Substrate Specificity of *O*-L-Lysylphosphatidylglycerol Synthetase. Enzymatic Studies on the Structure of *O*-L-Lysylphosphatidylglycerol*

W. J. Lennarz,† P. P. M. Bonsen, and L. L. M. van Deenen

ABSTRACT: A particulate enzyme fraction, prepared from crude extracts of *Staphylococcus aureus*, catalyzes the formation of L-[^{14}C]-*O*-lysylphosphatidylglycerol upon addition of L[^{14}C]lysyl-tRNA. Treatment of this enzyme fraction with organic solvents to remove endogenous lipids yields a preparation which has a marked requirement for the addition of phosphatidylglycerol in the enzymatic synthesis of L-[^{14}C]-*O*-lysylphosphatidylglycerol from L-[^{14}C]lysyl-tRNA. Specificity studies with a variety of natural and synthetic phospholipids indicate that the enzymatic transacylation reaction is highly specific for phosphatidylglycerol. A variety of analogs of phosphatidylglycerol, including 2′-deoxy- and 3′-deoxyphosphatidylglycerol, have been prepared with the use of phospholipase D. Tests on the ability of these compounds to serve as substrates in the enzymatic formation of lysylphospholipids have revealed that only 2′-deoxyphosphatidylglycerol (not 3′-deoxyphosphatidylglycerol) can serve as an acceptor of the lysyl group. These results indicate that the enzymatic aminoacylation occurs on the primary (3′) hydroxy group of the glycerol moiety of phosphatidylglycerol, and that the structure of the enzymatically formed lipid is 3-phosphatidyl-1′-(3′-*O*-L-lysyl)glycerol.

The occurrence of aminoacyl derivatives of phosphatidylglycerol in a variety of bacteria is now well established (Macfarlane, 1964; Houtsmuller and van Deenen, 1965; Lennarz, 1966). The amino acid most commonly found to be esterified to phosphatidylglycerol is lysine. A chemical synthesis of 3-phosphatidyl-1′-(3′-*O*-L-lysyl)glycerol (Bonsen *et al.*, 1966a, 1967) has confirmed the basic structure and stereochemical configuration assigned to the lysine-containing phospholipid (*O*-L-lysylphosphatidylglycerol) isolated from *Staphylococcus aureus* (Houtsmuller and van Deenen, 1965). However, studies on the products of enzymatic or chemical degradation of this lipid did not provide conclusive information about the position of the linkage of the lysyl group to the glyceryl moiety (2′ or 3′) in the natural phospholipid (*cf.* structure in Figure 1).

Recent studies (Lennarz *et al.*, 1966; Gould and Lennarz, 1967) have demonstrated that the biosynthesis of *O*-L-lysylphosphatidylglycerol involves the enzymatic transfer of lysine from lysyl-tRNA to phosphatidylglycerol (Figure 1). Preliminary experiments on the lipid substrate specificity of *O*-L-lysylphosphatidylglycerol synthetase suggested that this enzyme might serve as a useful tool in investigations concerned with the position of esterification of the lysyl moiety. In the present study, various synthetic or semisynthetic phospholipids related in chemical structure to phosphatidylglycerol were assayed for their suitability to serve as substrates in place of phosphatidylglycerol in this enzymatic reaction. The enzymatic transfer of the lysyl group from tRNA to lipid has been found to be specific for phosphatidylglycerol; the only analog of phosphatidylglycerol that serves as a lysine acceptor is 2′-deoxyphosphatidylglycerol. These results suggest that the enzymatic esterification occurs at the 3′ (primary) hydroxy group of phosphatidylglycerol to yield 3-phosphatidyl-1′-(3′-*O*-L-lysyl)glycerol (structure VI, Table I).

Experimental Section

3-Phosphatidyl-1′-glycerol (Bonsen *et al.*, 1966b), 3-phosphatidyl-1′-(2′,3′-isopropylidene)glycerol (Bonsen *et al.*, 1966b) 1,3-diphosphatidylglycerol (de Haas and van Deenen, 1965), and 3-phosphatidyl-(*rac*)-1′-glycerol 3′-phosphate (Bonsen and de Haas, 1967) were synthesized as described earlier. A number of analogs of phosphatidylglycerol were prepared from egg lecithin and an appropriate alcohol by means of a transphosphatidylation reaction catalyzed by phospholipase D as reported by Benson *et al.* (1965), Yang *et al.* (1967), Bartels and van Deenen (1966), Douce *et al.* (1966), and Dawson (1967). Crude preparations of phospholipase D from savoy cabbage (or Brussels sprouts) were obtained according to the procedure of Davidson and Long (1958). The following alcohols were used: ethylene glycol, racemic 1,2-propanediol, 1,3-propanediol, and 1,4-butanediol (all purchased

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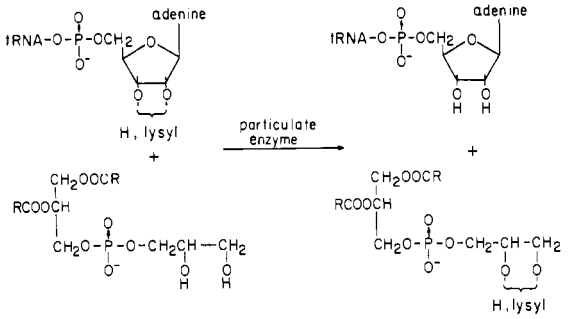

FIGURE 1: Reaction for the enzymatic synthesis of *O*-lysylphosphatidylglycerol.

from Fluka). The latter two alcohols were treated with sodium periodate in order to destroy traces of isomeric *vic*-glycols; following periodate treatment the compounds were subjected to final purification by distillation. In a typical experiment, 500 mg of egg lecithin dissolved in 50 ml of alcohol-free diethyl ether (freshly distilled over P_2O_5) was combined with 50 ml of a solution of phospholipase D in 0.1 M acetic acid–sodium acetate buffer (pH 5.5) containing 5×10^{-3} M calcium chloride. After addition of 5 g (about 100-fold excess) of one of the alcohols, the mixture was shaken at room temperature for 4 hr. Thin layer chromatography indicated a conversion of the lecithin into the desired compound (70–90% yield) and phosphatidic acid (30–10% yield). The reaction mixture was extracted several times with ether, and after removal of the solvent the residue was partitioned between 90% aqueous methanol and pentane. The pentane layer was found to contain most of the phosphatidic acid. The desired phospholipid present in the aqueous methanol was converted into the barium salt and purified by chromatography over silica columns using mixtures of chloroform–methanol as eluents. The phospholipids, which were obtained in final yields of 50–70%, revealed one spot on thin layer chromatograms developed in chloroform–methanol–ammonia (70:20:1.5, v/v) and on silica-impregnated paper with diisobutyl ketone–acetic acid–water (8:5:1, v/v) as solvent system. The R_F values of the analogs, depending on the alcohol used, were between those of phosphatidylglycerol and phosphatidic acid. The barium salts were converted into the sodium salts before use in the experiments with the bacterial enzymes. Lipids were suspended in H_2O (10 mg/ml) and dispersed by sonication for 2–3 min with a Bronwill 20-kcycle sonicator. Sonication of lipids containing unsaturated fatty acids was performed under nitrogen.

Lysyl-tRNA and particulate enzyme fractions of *S. aureus* and *Bacillus megaterium* were prepared as previously reported (Gould and Lennarz, 1967; Lennarz *et al.*, 1966). The particulate fraction obtained upon centrifugation of the crude extract at 100,000*g* for 1 hr was suspended in one-fourth volume (relative to the volume of crude extract subjected to centrifugation) of 0.02 M Tris·HCl–0.001 M mercaptoethanol (pH 7.0) (protein concentration, 15–30 mg/ml) and extracted with organic solvent to remove endogenous lipids. All organic solvents were maintained at −15° and all operations were performed in the cold room. To 9 ml of acetone, 0.5 ml of particulate enzyme solution was added dropwise with stirring over a period of 5–10 sec. The resulting suspension was centrifuged for 1–2 min in a clinical centri-

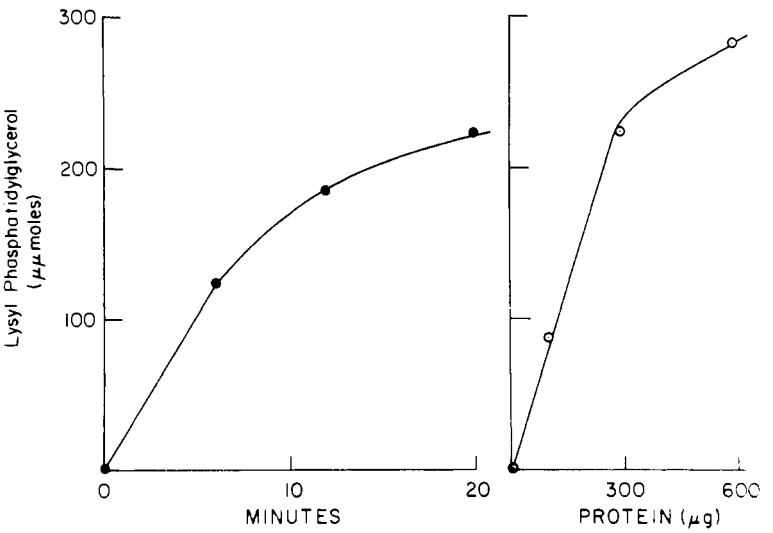

FIGURE 2: Dependence of lysylphosphatidylglycerol synthesis on time and on enzyme concentration. Except where variations are indicated, the complete system contained: L-[^{14}C]lysyl-tRNA, 392 μμmoles; phosphatidylglycerol, 354 mμmoles; Na^+ salt of total fatty acids of *S. aureus* or *Micrococcus lysodeikticus*, 750 mμmoles; $MgCl_2$, 3 μmoles; L-lysine, 0.1 μmole; KCl, 40 μmoles; Tris-maleate, 7.5 μmoles (pH 7.05); and enzyme, 296 μg. The final volume was 430 μl; incubation was carried out at 30° for 20 min.

TABLE I: Lipid Specificity in the Transfer of Lysine from [^{14}C]Lysyl-tRNA to Phospholipid.[a]

Lipid Added[b]	[^{14}C]Lysyl Lipid Formed ($\mu\mu$moles)
None	11.7–16.3
(I) R[c]–OCH_2CHCH_2OH (OH on middle C) synthetic	125.0
(I) R[c]–OCH_2CHCH_2OH (OH on middle C) natural (*S. aureus*)	137.0
(II) $ROCH_2CHCH_2OPO_3H_2$ (H, OH on middle C)	<25[d]
(III) $ROCH_2CHCH_2OR$ (OH on middle C)	<16
(IV) $ROCH_2C(CH_2)$ (O, O, $C(CH_3)_2$)	3.5
(V) $ROCH_2CH_2OH$	16.0
(VI) $ROCH_2CHCH_2OC(=O)CH(NH_2)(CH_2)_4NH_2$ (OH on middle C)	<16
(VII) $ROCH_2CH_2CH_2OH$	53.5
(VIII) $ROCH_2CHCH_3$ (H, OH)	12.3

[a] Incubation conditions as in Figure 2. [b] Lipids were tested in the range of 0.5–1.0 μmole. [c] R equals 1,2-diacylglycero-3-phosphoryl. [d] Values with different enzyme preparations varied from 16 (control values) to 25 $\mu\mu$moles. The high values may be due to the action of a phosphatidylglycerol phosphate phosphatase known to occur in bacterial extracts which could result in production of phosphatidylglycerol (Kanfer and Kennedy, 1964).

fuge, and the supernatant fluid was removed by decantation. The pellet was suspended in 10 ml of fresh diethyl ether and reisolated by centrifugation. The washed residue was suspended in 8 ml of $CHCl_3$ and the suspension was centrifuged for 1–2 min. After centrifugation, the resulting floating protein, contained in the upper 1 ml of $CHCl_3$, was carefully removed by means of a Pasteur pipet and transferred to a fresh tube. After addition of 6 ml of fresh $CHCl_3$ the suspension was centrifuged.

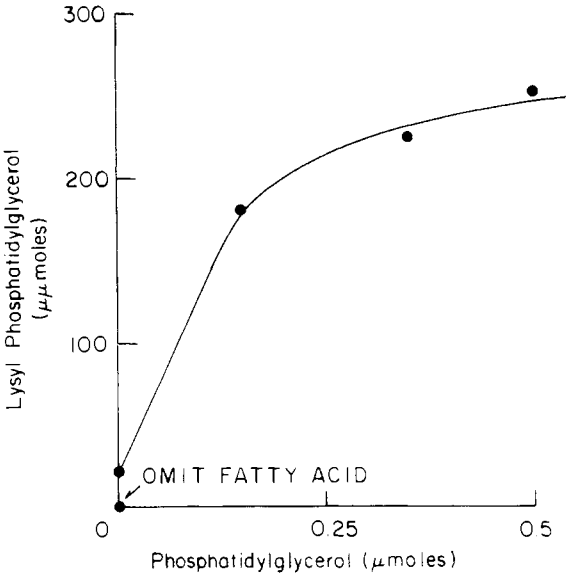

FIGURE 3: Dependence of lysylphosphatidylglycerol synthesis on phosphatidylglycerol concentration. Incubation conditions as in Figure 2.

The protein was again recovered as indicated above and transferred to a fresh tube, followed by addition of 8 ml of diethyl ether. After brief centrifugation the supernatant was discarded and the pellet was washed with 10 ml of diethyl ether. The ether wash was decanted and the pellet was immediately dried under high vacuum for 1 hr at 4°. The dry residue was suspended in 0.3–0.5 ml of 0.02 M Tris·HCl–0.001 M mercaptoethanol (pH 6.7) (protein concentration, 12–20 mg/ml). The specific activity of different preparations varied considerably; however, all preparations showed a marked dependence on added phosphatidylglycerol. The stimulation by phosphatidylglycerol varied from 5- to 25-fold. Whereas the particulate enzyme preparation retained full activity after storage at −20° for several weeks, frozen suspensions of the organic solvent extracted enzyme lost at least 50% of their activity in 3–5 days.

Results and Discussion

In the initial studies on the biosynthesis of *O*-L-lysylphosphatidylglycerol it was not possible to demonstrate that phosphatidylglycerol was a specific aminoacyl acceptor because the particulate enzyme preparation contained endogenous lipid (Lennarz *et al.*, 1966). Treatment of the particulate enzyme with organic solvents has afforded a preparation which requires addition of phosphatidylglycerol for synthesis of *O*-L-lysylphosphatidylglycerol. Maximal activity requires a medium of high ionic strength and the presence of an anionic surfactant such as the salt of a fatty acid. Studies on these requirements and on the physical state of the lipid substrate will be reported at a later date. The dependence of the reaction on time and enzyme concentration is shown

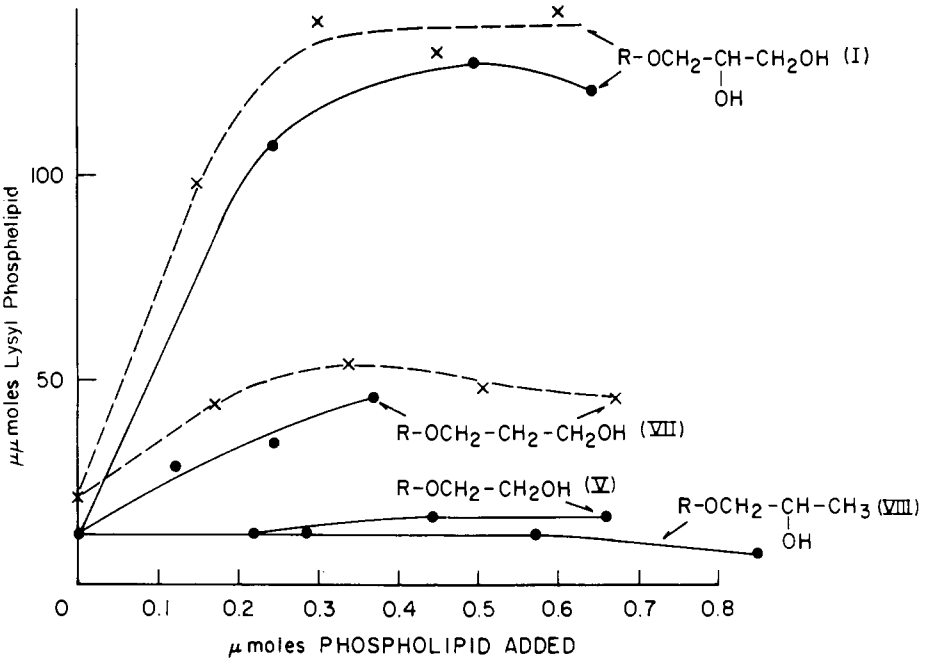

FIGURE 4: Dependence of lysyl lipid synthesis on added phosphatidylglycerol or phosphatidylglycerol analogs. Incubation conditions as in Figure 2. The solid and dashed lines represent results obtained in two separate experiments using different enzyme and lipid preparations.

in Figure 2. In Figure 3 the dependence on added phosphatidylglycerol (from *S. aureus*) is shown. A small but detectable level of synthesis is noted in the absence of exogenous phosphatidylglycerol provided that fatty acid salt is added. Upon addition of phosphatidylglycerol, fatty acid has little or no stimulatory effect.

Initial studies on the lipid specificity in the reaction indicated that the reaction was highly specific for phosphatidylglycerol, inasmuch as phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine, cardiolipin, and lysylphosphatidylglycerol did not act as acceptors of the lysyl group from lysyl-tRNA. In order to define further the structural requirements of the lipid substrate, studies with a variety of derivatives and analogs of phosphatidylglycerol were undertaken. The results are shown in Table I. Synthetic phosphatidylglycerol (I) was found to be active in accepting the lysyl moiety from lysyl-tRNA. The slight differences in activity between phosphatidylglycerol from bacterial and synthetic origin may be due to differences in fatty acid composition which could affect the micellar properties of the lipids in an aqueous environment. Phosphatidyl-(*rac*)-1′-glycero-3′-phosphate (II), a compound known to be a precursor in the biosynthesis of phosphatidylglycerol (Kiyasu *et al.*, 1963; Kanfer and Kennedy, 1964), did not give rise to the formation of a lysine-containing lipid. Similarly synthetic diphosphatidylglycerol (III) showed little or no activity as an acceptor of the lysyl group. Protection of both hydroxyl functions of phosphatidylglycerol destroyed the activity of the lipid as demonstrated by the results with the synthetic isopropylidene derivative IV. Also essentially inactive as an acceptor of the lysyl group was phosphatidylethylene glycol (V). Addition of *O*-lysylphosphatidylglycerol (VI) did not stimulate the incorporation of radioactive lysine into phospholipid, thus confirming the previous conclusion that an exchange reaction is not involved (Lennarz *et al.*, 1966). These results support the view that phosphatidylglycerol acts as a specific acceptor for lysine in the synthesis of *O*-lysylphosphatidylglycerol in *S. aureus*.

In contrast to these negative findings with a variety of derivatives and analogs of phosphatidylglycerol, phosphatidyl-(2′-deoxy)glycerol (VIII) was found to show significant activity in accepting the lysyl moiety from lysyl-tRNA. On the other hand, the isomeric compound phosphatidyl-(3′-deoxy)glycerol (VIII),[1] was found to be completely inactive as an acceptor. These results strongly suggest that the lysyl moiety is enzymatically esterified to the primary rather than to the secondary

[1] The transphosphatidylation reaction has been found to occur nearly exclusively with the primary hydroxyl functions of glycerol (Benson *et al.*, 1965; Bonsen *et al.*, 1966b; Dawson, 1967; Yang, *et al.*, 1967). However, it cannot be precluded that during the reaction with 1,2-propanediol a very small amount of esterification occurred at the secondary hydroxyl group, rather than the primary hydroxyl group. This reaction would lead to formation of the isomeric compound, phosphatidyl-2′-(1′-deoxy)glycerol, rather than VII. However, except for the unlikely possibility that small amounts of this isomer of VII act as an inhibitor of the enzymatic reaction, its presence in trace amounts would not alter any of the conclusions made in the present study.

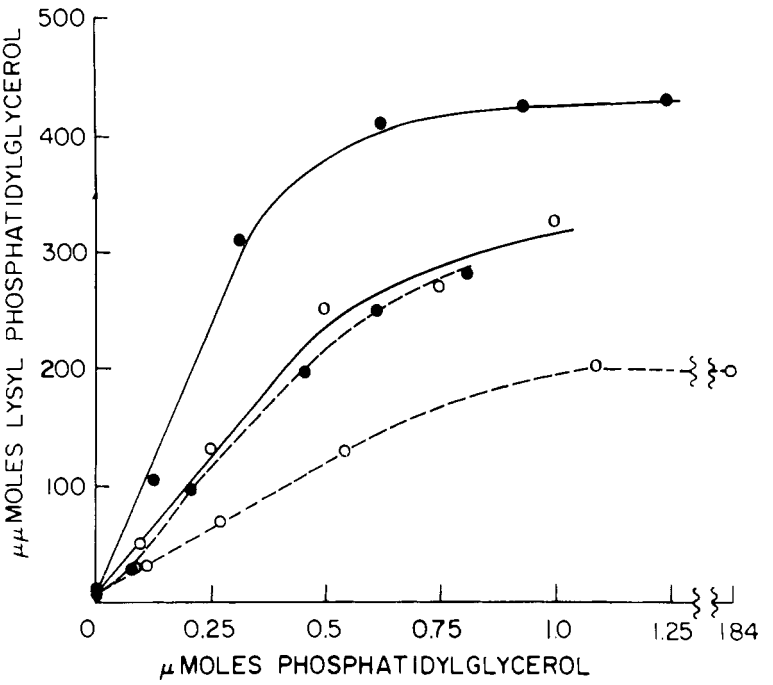

FIGURE 5: Comparison of lysyl group acceptor activity of phosphatidyl-(*rac*)-1′-glycerol prepared from egg lecithin with phosphatidylglycerol from *S. aureus*. Conditions and components were as in Figure 2 except that L-lysine and $MgCl_2$ were omitted and 60 μmoles of KCl, 580 μμmoles of [^{14}C]lysyl-tRNA, and 610 μg of protein were added. Incubation for 20 (solid line) or 10 min (dashed line). Either *S. aureus* phosphatidylglycerol (closed circles) or egg lecithin derived phosphatidylglycerol (open circles) was added as indicated.

hydroxyl group of phosphatidylglycerol (*cf.* structure VI, Table I).

The results of more extensive experiments in which analogs of phosphatidylglycerol were tested over a range of lipid concentrations are shown in Figure 4. From these results it is clear that the only analog that can act as a substrate for the lysylphosphatidylglycerol synthetase is 2′-deoxyphosphatidylglycerol. The lower activity of this compound, when compared to phosphatidylglycerol from *S. aureus*, may be ascribed to the absence of a secondary hydroxyl group which could play a role in the binding of substrate to enzyme, or may be due to differences in the fatty acid composition of the two lipids. The fatty acids of the lipids of *S. aureus* are known to contain a high proportion of C_{15} branched-chain fatty acids (Macfarlane, 1962), whereas 2′-deoxyphosphatidylglycerol contains the straight-chain saturated and unsaturated fatty acids typical of egg lecithin (Tattrie, 1959). In an attempt to clarify this point, a comparison of the acceptor activities of phosphatidylglycerol prepared from egg lecithin and phosphatidylglycerol from *S. aureus* was performed (Figure 5). It is evident that the lecithin-derived phosphatidylglycerol is a poorer acceptor of the lysyl group than phosphatidylglycerol from *S. aureus*. Unfortunately it is not possible to establish unambiguously that this difference is due to the differences in fatty acids, inasmuch as the 2′-hydroxyl group in the lecithin-derived phosphatidylglycerol is racemic (Yang *et al.*, 1967).

Comparable experiments with a lysylphosphatidylglycerol synthetase preparation from *B. megaterium* have yielded qualitatively similar results (W. J. Lennarz, unpublished studies).[2] The results of these studies with analogs of phosphatidylglycerol make it reasonable to conclude that the position of enzymatic esterification of phosphatidylglycerol is the 3′- rather than the 2′-hydroxyl group and, therefore, that the structure of the naturally occurring lipid is 3-phosphatidyl-1′-(3′-*O*-L-lysyl)glycerol. The latter conclusion, however, cannot be made with absolute certainty inasmuch as it is possible that the lysyl group, after enzymatic transfer to the 3′-hydroxyl group of the glyceryl moiety, undergoes chemical isomerization to yield an equilibrium mixture of the 2′ and 3′ isomers of *O*-L-lysylphosphatidylglycerol. Precedence for such a situation may be found in the apparent existence of aminoacyl esters of tRNA as an equilibrium mixture of the 2′- and 3′-aminoacyl esters of the ribosyl moiety (Sonnenbichler *et al.*, 1965; Griffin *et al.*, 1966). However, in the case of aminoacyl-tRNA, conditions for the establishment of such an equilibrium are more favorable than those in the present instance, inasmuch as both hydroxyl groups of the

[2] An additional potential substrate tested in studies with *B. megaterium* enzyme preparation was phosphatidyl-(4′-hydroxy)butanol. This compound was found to be essentially devoid of acceptor activity.

vicinal diol system of the ribosyl group are secondary, and are constrained into the *cis* configuration. In any event, it is hoped that further chemical studies will serve to clarify the question of lysyl group migration in lysylphosphatidylglycerol.

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