Membrane Lipids of *Mycoplasma gallisepticum*: A Disaturated Phosphatidylcholine and a Phosphatidylglycerol with an Unusual Positional Distribution of Fatty Acids†

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ABSTRACT: The lipid content of *Mycoplasma gallisepticum* depended on the growth phase of the culture, being high in cells harvested at the early logarithmic phase of growth and low in stationary phase cells. The phospholipid fraction is comprised of three major compounds, tentatively identified as sphingomyelin (SPM), phosphatidylcholine (PC), and phosphatidylglycerol (PG). The relative amounts of the three phospholipids depended on the concentration of horse serum in the growth medium. When grown with increasing serum concentrations, the relative content of PG decreased while that of PC and SPM increased. The SPM of *M. gallisepticum* appears to be incorporated unchanged from the growth medium. The PC of *M. gallisepticum* is a disaturated PC, differing from the 1-saturated, 2-unsaturated PC found in the growth medium. The disaturated PC is synthesized by the insertion of a saturated fatty acid at position 2 of lysophosphatidylcholine (lyso-PC), derived from exogenous PC of the growth medium, by what appears to be a deacylation–acylation enzymatic sequence. PG is the only phospholipid de novo synthesized by the organisms. It has an unusual positional distribution of fatty acids. Fatty acids with lower melting points are located primarily at position 1 and fatty acids with higher melting points at position 2 of the *sn*-glycerol 3-phosphate.

Being the only prokaryote that requires an external sterol source for growth (Edward & Fitzgerald, 1951; Razin, 1978), *Mycoplasma* is a unique model system for studying cholesterol transbilayer distribution and movement in biological membranes. In contrast to other membranous systems used for such studies, mycoplasmas offer the advantage of being cells capable of autonomous growth (Razin & Rottem, 1978). The studies so far presented were performed with cells of two *Mycoplasma* species: *M. gallisepticum* and *M. capricolum* (Bittman & Rottem, 1976; Rottem et al., 1978; Clejan et al., 1978). It is of considerable interest to extend these studies to other lipid constituents of the membrane, attempting to establish the influence of membrane polar lipids on cholesterol uptake, distribution, and movement. Very little is known so far on membrane lipids of these two organisms. In the present article, we describe the unusual characteristics of *M. gallisepticum* membrane lipids.

Materials and Methods

Growth of the Organism and Isolation of Membranes. *Mycoplasma gallisepticum* (strain A5969) was grown in an Edward medium (Razin & Rottem, 1976) containing 4% horse serum. To label membrane lipids, 0.002 μCi of [1-^{14}C]oleate (56 Ci/mol) or [1-^{14}C]palmitate (59 Ci/mol), both products of the Radiochemical Centre, Amersham, was added per mL of medium. For positional distribution studies, *M. gallisepticum* or *Acholeplasma laidlawii* cells were grown in a modified Edward medium in which 0.5% bovine serum albumin (Sigma, St. Louis, MO) treated with charcoal to remove fatty acids (Chen, 1967), 15 μg/mL cholesterol, and fatty acids replaced the horse serum. In order to determine the incorporation of radioactive choline derivatives, *M. gallisepticum* cells were grown in Edward medium containing 0.05 μCi/mL [^{3}H]palmitic acid (The Radiochemical Centre, Amersham, England) and approximately 1000 dpm/mL of either [*N-methyl*-^{14}C]choline (10 Ci/mol, New England Nuclear, Boston, MA), phosphatidyl[*N-methyl*-^{14}C]choline (50 Ci/mol, New England Nuclear, Boston, MA), ^{14}C-labeled phosphorylcholine, ^{14}C-labeled glycerophosphorylcholine, or ^{14}C-labeled lysophosphatidylcholine. The radioactive phosphorylcholine, glycerophosphorylcholine, and lysophosphatidylcholine were prepared from phosphatidyl[*N-methyl*-^{14}C]choline, by phospholipase C treatment (Rottem & Greenberg, 1975), alkaline hydrolysis in 0.25 M ethanolic KOH for 1 h at 70 °C followed by the separation of the water-soluble phosphate esters (Dittmer & Wells, 1969) and by phospholipase A_2 treatment, respectively. The cultures were incubated at 37 °C for 14–26 h and growth was followed by measuring the absorbance of the culture at 640 nm. Most experiments were performed with cultures at the mid-exponential phase of growth (A_{640} = 0.15–0.20) containing 70–90 μg of cell protein per mL. The cells were harvested by centrifugation at 12000*g* for 20 min, washed once, and resuspended in a cold 0.25 M NaCl solution. Membranes were prepared from cells by ultrasonic treatment of washed cell suspensions containing 0.1 mg of cell protein per mL of 6 mM NaCl solution, for 3 min at 0 °C in a W-350 Heat Systems sonicator operated at 50% duty cycles at 200 W. The membranes were collected by centrifugation at 34000*g* for 30 min, washed once, and resuspended in 0.25 M NaCl solution.

Lipid Analyses. Lipids were extracted from intact cells, isolated membranes, or freeze-dried growth medium preparations by the method of Bligh & Dyer (1969). The solvent was evaporated under a stream of nitrogen and traces of water were removed by freeze-drying of the preparation for 2 h. The dried lipids were weighed and redissolved in 0.5–1.0 mL of chloroform. Neutral lipids were separated from polar lipids by silicic acid chromatography (Rottem & Razin, 1973). Neutral lipids were chromatographed on silica gel G plates developed at room temperature with benzene–diethyl ether–ethanol–acetic acid (50:40:2:0.2 by vol) followed by hexane–diethyl ether (94:6 by vol). Polar lipids were separated on silica gel HR (Kiesel-gel 60 HR, Merck, Darmstadt, West Germany) plates developed at 4 °C with chloroform–methanol–water (65:25:4 by vol). For thin-layer chromatography of total membrane lipids on silica gel HR plates, the plates were first developed at room temperature with petroleum ether (bp 40–60 °C)–acetone (3:1 by vol) and then at 4 °C with chloroform–methanol–water (65:25:4 by vol). Lipid spots were

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Table I: Properties of the Major Phospholipids of *M. gallisepticum*

compd	R_f[a]	lipid phosphorus (nmol/μg of cell protein)	% of total	radioact. (dpm/nmol of lipid phosphorus), cells grown with[b]		fatty acid content (mol/mol of lipid phosphorus)	glycerol content (mol/mol of lipid phosphorus)
				[1-^{14}C]oleate	[1-^{14}C]palmitate		
A	0.22	16.5	19.0	10	10	ND[c]	0
B	0.29	25.5	30.0	20	360	2.0	0.9
C	0.44	40.0	46.5	750	580	1.9	1.9

[a] The developing solvent system was chloroform–methanol–water (65:25:4 by volume). [b] Cells were grown to an absorbance of A_{640} = 0.20. [c] Not determined.

detected by iodine vapor, phospholipid spots by the molybdate spray reagent (Dittmer & Lester, 1964), α-glycol-containing lipids by the periodate/Schiff reagent (Shaw, 1968), glycolipids by the anthrone reagent (Van Gent et al., 1973), and choline-containing lipids by the Dragendorf reagent (Wagner et al., 1961). For determining radioactivity in the lipid spots, they were scraped off the plate into scintillation vials containing 5 mL of toluene scintillation liquor. The radioactivity was measured in a Model 2650 Packard Tri-Carb scintillation spectrometer and expressed as decompositions/min (dpm). Sensitivity of the phospholipids to alkali was determined by incubating 0.1–0.5 mg of lipid in 1 mL of 0.5 N KOH in methanol for 30 min at 37 °C. Methyl esters of the fatty acids were prepared by heating the lipid samples in 14% boron trifluoride in methanol (Sigma, St. Louis, MO) at 72 °C for 15 min. The resulting methyl esters were extracted with *n*-hexane and subjected to gas–liquid chromatography in a Packard Model 840 instrument equipped with a polar column (200 × 0.3 cm, 15% diethylene glycol adipate on Chromosorb W). Fatty acids were identified by their retention time relative to that of standard methyl ester mixtures (Supelco Inc., Belofonte, PA). For quantitative determination of the fatty acids, 20 nmol of pentadecanoic acid was added to the lipid samples prior to the transmethylation. By assuming an almost equal peak area/mol ratio for the major fatty acid methyl esters present in *M. gallisepticum* preparation, the concentration of each methyl fatty acid (in nmol) was calculated by dividing its peak area by that of methyl pentadecanoate and multiplying the result by 20.

Phospholipase A_2 Treatment. PG[1] preparations (0.4–0.5 μmol) from *M. gallisepticum* or *A. laidlawii* cells or PC preparations (0.3–0.4 μmol) from *M. gallisepticum* or from the growth medium were scraped off thin-layer chromatography plates into test tubes containing in a total volume of 1 mL: 50 mM Tris-HCl, pH 7.4, 25 mM $CaCl_2$, and 1.25 mg of bovine serum albumin. Aliquots (0.1 mL) of a solution (1 mg/mL in deionized water) of purified phospholipase A_2 from pig pancreas, kindly provided by Dr. J. A. F. Op den Kamp, were then added and the test tubes were incubated at 37 °C for 2 h. The reaction was stopped by adding 1 mL of 0.1 M ethylenediaminetetraacetic acid, and lipids were extracted from the reaction mixture as described by Op den Kamp et al. (1972). The lyso compounds, free fatty acids, and residual undigested phospholipids were separated on silica gel HR plates and their fatty acid composition was determined as described above.

Analytical Methods. Protein was determined according to Lowry et al. (1951). Total phosphorus in the lipid fraction was determined by the method of Ames (1966) after digestion of the sample with an ethanolic solution of $Mg(NO_3)_2$. In order to determine phosphorus in phospholipid spots resolved by thin-layer chromatography, the spots were scraped off the plate into test tubes and digested with 0.5 mL of the ethanolic $Mg(NO_3)_2$ solution in the presence of the silica gel. The total cholesterol concentration in the extracted lipids was measured colorimetrically (Rudel & Morris, 1973). Unesterified cholesterol was separated from esterified cholesterol and their relative concentrations were determined as described previously (Bittman & Rottem, 1976). Glycerol was determined according to Bublitz & Kennedy (1954). Nuclear magnetic resonance spectra were obtained by using a Bruker W-60 NMR spectrometer.

Results

The lipid content of *Mycoplasma gallisepticum* strain A5969 depended on the growth phase of the culture, being high (160 μg/mg of cell protein) in the early logarithmic phase of growth and low (90 μg/mg of cell protein) in the stationary phase cells. Essentially all of the lipids were found to be located in the cell membrane. About 40% of the lipids were eluted from a silicic acid column by chloroform, comprising of the neutral lipids. When this fraction was chromatographed on silica gel G plates, it was found to contain unesterified cholesterol (28–50 μg/mg of cell protein), esterified cholesterol (3–5 μg/mg of cell protein), and glycerides (7–10 μg/mg of cell protein). The amount of glycerides was obtained by adding the amount of the polar lipids recovered to that of total cholesterol and then subtracting this value from the weight of the starting material. This assumes 100% recovery of polar lipid and cholesterol from the thin-layer chromatography plates. When the cells were grown with radioactive palmitic or oleic acids, 10–15% of the label was found in the neutral lipid fraction, mainly in diglycerides (6–8% of the total) and free fatty acids (3–5% of the total). Since the addition of the radioactive fatty acid to the growth medium failed to label the cholesterol esters, it seems that the esters are not synthesized by the organisms but are incorporated from the growth medium.

About 60% of the total lipids were eluted from the silicic acid column with chloroform/methanol (1:1 by vol) and hence constitute polar lipids. Thin-layer chromatography of the polar lipid fraction on silica gel HR plates revealed that the polar lipid fraction is constituted of three major spots designated A, B, and C (Table I). All spots reacted with the molybdate reagent used to detect phosphorus-containing lipids, but none of them reacted with the anthrone reagent which detects glycolipids or with ninhydrin reagent used to detect amino lipids. Likewise spots A and B reacted positively with the Dragendorf reagent but had a negative periodate/Schiff reaction, whereas spot C gave a positive periodate/Schiff reaction but a negative reaction with the Dragendorf reagent. Compound A resisted hydrolysis by alkali and phospholipase A_2, while compounds B and C were sensitive to both treatments. Additional biochemical properties of the three

[1] Abbreviations used: SPM, sphingomyelin; PC, phosphatidylcholine; PG, phosphatidylglycerol; lyso-PG, lysophosphatidylglycerol; lyso-PC, lysophosphatidylcholine.

Table II: Fatty Acid Composition of Phosphatidylcholine and Lysophosphatidylcholine Preparations from *M. gallisepticum* Cells Grown with Various Fatty Acids

	PC prep from cells grown with[b]				lyso-PC prep from[c] cells grown with			
fatty acid[a]	14:0 + 16:0	16:0 + 18:1	16:0 + 18:2	growth medium	14:0 + 16:0	16:0 + 18:1	16:0 + 18:2	growth medium
14:0	37.4	2.0	0.5	1.0	<0.5	<0.5	<0.5	<0.5
16:0	24.9	56.9	56.8	15.8	34.0	35.0	37.0	33.0
18:0	37.7	36.0	32.7	31.9	65.0	64.0	62.0	65.0
18:1	<0.5	2.5	0.6	11.4	<0.5	<0.5	<0.5	<0.5
18:2	<0.5	<0.5	<0.5	41.5	<0.5	<0.5	<0.5	<0.5

[a] The first number indicates chain length and the second, the number of double bonds. [b] Cells were grown in Edward Medium containing 0.5% bovine serum albumin, 15 μg/mL cholesterol, and 40 μM of each of the fatty acids. [c] Lysophosphatidylcholine preparations were obtained by treating phosphatidylcholine with phospholipase A_2.

compounds are summarized in Table I. Accordingly, the compounds were tentatively identified as SPM (A), PC (B), and PG (C). This identification was also supported by the comigration of the three components with commercially available standards in chloroform–methanol–acetic acid–water (65:25:1:4 by vol) and chloroform–methanol–ammonia–water (60:35:2.5:2.5 by vol) solvent systems, and in a two-dimensional system (Yavin & Zutra, 1977). The identification of compound B as PC was confirmed by nuclear magnetic resonance spectroscopy in deuterated chloroform which revealed a peak at 3.26 ppm downfield to tetramethylsilane, typical of $-N^+(CH_3)_3$ protons (Haque et al., 1972). The ratio of the peak area of the major methylene proton at 1.26 ppm to the peak area of the $-N^+(CH_3)_3$ protons was 3.0 for component B as compared with 2.5 for a control of dimyristoyl-PC. This difference is apparently due to the longer chain fatty acids of component B (mainly palmitic and stearic acids).

The three phospholipids comprised over 95% of the total *M. gallisepticum* phospholipids as judged from their lipid phosphorus content (Table I). Although the phospholipid content varied with the age of the culture (Rottem et al., 1978), the relative amounts of each of the three phospholipids remained constant throughout the growth cycle. Table I also shows that when the cells were grown with a radioactive fatty acid, SPM remained unlabeled, PC was labeled by radioactive palmitate but not by oleate, while PG was labeled by both palmitate and oleate. This suggests that SPM is taken up from the growth medium and is not synthesized by the cells. In fact, by changing the horse serum concentrations in the growth medium, SPM content of *M. gallisepticum* was most dramatically affected (Figure 1). Increasing the horse serum concentration of the growth medium also resulted in an increase in the PC content of *M. gallisepticum* (Figure 1). The possibility that the PC found in *M. gallisepticum* membranes is taken up unchanged from the growth medium is unlikely since, when cells were grown with radioactive palmitic acid, a considerable amount of radioactivity was found in the PC fraction (Table I). Moreover, the fatty acid composition of the PC fraction of *M. gallisepticum* differed from that of the PC fraction extracted from the growth medium (Table II). The PC of *M. gallisepticum* contained mainly saturated fatty acids, whereas PC from the growth medium contained approximately equal amounts of saturated and unsaturated fatty acids. Table II also shows that the fatty acid composition of *M. gallisepticum* PC could be varied to a considerable extent, depending on the exogenous saturated free fatty acids added to the growth medium. But, when *M. gallisepticum* PC preparations were treated with phospholipase A_2, the fatty acid composition of the lyso-PC obtained was always the same (Table II). Stearic acid (18:0) appeared as a major fatty acid

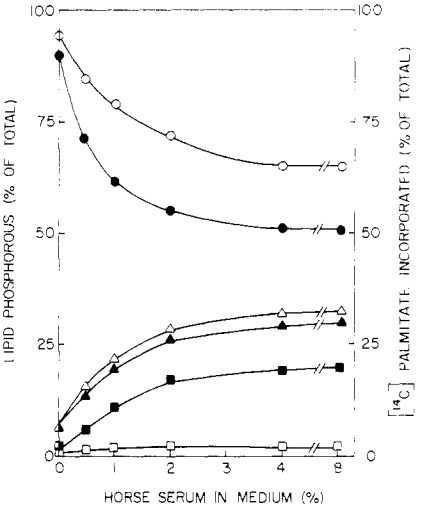

FIGURE 1: The effect of horse serum concentration in the growth medium on the relative amounts of *M. gallisepticum* phospholipids. (Open symbols) Radioactivity derived from ^{14}C-labeled palmitic acid added to the growth medium. (Closed symbols) Lipid phosphorus. (○ and ●) Phosphatidylglycerol; (△ and ▲) phosphatidylcholine; (□ and ■) sphingomyelin.

Table III: Incorporation of ^{14}C-Labeled Choline Derivatives and [^3H]Palmitic Acid into Phosphatidylcholine of *M. gallisepticum*[a]

^{14}C-labeled compd added to medium	radioact. in PC: ^{14}C (dpm)	^3H (dpm)
choline	106	31 000
phosphorylcholine	92	29 600
glycerophosphorylcholine	80	30 000
lyso-PC	12 700	31 250
PC	26 380	30 240

[a] Cells were grown to an absorbance of $A_{640} = 0.18$ in Edward medium containing 0.05 μCi/mL of [^3H]palmitic acid and 1000 dpm/mL of either [*N-methyl*-^{14}C]choline, phosphatidyl[*N-methyl*-^{14}C]choline, ^{14}C-labeled phosphorylcholine, ^{14}C-labeled glycerophosphorylcholine, or ^{14}C-labeled lysophosphatidylcholine. The three latter compounds were prepared from phosphatidyl[*N-methyl*-^{14}C]choline as described in the text.

of *M. gallisepticum* PC. A high stearic acid content (~35% of total fatty acids) was found even in PC preparations from cells grown without exogenously supplied stearic acid. Likewise, after phospholipase A_2 treatment the stearic acid residue of *M. gallisepticum* PC was almost fully recovered in lyso-PC (Table II), whereas radioactivity of *M. gallisepticum* PC, derived from ^{14}C-labeled palmitate added to the growth medium, was fully recovered in the free fatty acid fraction (not shown in the table). These findings indicate that

Table IV: Positional Distribution of Fatty Acyl Chains in Phosphatidylglycerol Preparations from *M. gallisepticum* and *A. laidlawii*[a]

fatty acid[b]	fatty acid of *M. gallisepticum* phosphatidylglycerol (mol %)			fatty acid of *A. laidlawii* phosphatidylglycerol (mol %)		
		treated with phospholipase A_2			treated with phospholipase A_2	
	untreated	lyso-PG	free fatty acids	untreated	lyso-PG	free fatty acids
14:0	1.6	1.8	1.6	3.4	4.6	3.8
16:0	49.0	10.1	84.1	31.5	66.7	8.6
18:0	1.4	<0.5	3.2	6.9	14.3	1.8
18:1	47.7	83.2	11.1	61.6	19.1	87.5

[a] The phosphatidylglycerol preparations were obtained from cells grown in Edward medium containing 0.5% bovine serum albumin, 15 μg/mL cholesterol, palmitic and oleic acids (20 μM each for *M. gallisepticum*; 20 μM palmitate and 40 μM oleate for *A. laidawii*). The positional distribution was determined by using phospholipase A_2 as described in the text. The fatty acid content of lyso-PG represents the fatty acyl chains at position 1, whereas the fatty acid content of the free fatty acid fraction represents the fatty acyl chains at position 2. [b] The first number indicates chain length and the second, the number of double bonds.

M. gallisepticum utilizes a stearic acid rich lipid backbone, such as lyso-PC, for the synthesis of its highly saturated PC. Table III shows the incorporation of radioactive choline derivatives into the PC of *M. gallisepticum*. The incorporation was not affected by replacing the active horse serum component of the growth medium by heat-inactivated (56 °C for 1 h) serum. The table shows that a double-labeled PC was synthesized by *M. gallisepticum* cells grown with [^{3}H]palmitate and either ^{14}C-labeled PC or ^{14}C-labeled lyso-PC, but not with ^{14}C-labeled choline, phosphorylcholine, or glycerophosphorylcholine. The double-labeled PC spot obtained when ^{14}C-labeled PC was added to the growth medium was not due to comigration of a newly synthesized ^{3}H-labeled *M. gallisepticum* PC and the ^{14}C-labeled exogenous egg PC, since the PC spot contained only a disaturated PC.

The positional distribution of fatty acyl chains in the PG of *M. gallisepticum* is presented in Table IV. The results are compared with those obtained with a PG preparation of *Acholeplasma laidlawii*. Since the positional distribution of the fatty acids is relative, depending upon the nature and concentration of the other fatty acids present in the phospholipid (Saito & McElhaney, 1978), the fatty acid composition of the PG from both *A. laidlawii* and *M. gallisepticum* was standardized to contain the same fatty acids at approximately the same concentrations by growing the cells in a fatty acid poor medium containing exogenously supplied palmitic and oleic acids. The positional distribution was studied with the aid of a pancreatic phospholipase A_2, which catalyzes the hydrolysis of fatty acid ester linkages exclusively at the 2 position. Thus, the fatty acids present in the lyso-PG represent the fatty acids located in the 1 position, whereas the free fatty acids liberated by the enzyme treatment represent the fatty acids located at the 2 position. As seen in the table, the positional distribution of fatty acids in PG preparations from *A. laidlawii* is in accordance with that found elsewhere in nature. The unsaturated oleic acid was found primarily in the 2 position, whereas the saturated palmitic acid was found in the 1 position. In the case of *M. gallisepticum*, however, a notable exception was encountered. Unsaturated fatty acids were found predominantly in the 1 position, and saturated fatty acids were found more abundantly in the 2 position. Therefore, the saturated to unsaturated fatty acids ratio was high (7.75) in fatty acids occupying position 2 and low (0.14) in fatty acids of position 1, as opposed to the control *A. laidlawii* preparation, which showed a low ratio in position 2 and a high ratio in position 1. The unusual positional distribution of fatty acids in the PG preparation of *M. gallisepticum* is clearly expressed when the positional specificity of palmitic and oleic acids (the two major fatty acids constituting the PG preparations) was calculated as the ratio of the quantity of each of the fatty acids present at the 1 position to the quantity at position 2. A position 1/position 2 ratio of >1.0 indicates a preferential esterification at the 1 position. A position 1/position 2 ratio of <1.0 indicates esterification at position 2. In *M. gallisepticum* preparations, the position 1/position 2 ratio of palmitic acid was 0.2 and that of oleic acid was 7.7. The position 1/position 2 ratio was further used to determine the positional preference of various exogenous fatty acids in *M. gallisepticum* PG (Table V). The results indicated that the shorter the chain length, the higher the preference toward the 1 position within the chosen series. Among the three acids having the same carbon number, the preference toward the 1 position is in the order of cis-unsaturated > trans-unsaturated > saturated fatty acids.

Table V: Positional Preference of Various Exogenous Fatty Acids in Phosphatidylglycerol of *M. gallisepticum*[a]

no. of carbons of fatty acid	position 1/position 2 ratio of		
	saturated fatty acids	monounsaturated fatty acids	
		trans isomer	cis isomer
14	5.9	ND[b]	ND[b]
16	0.4	4.7	10.0
18	0.2	1.9	2.7

[a] Phosphatidylglycerol was obtained from *M. gallisepticum* cells grown in a medium containing 0.5% bovine serum albumin, myristic, palmitic, and stearic acid (15 μM each), or palmitelaidic and elaidic acid (22.5 μM each). The positional distribution was determined by using pancreatic phospholipase A_2 as described in the text. The position 1/position 2 ratios of the saturated fatty acids are those calculated for phosphatidylglycerol from cells grown with saturated plus cis-unsaturated fatty acid mixture. [b] Not determined because of an extremely low incorporation of the tetradecenoic acid cis and trans isomers.

Discussion

Phospholipids and cholesterol are the major lipids in *M. gallisepticum* membranes. The high cholesterol content is to be expected from a cholesterol-requiring mycoplasma (Razin, 1978). Neutral lipids constitute a minor fraction and glycolipids were not detected. The phospholipid composition of *M. gallisepticum* is rather simple and resembles that of *M. hominis* (Rottem & Razin, 1973) in comprising of one major de novo synthesized phospholipid identified as PG, the most common phospholipid in mycoplasmas (Smith, 1979). When grown in a medium which does not contain serum, this phospholipid comprises 95% of membrane phospholipids but, when grown in the ordinary mycoplasma media that contain horse serum, significant amounts of SPM and PC were detected in the cell membrane, depending on the serum concentration in the growth medium. SPM and PC are un-

common lipids in wall-covered bacteria but were found to be incorporated unchanged from the growth medium in several species of the wall-less mycoplasmas (Plackett & Rodwell, 1970; Freeman et al., 1976). Our study shows that in *M. gallisepticum* SPM is incorporated unchanged from the growth medium, but the PC is modified by the insertion of saturated fatty acids at position 2 of the *sn*-glycerol 3-phosphate, presumably by a deacylation–reacylation enzymatic sequence. This conclusion is based on the following observations: (a) *M. gallisepticum* PC differs in its fatty acid composition from PC of the growth medium; (b) it is possible to label position 2 of *M. gallisepticum* PC by adding radioactive palmitate to the growth medium; (c) it is possible to introduce controlled variation in the fatty acid composition at position 2 by changing the free saturated fatty acids of the growth medium; (d) it is possible to obtain a double-labeled disaturated PC by growing *M. gallisepticum* cells with ^{14}C-labeled lyso-PC and ^{3}H-labeled palmitic acid. It is interesting to note that a disaturated PC was also found recently in the cell membrane of *M. pneumoniae* (S. Rottem, unpublished). A deacylation–reacylation enzymatic sequence will require the hydrolysis of the ester bond at position 2 of the PC of the growth medium by an enzyme present either in *M. gallisepticum* cells or in the growth medium. Since heating the horse serum component of the growth medium to 56 °C for 1 h or replacing it by bovine serum albumin, fatty acids, cholesterol, and egg PC had almost no effect on the disaturated PC formed by the cells, it is more likely that the hydrolysis of the ester bond at position 2 is due to an endogenic phospholipase activity. Attempts to demonstrate such activity in isolated *M. gallisepticum* membrane preparations have failed thus far (O. Markowitz, unpublished results).

Phospholipids derived from a variety of natural sources, with the exception found in *Clostridium butyricum* (Hildebrand & Law, 1964), show a nonrandom distribution of fatty acids: saturated fatty acids are located at position 1 of the glycerol, while unsaturated fatty acids are usually found at position 2 (Van Deenen, 1965; Cronan & Gelman, 1975). Until recently, very little was known about the positional distribution of fatty acids in membrane lipids of mycoplasmas. Previous studies on *Acholeplasma laidlawii* B (McElhaney & Tourtellotte, 1970; Saito et al., 1977) showed that the fatty acid positional distribution in phospho- and glycolipids of this organism is in accord with that found elsewhere in nature. Our present study on *M. gallisepticum* shows that in this organism the major membrane phospholipid, PG, shows an unusual distribution of fatty acids. When *M. gallisepticum* cells were grown in a medium containing oleic and palmitic acids, oleate was found more abundantly in position 1, while palmitate was found in position 2. The correlation between the positional specificity of a given fatty acid and the relative strength of the apolar attractive interactions of its hydrocarbon chain suggests that the enzyme(s) catalyzing the acylation of the 1 and 2 position of *sn*-glycerol 3-phosphate in *M. gallisepticum* appear(s) to function by recognizing the physical properties of the fatty acids, rather than the chemical properties or electronic configurations of the fatty acid hydrocarbon chains. An unusual positional distribution of fatty acids with fatty acids having lower melting points at position 1 while those with higher melting points at position 2 was recently found in de novo synthesized membrane phospholipids of seven other *Mycoplasma* species (S. Rottem, unpublished). It seems that an unusual positional distribution is a general characteristic of the *Mycoplasma* species. These species differ from the *Acholeplasma* species and from other prokaryotic cells in their growth requirement for cholesterol (Razin & Rottem, 1978; Razin, 1978). Cholesterol is incorporated into the cell membrane, reaching levels of about 50 mol % of the total membrane lipids. It can be suggested that the requirement for cholesterol and its high content in the *Mycoplasma* species is associated in some way with the presence of phospholipids with unusual positional distribution. Such association might be due to an effect of the high cholesterol content on the specificity of the acyltransferases located in the cell membrane of mycoplasmas (Rottem & Greenberg, 1975). On the other hand, one can argue that the differences in the physical properties of the membrane imposed by the differences in the positional distribution result in the strict requirement of the *Mycoplasma* species for cholesterol. The latter possibility is to some extent unlikely in view of the data with artificial membrane systems (Demel et al., 1972; De Kruyff et al., 1973), showing that the interaction of cholesterol with both structural isomers of phosphatidylcholine with monounsaturated fatty acids at positions 1 or 2 was similar.

A model for the phospholipid–cholesterol complex in a membrane was recently presented by Huang (1978) based on the structural properties of phospholipid and cholesterol molecules. Accordingly, the β face of cholesterol is packed in close contact to the unsaturated fatty acyl chain esterified at position 2 of PC, while the α face participates in strong van der Waals interactions with the saturated acyl chain at position 1. Since in the cholesterol-requiring *Mycoplasma* species unsaturated fatty acids seem to be commonly in position 1 of the de novo synthesized phospholipids, the β face of the steroid nucleus will interact preferentially with the acyl chain esterified at position 1 of the phospholipids. Therefore, the carbonyl oxygen of position 1, suggested by Huang (1978) to be engaged in hydrogen bonding with the equatorial hydroxyl group of the cholesterol, is less common for such interaction in *Mycoplasma*.

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Electron Paramagnetic Resonance Analyses of Horseradish Peroxidase in Situ and after Purification†

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ABSTRACT: We present EPR spectra of freshly harvested horseradish root, partially purified root extract, and pure horseradish peroxidase isoenzymes A2 and C2 at various values of pH, temperature, and power. They exhibit signals in the $g \simeq 6$ and $g \simeq 5$ region, typical of high-spin and *quantum* mixed-spin heme iron, respectively, with a greater proportion of $g \simeq 5$ signal present in the root and root extract samples than is seen in the spectra of most samples of pure A2 and C2 isoenzymes. The addition of hydroquinone to A2 or C2 causes an increase in the *quantum* mixed-spin signal and an accompanying decrease in the high-spin signal. Tests for adhering donor in the purified isoenzymes were negative, implying that the *quantum* mixed-spin signals, $g \simeq 5$, originate from free horseradish peroxidase. The addition of hydrogen peroxide decreases the $g \simeq 5$ and $g \simeq 6$ signals in parallel with each other and the increase in the free radical signal at $g \simeq 2$. pH titration of A2 or C2 results in reversible transitions between various high-spin, *quantum* mixed-spin, and low-spin EPR spectral species. The variation in the relative amounts of *quantum* mixed-spin and high-spin species monitored in different horseradish peroxidase preparations and the response to conditions of donor and pH show that the protein conformation is sensitive to perturbation imposed upon it during and after purification. The implications of *quantum* mixed-spin properties for the peroxidase function of the enzyme are discussed in the context of the model for the iron–ligand configuration inferred from magnetic studies of *quantum* mixed-spin heme proteins.

The magnetic properties of horseradish peroxidase have been analyzed in a number of papers, including EPR studies under various conditions (Morita & Mason, 1965; Blumberg et al., 1968; Douzou & Leterrier, 1970; Tamura & Hori, 1972; Critchlow & Dunford, 1972; Schonbaum, 1973; Aasa et al., 1975; Leigh et al., 1975). Although qualitatively similar, the results on the free enzyme have differed in both spectral details and interpretation. In particular, an EPR absorption at $g \simeq 5$ has varied considerably from one preparation to another. This fact, the uncommon nature of this signal in heme protein EPR spectra, and the observation that the signal is reinforced by aromatic hydrogen donors raised the question whether the $g \simeq 5$ signal is caused by the free HRP[1] itself or by a complex with some substance from the root.

The EPR spectrum of HRP has been interpreted as originating from a thermal or chemical mixture of high- and low-spin forms of HRP (Tamura, 1971; Tamura & Hori,

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[1] Abbreviations used: HRP, horseradish peroxidase; RZ, A_{402}/A_{280} (Reinheitszahl).

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