D- and L-Alanylphosphatidylglycerols from *Mycoplasma laidlawii*, Strain B*

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ABSTRACT: *Mycoplasma laidlawii*, strain B, when acidified in culture after normal growth, was found to contain ninhydrin-positive phospholipids identified as *O*-amino acid esters of phosphatidylglycerol. The amino acids esterified were identified as alanine, glutamic acid, glycine, leucine–isoleucine, lysine, and tyrosine. Alanine, the principal amino acid, was found by analysis with stereospecific D- and L-amino acid oxidases to be present in a nonracemic mixture of about 2:1, respectively.

Biosynthesis of L-alanylphosphatidylglycerol was found to involve a membrane-associated enzymatic transesterification of the amino acid from L-alanyl transfer ribonucleic acid, was ribonuclease-sensitive, and required addition of exogenous phosphatidylglycerol. Biosynthesis of D-alanylphosphatidylglycerol did not involve transfer ribonucleic acid and was ribonuclease resistant but required phosphatidylglycerol. Formation of D-alanylphosphatidylglycerol occurred *via* a complex of D-alanine with adenosine triphosphate and an amino acid "activating" enzyme. Biosynthesis of isomeric alanylphosphatidylglycerols was noncompetitive and stereospecific and the product configuration was maintained.

The presence of ninhydrin-reactive phospholipids other than phosphatidylethanolamine was first reported in an acidified culture of *Clostridium welchii* by MacFarlane (1962). By analysis of products of mild alkaline hydrolysis, phosphorus:nitrogen atomic ratios, and chemical reactivities, these compounds were identified as *O*-amino acid esters of phosphatidylglycerol, or, as termed by MacFarlane, "lipoaminoacids." The amino acids present were identified as predominantly alanine, plus lysine, glutamic acid, aspartic acid, and possibly arginine or histidine. Positional ester linkage of the amino acid was arbitrarily assigned to the α' (3′) carbon of the unacylated glycerol of phosphatidylglycerol. The assigned structure of the type compound and products found on mild alkaline hydrolysis are shown in Figure 1. Subsequently, additional workers reported the presence of amino acid esters of phosphatidylglycerol in a variety of gram-positive bacteria as well as in a lesser number of gram-negative organisms (reviewed by Kates, 1964). Although the type of amino acid present seems genus or species specific, in general L-alanine and L-lysine were reported most frequently present and quantitatively predominant. All amino acids exist as the L isomers except for the apparent presence of D-alanylphosphatidylglycerol in *Leuconostoc mesenteroides* (Ikawa, 1963). Appearance of the amino acid esters of phosphatidylglycerol is dependent upon acidic conditions, either through acidification of the culture medium by organic acid end products of metabolism, or by addition of mineral acid. Biosynthesis of the esters of the L-amino acids has been demonstrated (Lennarz *et al.*, 1966; Gould and Lennarz, 1967) to involve the mechanism of amino acid "activation" for protein synthesis: (I) (amino acid specific) aminoacyl-tRNA synthetase + amino acid + ATP $\rightleftarrows$ AMP–amino acid–enzyme complex + PP_i; (II) AMP–amino acid-tRNA synthetase complex + (amino acid specific) tRNA $\rightarrow$ AMP + enzyme + aminoacyl-tRNA. The "activated" amino acid is then transesterified to phosphatidylglycerol by a membrane-associated "synthetase" enzyme; (III) aminoacyl-tRNA + phosphatidylglycerol (aminoacylphosphatidylglycerol synthetase) $\rightarrow$ aminoacylphosphatidylglycerol + tRNA. Recently the structure of the aminoacylphosphatidylglycerols proposed by MacFarlane (1962) has been proven by Lennarz *et al.* (1967), using chemically synthesized analogs of phosphatidylglycerol (particularly 2′- and 3′-deoxyphosphatidylglycerol) as substrates for transesterification of amino acid from aminoacyl-tRNA by aminoacylphosphatidylglycerol synthetase. Further, definitive proof of structure was adduced by Bonsen *et al.* (1967) and by Molotkovsky and Bergelson (1968) who compared chromatographic, chemical, and enzyme substrate qualities of chemically synthesized and natural aminoacylphosphatidylglycerols, and identified the natural compounds as 1,2-diacylglycerol-3-phosphoryl-(3′-*O*-aminoacyl)glycerol.

The presence of phosphatidylglycerol in two strains of *Mycoplasma* (Smith and Koostra, 1967; Shaw *et al.*, 1968) suggested the possibility of formation of aminoacylphosphatidylglycerols by mycoplasmas under acidic conditions. Identification of such compounds, examination of the stereoconfiguration of the amino acid(s) and mechanism of their incorporation into the esters, are reported in this study.

Materials and Methods

Growth of Organisms, Extraction of Lipids. Mycoplasma laidlawii, strain B, was grown as described by Smith and Henrikson (1965) except that after 22-hr growth at 37°, the cultures were acidified by addition of 5.5 ml of sterile concentrated HCl per 2 l. of culture, lowering the pH to approxi-

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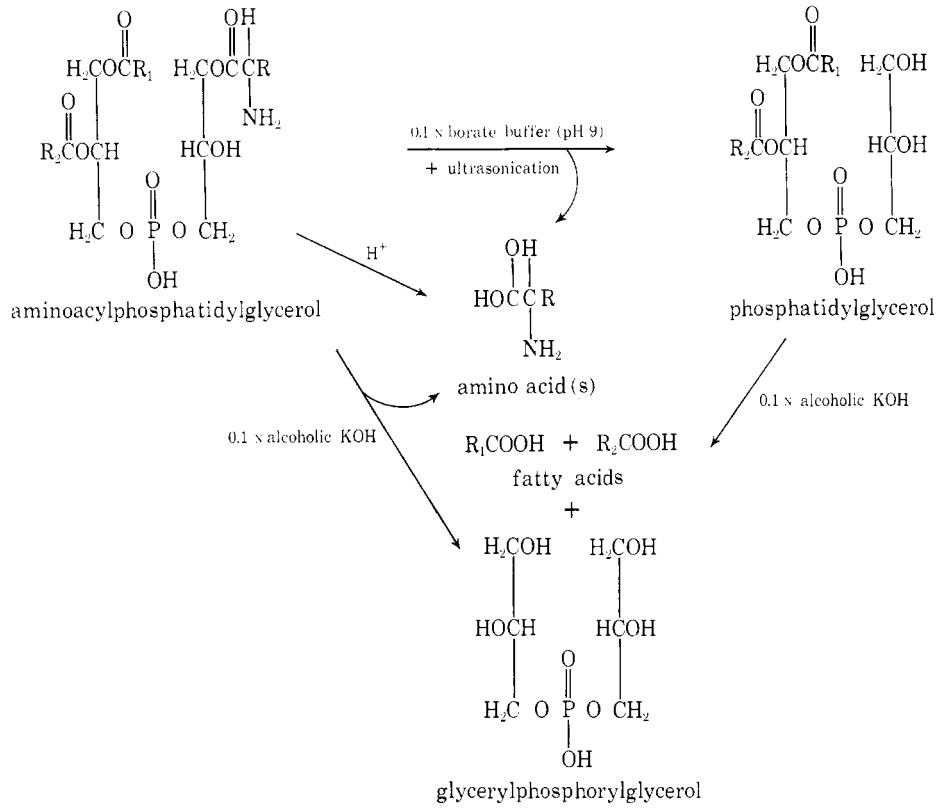

FIGURE 1: Reaction scheme for the degradation of *O*-aminoacylphosphatidylglycerol.

mately 5. Acidified cultures were incubated for an additional 2 hr at 37°. *Staphylococcus aureus* FDA 209 (ATCC 6538), when used as a source of lysylphosphatidylglycerol for a standard or control, was grown as described by Houtsmuller and van Deenen (1965) except for omission of [^{32}P]orthophosphate. The organisms were harvested with a Sharples continuous-flow centrifuge and further concentrated to a pellet by centrifugation at 15,000*g* for 30 min. Subsequent treatment maintained acidic conditions throughout following Houtsmuller and van Deenen (1965); cells (0.5–2.0 g) were suspended in 30 ml of 0.2 M acetate buffer (pH 4.5), and 30 ml of chloroform and 65 ml of methanol were added, forming a single-phase system (Bligh and Dyer, 1959). The suspension was stored 2–3 hr at 2° and the cells were collected by centrifugation or by filtration through Whatman No. 1 filter paper. The extraction was repeated three times. The extracts were combined, and 30 ml of water and 30 ml of chloroform were added, forming a biphasic system. After standing 1–2 hr at room temperature or 2° for phase separation, the water layer was discarded and the chloroform layer was dried under reduced pressure on a rotary evaporator.

Separation of Lipids on Silicic Acid. The dried lipid extract was dissolved in 10 ml of chloroform and applied to a column of 7 g of activated silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.) made with chloroform. Separation was accomplished with the following eluents: chloroform, 100 ml; chloroform–methanol (98:2), 100 ml; chloroform–methanol (96:4), 100 ml; chloroform–methanol (92:8), 200 ml; chloroform–methanol (3:1), 200 ml; chloroform–methanol (1:1), 100 ml (all solvent ratios being v/v). The fractions were dried *in vacuo*.

Thin-Layer Chromatography. The purity of each fraction was determined by thin-layer chromatography on glass plates (C. Desaga–Brinkman, Westbury, N. Y.) coated with a layer 250 μ thick of silica gel G (E. Merck AG, Darmstadt, Germany) and activated 1 hr at 100° before use. Samples were applied in chloroform, dried, and the chromatograms were developed in chloroform–methanol–acetic acid–water (65:25:8:4) or 2,6-dimethyl-4-heptanone(diisobutyl ketone)–glacial acetic acid–water (40:25:5) (Nichols, 1963). Following solvent flow to 13 cm from the origin, lipids were visualized by spraying with water or by exposure to iodine vapor (Smith and Koostra, 1967). Phosphorus was detected by spraying with the ammonium molybdate–perchloric acid reagent (Hanes and Isherwood, 1949), and amino acids by spraying with 0.2% ninhydrin in water-saturated 1-butanol followed by heating in a 100° oven for 10 min (Block *et al.*, 1958).

Analytical Procedures. Ninhydrin-reactive phospholipids which were presumed amino acid esters of phosphatidylglycerol were hydrolyzed by several means (Figure 1): (1) total hydrolysis by refluxing 48 hr in 2 N HCl, for glycerol analysis; (2) total acid hydrolysis in 6 N HCl at 100° for 3 hr, followed by paper chromatographic separation of water-soluble products; (3) very mild alkaline hydrolysis with ultrasonic emulsification in 0.1 M borate buffer (pH 9) (Houtsmuller and van Deenen, 1965) with a 10-kc Raytheon sonic oscillator, followed by incubation at room temperature 30 min and paper chromatographic separation of water- and ether-soluble products; (4) mild saponification in 0.1 N methanolic KOH (or NaOH) at 37° for 20 min (Benson and Maruo, 1958) followed by neutralization with Dowex 50

H^+ and paper chromatographic separation of the products. Deacylation products were chromatographed by the ascending technique on Whatman No. 1 paper strips in four solvent systems: phenol–water (100:38) (solvent A), propanol–aqueous ammonia (specific gravity 0.88)–water (6:3:1) (B), phenol–glacial acetic acid–absolute ethanol–water (40:5:6:10) (C), and butanol–propionic acid–water (142:71:100) (D). Spots were located by spraying with the Hanes–Isherwood reagent. Identification of spots was made by comparison of R_F values with those of phosphatidylglycerol isolated from *Staphylococcus aureus*, (bis)phosphatidylglycerol (Sylvana Chemical Co.), and phosphatidic acid, and their mild alkaline deacylation products. Phosphatidic acid was isolated from egg lecithin (Nutritional Biochemical Corp., Cleveland, Ohio) by the method of Kates (1955) using phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) isolated from carrots or cabbage leaves (Kates, 1954). Ninhydrin-positive products were chromatographed by the descending technique on Whatman No. 1 paper strips in five solvent systems: 1-propanol–ammonium hydroxide (specific gravity 0.88)–water (6:3:1) (solvent I); 1-butanol–glacial acetic acid–water (4:1:5) (upper layer) (II); 1-butanol (110 ml), then water-saturated and 30 ml of glacial acetic acid added (III); pyridine–isoamyl alcohol–diethylamine (10:10:7:3) (IV); 1-butanol–benzyl alcohol (1:1) then water-saturated, run in diethylamine atmosphere (V). Commercial L- or DL-amino acids were chromatographed as standards. Spots were located by spraying with the ninhydrin reagent followed by heating 10 min at 100°. Samples of the deacylated or intact lipid were hydrolyzed by refluxing 48 hr in 2 N hydrochloric acid (Hanahan and Olley, 1958) and used for glycerol analysis. Glycerol was determined by phosphorylation with ATP (Sigma Chemical Co.) and glycerol kinase (EC 2.7.1.30) (Sigma, from *Candida mycoderma*) followed by reduction of NAD (Sigma Chemical Co.) by α-glycerophosphate dehydrogenase (Calbiochem, Los Angeles, Calif., A grade, from rabbit muscle) (Wieland, 1963). Free amino groups were determined with ninhydrin (Fraenkel-Conrat, 1957), phosphorus by the method of King (1932), and fatty acid esters by hydroxamate formation in presence of ferric perchlorate (Rapport and Alonzo, 1955).

Configurational Analysis of Amino Acids. For configurational analysis of the amino acids esterified to phosphatidylglycerol, the water-soluble products of total acid hydrolysis or pH 9 alkaline hydrolysis were neutralized and used as substrates of the stereospecific oxidative deaminases, D-amino acid oxidase (EC 1.4.3.3) (crude, from hog kidney, Sigma Chemical Co.) and L-amino acid oxidase (EC 1.4.3.2) (Sigma Type I, crude venom from *Crotalus adamanteus*), in a Warburg apparatus gassed with oxygen (Boulanger and Osteux, 1963). Water-soluble products of total acid hydrolysis of lysylphosphatidylglycerol from *S. aureus* (Houtsmuller and van Deenen, 1965) were used as a control, and D- and L-alanine and D- and L-lysine (Nutritional Biochemicals Corp.) were used as standards. Activities of the two enzymes with DL-alanine were made equivalent by adjustment of enzyme concentrations. Enzymic activity was followed by measurement of oxygen uptake over a 2-hr period at 37°. The reaction mixtures were deproteinized, and the α-keto acids produced were converted into their respective 2,4-dinitrophenylhydrazones (Boulanger and Osteux, 1963) and extracted into acidic ether or ethyl acetate. The hydrazones were separated chromatographically on Whatman No. 1 paper (descending technique) in 1-butanol–ethanol–0.5 N ammonia (7:1:2) (solvent VI) and in 1-butanol–ethanol–water (4:5:1) (solvent VII).

Isolation and Assay of Aminoacyl Transfer Ribonucleic Acid Synthetases. Aminoacyl transfer ribonucleic acid (aminoacyl-tRNA) synthetases were isolated from *Escherichia coli* B (late logarithmic growth phase, General Biochemicals, Chagrin Falls, Ohio) and from a 24-hr culture of *M. laidlawii* according to Calendar and Berg (1966) through the ammonium sulfate and dialysis step (step III). The aminoacyl-tRNA synthetases also were isolated from *M. laidlawii* B by the method of Gasior (1966). Activity of the enzymes with D- and L-alanine and L-leucine as substrates was determined in the exchange reaction (DeMoss and Novelli, 1956). The assay utilizes the reversibility of reaction I; on addition of [^{32}P]PP_i to the components of the reaction, [^{32}P]ATP can be isolated from the products and quantitated. Formation of [^{32}P]PP_i was accomplished by pyrolysis of pH 9 sodium [^{32}P]-orthophosphate (neutralized [^{32}P]orthophosphoric acid, New England Nuclear Corp.) in a muffle furnace at 400° for 1 hr, followed by separation of PP_i from ortho- and polyphosphates on Dowex 1 (Cl^-) by elution with 0.05 M NaCl in 0.01 N HCl (Peng, 1956). Pyrophosphate and orthophosphate were determined by the cysteine modification of the Fiske–Subbarow phosphate determination (Flynn *et al.*, 1954) and ^{32}P radioactivity was quantitated by liquid scintillation counting.

Isolation of Complexes of Adenosine Monophosphate (AMP), Amino Acid, and Aminoacyl Transfer Ribonucleic Acid Synthetases. Isolation of the product of the first step of amino acid "activation" (*i.e.*, AMP–aminoacyl–enzyme complex, reaction I) was performed using the reaction conditions of Norris and Berg (1964) except for omission of tritium-labeled ATP. The substrates utilized were D-[1-^{14}C]alanine (Calbiochem, specific activity 9.1 mCi/mmole), and L-[U-^{14}C]-alanine and L-[U-^{14}C]leucine (New England Nuclear Corp., specific activity 10 mCi/mmole). The enzyme–substrate complexes were isolated by gel filtration through a column (1 cm diameter by 30 cm long) of Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) as described by Allende *et al.* (1964). Samples of the eluate (0.01 M Tris-chloride buffer (pH 7.4), 0.75-ml fractions) were assayed for ATP-[^{32}P]PP_i-exchange activity (0.25 ml) (DeMoss and Novelli 1956) and for [^{14}C]amino acid (0.50 ml) by counting in the TriCarb liquid scintillation counter. Controls consisted of the reaction mixture containing heat-inactivated enzyme protein.

Reaction of Complexes of AMP, Amino Acid, and Aminoacyl Transfer Ribonucleic Acid Synthetases with Transfer Ribonucleic Acids. Crude tRNA was isolated from a 24-hr culture of *M. laidlawii* B by a modified Kirby phenol extraction (Holley *et al.*, 1961). The transfer of [^{14}C]amino acids from the AMP–aminoacyl–enzyme complex to tRNA (reaction II) used the reaction conditions of Norris and Berg (1964) and incubation at 0° for 15 min. The aminoacyl-tRNA was precipitated by addition of 2 ml of ice-cold 10% (w/v) trichloroacetic acid, followed by filtration on a sintered glass filter fitted with a paper disk (Whatman No. 3MM chromatography paper, 2.6 cm diameter) and successive washings (*ca.* 3 ml each) with 3% (v/v) perchloric acid (four times), ethanol (four times), and ether (two times) (Makman, 1966). The disks were placed in vials, dried, and counted.

Single-Step Formation and Isolation of Aminoacyl Transfer Ribonucleic Acid. When isolation of the intermediate AMP–aminoacyl-tRNA complex was not necessary (*i.e.*, when reaction I and II could be combined), formation of [^{14}C]-aminoacyl-tRNA used the reaction conditions of Zubay (1966). Products of the reaction were precipitated with cold 10% trichloroacetic acid, washed, and counted as described (Makman, 1966). Ribonuclease treatment utilized 200 μg of pancreatic RNase (EC 2.7.7.16; RNase A, Worthington Biochemical Corp.) in 0.1 M Tris-chloride (pH 7.0) for 30 min at 37°.

Analysis of the Structure of the Aminoacyl Transfer Ribonucleic Acids with Ribonuclease. Aminoacyl-tRNA formed by the methods described above was used as substrate for ribonuclease. Conditions utilized were modified slightly from Zachau *et al.* (1958) and Preiss *et al.* (1959). The reaction was carried out in 0.1 M Tris-maleate buffer (pH 6.0) for 15 min at 30°. Products of the reaction were separated by paper chromatography on Whatman No. 1 paper (descending technique) in 2-butanol–formic acid (88%)–water (7:1:2) (solvent VIII), in 1-propanol–ammonium hydroxide (specific gravity 0.88)–water (6:3:1) (solvent I), and in 1-butanol–glacial acetic acid–water (4:1:5) (upper layer) (solvent II), and electrophoretically on Whatman No. 3MM paper in 0.02 M sodium citrate buffer (pH 3.1) at 14 V/cm at 3° (Berg, 1958). Standards employed were 2′(3′)-L-alanyl-AMP, 2′(3′)-D-alanyl-AMP, and 2′(3′)-L-leucyl-AMP, synthesized from the respective amino acids in the presence of phosphorus pentachloride and AMP similar to Wieland and Pfleiderer (1958). Also used as standards were the corresponding 2′(3′)-aminoacyladenosines, formed by dephosphorylation of the 2′(3′)aminoacyl-AMP's with acid phosphatase (EC 3.1.3.2) (Worthington Biochemical Corp.) in 0.1 M acetate buffer (pH 5.0) for 30 min at 37°. Detection of spots was accomplished by observation under ultraviolet light, with ninhydrin, and by passing the strips through a radiochromatogram scanner (Atomic Accessories, Inc., Valley Stream, N. Y.).

Formation of [^{14}C]Aminoacylphosphatidylglycerol from [^{14}C]Aminoacyl Transfer Ribonucleic Acid. Conditions for formation of aminoacylphosphatidylglycerol from aminoacyl-tRNA and phosphatidylglycerol (reaction III) were modified from Gould and Lennarz (1967). Source of aminoacylphosphatidylglycerol synthetase was a particulate (membrane) fraction of *M. laidlawii.* Cells harvested from 24-hr cultures were disrupted by sonication or osmotically by suspending in deionized water 1 hr at 37°. The latter preparation was washed with deionized water and centrifuged three successive times. A suspension containing 15 mg of protein (Lowry *et al.*, 1951) in a volume of 1.0 ml was added to a reaction system containing Tris-chloride (pH 7.0), 10 μmoles; $MgCl_2$, 5.5 μmoles; ATP (pH 7.0), 8.0 μmoles; phosphoenolpyruvic acid (Sigma), 5.0 μmoles; pyruvate kinase (EC 2.7.1.40) (Sigma, type II, from rabbit skeletal muscle), 0.01 ml; [^{14}C]aminoacyl-tRNA (*ca.* 100,000 counts), 500 μg; sonicated emulsion of phosphatidylglycerol isolated from *M. laidlawii*, 2 mg. The total volume was 2.0 ml. Incubation was at 37° for 30 min unless aliquot samples were withdrawn at timed intervals. Controls consisted of parallel determinations in which the enzyme had been denatured at 100° for 15 min. When present, 100 μg of RNase was added.

In some of the experiments the biosynthesis of [^{14}C]-aminoacylphosphatidylglycerol was not performed using isolated [^{14}C]aminoacyl-tRNA; instead, the phosphatidylglycerol and aminoacylphosphatidylglycerol synthetase preparations were omitted initially. Substituted for preformed [^{14}C]aminoacyl-tRNA was 1 mg of tRNA plus 0.03 μmole (*ca.* 400,000 counts) of [^{14}C]amino acid. The amino acid was "activated" (*i.e.*, esterified to tRNA, reactions I and II) during a preliminary incubation for 10 min at 37°. Then, following addition of 2 mg of phosphatidylglycerol and 15 mg of whole cell lysate containing the particulate aminoacyl-phosphatidylglycerol synthetase, the mixture (total volume, 2.0 ml) was further incubated at 37° for 15 min, to allow reaction III to occur. These latter experiments thus comprised the summation of biosynthetic reactions I, II, and III in a single reaction vessel.

For kinetic biosynthetic studies, aliquots of 0.2 ml each were removed at intervals from the reaction mixtures described above.

The reaction was stopped by addition of an equal volume of 10% (w/v) trichloroacetic acid. To each volume of aqueous solution was added 1.2 volumes each of chloroform and methanol, forming a two-phase system (Bligh and Dyer, 1959). After centrifugation for 10 min at 2000*g*, the lower (organic) phase was removed. The aqueous phase was re-extracted twice with 1.5 volumes of chloroform, and the organic solvents were pooled and evaporated to dryness under reduced pressure. The samples were spotted onto thin-layer plates of silica gel G which were developed in chloroform–methanol–acetic acid–water (65:25:8:4) (Nichols, 1963). Lipids and amino acids were located as previously described. Spots corresponding to phosphatidylglycerol and aminoacylphosphatidylglycerol, as well as the origins, were scraped from the plates into vials and counted in the scintillation counter.

Component Ratio Analysis and Stereoisomeric Analysis of in Vitro Synthesized Alanylphosphatidylglycerols. Isomeric [^{14}C]alanylphosphatidylglycerols synthesized enzymatically *in vitro* as described in the preceding section except for addition of 1 μmole of unlabeled isomeric alanine were analyzed for component glycerol, fatty acid esters, amino acid nitrogen, and phosphorus as previously enumerated. The amino acids were analyzed for their stereoconfiguration by means of the stereospecific amino acid oxidases, and the radioactivity of the [^{14}C]pyruvate produced was determined in the scintillation counter.

Competition of Isomers of [^{14}C]Alanine for Incorporation in Alanylphosphatidylglycerol. To examine for competition between the isomers of alanine used in the *in vitro* enzymatic system for synthesis of alanylphosphatidylglycerol, an experiment was made using as substrates for the synthetase enzyme system L-[^{14}C]alanine and several concentrations of added unlabeled D-alanine. The converse experiment consisted of D-[^{14}C]alanine plus several concentrations of unlabeled L-alanine or L-alanyl-tRNA. The products were isolated and analyzed for [^{14}C]amino acid as described above.

Results

Chromatographic Identification of Aminoacylphosphatidylglycerols in Mycoplasma. Lipid extracts of cultures of *M. laidlawii* grown to the late logarithmic growth phase in appropriate media and then acidified to pH 5.0 with mineral

TABLE I: Elution Pattern of *M. laidlawii* Glycolipids and Phospholipids on Silicic Acid.

Solvent	Ratio v/v	Lipid Eluted
Chloroform–methanol	89:2	Glycolipid; phosphatidylglucose
Chloroform–methanol	96:4	Phosphatidylglycerol
Chloroform–methanol	92:8	Phosphatidylglycerol
Chloroform–methanol	3:1	Aminoacylphosphatidylglycerol
Chloroform–methanol	1:1	Aminoacylphosphatidylglycerol

acid and incubated an additional 2 hr were found to contain a ninhydrin-positive component not present in cultures grown under the usual slightly alkaline (pH 8.0) conditions. Although the viability of the organisms decreases, no lysis occurred as measured by reduction of cell yield or leakage of protein into the medium. Because of the reported alkaline lability of esters of amino acids with phosphatidylglycerol (Houtsmuller and van Deenen, 1965), acidic conditions were maintained throughout the lipid extraction, and chromatographic procedures were performed using acidic solvents. The elution pattern on silicic acid of the glycolipids and phospholipids of acidified *M. laidlawii* (Table I) revealed that the ninhydrin-positive component was eluted at a higher solvent ratio of methanol to chloroform than any of the phospholipids of alkaline-grown cells (Smith and Henrikson, 1965). Elution of the ninhydrin-positive component at this solvent ratio chloroform–methanol (3:1) was consonant with the elution behavior of lysylphosphatidylglycerol of *Staphylococcus aureus* reported by Houtsmuller and van Deenen (1965), and provided the first tentative indication that the examined compound was an aminoacylphosphatidylglycerol. The chromatographic behavior (R_F 0.53) of this compound on silica gel G in an acidic solvent was indicative of a material less acidic than phosphatidylglycerol (R_F 0.62). The ninhydrin-positive fraction eluted from silicic acid with 3:1 and 1:1 chloroform–methanol was found to be chromatographically pure on thin-layer chromatography; therefore, this fraction was used without further purification.

TABLE II: R_F Values of Phospholipids and Deacylation Products.

Phospholipids	R_F in Solvent A	B	C	D
Phosphatidic acid	0.25	0.55	0.85	0.42
(Bis)phosphatidylglycerol	0.23	0.88	0.41	0.30
Phosphatidylglycerol	0.32	0.75	0.44	0.36
Ninhydrin-positive phospholipid from *M. laidlawii*	0.15	0.73	0.31	0.17
Product of Mild Deacylation[a]				
α-Glycerol phosphate[b]	0.28	0.35	0.54	0.08
GPGPG[c]	0.31	0.45	0.28	0.14
GPG[d]	0.39	0.59	0.11	0.36
Deacylation product of ninhydrin-positive phospholipid from *M. laidlawii*	0.39	0.60	0.11	0.35

[a] Deacylation in 0.1 N potassium hydroxide 10 min at 37°. [b] Produced by deacylation of phosphatidic acid. [c] GPGPG = glycerylphosphorylglycerylphosphorylglycerol. [d] GPG = glycerylphosphorylglycerol.

TABLE III: R_F Values of Amino Acids and Ninhydrin-Positive Water-Soluble Products of Hydrolysis of *O*-Aminoacylphosphatidylglycerols from *M. laidlawii*.

	R_F in Solvent[a] I	II	III	IV	V
Hydrolysis product of *M. laidlawii*[b]	0.27	0.11	0.05	0.06	0.04
	0.37	0.16	0.16	0.16	
	0.48	0.27	0.23	0.19	0.12
	0.79	0.49	0.31	0.28	0.26
		0.62	0.44	0.40	0.32
			0.66	0.50	0.48
Amino Acid Standards					
Alanine	0.48	0.17	0.32	0.30	0.13
Glutamic acid	0.25	0.10	0.20	0.03	0.04
Glycine	0.35	0.10	0.19	0.18	0.07
Isoleucine	0.78	0.67	0.67	0.48	0.44
Leucine	0.76	0.68	0.67	0.47	0.46
Lysine	0.41	0.28	0.07	0.09	0.33
Tyrosine	0.49	0.49	0.67	0.26	0.27

[a] Each value represents a minimum of two determinations. [b] Hydrolysis by total acid hydrolysis and by deacylation in 0.1 N alcoholic KOH or pH 9 borate buffer.

Chemical Analyses. Analyses of the intact phospholipid gave a molar ratio of glycerol–fatty acid ester–amino acid nitrogen–phosphorus at 2.08:2.11:0.98:1.00, respectively. These results are in agreement with those expected for an aminoacylphosphatidylglycerol.

Ultrasonic emulsification at pH 9.0 yielded a water-soluble ninhydrin-positive component and an ether-soluble component. The product of mild deacylation of this ether-soluble fraction was identical with the product of mild deacylation of the parent compound, except for the presence of water-soluble ninhydrin-positive material in the latter. Finally, total acid hydrolysis in 6 N hydrochloric acid for 3 hr at 100° yielded nearly entirely water-soluble, ninhydrin-positive products. The anticipated products of these hydrolyses is given in Figure 1.

The intact aminoacylphosphatidylglycerol and the deacylation products were chromatographed in four solvent systems. Phosphatidic acid, phosphatidylglycerol, and (bis)phosphatidylglycerol isolated from natural sources, and their

TABLE IV: Oxidative Deamination of Amino Acids from *O*-Aminoacylphosphatidylglycerols of *M. laidlawii*.[a]

Substrate	O_2 Uptake (μl)		R_F Values of Hydrazones of α-Keto Acid	
			Solvents	
	D-Amino Acid Oxidase	L-Amino Acid Oxidase	VI	VII
Amino acids from aminoacyl-PG				
M. laidlawii B				
Total acid hydrolysis	76	36	0.46	0.45
Mild alkaline hydrolysis	92	40	0.48	0.46
S. aureus				
Total acid hydrolysis	4	10	0.45	0.55
Mild alkaline hydrolysis	0	12	0.44	0.55
Standard amino acid (20 μmoles)				
D-Alanine	134	0	0.46	0.46
L-Alanine	0	136	0 46	0.48
D-Lysine	10	0	0.42	0.50
L-Lysine	0	8	0.43	0.51

[a] See Materials and Methods for experimental procedures.

products of mild deacylation, were chromatographed as standards. The results are shown in Table II. Chromatography of the intact aminoacylphosphatidylglycerol in ammoniacal solvent B resulted in the production of a material with the same R_F value as that of phosphatidylglycerol, while chromatography in the other, acidic, solvents produced spots corresponding to none of the standard phospholipids. The results are interpreted as showing that under the alkaline conditions of chromatography in solvent B the aminoacylphosphatidylglycerol was hydrolyzed to its constituent parts, amino acid(s) and phosphatidylglycerol, while in the acidic solvents the structural integrity of the aminoacylphosphatidylglycerol was maintained. Chromatography of the products of mild deacylation produced spots corresponding to glycerolphosphorylglycerol. The characteristic alkaline lability and the results of chromatography of the intact ninhydrin-positive phospholipid and its mild alkaline hydrolysis product corroborate the results of the analysis of the glycerol:fatty acid ester:amino acid nitrogen:phosphorus molar ratios, and indicate that the material examined is an *O*-amino acid ester of phosphatidylglycerol. No analysis was made of the fatty acids released from the phosphoglyceride by alkaline hydrolysis; it is presumed that they consist predominantly of palmitic and myristic acids, typical of the fatty acids of the other phosphoglycerides of this organism (Smith and Henrikson, 1965).

Paper chromatography in five solvent systems of the water-soluble products of hydrolysis of the aminoacylphosphatidylglycerol from *M. laidlawii* yielded several ninhydrin spots which by comparison with standards, were identified (Table III) as alanine, glutamic acid, glycine, isoleucine or leucine, lysine, and tyrosine. A quantitative preponderance of alanine was observed. All the above amino acids appeared to be covalently bonded since added free amino acids did not migrate with the lipid and liberation of free amino acids required mild alkaline hydrolysis.

Configurational Analysis of Amino Acids. Analysis of the water-soluble components of hydrolysates of aminoacylphosphatidylglycerol from *M. laidlawii* using D- and L-amino acid oxidases of equivalent activities on DL-alanine revealed activity of both enzymes in approximately a 2:1 ratio, respectively (Table IV). Products of each were identified by the chromatographic behavior of their dinitrophenylhydrazones in two solvent systems. Thus D- and L-alanine were shown to be present in approximately a 2:1 molar ratio. In *S. aureus*, used as a control and in which L-lysine is the only amino acid esterified to phosphatidylglycerol (Houtsmuller and van Deenen, 1965; Lennarz *et al.*, 1966), L-amino acid oxidase yielded a product, presumably α-keto-ϵ-aminocaproic acid. Slight activity by D-amino acid oxidase on the acid hydrolysate of *S. aureus* apparently was due to racemization during hydrolysis.

Amino Acid "Activation." The "activating" mechanism (reactions I and II) applies only to the L isomers of the amino acids. If D-alanylphosphatidylglycerol is present in *M. laidlawii* and is not formed by racemization of L-alanylphosphatidylglycerol (see below for experimental examination of this possibility) biosynthesis of D-alanylphosphatidylglycerol must occur by some other mechanism. Baddiley and Neuhaus (1960) have reported an enzymic activation of D-alanine in *Lactobacillus arabinosus* involving formation of an amino acid–AMP–enzyme complex analogous, if not similar, to "activation" of the L-amino acids. Such a mechanism apparently is involved in "activation" of D-alanine and D-glutamic acid in *Mycobacterium phlei*, and of valine in *Bacillus brevis* which synthesizes the D-amino acid containing polypeptide antibiotics gramicidin and tyrocidine (Okuda *et al.*, 1964); but is seemingly not involved in incorporation of D-glutamic acid or D-alanine and D-alanyl-D-alanine into the mucopeptide of *S. aureus* (Ito and Strominger, 1962). The latter authors report evidence of a different enzyme–substrate complex, involving amino acid–phosphate–enzyme. This mechanism,

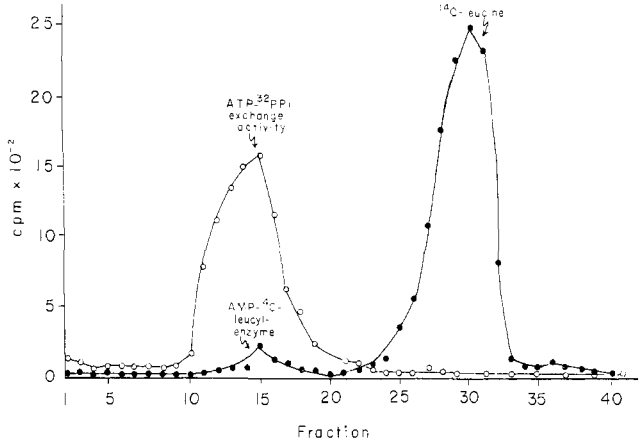

FIGURE 2: Separation of adenosine monophosphate L-[^{14}C]-Leu-tRNA complex of Sephadex G-50. Formation of the complex used the following reaction mixture: pH 7.5 potassium phosphate, 0.4 μmole; magnesium chloride, 1.0 μmole; 2-mercaptoethanol, 2.0 μmoles; pH 7.0 ATP, 0.10 μmole; L-[^{14}C]leucine, 0.01 μmole (about 125,000 counts); aminoacyltransfer ribonucleic acid synthetase enzymes, 3 mg of protein. Total volume, 1.0 ml. Incubation was for 15 min at 37° then the reaction mixture was placed on a column (1 cm diameter by 30 cm in length) of Sephadex G-50 (coarse) previously equilibrated with 0.01 M Tris-chloride (pH 7.5). Fractions (0.75 ml each) eluted with the same buffer were assayed for ^{14}C radioactivity (0.25 ml) and (0.50 ml) for activity in the ATP-[^{32}P]pyrophosphate-exchange reaction from which [^{32}P]ATP formed was quantitated. Substrate for the latter assay was L-leucine.

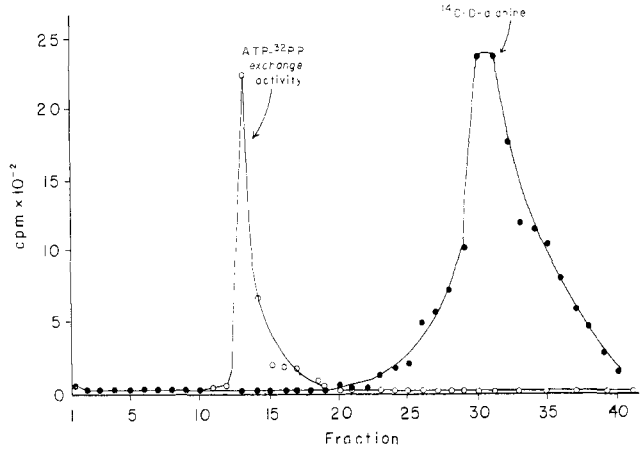

FIGURE 4: Attempted separation of adenosine monophosphate–D-[^{14}C]Ala-tRNA synthetase complex on Sephadex G-50. Incubation conditions as in Figure 2 except for replacement of L-[^{14}C]leucine by D-[^{14}C]alanine in the complex-forming reaction, and replacement of L-leucine by D-alanine in the ATP-[^{32}P]pyrophosphate exchange reaction.

or one similar, has also been reported active in synthesis of glutamine (Krishnaswamy *et al.*, 1962). Elucidation of the mechanism of "activation" of D-alanine thus became of major importance.

Gasior (1966) reported separation of the enzymes for "activation" of D- and L-alanine and glutamic acid from *M. phlei*. The ATP-[^{32}P]PP$_i$-exchange reaction was used as the assay of enzyme activity. The "activating" enzyme for

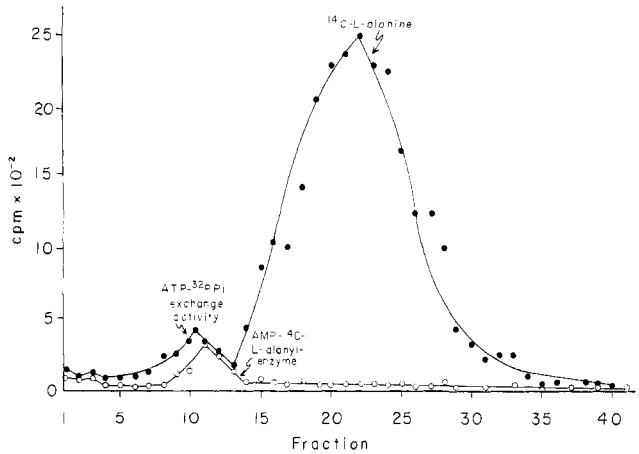

FIGURE 3: Separation of adenosine monophosphate–L-[^{14}C]Ala-tRNA synthetase complex on Sephadex G-50. Incubation conditions as in Figure 2 except for replacement of L-[^{14}C]leucine by L-[^{14}C]alanine in the complex-forming reaction; and replacement of L-leucine by L-alanine in the ATP-[^{32}P]pyrophosphate-exchange reaction.

the L isomers were precipitable at pH 4.5 while those for the D isomers were found in the supernatant from the pH 4.5 treatment and were precipitable by ammonium sulfate at 35–70% saturation. In such an experiment with *M. laidlawii*, no activity was found in the pH 4.5 precipitate and thus no fractionation was achieved, although an appreciable quantity of protein precipitated with the pH 4.5 fraction and the subsequent ammonium sulfate precipitate was of high specific activity in the ATP-[^{32}P]PP$_i$-exchange reaction. Thereafter, the "activating" enzymes were isolated by the method of Calendar and Berg (1966).

Isolation of AMP–Aminoacyl–Enzyme Complexes. Following a short incubation for formation of the enzyme–substrate complex in a system composed of L-[^{14}C]leucine, ATP, and crude aminoacyl-tRNA synthetases from *M. laidlawii*, the mixture was separated by gel filtration on Sephadex G-50. Aliquots assayed for ^{14}C revealed an initial small peak plus a subsequent larger peak (Figure 2). Assays of the fractions for enzyme activity in the ATP-[^{32}P]PP$_i$-exchange reaction using L-leucine as substrate demonstrated activity in a peak coinciding with, and surrounding, the initial [^{14}C]amino acid peak. The results are interpreted as demonstrating isolation of the AMP–aminoacyl–enzyme complex for leucine. A similar experiment, using L-alanine in both the AMP–aminoacyl–enzyme reaction and the ATP-[^{32}P]PP$_i$-exchange reaction (Figure 3), gave similar results, of the same import. Performing this experiment with D-alanine as substrate for both reactions gave (Figure 4) a distribution of the exchange reaction activity, very much narrower and sharper than that of the exchange reactions for L-leucine or L-alanine, and no AMP–D-alanine–enzyme complex was isolable in three experiments. Nonisolability of the AMP–D-alanine–enzyme complex indicates a qualitative difference of the D-alanine "activating" enzyme as compared with those for the L isomers. The presence of activity in the ATP-[^{32}P]PP$_i$-exchange reaction signifies that such an enzyme–substrate complex is formed although it is unstable under the conditions used. Presence of the exchange reaction indicates, secondly, that the mechanism of activation of D-alanine in *M. laidlawii*

TABLE V: Transfer of [^{14}C]Amino Acid from AMP–[^{14}C]-Aminoacyl–Enzyme Complex to tRNA.[a]

[^{14}C]Aminoacyl–AMP–Enzyme Complex Containing		Incorp into Acid-Precipitable [^{14}C]Aminoacyl-tRNA	
Amino Acid	cpm	cpm	%
D-Alanine[b]	0	40	<0.01
L-Alanine	2300	2240	97
L-Leucine	960	890	93

[a] The reaction mixture contained pH 7.0 sodium cacocylate buffer, 50 μmoles; 2-mercaptoethanol, 5 μmoles; potassium chloride, 5 μmoles; tRNA, 1 mg; [^{14}C]aminoacyl–AMP–enzyme complex as listed. Total volume, 0.5 ml. Incubation was for 15 min at 0°. The reaction was stopped by addition of 0.5 ml of cold 10% trichloroacetic acid. The product was filtered and washed successively (3 ml each) with 3% perchloric acid (four times), ethanol (four times), and ether (two times). [b] D-[^{14}C]Alanyl–AMP–enzyme complex was not isolable under the conditions used; fractions on Sephadex G-50 corresponding to such a complex and having activity in the ATP–[^{32}P]PP_i-exchange reaction with D-alanine as substrate were used in the reactions. Added to the transfer reaction mixture was 0.01 μmole of D-[^{14}C]alanine (about 130,000 counts).

TABLE VI: Single-Step Formation, and Isolation, of [^{14}C]-Aminoacyl Transfer Ribonucleic Acid.[a]

[^{14}C]Amino Acid Incorp into Acid-Precipitable [^{14}C]Aminoacyl-tRNA			[^{14}C]Amino Acid Precipitable as [^{14}C]-Aminoacyl-tRNA Following Ribonuclease Treatment
Amino Acid	cpm	% Incorp	cpm
D-Alanine	36	0.01	34
L-Alanine	83,800	2.1	208
L-Leucine	72,700	1.8	62

[a] The reaction mixture consisted of tRNA, 4 mg; pH 7.0 Tris-chloride, 10 μmoles; magnesium chloride, 5.5 μmoles; pH 7.0 ATP, 8.0 μmoles; phosphoenolpyruvate, 5.0 μmoles; pyruvate kinase, 30 μg; [^{14}C]amino acid, 7 mci/mmole, 0.03 μmole (about 400,000 counts); aminoacyl-tRNA synthetases, 2–4 mg. Total volume, 1 ml. After 10-min incubation at 37°, the reaction was stopped by addition of 1 ml of cold 10% trichloroacetic acid. The product was filtered and washed successively (3 ml each) with 3% perchloric acid (four times), ethanol (four times) and ether (two times). When present, 100 g of RNase was added prior to incubation of the reaction mixture.

involves formation of the AMP–amino acid–enzyme complex, rather than the alternative amino acid–phosphate–enzyme complex.

Transfer of the "Activated" Amino Acids to tRNA. Samples corresponding to the AMP–amino acid–enzyme complex on Sephadex for each of the three amino acids, were pooled and cooled to 0°, and tRNA was added. After incubation for 15 min at 0°, the acid-precipitable components of the mixture were washed, dried, and counted for ^{14}C radioactivity. As seen in Table V, the transfer of [^{14}C]amino acid to tRNA was 90% or more effective in the cases of L-leucine and L-alanine, indicative of the high affinity of the "activated" L-amino acids for tRNA, but was ineffective with D-alanine, even on addition of D-[^{14}C]alanine to the incubation mixture, suggesting that the tRNA mixture contained no fraction capable of accepting D-alanine.

Ribonuclease Sensitivity of Acid-Precipitable Transfer Ribonucleic Acid. In Table VI is presented the same experiment as in Table V, except that isolation of the AMP–aminoacyl–enzyme complex was omitted and both steps of amino acid "activation," *i.e.*, reactions I and II, were combined. Incorporation of L-[^{14}C]alanine and L-leucine into the acid-precipitable fraction approximated 2% of the input radioactivity, and was destroyed by ribonuclease. As previously, no D-[^{14}C]alanine became acid precipitable.

Isolation of Product of Ribonuclease Action on Aminoacyl-tRNA. Ribonuclease, with substrate specificity for the 5′-phosphate of a purine adjacent to a pyridine in RNA, will hydrolyze the terminal cydityl-(3′→5′)-adenylamino acid common to all aminoacyl-tRNA's to yield aminoacyl-2′(3′)-adenosine (Zachau *et al.*, 1958; Preiss *et al.*, 1959). Use was made of this property to demonstrate esterification of L-leucine and L-alanine but not of D-alanine to tRNA (Table VII). Separation of products was done by paper chromatography in three solvent systems and by paper electrophoresis. Comparison was made with synthesized appropriate standards.

Formation of [^{14}C]Aminoacylphosphatidylglycerol. Synthesis of [^{14}C]aminoacylphosphatidylglycerol and determination of requirements for the reaction used a modification of the system of Lennarz *et al.* (1966). Generally incorporation of [^{14}C]amino acid was rather low even under optimal conditions, amounting to about 1%. In Table VIII, expt I, the source of L-[^{14}C]alanine was preformed L-[^{14}C]-alanyl-tRNA. Source of aminoacylphosphatidylglycerol synthetase was washed *M. laidlawii* membranes. The [^{14}C]-alanylphosphatidylglycerol thus synthesized was extracted from the reaction mixture with organic solvents which were dried, and the lipid was separated by thin-layer chromatography from any free [^{14}C]alanine and [^{14}C]alanyl-tRNA, which may have been carried over during the extraction and which moved only slightly in the solvent used. Thus the carry-over contamination of [^{14}C]aminoacylphosphatidylglycerol on thin-layer chromatograms by [^{14}C]amino acid or [^{14}C]aminoacyl-tRNA was less than 0.05%.

The results of the experiments demonstrated a requirement of the system for L-[^{14}C]alanyl-tRNA and for exogenous phosphatidylglycerol. Other lipids could not substitute for phosphatidylglycerol in the reaction and may have been

TABLE VII: Paper Chromatography and Electrophoresis of Aminoacyladenosine Monophosphates, Aminoacyl Transfer Ribonucleic Acids, and Related Compounds.

Substance	R_F in Solvent			Electrophoretic Migration at pH 3.1, 14 V/cm, 3 hr
	VIII	I	II	
Adenine	0.48	0.66	0.46	Cathodic, 6 cm
Adenosine	0.48	0.64	0.27	Cathodic, 7–7.5 cm
AMP	0.18	0.24	0.03	Anodic, 1 cm
D-Alanine	0.39	0.51	0.33	Cathodic, 1.5 cm
D-Alanyl-2′(3′)-AMP	0.10	0.24	0.03	Cathodic, 0.5 cm
D-Alanyl-2′(3′)-adenosine	0.06	0.38	0.18	Cathodic, 3.5 cm
"Activated" D-alanine[a]	0	0	0	Cathodic, 3.5 cm
RNase-treated "activated" D-alanine	0	0	0	Cathodic, 3.5 cm
L-Alanine	0.39	0.48	0.34	Cathodic, 1 cm
L-Alanyl-2′(3′)-AMP	0.10	0.22	0.03	Cathodic, 0.5 cm
L-Alanyl-2′(3′)-adenosine	0.15	0.40	0.17	Cathodic, 3–4 cm
L-Alanyl-tRNA	0	0	0	Cathodic, 3–3.5 cm
RNase-treated L-alanyl-tRNA	0	0[b]	0[b]	
	0.15[b]	0.41[b]	0.18[b]	
	0.38[c]	0.49[c]	0.35[c]	Cathodic, 0.5 cm[b,c]
L-Leucine	0.70	0.77	0.67	Cathodic, 1–1.5 cm
L-Leucyl-2′(3′)-AMP	0.62	0.32	0.03	Cathodic, 0.5 cm
L-Leucyl-2′(3′)-adenosine	0.47	0.51	0.27	Cathodic, 3.5 cm
L-Leucyl-tRNA	0	0	0	Cathodic, 3–3.5 cm
RNase-treated L-leucyl-tRNA	0[b]	0[b]	0[b]	
	0.48[b]	0.51[b]	0.28[b]	
	0.70[c]	0.78[c]	0.67[c]	Cathodic, 0.5 cm[b] 1.5 cm[c]

[a] "Activated" D-alanine treated with ATP, aminoacyl-tRNA synthetases, and tRNA. [b] Spot located under ultraviolet light. [c] Spot located with ninhydrin and/or by radioisotopic scanning of [^{14}C]amino acid.

inhibitory. Incorporation of counts from L-[^{14}C]alanyl-tRNA was highly sensitive to addition of ribonuclease.

In Table VIII (expt II) whole cell lysate was substituted for washed cell membrane as source of aminoacylphosphatidylglycerol synthetase, and L-[^{14}C]alanine and 1 mg of tRNA were substituted for preformed L-[^{14}C]alanyl-tRNA. Omission of tRNA or addition of ribonuclease reduced incorporation of L-[^{14}C]alanine counts into the lipid.

Table IX contains the data for the incorporation of D-alanine in an experiment like that immediately preceding; unlike its predecessor, this experiment was insensitive to omission or hydrolytic destruction of tRNA. Unsubstitutable requirement for exogenous phosphatidylglycerol again was observed.

The kinetics of formation of L- and D-alanylphosphatidylglycerol were examined on timed aliquots of the reactions described under expt II, Table VIII (L-isomer) and Table IX (D isomer). The results in Figure 5 show that at 75 min the biosynthesis of the L isomer is still increasing, but that synthesis of the D isomer reached a peak at 30 min and declined to its zero time value by 75 min.

Stereospecificity of Alanylphosphatidylglycerol Synthesis. This experiment was designed to test the possibility of racemization of the isomers of alanine in alanylphosphatidylglycerol during or after synthesis from phosphatidylglycerol and the appropriately "activated" amino acid. The experiment consisted simply of quantitation of the radioactive pyruvic acid produced by the stereospecific amino acid oxidases on the [^{14}C]amino acids released by pH 9.0 hydrolysis of *in vitro* enzymatically synthesized isomeric [^{14}C]alanylphosphatidylglycerols. The results, in Table X, reveal essentially complete stereospecificity.

Isomer Competition for Synthesis of Alanylphosphatidylglycerol. Addition of unlabeled D-alanine to a system for synthesis of L-[^{14}C]alanylphosphatidylglycerol and *vice versa* revealed (Table XI) lack of competition of an "unactivated" isomer for its opposite "activated" isomer. Slight indication, in expt II, of competition for incorporation of L-[^{14}C]alanine by D-alanine suggests a possible depletion of substrate phosphatidylglycerol.

Discussion

M. laidlawii, when incubated under acidic conditions, contains *O*-amino acid esters of phosphatidylglycerol. A remarkably large variety of amino acids is found in such esters of *M. laidlawii*. With the exception of the report by Ikawa (1963) of the presence of D-alanine in the lipids of *Leuconostoc mesenteroides*, the presence of D-alanine among these esters of *M. laidlawii* is unique. Similar presence of D-alanine in the peptidoglycan of cell walls of gram-positive bacteria suggests the entertaining possibility that the con-

TABLE VIII: Requirements for Formation of L-Alanylphosphatidylglycerol by *M. laidlawii*.

Expt	Conditions	L-Alanylphosphatidylglycerol (cpm)
I[a]	Complete system, 0 min	110
	Complete system, 30 min	1360
	Complete system minus [^{14}C]L-alanyl-tRNA plus [^{14}C]alanine	50
	Complete system minus phosphatidylglycerol	150
	Complete system minus phosphatidylglycerol, plus phosphatidic acid or diglyceride	75
	Complete system plus ribonuclease	125
	Complete system boiled enzyme	70
II[b]	Complete system, 30 min	1240
	Complete system minus tRNA	95
	Complete system plus ribonuclease	210
	Complete system boiled enzyme	105

[a] In expt I, the complete system included pH 7.0 Tris-chloride, 100 μmoles; magnesium chloride, 5.0 μmoles; (pH 7.0) ATP, 8.0 μmoles; phosphoenolpyruvate, 5.0 μmoles; pyruvate kinase, 30 μg; L-[^{14}C]alanyl-tRNA (about 100,000 counts), 500 μg; sonicated emulsion of phosphatidylglycerol or other lipid, 2 mg; washed membrane preparation containing aminoacylphosphatidylglycerol synthetase; and 15 mg of protein. Total volume, 2.0 ml. Incubation was for 30 min at 37° unless otherwise noted. The reaction was stopped by addition of 2.0 ml of 10% (w/v) trichloroacetic acid. The lipids were extracted with chloroform–methanol, separated by thin-layer chromatography, and the areas of the chromatograms corresponding to aminoacylphosphatidylglycerol were scraped into vials and counted for ^{14}C activity. When present, 100 μg of RNase was added. [b] Experiment II was modified from expt I as follows: L-[^{14}C]alanyl-tRNA and washed enzymes were omitted; substituted were 1 mg of tRNA, 0.03 μmole of L-[^{14}C]alanine (about 400,000 counts), and whole cell lysate equivalent to 15 mg of protein. Conditions of incubation and subsequent procedures and analysis were as in expt I.

figuration of the glutamic acid moiety present among the amino acid assortment from aminoacylphosphatidylglycerols of both the wall-less *M. laidlawii* and *M. hominis* strain 07 (Smith and Koostra, 1967) also be D, like that of the glutamic acid (present as glutamine) of the peptidoglycan. However, the configuration of glutamic acid is not readily determinable with amino acid oxidases because of their low activity with glutamate as a substrate (Boulanger and Osteux, 1963). A D-amino acid oxidase from octopus hepatopancreas which is reported to be active on D-glutamate (Blashko and Hawkins, 1952) was not available. Alternative analytical methods, including bacterial D-glutamate transaminases (Thorne and Molnar, 1955; Thorne *et al.*, 1955), may be useful in such determinations. The presence of D-alanine and, if present,

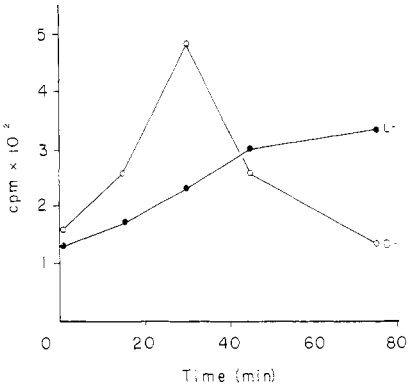

FIGURE 5: Kinetics of incorporation of [^{14}C]alanine isomers into isomeric alanylphosphatidylglycerols of *M. laidlawii*. Reaction mixture consisted of: pH 7.0 Tris-chloride, 10.0 μmoles; magnesium chloride, 5.0 μmoles; pH 7.0 ATP, 8.0 μmoles; phosphoenolpyruvate, 5.0 μmoles; pyruvate kinase, 30 μg; D- or L-[^{14}C]alanine, 0.03 μmole (about 400,000 counts); tRNA, 1 mg; sonicated emulsion of phosphatidylglycerol, 2 mg; and whole cell lysate equivalent to 15 mg of protein. The reaction was carried out in a total volume of 2.0 ml and at 37°. At the time intervals noted, a 0.2-ml sample was removed and the reaction was stopped by addition of an equal volume of 10% (w/v) trichloroacetic acid. The lipids were extracted with chloroform–methanol, separated by thin-layer chromatography, and areas of the chromatograms corresponding to aminoacylphosphatidylglycerol were scraped into vials and counted for ^{14}C activity.

D-glutamic acid, of aminoacylphosphatidylglycerols from mycoplasmas stimulates speculation regarding a possible relationship of mycoplasmas to bacteria and L forms. To date, no analysis has been made of the comparative aminoacylphosphatidylglycerol synthetases activity of a bacterium,

TABLE IX: Requirements for Formation of D-Alanylphosphatidylglycerol by *M. laidlawii*.

Conditions	D-Alanylphosphatidylglycerol (cpm)
Complete system,[a] 0 min	145
Complete system	2860
Complete system minus tRNA	2795
Complete system plus RNase	2890
Complete system minus phosphatidylglycerol	285
Complete system minus phosphatidylglycerol; plus phosphatidic acid or diglyceride	125
Complete system boiled enzymes	110

[a] The complete system included pH 7.0 Tris-chloride, 100 μmoles; magnesium chloride, 5.0 μmoles; pH 7.0 ATP, 8.0 μmoles; phosphoenolpyruvate, 5.0 μmoles; pyruvate kinase, 30 μg; D-[^{14}C]alanine, 0.03 μmole (about 400,000 counts); tRNA, 1 mg; sonicated emulsion of phosphatidylglycerol or other lipid, 2 mg; and whole cell lysate equivalent to 15 mg of protein. Total volume, 2.0 ml. Incubation was for 30 min at 37° unless otherwise noted. Subsequent procedures and analysis were identical with those in expt I of Table VIII.

TABLE X: Analysis of Components of *in Vitro* Enzymatically Synthesized Isomeric [^{14}C]Alanylphosphatidylglycerols.

[^{14}C]Alanylphosphatidylglycerols[a]	Component Analysis, Molar Ratio				[^{14}C]Pyruvate Produced by Action of	
	Glycerol	Fatty Acid Esters	Amino Acid Nitrogen	Phosphorus	D-Amino Acid Oxidase (cpm)	L-Amino Acid Oxidase (cpm)
D isomer	1.98	2.05	1.02	1.00	770	20
L isomer	2.03	1.90	0.90	1.00	35	680

[a] L-[^{14}C]Alanylphosphatidylglycerol was synthesized and isolated using the conditions described in expt II, Table VIII, except for addition of 1 μmole of nonisotopic L-alanine; D-[^{14}C]alanylphosphatidylglycerol was synthesized and isolated using the conditions of Table VI except for addition of 1 μmole of nonisotopic D-alanine. Specific activity of each was about 1000 cpm/μmole.

TABLE XI: [^{14}C]Alanine Isomer Competition for Synthesis of [^{14}C]Alanylphosphatidylglycerol.

Experiment	Conditions	[^{14}C]Alanylphosphatidylglycerol (cpm)
I. Formation of L-[^{14}C]alanylphosphatidylglycerol[a]	Complete system, no D-alanine	1480
	Complete system, 0.01 mM D-alanine	1440
	Complete system, 0.10 mM D-alanine	1540
	Complete system, 1.00 mM D-alanine	1480
	Complete system, 0.10 mM L-alanine	255
II. Formation of L-[^{14}C]alanylphosphatidylglycerol[b]	Complete system, no D-alanine	1305
	Complete system, 0.01 mM D-alanine	1270
	Complete system 0.10 mM D-alanine	1190
	Complete system 1.00 mM D-alanine	1100
	Complete system 0.10 mM L-alanine	205
III. Formation of D-[^{14}C]alanylphosphatidylglycerol[c]	Complete system, no L-alanine	3140
	Complete system, 0.01 mM L-alanine	3100
	Complete system, 0.10 mM L-alanine	2780
	Complete system, 1.00 mM L-alanine	2510
	Complete system, 0.10 mM D-alanine	420
	Complete system, 1 μmole L-alanyl-tRNA	2675

[a] The experiment used isolated L-[^{14}C]alanyl-tRNA and the particular synthetase enzyme, as described in expt I of Table VIII. [b] The experiment used the complete activating enzyme system plus ATP and tRNA, and the particulate synthetase enzyme, as described in expt II of Table VIII. [c] The experiment used the complete activating enzyme system plus ATP and tRNA and the particulate synthetase enzyme, as described in Table IX. The quantities of L-[^{14}C]alanyl-tRNA and [^{14}C]alanine used were the same as noted in Table VIII.

e.g., *S. aureus* or *Bacillus megaterium*, and its L form. Such studies may aid to elucidate the taxonomy of the mycoplasmas.

The origin of D-alanine in *M. laidlawii* is unknown. Alanine racemase has been reported to be absent from *M. laidlawii* (Plapp, 1963), as have L-glutamic–oxalacetic and L-glutamic–pyruvic transaminase (Razin and Cohen, 1963). Other possibilities exist, however, including that of racemization of glutamate by a glutamic acid racemase and/or D-glutamic acid transaminase (Thorne *et al.*, 1955; Thorne and Molnar, 1955). Further investigations are necessary into the metabolism of D-amino acid(s) of mycoplasmas.

Biosynthesis of aminoacylphosphatidylglycerols of mycoplasmas, like those of the several other organisms characterized by Lennarz and coworkers, requires several factors. These include "activation" of the amino acid *via* aminoacyl-tRNA in the case of the L isomer of alanine examined in these experiments. Although the emphasis of this report is upon biosynthesis of alanylphosphatidylglycerol, it would

seem a safe assumption that the mechanism of biosynthesis of the esters of the several other amino acids with phosphatidylglycerol also proceeds through their "activation" as esters of tRNA. Such has been shown to be the case by Lennarz's group for L-lysine, L-alanine, and, apparently, for L-histidine and L-arginine. The "activation" of D-alanine represents a special case, unlike that of the L isomers. The presence of activity in the ATP-[^{32}P]PP$_i$-exchange reaction by D-alanine with the activating enzymes indicates that in *M. laidlawii* the activation of D-alanine takes place *via* an AMP–aminoacyl–enzyme complex. The narrow distribution of the enzyme activity on Sephadex marks a difference of this enzyme compared with the enzymes for activation of L-alanine and L-leucine. A second indication of a difference of these enzymes is the nonisolability of the AMP–D-alanine–enzyme complex, while the complexes incorporating L-alanine and L-leucine are sufficiently stable to permit their isolation. The absence of a tRNA species with acceptor activity was evidenced by lack of requirement for tRNA and the lack of inhibition by ribonuclease in the D-alanine "activating" system as determined in the aminoacylphosphatidylglycerol synthetase reaction. Additional differences should be expected to be revealed on further investigation. Failures to fractionate the D and L isomer activating enzymes of *M. laidlawii*, as has been reported by Gasior (1966) to be successful for those enzymes of *Mycobacterium phlei*, probably reflect species differences.

The usual additional requirement of exogenous phosphatidylglycerol for aminoacylphosphatidylglycerol synthesis was necessary for incorporation of either alanine isomer in *M. laidlawii*. This requirement could not be replaced by phosphatidic acid or by diglyceride.

Stereospecificity of the synthesis and stability of the stereoconfiguration of isomeric alanylphosphatidylglycerols of *M. laidlawii* are obvious from the competition and amino acid oxidase analytical experiments.

The possible function of aminoacylphosphatidylglycerols in *Mycoplasma* is unknown. Since the species examined are incapable of growth at pH 5, the pH used to induce formation of the aminoacylphosphatidylglycerols, and since lipid extracts of cultures grown under the usual slightly alkaline (pH 8.0) culture conditions contain no ninhydrin-reactive phospholipids (Smith and Henrikson, 1965; Shaw *et al.*, 1968), it would appear that production of the amino acid esters of phosphatidylglycerol is not a physiological response, or rather is not a response to physiological conditions. The organisms, unlike, *e.g.*, *S. aureus*, do not produce sufficient acidic metabolic end products to lower the pH of the medium below neutrality and indeed could not grow and divide at a pH much below neutrality.

An additional function for the amino acid esters of phosphatidylglycerol is as carriers of amino acids, in membrane transport. Mitigating against this hypothesis, however, is the slow rate of synthesis of the esters. A similar hypothetical function is for synthesis of membrane protein or other amino acid containing components, particularly of the cell membrane. The use of the energy of the aminoacyl-tRNA bond, which is significantly higher than that of the ester bond of aminoacylphosphatidylglycerol, represents an energy loss not compatible with the usual cell economy.

A final and possibly the most attractive hypothesis for function of aminoacylphosphatidylglycerols is that of preservation of membrane charge or architecture under adverse conditions of pH. Formation of the amino acid esters of phosphatidylglycerol according to this hypothesis would represent a buffering function. The formation of the esters may represent an attempt at neutralization of charge in an attempt to maintain membrane integrity. The maintenance of the original bacterial shape by protoplasts of *Bacillus megatherium* and *B. subtilis* when harvested from an acidic environment and containing lysyl- and glucosaminylphosphatidylglycerols may directly relate to this possible function (J. A. F. Op den Kamp, personal communication).

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Studies on the Effect of Cortisone on Rat Liver Transfer Ribonucleic Acid*

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ABSTRACT: The effect of cortisone on the population of rat liver transfer ribonucleic acid and aminoacyl transfer ribonucleic acid synthetases was studied. The 20–30% increase in labeling of transfer ribonucleic acid, following cortisone administration, seemed to represent a general increase in most or all types of rat liver transfer ribonucleic acid, rather than a preferential increase in the synthesis of a few transfer ribonucleic acids. No gross differences in relative abundance of amino acid specific transfer ribonucleic acids were apparent in assays of amino acid acceptance capacities of transfer ribonucleic acid obtained from control and cortisone-injected rats. Cortisone did not produce detectable changes in the activities of liver aminoacyl transfer ribonucleic acid synthetases, nor did the hormone form a stable complex with liver transfer ribonucleic acid *in vitro* or *in vivo*. In addition, no changes were found in the isoaccepting species of leucine transfer ribonucleic acid or tyrosine transfer ribonucleic acid, after cortisone administration.

Among the early and most pronounced effects of glucocorticoids is the induction of several hepatic enzyme activities (Knox, 1951) which is due to hormonal stimulation in the rate of their biogenesis (Kenney, 1962; Schimke *et al.*, 1965a,b) leading to elevated levels of these enzyme proteins (Feigelson, and Greengard, 1962; Kenney, 1962) in the hepatic cytosol. Although previous reports have attempted to elucidate the site of action of glucocorticoid hormones at both the translational (Tomkins *et al.*, 1965; Kenney and Albritton, 1965) and the transcriptional (Feigelson and Feigelson, 1965; Koide, 1969) level, the primary target of these hormones remains unknown. Results from several laboratories in the last several years have suggested strongly that increased enzyme synthesis in response to corticosteroids in mammalian liver is secondary to the hormonal stimulation of RNA synthesis (Greengard and Acs, 1962; Kenney and Kull, 1963; Yu and Feigelson, 1969a; Feigelson and Hanoune, 1969). With ^{32}P as a precursor, a two–threefold stimulation in the *de novo* synthesis of all RNA species, including tRNA, has been reported to occur during the first 3–4 hr after injection of corticosteroids (Feigelson *et al.*, 1962; Wicks *et al.*, 1965). This stimulation is also apparent, though less pronounced,

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