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Lipid Interconversions in Aging *Mycoplasma capricolum* Cultures

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During the progression of *Mycoplasma capricolum* cultures from the early exponential to the stationary phase of growth, a decrease in the phospholipid-to-protein ratio and increases in both the unsaturated-to-saturated fatty acid ratio and the diphosphatidylglycerol (DPG)-to-phosphatidylglycerol (PG) ratio were found. The freedom of motion of spin-labeled fatty acids incorporated into the membrane remained unchanged throughout the growth cycle. The increase in DPG was almost stoichiometric with the decrease in PG. Furthermore, exogenous PG added to the medium was incorporated by the cells and partially converted to DPG. The DPG that was accumulated upon aging was always more unsaturated than the PG. This accumulation was enhanced in palmitic acid-poor media, but was inhibited even in aged cells when the cells were grown in palmitic acid-rich media, suggesting that the accumulation of DPG upon aging was associated with changes in the fatty acid composition of membrane lipids rather than with the transition of the cells from the exponential- to stationary-growth phase.

The de novo-synthesized phospholipids of *Mycoplasma capricolum* are rather simple, being composed primarily of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) (11). This organism, like other mycoplasmas, is a fatty acid and cholesterol auxotroph (6, 25) and requires both a saturated and an unsaturated fatty acid. When mycoplasmas are grown in ordinary media that contain horse serum, significant amounts of exogenous phospholipids, mainly phosphatidylcholine and sphingomyelin, are incorporated into the cell membranes (16, 19). In a previous study (11), we have shown that a main consequence of the incorporation of exogenous phosphatidylcholine into *M. capricolum* cells is a marked increase in the DPG content of the membrane lipids. Changes in DPG content have been observed in a variety of bacteria, primarily in relation to growth phases (5), cell division (5, 14), and sporulation (21). Because DPG is synthesized mainly by the conversion of two PG molecules to DPG (12), it has been suggested that the PG-to-DPG conversion has a vital role in cell growth (5).

In the present paper, we report on changes in membrane lipids that occurred with the aging of *M. capricolum* cultures and show that an increase in the DPG-to-PG ratio, which occurred under some growth conditions, was associated with an increase in the unsaturation of membrane lipids rather than with the stage of growth per se.

MATERIALS AND METHODS

Organisms and growth conditions. *M. capricolum* (KID) was grown in a modified Edward medium (20) supplemented with 0.5% delipidated bovine serum albumin, cholesterol (20 μg/ml), and oleic and palmitic acids (10 μg of each per ml). To delipidate the albumin, 50 g of bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, Mo.) was extracted three times with 200 ml of chloroform-methanol (1:1 [vol/vol]) at 4°C for 1 h with constant stirring. The extracted material was collected and dried overnight at room temperature, dissolved in deionized water, and treated with charcoal as described before (15). Membrane lipids were labeled by growing the organisms with 0.002 μCi of [1-^{14}C]palmitic acid per ml (50 to 60 Ci/mol), 0.002 μCi of [1-^{14}C]oleic acid per ml (50 Ci/mol), 0.02 μCi of [9,10-*n*-^{3}H]palmitic acid per ml (500 Ci/mol), or 0.02 μCi of [2-^{14}C]glycerol per ml (10 to 20 Ci/mol). All radioactive lipid precursors were products from the Radiochemical Centre, Amersham, England. In some experiments, the cells were grown with radioactive exogenous PG or DPG. The radioactive exogenous phospholipids were obtained by growing *M. capricolum* cells to late exponential growth in medium containing [^{3}H]palmitate plus [^{14}C]glycerol. Lipids were then extracted from the cell pellets, and PG and DPG were purified and added to the growth medium as described before (11). Growth at 37°C was monitored by measuring the A_{640}. At the required phase of growth, the cells were harvested by centrifugation at 12,000 × *g* for 15 min, washed once, and suspended in cold 0.25 M NaCl. Membranes were isolated from the washed cells by osmotic lysis of the organisms in 40 volumes of deionized water at 37°C (20). The density of the membrane preparations was determined as described before (20).

Lipid analyses. Lipids were extracted from intact cells or isolated membranes by the method of Bligh and Dyer (4). The solvents were evaporated under a stream of nitrogen and dissolved in 0.1 to 0.5 ml of chloroform-methanol (2:1 [vol/vol]). Thin-layer chromatography of total membrane lipids was performed on silica gel HF plates; the plates were first developed at room temperature with light petroleum (bp 40 to 60°C)-acetone (3:1 [vol/vol]) and then at 4°C with chloroform-methanol-water (65:25:4 [vol/vol/vol]). Methyl esters of fatty acids were prepared by heating the lipid samples in 14% boron trifluoride (Sigma) in methanol at 80°C for 15 min. The resulting methyl esters were extracted with *n*-hexane and subjected to gas-liquid chromatography in a Packard-Becker Co. (Delft, The Netherlands) model 537 gas-liquid chromatograph equipped with a polar column (200 by 0.3 cm; 10% SP-2340 [Cyanosilicone] on 100/120 Chromosorb WAW). Fatty acids were identified by their retention times relative to those in a standard methyl ester mixture (Supelco Inc., Bellefonte, Pa.).

Analytical methods. Protein levels in cell and membrane preparations were determined by the method of Lowry et al.

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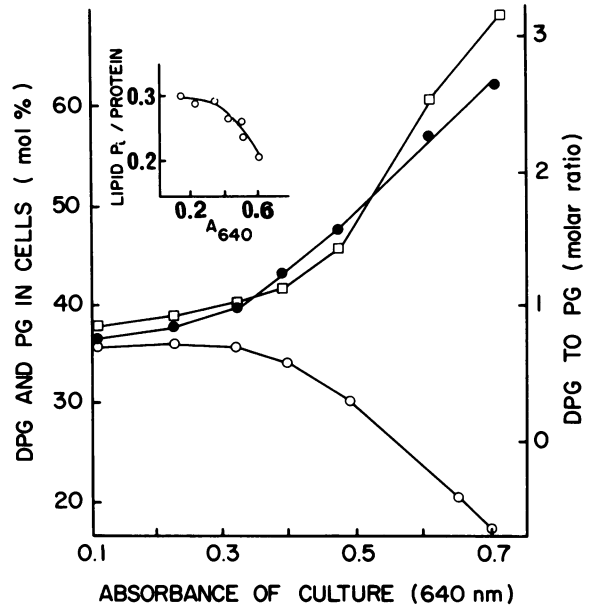

FIG. 1. Effect of aging on the relative amounts of PG and DPG of *M. capricolum* cultures. ○, PG; □, DPG; ●, DPG-to-PG ratio. The inset shows the decrease in the ratio of phospholipid-to-protein upon aging. The results are expressed as micromoles of lipid P_i per milligram of protein.

(13). Total amounts of phosphorus in the lipid fractions and in phospholipid spots resolved by thin-layer chromatography were determined by the method of Ames (2), after digestion of the sample with an ethanolic solution of $Mg(NO_3)_2$. To determine radioactivity levels in the lipid spots, the lipids were scraped from the thin-layer chromatography plate into scintillation vials containing 5 ml of toluene scintillation liquor. The radioactivity was measured in a Packard Tricarb scintillation spectrometer (model 2650). For electron paramagnetic-spin resonance (EPR) analyses, *M. capricolum* membrane preparations were labeled with *N*-oxyl-4,4-dimethyl-oxazolidine derivatives of ketostearic acid (5-doxylstearate or 12-doxylstearate) Syva, Palo Alto, Calif.) as previously described (7). EPR analyses were carried out in a Varian E-4 spectrometer equipped with a temperature control accessory. The freedom of motion of the spin-labeled fatty acids was assessed from the order parameter (*S*) (9). *S* is related to the mean angular deviation of the labeled fatty acid chain from its average orientation in the membrane.

RESULTS

During the progression of growth of *M. capricolum* in a medium in which the serum component was replaced for by 0.5% lipid-preextracted bovine serum albumin–cholesterol (20 μg/ml)–oleic and palmitic acids (10 μg of each per ml), the total phospholipid-to-membrane protein ratio was essentially unchanged during the early-exponential-growth phase but decreased at the late exponential and stationary phases of growth (Fig. 1, inset). The lower phospholipid-to-protein ratio in membranes from aged cells affected the densities of the membrane preparations. Thus, membranes from cells harvested at the stationary phase of growth (A_{640}, 0.6) had a higher density (1.18 ± 0.01 g/ml) compared with membranes obtained from early exponential cells (A_{640}, 0.2; density, 1.15 ± 0.01 g/ml). However, the physical states of membranes from cells harvested at the various growth phases were very similar. The physical state was assessed from the freedom of motion of spin-labeled fatty acids in the membranes. When membranes from early and late exponential phases as well as from stationary-phase cells were labeled with 5-doxylstearate or 12-doxylstearate, the EPR spectra obtained were single-component spectra with almost identical *S* values. With spin-labeled membranes analyzed at 37°C, *S* values of 0.46 ± 0.01 for membranes labeled with 12-doxylstearate and 0.65 ± 0.01 for membranes labeled with 5-doxylstearate were obtained.

When cells were grown in a serum-free medium, the major de novo-synthesized phospholipids were PG and DPG. These phospholipids accounted for 75 ± 5% of the total polar lipid fraction. With an increase in density of *M. capricolum* cultures, the relative amounts of PG and DPG were changed (Fig. 1). The amount of PG was decreased followed by a concomitant increase in the amount of DPG. As a result, the DPG-to-PG molar ratio was markedly increased. To determine whether these changes were a consequence of cells entering the stationary phase of growth, the exponential-growth phase was extended by controlling the pH of the growth medium. Typical growth curves in uncontrolled and pH-controlled media are shown in Fig. 2. Under uncontrolled conditions the increase in absorbance of the culture was exponential up to 24 h of incubation at 37°C, reaching a maximum A_{640} of 0.50 to 0.55. When a constant pH of 7.5 was maintained throughout the growth cycle, the exponential phase of growth was extended to 26 h with a maximal A_{640} of 0.80 to 0.85. Lipid analyses of the cell preparations grown under the uncontrolled and pH-controlled conditions revealed that changes in DPG-to-PG ratios were associated with changes in the density of the culture per se, rather than with changes in the growth phase of the cells. Thus, the DPG-to-PG ratio in cells grown in an uncontrolled medium

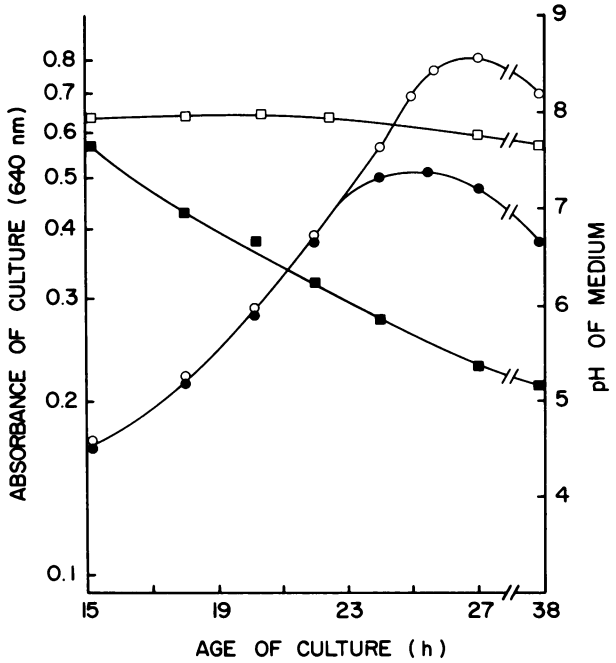

FIG. 2. Growth curve of *M. capricolum* under pH-controlled (open symbols) and uncontrolled (closed symbols) conditions. pH was controlled by titrating the excess acid with 0.1 N NaOH. ○ and ●, A_{640}; □ and ■, pH of medium.

TABLE 1. Effect of fatty acids added to the medium on the DPG-to-PG ratio in *M. capricolum* cells harvested at various growth phases[a]

Concn of fatty acid added (μg/ml)		DPG-to-PG molar ratio at A_{640}:			Unsaturated-to-saturated fatty acid ratio at A_{640}:		
Palmitic acid	Oleic acid	0.18 ± 0.03	0.37 ± 0.5	0.57 ± 0.05	0.18 ± 0.03	0.37 ± 0.03	0.57 ± 0.05
15	5	0.9	0.8	0.8	0.5	0.6	0.6
10	10	1.0	1.3	2.6	0.6	0.7	1.3
5	15	1.1	3.5	4.4	0.6	1.5	2.0
30	10	0.7	0.8	0.7	0.5	0.5	0.6
20	20	0.9	1.0	1.0	0.5	0.6	0.5
10	30	0.8	0.7	0.7	0.8	0.6	0.7

[a] Cells were grown in a medium containing 0.5% albumin, 20 μg of cholesterol per ml, and various amounts of oleic and palmitic acids. The cells were harvested at the early (A_{640}, 0.18 ± 0.03), mid- (A_{640}, 0.37 ± 0.05), and late (A_{640}, 0.57 ± 0.05) exponential phases of growth. Phospholipid and fatty acid analyses were performed as described in Materials and Methods. The unsaturated-to-saturated fatty acid ratio is of the total lipid fraction.

and harvested at the stationary phase of growth (A_{640}, ≈0.5) was practically identical to the ratio in mid-exponential-phase cells grown to an identical absorbance in a pH-controlled medium.

Since *M. capricolum* can neither synthesize nor modify long-chain fatty acids, these cells depend on an exogenous supply of fatty acid in the growth medium. This requirement is usually met by a mixture of palmitic and oleic acids. All results so far described were obtained in a standard medium containing palmitic and oleic acids in a ratio of 1.0 (10 μg of each per ml). However, varying the palmitic-to-oleic acid ratio in the medium without changing the total fatty acid concentration (20 μg/ml) had a pronounced effect on the DPG-to-PG ratio. Cells grown in a medium with a higher palmitic-to-oleic acid ratio (15 μg of palmitic acid per ml plus 5 μg of oleic acid per ml) showed very little variation in their DPG-to-PG ratio upon aging, whereas cells grown with a lower palmitic-to-oleic acid ratio (5 μg of palmitic acid per ml plus 15 μg of oleic acid per ml) showed a pronounced increase in their DPG-to-PG ratio (Table 1). Growing the cells with either a low or high palmitic-to-oleic acid ratio had almost no effect on the phospholipid-to-protein ratio in the membranes or on the physical state of membrane lipids (Table 2). When the final concentration of fatty acids in the growth medium was increased from 20 to 40 μg/ml, the increase in the DPG-to-PG ratio upon aging was slight even in cells grown in a medium containing a low palmitic-to-oleic acid ratio (Table 1). As the growth curves of all cultures analyzed were almost identical, it seems that the increase in the DPG-to-PG ratio with the aging of *M. capricolum* depended to a large extent on the palmitic and oleic acid content of the growth medium.

The specific labeling of PG and DPG by radioactive fatty acids added to the growth medium is presented in Table 3. When cells were grown with radioactive palmitate, the labeling of the PG fraction was considerably higher than that of the DPG fraction, whereas when cells were grown with radioactive oleate the labeling of the PG fractions was lower. The more unsaturated nature of the DPG fraction was further supported by fatty acid analyses by gas-liquid chromatography of the isolated PG and DPG fractions. The unsaturated-to-saturated fatty acid ratios of the PG and DPG fractions from stationary-phase *M. capricolum* cells (A_{640}, 0.6) were 0.88 and 1.28, respectively. The results of a pulse-chase experiment in which the cells were grown with radioactive palmitate to an A_{640} of 0.20, washed, and suspended in a fresh unlabeled medium are shown in Fig. 3. Growth was then continued for 6 h to the end of the exponential phase of growth (A_{640}, 0.55). Throughout the growth period, the radioactivity of the PG pool was decreased, accompanied by an increase in the radioactivity of the DPG pool (Fig. 3). Growing *M. capricolum* cells with both [^{3}H]palmitate and [^{14}C]glycerol after the chase in an unlabeled medium enabled us to determine the turnover rate of the fatty acid and glycerol moieties of the total membrane phospholipid. As is apparent from the inset to Fig. 3, the turnover rate of the glycerol moiety was 2.6 to 3.0 times higher than that of palmitic acid. Turnover rates identical to that of the palmitic acid moiety were obtained with oleic and elaidic acids (data not shown).

Because *M. capricolum* cells are capable of incorporated exogenous phospholipid from the growth medium (11), the incorporation of radioactive PG and DPG added to the growth media was investigated. When the cells were grown with PG labeled with [^{3}H]palmitate and [^{14}C]glycerol, 5 ±1% of the radioactivity added to the growth medium was incorporated by the organisms (Table 4). Two-thirds of the incorporated radioactive PG remained unchanged, whereas about one-third was converted to DPG that showed a ^{3}H-to-^{14}C ratio significantly higher than that in the PG pool. When the cells were grown with exogenous radioactive DPG, about 2 ± 1% of the DPG was incorporated by the cells, but practically all of the radioactivity was recovered in the DPG fraction (data not shown).

DISCUSSION

In most bacterial systems investigated, the lipid-to-protein ratio in the cytoplasmic membrane remains constant at various phases of growth or under various growth conditions (5). Our results, however, show that the phospholipid-to-protein ratio in the membrane of *M. capricolum* was dramatically decreased with the aging of the culture. We previously described a progressive decrease in the lipid-to-protein ratio of *M. hominis* (23) and attributed this decrease to an increase in the membrane protein content rather than to a decrease in the membrane lipid content. With the decrease in the lipid-to-protein ratio in aged *M. hominis* cells, significant changes in the organization of membrane proteins, in particular proteins located on the inner membrane surface, were found (1). Likewise, the bulk of membrane lipids of the aged *M. hominis* were more rigid (23). It was postulated that the higher rigidity was caused by the higher amounts of proteins that penetrated deeply into the bilayer, immobilizing the membrane lipids (24). In *M. capricolum*, however, although the lipid-to-protein ratio in aged cells was markedly decreased, the freedom of motion of spin-labeled probes in the lipid domain remained unchanged, suggesting that in this organism lipid-protein interactions did not alter the average freedom of motion of spin-labeled probes. The constancy of the freedom of motion of the spin-labeled probes, even in cells in which the percentage of *cis*-unsaturated fatty acids

TABLE 2. Effect of fatty acids added to the medium on the phospholipid-to-protein ratio in the cell membrane and on the physical state of membrane lipids of aging *M. capricolum* cells[a]

Concn of fatty acids added (μg/ml)		A_{640} of culture	Lipid P_i/protein ratio (μmol/mg)	*S*	
Palmitic acid	Oleic acid			5-Doxylstearate	12-Doxylstearate
15	5	0.20	0.32	0.65	0.46
		0.35	0.28	0.64	0.46
		0.52	0.23	0.65	0.45
5	15	0.18	0.31	0.65	0.44
		0.42	0.26	0.65	0.45
		0.62	0.22	0.63	0.45

[a] Cells were grown in a medium containing 0.5% albumin, oleic and palmitic acids (5 or 15 μg of each per ml), and 20 μg of cholesterol per ml. Membrane isolation and lipid analyses were performed as described in Materials and Methods. EPR spectrometry of 5-doxylstearate or 12-doxylstearate was performed at 37°C.

was increased upon aging (cells grown with 15 μg of oleate per ml and 5 μg of palmitate per ml), suggests that in aging *M. capricolum* cells the increase in unsaturation, known to increase motion freedom in biological membranes (5, 22), was counterbalanced by a yet unknown factor. Because the rigidifying effect of cholesterol in biological membranes is well established (6, 22), this factor may have been an increase in the cholesterol content in the membranes. An increase in the incorporation of cholesterol into mycoplasmas has been shown to occur when the unsaturation of membrane polar lipids is increased (18).

Our results suggest that, as in other microbial systems (5, 12), in *M. capricolum*, PG serves as the only precursor for the synthesis of DPG. This conclusion is based on observations that the progressive decrease in PG with the aging of *M. capricolum* cultures was almost stoichiometric with the increase in DPG and on the pulse-chase results showing that, after chasing, the PG was turned over very quickly, apparently owing to its conversion to DPG. Furthermore, as mycoplasmas appear to be novel in the latitude they display for the incorporation of ready-made exogenous phospholipids from the growth medium (19), it was possible to monitor the fate of exogenous PG added to the growth medium. Our results showed unequivocally that the exogenous PG that was incorporated into the cells was partially converted to DPG. The increase in the [^{3}H]palmitate-to-[^{14}C]glycerol ratio in the newly formed DPG was apparently a result of the loss of a glycerol moiety after the synthesis of DPG from two molecules of PG. Because PG taken up from the growth media was first incorporated into the outer leaflet of the lipid bilayer, it is possible that the PG incorporated was rapidly translocated to the inner half of the bilayer where phospholipid biosynthesis occurs (5).

In several bacteria the turnover of PG occurs in a cyclic manner involving the conversion of two PG molecules into DPG and the rapid hydrolysis of DPG by a DPG-specific phospholipase D to yield PG and phosphatidic acid (3). Because phospholipase D activity was not detected in *M. capricolum* membranes (L. Adar and S. Rottem, unpublished results) and exogenous DPG added to the growth medium was incorporated by the cells and quantitatively recovered in the membrane DPG fraction, it seems that, in *M. capricolum*, DPG hydrolysis by phospholipase D did not occur.

The accumulation of DPG in the stationary phase of growth seems to be universal among bacteria (5, 21) and has been described previously for *Acholeplasma laidlawii* and *Mycoplasma mycoides* subsp. *mycoides* (16, 25). Because such accumulation can be triggered by inhibitors of cell division in *Escherichia coli*, it has been postulated that the PG-to-DPG conversion plays a vital role in bacterial cell growth and division (14). Our observations that the cell growth of *M. capricolum* was uniform under culture conditions in which the PG-to-DPG ratio was markedly varied

TABLE 3. Specific labeling in the PG and DPG fractions of *M. capricolum* cells grown with radioactive fatty acids[a]

Radioactive fatty acid added to the medium	A_{640} of culture	Specific labeling (cpm/nmol of lipid P_i) in:	
		PG	DPG
[1-^{14}C]palmitate	0.21	105 ± 10	70 ± 4
	0.60	70 ± 4	44 ± 3
[1-^{14}C]oleate	0.22	120 ± 8	156 ± 10
	0.60	80 ± 5	106 ± 5

[a] Cells were grown in a medium containing 0.5% albumin, oleic and palmitic acids (10 μg of each per ml), cholesterol (20 μg/ml), and either [1-^{14}C]palmitate or [1-^{14}C]oleate. The cells were collected at the early (A_{640}, 0.21 to 0.22) or late (A_{640}, 0.60) exponential phase of growth, extracted, and analyzed as described in Materials and Methods.

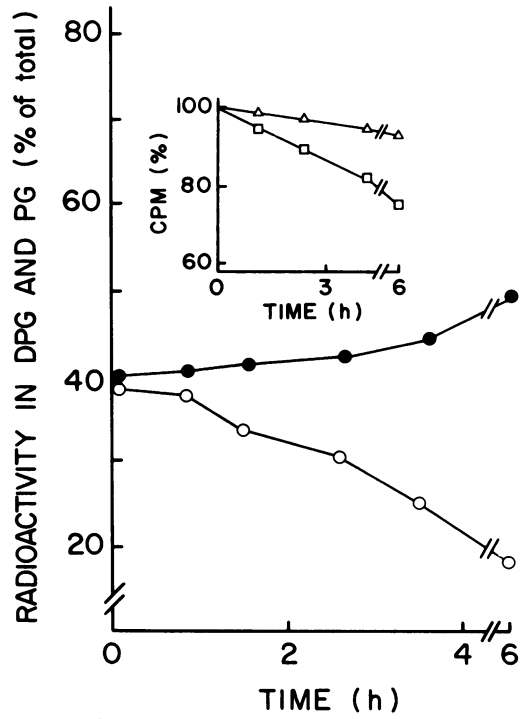

FIG. 3. Turnover of radiolabeled phospholipids in *M. capricolum*. The cells were grown with [1-^{14}C]palmitate. After 10 h of incubation, the cells were sedimented, washed, and suspended in a fresh unlabeled medium. Growth was continued, and at the time intervals indicated, samples were withdrawn and lipids were extracted and analyzed as described in Materials and Methods. ○, Radioactivity in PG fraction; ●, radioactivity in DPG fraction; inset, turnover of the fatty acid and glycerol moieties of the total phospholipid fraction. The cells were grown with [9,10-*n*-^{3}H]palmitate and [2-^{14}C]glycerol and treated as described above. Results are presented as the percentages of total radioactivity that remained in the lipid fraction. □, Glycerol; △, palmitate.

TABLE 4. Incorporation of radioactive PG into the lipid fractions of *M. capricolum*[a]

Lipid fractions	Radioactivity (cpm) in lipid fractions from growth medium		Growth medium $^3H/^{14}C$ ratio	Radioactivity (cpm) in lipid fractions from *M. capricolum*		*M. capricolum* $^3H/^{14}C$ ratio
	^3H	^{14}C		^3H	^{14}C	
Total Lipids	355,000	44,000	8.1	166,000	20,000	8.4
PG	336,000	42,000	8.0	114,000	15,000	7.6
DPG	Traces	Traces		52,000	5,500	9.5

[a] Cells were grown in a medium containing 0.5% albumin, oleic acid and palmitic acids (10 μg of each per ml), 20 μg of cholesterol per ml, and radioactive PG (5 μg/ml) labeled with ^{14}C at the glycerol moiety and with ^3H at the palmitic acid moiety. Lipids were extracted and analyzed as described in Materials and Methods.

suggests that the ratio of these two phospholipids is not a crucial factor in normal cell growth, supporting the findings of Raetz and co-workers (17). It appears that the PG-to-DPG conversion in aged *M. capricolum* cells is associated with changes in the fatty acid composition of membrane lipids. As the cells aged, the unsaturated-to-saturated fatty acid ratio in *M. capricolum* membranes was increased, apparently due to the exhaustion of palmitic acid from the growth medium rather than to the cells entering late phases of growth. When the unsaturated-to-saturated fatty acid ratio was maintained unchanged by supplementing the growth medium with excess palmitic acid, DPG accumulation was completely inhibited even in aged cells. The requirement for more unsaturated phospholipids for the biosynthesis of DPG may be associated with our finding that the DPG formed, although synthesized from preexisting PG residues was more unsaturated than the bulk membrane PG. Thus, DPG was more efficiently labeled by oleic acid, and its unsaturated-to-saturated fatty acid ratio was significantly higher than that of PG. The more unsaturated nature of DPG suggests that only the more unsaturated pool of PG served as a precursor. Various PG pools differing in the degree of unsaturation in mycoplasmas have previously been described (22). It is conceivable that in a saturated-fatty-acid-poor medium, the size of the more unsaturated PG pool will be increased. We cannot exclude the possibility however, that for the PG-to-DPG conversion, a highly fluid membrane is required. Although the overall fluidity of the bulk membrane lipids of *M. capricolum* seemed to be unchanged throughout the growth cycle, the possibility that distinct compartments differing in lipid composition exist within the membrane cannot be ruled out.

The physiological function of the PG-to-DPG conversion is not known. However, since DPG may be induced in the presence of cytosolic Ca^{2+} to form nonlamellar phases (8, 26), it has been suggested that the DPG-to-PG increase is part of a control mechanism to maintain an intermediate membrane lipid structure (11, 26). These structures would contain a balanced mixture of bilayer and nonbilayer lipids that would have to satisfy the structural role as well as participate in various membrane-mediated processes (10, 26). In *A. laidlawii* such balance is maintained by varying the molar ratio between monoglucosyldiglyceride and diglucosyldiglyceride as well as that between ionic and nonionic membrane lipids (28, 29). These variations occur as a response to the fatty acid composition of the growth medium, cholesterol content, and changes in growth temperature and have been explained by using the lipid-molecular-geometry hypothesis (27, 28).

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