1′-LPG that was substrate for the transacylase. First, *sn*-3:s*n*-1′-PG was hydrolyzed by the partially purified PLA for 60 min prior to the addition of the transacylase (Figure 4). At various times after the addition of the transacylase and acyl donor lipid (0−90 min), the reactions were quenched, the phospholipids extracted, and the radiolabeled products quantitated. The zero time represents the point at which the aliquot of the transacylase was added. The amount of BMP synthesized increased up to 60 min of incubation, at which time over 20% of the total radiolabeled lipid is BMP. The percentage of LPG remained relatively constant while PG decreased, indicating that PLA activity remained active. Little APG accumulated, suggesting that the second acylation of either glycerol is not a major reaction.

The second approach is presented in Figure 5. In this study, the PLA was allowed to hydrolyze *sn*-3:*sn*-1′-PG for 0−90 min prior to the addition of the transacylase for the

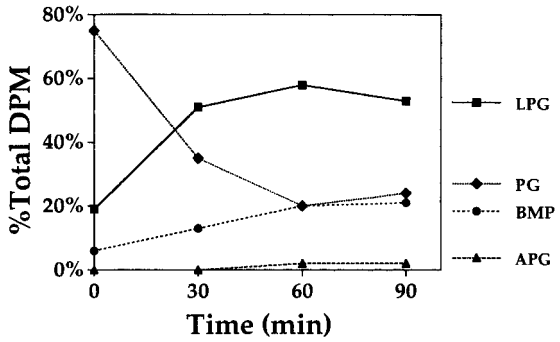


FIGURE 5: Recombination of the transacylase and PLA activities. [1,2,3-^{3}H]PG (1×10^5 cpm, 100 pmol) and 30 nmol of acyl donor lipid were incubated with the PLA (1 μg) alone for a variable time before the addition of the transacylase (5 μg) for 60 min. The time scale represents the length of time before the addition of the transacylase pool protein.

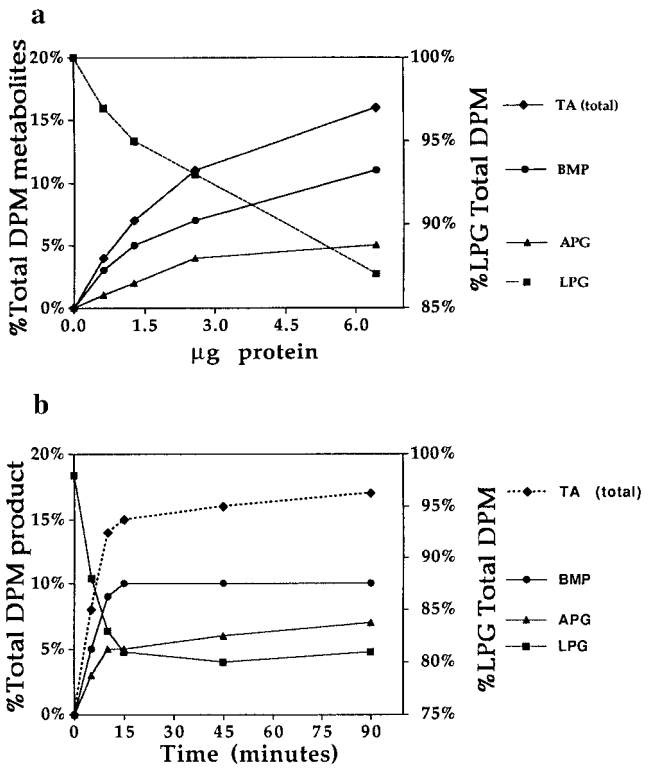


FIGURE 6: (a) Effect of protein concentration on transacylase. [1,2,3-^{3}H]-*sn*-3:*rac*-LPG (1×10^5 dpm, 100 pmol) and 25 nmol of acyl donor lipid were incubated with varying amounts of the transacylase pool protein for 30 min. The total transacylase activity was calculated as the sum of BMP and APG formation. (b) Time-dependent transacylase activity. [1,2,3-^{3}H]-*sn*-3:*rac*-LPG (1×10^5 dpm, 100 pmol) and 25 nmol of acyl donor lipid were incubated with 5 μg of the partially purified transacylase at 37 °C and pH 4.5. At various times, the reactions were quenched by extracting phospholipid and quantitated as described in Materials and Methods.

fixed time of 60 min. At all times, LPG was the major metabolite isolated. Importantly, there was a time-dependent increase in the amount of *sn*-3:*sn*-1′-BMP formed. These studies demonstrated that the product from the phospholipase activity was a suitable substrate for the transacylase activity in the formation of BMP.

The protein dependency of the transacylase was studied over a 30 min time period using [1,2,3-^{3}H]-*sn*-3:*sn*-1′-LPG and RAW 264.7 cell lipid as the acyl donor (Figure 6a). There was a linear increase in *sn*-3:*rac*-BMP synthesized up to 1.4 μg; above this concentration, there is a more gradual increase in *sn*-3:*rac*-BMP. The reduction in *sn*-3:*rac*-LPG

Table 2: Substrate Specificity of the PLA[a]

phospholipid	% total hydrolysis		
		PLA_2	PLA_1
PC	13	3	10
PE	23	8	15
PG	48	13	35
PA	3		
PI	0		
BMP	0		

[a] The substrate specificity of the partially purified phospholipase was examined by incubating 5 nmol of each phospholipid with 5 μg of the phospholipase for 45 min at pH 4.5. The total hydrolysis was determined as the sum of the percent of total radioactivity in the assay received as either radiolabeled fatty acid or radiolabeled lysophospholipid. PLA_1 activity is defined as the release of [^{14}C]C16:0 from PC and PG or the generation of [^{14}C]LPE from PE, whereas PLA_2 is defined as the formation of radiolabeled LPC and LPG or the release of [^{14}C]C18:2 from PE. Abbreviations: PC, 1-[^{14}C]C16:0-2-acyl-PC; PE, 1-acyl-2-[^{14}C]C18:2-PE; PG, 1-[^{14}C]C16:0-2-acyl-PG; PA, 1,2-diacyl-[1,2,3-*glycerol*-^{3}H]-PA; PI, 1-acyl-2-[^{3}H]C18:1-PI; BMP, [1,2,3-^{3}H]-*sn*-3:*sn*-1′-BMP.

was approximately equal to the increase in the sum of *sn*-3:*rac*-BMP and *sn*-3:*rac*-APG. The accumulation of *sn*-3:*rac*-APG amounted to about 5% of the total radiolabel, and no PG was noted at any protein concentration.

The transacylase was also monitored with respect to time using 5.0 μg of partially purified transacylase protein (Figure 6b). There was a rapid increase in *sn*-3:*rac*-BMP synthesis through the first 10 min, but then the level remained relatively constant throughout the remainder of the experiment. A precursor−product relationship could be shown between the *sn*-3:*rac*-LPG and *sn*-3:*rac*-BMP as seen by the rapid decrease in the *sn*-3:*rac*-LPG during the first 15 min of incubation. Interestingly, the synthesis of *sn*-3:*rac*-APG was as rapid as the synthesis of BMP even though the reacylation presumably uses BMP as the precursor to APG. At no point did *sn*-3:*rac*-PG accumulate, as found in Figure 6a.

The effect of pH on the conversion of *sn*-3:*rac*-LPG to *sn*-3:*rac*-BMP was monitored using the partially purified transacylase. The enzyme was most active at pH values between 3.5 and 5.0, where 30% of the *sn*-3:*rac*-LPG was converted to *sn-3:rac*-BMP (data not shown).

Further studies of the transacylase required some knowledge of the PLA(s) that formed the precursor for transacylation, LPG. The partially purified phospholipase hydrolyzes PG preferentially (Table 2). Both PE and PC were hydrolyzed, while PI, PA and *sn*-1:*sn*-1′-BMP were poor substrates. The optimal activity of the phospholipase was found at 4.5 where 63% of the PG was hydrolyzed [data not shown and Franson and Waite (1973)]. Since both radiolabeled fatty acid and lysolipid were found, the partially purified PLA preparation may have contained both PLA_1 and PLA_2 activity. Phosphatidylglycerol was synthesized with either [^{14}C]C16:0 esterified at the *sn*-1 position or [^{3}H]C18:2 esterified at the *sn*-2 position so these two phospholipase activities could be examined further. The hydrolysis of this mixed labeled substrate was studied with respect to time (Figure 7). The level of PLA_1 activity was measured by the release of [^{14}C]C16:0 and [^{3}H]LPG, while PLA_2 was determined by the release of [^{3}H]C18:2 and [^{14}C]LPG. This experiment demonstrated that both PLA_1 and PLA_2 activities are present. Since our previous work showed that alveolar macrophage granules contain separate PLA_1 and PLA_2, we

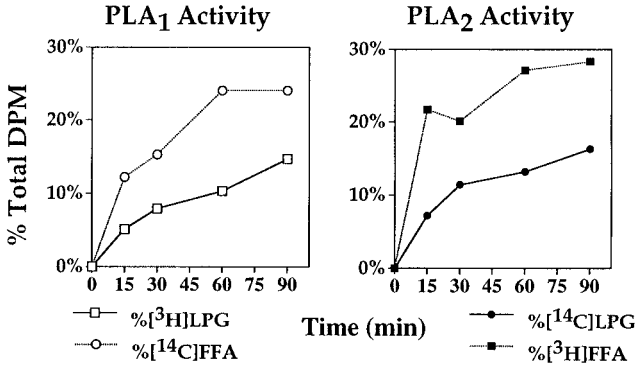


FIGURE 7: Hydrolysis of 1-[^{14}C]C16:0-2-[^{3}H]C18:2-PG as a function of time. PG was synthesized with either [^{14}C]palmitate at the *sn*-1 position or [^{3}H]linoleate esterified at the *sn*-2 position as described in Materials and Methods. Five nanomoles of mixed radiolabeled PG (1×10^5 dpm ^{3}H and 0.25×10^4 dpm ^{14}C) was incubated with 1 μg of the PLA preparation for 0−60 min at pH 4.5. Radioactive lipids were quantitated by TLC resolved in system A.

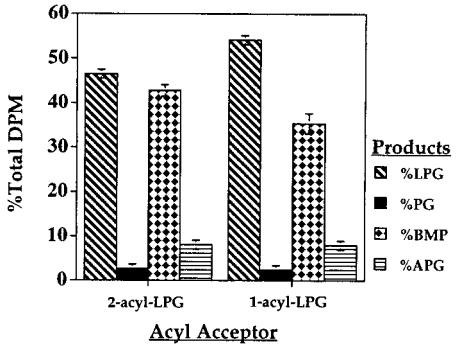


FIGURE 8: Positional specificity of the transacylase. 2-Acyl[1,2,3-^{3}H]LPG was formed by hydrolysis of [1,2,3-^{3}H]PG by the lipase of *R. delemar*, and 1-acyl[1,2,3-^{3}H]LPG was formed by porcine pancreatic PLA_2 hydrolysis of [1,2,3-^{3}H]PG. RAW 264.7 lipid (30 nmol) which was used as the acyl donor and [1,2,3-^{3}H]LPG (1×10^5 dpm, 100 pmol) were sonically dispersed together before the addition of transacylase preparation (5 μg) for 45 min at pH 4.5. Reactions were stopped, and radiolabeled products were quantitated as in Figure 3a. Results are expressed as a percentage of the total radioactivity in the assay.

believe that the activity here is catalyzed by two enzymes (Franson & Waite, 1973). The percentage of [^{3}H]LPG and [^{14}C]LPG recovered was approximately equal at all times tested during the experiment. The percentages of radiolabeled fatty acid are also similar. These results suggest that there is a lysophospholipase present as well, since the major radiolabeled metabolite recovered using either substrate was free fatty acid. This observed lysophospholipase activity may be a separate enzyme or may be an inherent property of the PLAs.

Since both PLA_1 and PLA_2 activities are present, it is important to determine if the transacylase has a preference for LPG with the acyl group esterified at the *sn*-1 or the *sn*-2 position. The acyl-LPG was prepared as before by porcine pancreatic PLA_2 hydrolysis. The 2-acyl-LPG was synthesized in a similar fashion using the lipase from *R. delemar* which cleaves only primary esters. The hydrolysis buffer contained borate to minimize acyl migration that could cause the 2-acyl-LPG to isomerize to 1-acyl-LPG. Controls run with purified PLA_2 and lipase demonstrated that little acyl migration had occurred. The acylation of the two isomers of LPG to BMP, described in Figure 8, shows that the partially purified transacylase does not differentiate with regard to location of the acyl group.

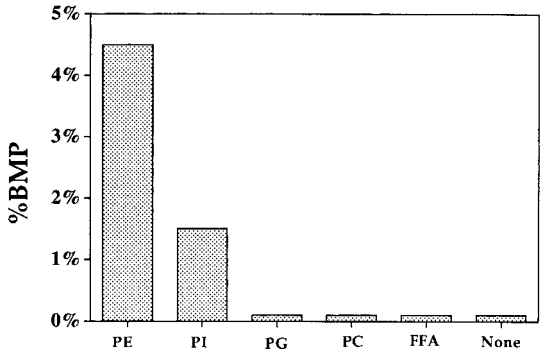


FIGURE 9: Reconstitution of transacylase activity with single phospholipid classes. [1,2,3-^{3}H]-*sn*-3:*rac*-LPG (1×10^5 dpm, 100 pmol) and 1 nmol of various individual lipid classes were incubated with 5 μg of the partially purified transacylase for 45 min at pH 4.5. The results are expressed as the percentage of total radioactivity.

Table 3: Uptake and Metabolism of PG and PG Analogs by RAW 264.7 Cells[a]

precursor	% dpm cell associated	% conversion to product	% *sn*-1:*rac* product	% *sn*-3:*rac* product
[1-^{3}H]-*sn*-3:*rac*-PG	45	45	90	10
[1-^{3}H]-*sn*-3:*rac*-LPG	72	75	90	10
[1-^{3}H]PEG	60	7	80	20
[1-^{3}H]P(1,2)PD	64	5	77	23
[1,2,3-^{3}H]lysoP(1,2)PD	43	15	NA[b]	NA
[1-^{3}H]P(1,3)PD	54	1	NA	NA
[1,2,3-^{3}H]lysoP(1,3)PD	45	<1	NA	NA

[a] RAW 264.7 cells were cultured in 35 mm^2 culture dishes with 5×10^5 dpm [1,2,3-^{3}H]PG, [1,2,3-^{3}H]P(1,3)PD, [1,2,3-^{3}H]P(1,2)PD, or [1,2,3-^{3}H]PEG for 20 h in 2.5 mL of medium. The cells were then extracted by a modification of the method of Bligh and Dyer. The chloroform layer was resolved by TLC using system C, and BMP or its analogs were scraped and quantitated by liquid scintillation. [b] NA, not assayed.

A study was conducted to examine the acylation of [1,2,3-^{3}H]-*sn*-3:*rac*-LPG to form [1,2,3-^{3}H]BMP using individual phospholipid species, or free fatty acid, as acyl donors. PE and, to a lesser extent, PI functioned as acyl donors, whereas other phospholipids were not suitable substrates (Figure 9). These data contrast with what was observed using the intact fraction 1, where PC and PG (as well as PE) acted as acyl donors (data not shown). This may be due to the loss of additional transacylase(s) in delipidation or chromatography.

The location of the acyl groups esterified to BMP is depicted at the *sn*-2 and *sn*-2′ positions in Scheme 1 primarily on the basis of the prevalence of unsaturated fatty acids esterified at secondary positions of the glycerols. In order to determine the location fatty acid esterification, monohydroxylated analogs of PG were synthesized. Table 3 describes the results of the metabolism of these monohydroxylated PG analogs by the RAW 264.7 cells after 18 h. Each of the compounds tested was readily taken up by the cell as described by the percent of cell-associated radiolabel. Lyso-monohydroxylated analogs were not stereospecifically labeled; therefore, the stereoconfiguration of products could not be determined. As described in earlier results, both *sn*-3:*sn*-1′-PG and 1-acyl-*sn*-3:*rac*-LPG were readily converted to *sn*-1:*rac*-BMP as the product. All three analogs that contain a free hydroxyl on the carbon adjacent to the phosphoester [P(1,2)PD, LP(1,2)PD, and *sn*-3-PEG] were converted to the corresponding BMP analog, although the extent of their metabolism was 10−20% of that of the parent compound. Also, the backbone glycerol of these products was determined to have an *sn*-1 stereoconfiguration. This suggests that the P(1,2)PD and PEG can be metabolized by the same pathway as is PG but that the head group modification reduces the extent of metabolism. Only 1% of P(1,3)PD or LP(1,3)PD was metabolized to the BMP analog. These results, therefore, suggest that the transacylase has a preference for the *sn*-2′ position of the head group glycerol. Although not shown in the table, much of each monohydroxylated analog was converted to PC. Previously, Waite et al. (1987) found that the macrophage converted alkyl-PG to alkyl-PC and not to BMP. It seems that the polar head group as well as the radyl composition may determine the intracellular fate of internalized PG.

Table 4: *In Vitro* Metabolism of Monohydroxylated Analogs[a]

lysophospholipid	% BMP analog
LPG	14
LPEG	5
LP(1,3)PD	9
LP[(R)1,2]PD	9
LP[(S)1,2]PD	12

[a] LPG or lyso derivatives of monohydroxylated (1×10^5 dpm, 1 nmol) and 30 nmol of RAW 264.7 lipid was sonically dispersed in water. The partially purified transacylase (25 μg) was added, and the pH was lowered with the addition of 20 μL of 1 M sodium acetate (pH 4.5). Phospholipids were extracted as before after 45 min in a shaking 37 °C water bath and resolved with chromatography system D. Radioactive products were scraped, and radioactivity was quantitated by liquid scintillation counting.

When these PG analogs were used as substrates for the transacylase preparation, all were acylated (Table 4). In this case, the 1,2- and 1,3-propanediol derivatives were acylated to the same extent and were somewhat better substrates than the ethylene glycol derivative. It appears, therefore, that the transacylase does not prefer either of the two hydroxyls of the head group moiety and that differences obtained with intact cells (Table 3) are not at the enzyme level.

DISCUSSION

In this study, we have separated and partially purified enzymes that we postulate catalyze the first two steps in the conversion of PG to *sn*-1-BMP (Scheme 1). The combination of these two enzymes yields the *sn*-3-BMP, a proposed intermediate in the synthesis of *sn*-1-BMP. These two, PLAs and LPG transacylase, are recovered primarily in a lipid-filled, lysosome-enriched fraction of the RAW 264.7 cells. Both enzymes are optimally active in the acidic pH range, as expected of lysosomal enzymes. Thus far, we have not been successful in reconstituting the complete system that would yield *sn*-1-BMP. The presumed reorienting enzyme (step 3, Scheme 1) is yet to be detected.

We previously found both PLA_1 and PLA_2 in rat liver lysosomes and BAL macrophage granules (Franson et al., 1970; Franson & Waite, 1973). While the evidence presented here for two separate enzymes for the RAW 264.7 cells is less direct, the currently used PLA preparation has properties similar to those of the BAL macrophage. The PLA_1 has been purified and characterized by us and by the group of Hostetler (Huterer et al., 1993; Robinson & Waite, 1983; Hostetler et al., 1982). Both groups found that the observed specificity toward different substrates was dependent on the physical state of the substrate. Detergents and cations drastically altered the activity of the PLA_1 (Robinson & Waite, 1983). The latter effect could be attributed to a change in the ζ potential (surface charge) of the substrate.

Hostetler et al. (1982) reported that the purified PLA_1 also had significant lysophospholipase and transacylase activity, contrary to our observations with this enzyme. While we do not know the reason for this discrepancy, the method of assay may be responsible. Here we find that the preparation of PLAs has slight lysophospholipase activity since the amount of fatty acid formed is somewhat greater than the amount of LPG. We found little, if any, transacylase activity in the PLA pool (Figure 3).

The transacylase activity we report here was previously described by Hostetler's and Wherret's groups in lysosomal preparations (Frentzen-Bertrams & Dubuch, 1981; Huterer et al., 1993; Matsuzawa et al., 1978). Although no purification was carried out, both groups reported this activity to be a transacylase that is CoA- and ATP-independent, results that are confirmed in our present study. Each group reported differences in the phospholipid acyl donor, although all reports demonstrate that the transacylases lack absolute donor specificity. It is not clear at this point if indeed the same transacylases have been studied by the three groups. Huterer et al. (1993) found that a partially purified preparation of PLA_1 from lysosomes could carry out a transacylation in which the acyl group from position 1 of PC was transferred to BMP, forming APG. Our studies did not show such an activity, and only when the partially purified preparation of transacylase was added to the PLA preparation was there significant formation of BMP from PG. It is possible that the PLA_1 preparation used by Huterer did not have the transacylase removed.

One of the interesting features of the transacylase is the lack of LPG acylation to form PG. It would be predicted that this enzyme, capable of acylating BMP to form APG, could acylate the backbone to form PG. The explanation we favor at this time is a stereospecificity requirement for a glycerol moiety with the phosphate at the *sn*-1 position. Such an interpretation would favor a transacylase functioning at the second transacylation step in which *sn*-1-LPG is converted to the final product, *sn*-1-BMP.

The evidence we present here and reported earlier supports the interpretation that acylation of LPG occurs primarily at the secondary hydroxyl of the head group glycerol (Mason et al., 1972). Hydrolysis of BMP labeled *in vivo* with arachidonic acid indicates that 60−70% of the arachidonic acid is in the secondary position of the glycerols (Cochran et al., 1985). Our report here clearly shows that PG analogs that lack a hydroxyl adjacent to the phosphate ester are taken up by the cell as readily as PG yet are not converted to BMP analogs. The puzzling observation, however, is the apparent nondiscrimination when the same substrates are used with the transacylase preparation. In this case, the propanediol analogs are all readily acylated. We must conclude that the apparent preference *in vivo* for substrates with a free hydroxyl adjacent to the phosphate ester is the result of the mechanism of uptake and/or trafficking within the cell. Such speculation is favored by our earlier finding that 1-alkyl-PG is readily taken up by the cell but converted to PE and PC (Waite et al., 1987). The synthesis of these compounds occurred in the endoplasmic reticulum primarily rather than in the lysosomal compartment where BMP synthesis is thought to occur (Mason et al., 1972; Bleistein et al., 1980; Frentzen-Bertrams & Dubuch, 1981; Matsuzawa et al., 1978; Huterer & Wherret, 1989, 1990; Hostetler et al., 1992). This speculation will require studies with fluorescently labeled substrates for verification of the cellular site of metabolism.

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