J. Membrane Biol. 56, 49–53 (1980)

The Journal of
Membrane Biology

Modification of Cell Membrane Lipids in *Micrococcus lysodeikticus* Induced by Pantoyl Lactone

John H. Johnson, E.A. Grula, Richard Staerkel, B.M. Fung, and B.L. Mangum

Department of Cell, Molecular and Developmental Biology, Oklahoma State University, Stillwater, Oklahoma 74078, and Department of Chemistry, Oklahoma University, Norman, Oklahoma 73069

Summary. Growth of *Micrococcus lysodeikticus* in the presence of pantoyl lactone brings about both qualitative and quantitative changes in cell membrane lipids. Significant amounts of the two major phospholipids (phosphatidylglycerol and diphosphatidylglycerol) are converted to lyso forms; the largest conversion occurs in the phosphatidylglycerol. In addition, amounts of several phospholipid fatty acids are changed. Physical alteration of the cell membrane can be demonstrated using differential scanning calorimetry. Although growth and transport are significantly inhibited when pantoyl lactone is present, cells possessing altered cell membrane phospholipids and phospholipid fatty acids, brought about by growth in the presence of pantoyl lactone, transport D-alanine, L-glutamic and L-aspartic acid normally when washed free of the pantoyl lactone.

Key words: *Micrococcus lysodeikticus*; cell division, cell membrane, phospholipids, fatty acids, pantoyl lactone, transport, lysophospholipids.

Cell division in both Gram-positive and Gram-negative bacteria is sensitive to a diverse group of agents including several D-amino acids, vancomycin, penicillin, D-cycloserine, mitomycin *c*, aminopterin, hydroxylamine and non-ionizing as well as ionizing radiation. One compound, pantoyl lactone, reverses and/or prevents the cell division inhibition regardless of the inducing agent (Grula, 1960; Grula & Grula, 1962; 1964; Grula & King, 1970; 1972; Adler & Hardigree, 1965). The positive effects of pantoyl lactone on cell division are usually observed within one generation (about 1 hr). Evidence indicates that the beneficial solvating effects on cell membrane components could result from the hydrogen and van der Waals bonding abilities of pantoyl lactone (Johnson et al., 1978).

Along with the short-term effects on growth and division of *Micrococcus lysodeikticus* and *Erwinia carotovora*, we have studied the effects of prolonged growth in the presence of pantoyl lactone on membrane components. Profound effects of pantoyl lactone on cell membrane lipid composition have been observed, and these changes, as well as their consequences on transport in *M. lysodeikticus*, are the subject of this paper.

Materials and Methods

Conditions for growth and handling of *M. lysodeikticus* disIIp$^+$ have been described elsewhere (King & Grula, 1972). Pantoyl lactone was added to the growth medium to a final concentration of 0.15 M after 12 hr growth. Cell membranes were isolated according to the procedures of Butler, Smith and Grula (1967), and washed once in deionized water before lyophilization.

Total lipid extractions were performed by the method of Folch, Lees and Sloane-Stanley (1957). Phospholipids were fractionated, using thin-layer chromatography on glass plates coated with silica gel G (EM Reagents, Westbury, N.Y.). After development in a solvent system of chloroform methanol water (65:25:4, vol/vol/vol), phosphate-containing lipids were located using $FeCl_3$-sulfosalicyclic acid (Wade & Morgan, 1953). Final identifications were made by comparing R_f values with known standards (Sigma Chemical Co., St. Louis) and reaction with periodate-Schiff reagent (Shaw, 1968). Lysophospholipids were identified in the same manner except that standards and control cell membrane fractions were treated with phospholipase-A_2 (Type I, Sigma Chemical Co., St. Louis). The percentage composition of phospholipids were determined using labeled lipids obtained after growth in 100 ml of medium containing 2 μCi each of L-aspartic acid-U-[^{14}C] (154 mCi/mM) and L-glutamic acid-U-[^{14}C] (206 mCi/mM). Phospholipids used for fatty acid analyses were eluted from the thin-layer plates with chloroform/methanol (2:1, vol/vol).

Fatty acid methylation was accomplished using a modification of the procedures of Nelson and Freeman (1960). Methylation was carried out in the presence of 2% H_2SO_4 and the resulting methyl esters were extracted into hexane. Identification of the fatty acids was accomplished using mass spectroscopy and gas liquid chromatography. Mass spectra were evaluated according to the criteria of Rhyage and Stenhagen (1960). The gas-liquid chromato-

0022-2631/80/0056-0049 $01.00

graph was a Perkin-Elmer 990 equipped with a hydrogen flame detector. A glass column (6′ × 1/4″) packed with 20% diethylene glycol succinate on a Chromosorb support was used. Quantitation was achieved by comparing extractions of both internal and external standards to samples extracted from membranes and whole cells of known dry weight.

Transport of D-alanine-U-[^{14}C], L-glutamic acid-U-[^{14}C] and L-aspartic acid-U-[^{14}C] was measured as previously described (Grula & King, 1971). Differential scanning calorimetry of isolated membranes and whole cells was accomplished using a Perkin-Elmer DSC-2 unit. The sensitivity of the system was 0.2 mcal/sec and the heating rate was 5°/min. All preparations were sealed in stainless steel sample pans (38–40 µl) after centrifugation in ethylene glycol-0.85% NaCl (50:50, vol/vol).

Results

Fatty Acid Composition of the Total Lipid Extract

Table 1 shows the fatty acids present in the chloroform/methanol extracts from control cells and cells grown in the presence of pantoyl lactone for varying lengths of time. Although the beneficial effects on growth of *M. lysodeikticus* in the presence of pantoyl lactone are observed within 1 hr, no changes can be detected in lipid composition until 4 hr growth in the presence of the compound and the changes are still minimal.

After 6 hr growth in the presence of pantoyl lactone, the *ai*-15:0 fatty acid, which is the major fatty acid of this organism, is decreased from 68% (47.3 µg/mg cell dry wt) to 49% (27.9 µg/mg cell dry wt), whereas the *i*16:0 and 16:1 fatty acid are increased from 13% (8.9 µg/mg cell dry wt) to 27% (15.7 µg/mg cell dry wt) and 1% (0.7 µg/mg cell dry wt) to 4% (2.5 µg/mg cell dry wt), respectively.

After 12 hr growth, the *ai*-15:0 fatty acid is decreased even further; in addition, less than one-half of the amount of fatty acids found in control cells is present. These same changes are observed in cells grown in the presence of both pantoyl lactone and D-cycloserine; therefore, only data from cells grown in the presence of pantoyl lactone are shown.

Fatty Acids of Isolated Membranes

Isolated cell membranes from cells grown in the presence of pantoyl lactone for 6 hr exhibit fatty acid compositions very similar to those obtained using whole cells for extraction (Table 2). A decrease in the relative amount of the *ai*-15:0 fatty acid from about 65% of total fatty acid in the control cell membranes to 49% of the total in cell membranes from cells grown in the presence of pantoyl lactone reflects about the same degree of change observed using whole cell extractions. Likewise, the increase in the *i*-16:0 fatty acid from about 11% of the total fatty acid in control cell membranes to about 25% of the total in cell membranes from pantoyl lactone-grown cells is of the same order of magnitude as seen using whole cells. Variations (dry wt basis) in the content

Table 1. Fatty acids in total lipid extracts from whole cells of *M. lysodeikticus*

Fatty acid[a]	Control cells (µg/mg cell dry wt)	Pantoyl lactone-grown cells[b] (µg/mg cell dry wt)	Control cells (µg/mg cell dry wt)	Pantoyl lactone-grown cells[c] (µg/mg cell dry wt)
	18 hr growth		24 hr growth	
i-14:0	1.3	1.0	1.2	1.2
14:0	1.5	0.5	1.5	0.6
ai-15:0	47.3	27.9	46.9	13.2
15:0	0.7	0.6	0.7	0.3
i-16:0	8.9	15.7	8.3	11.4
16:0	5.9	6.0	6.3	4.0
16:1	0.7	2.5	0.7	0.9
ai-17:0	3.7	3.0	3.2	1.3
Total	70.0	57.2	68.8	32.9

i = iso configuration; *ai* = anteiso configuration (assigned according to Rhyage and Stenhagen, 1960).

[a] Trace amounts (less than 1% of the total) of 12:0, 13:0, 18:0, 18:1 and *ai* 19:0 plus an unknown methylester exiting between *ai* 17:0 and 18:0 are also present.

[b] Pantoyl lactone was added after 12 hr growth; cells were harvested 6 hr later.

[c] Pantoyl lactone was added after 12 hr growth; cells were harvested 12 hr later.

Table 2. Fatty acid content of cell membranes from *M. lysodeikticus* grown in the presence or absence of pantoyl lactone

Fatty acid[a]	Control cell membrane (µg/mg cell membrane dry wt)	% of total	Pantoyl lactone-grown cell membrane (µg/mg cell membrane dry wt)[b]	% of total
i-14:0	3.9	2.0	2.0	1.8
14:0	3.9	2.0	1.1	1.0
ai-15:0	126.8	65.4	55.8	49.3
15:0	3.9	2.0	1.0	1.0
i-16:0	21.0	10.8	28.4	25.0
16:0	13.5	6.9	8.0	7.1
16:1	9.5	4.9	5.1	4.5
ai-17:0	9.5	4.9	10.6	9.4
Total	192.0		112.0	

[a] Designations are the same as given in Table 1.

[b] Pantoyl lactone was added after 12 hr growth; cells were harvested 6 hr later.

of 16:0 (down 41%) and 16:1 (down 47%) are also seen. The total fatty acid content of cell membranes isolated from cells grown in the presence of pantoyl lactone is also decreased. This indicates that the fatty acid alterations are not simply due to decreases in cellular free fatty acid pools. In addition, leakage of lipids into the growth medium does not occur in the presence of pantoyl lactone (data not shown).

Membrane Phospholipids and Their Constituent Fatty Acids

When the phospholipids from control cells and cells grown in the presence of pantoyl lactone (and D-cycloserine plus pantoyl lactone) are separated using thin-layer chromatography, a change in the phospholipid spectrum can readily be observed in cells exposed to pantoyl lactone (Table 3). In addition to an increase in diphosphatidylglycerol, significant amounts of lysodiphosphatidylglycerol are also present. Further, the amount of phosphatidylglycerol is significantly decreased. Reaction with the periodate-Schiff reagent shows that most, if not all, of the phosphatidylglycerol is present as lysophosphatidylglycerol. Because the R_f value of this compound is very close to that of phosphatidylinositol, satisfactory quantitation was difficult to accomplish.

Fatty acids present in each of the phospholipids obtained from control and pantoyl lactone-grown cells are also shown in Table 3. The fatty acid profiles and content of the individual phospholipids are very similar to those seen when total extracts from either control or pantoyl lactone-grown whole cells or isolated cell membranes are analyzed (*compare* with data given in Tables 1 and 2). Phosphatidylinositol is an exception. Content of palmitic acid in this phospholipid is significantly greater than that seen in the other two phospholipids (control cells). It may be significant that a greater relative amount of this fatty acid is always present in phosphatidylinositol isolated from cells grown in the presence of pantoyl lactone.

Another difference noted in the fatty acid content of individual phospholipids of pantoyl lactone-grown cells relates to the *i*-16:1 fatty acid. When either whole cells or isolated cell membranes are extracted using chloroform/methanol, more of this fatty acid is present than is seen when phospholipids are individually analyzed. Small amounts of this fatty acid exist free in the cell pool and in association with carotenoids; however, these amounts are not sufficient to make up for the differences observed.

An unidentified compound which migrates to a slightly lower R_f (about 0.7) value than DPTG is present only in cells grown in the presence of pantoyl lactone. This compound contains no fatty acids, carbohydrate, phosphate, or free amino groups, and gives a blue-white fluorescence emission under ultraviolet irradiation. It is not pantoyl lactone as judged by lactone indicator reagents or R_f values.

Table 3. Fatty acids of the individual phospholipids in membranes from control cells and cells grown in the presence of pantoyl lactone[a]

Phospholipid	% Total lipid	μg/mg memb.wt.	*i*14:0	14:0	*ai*-15:0	15:0	*i*16:0	16:0	16:1	*ai*-17:0
DPG										
Control	38.7	100.9	1.0	2.0	65.8	2.0	12.1	8.1	2.9	5.9
PL-grown	52.7	86.3	2.0	4.0	46.3	2.0	21.1	16.1	4.8	2.1
l-DPG										
Control	Not detected	–								
PL-grown	20.4	33.4	1.0	2.0	43.0	1.0	16.3	26.1	4.9	3.2
PG										
Control	40.7	106.1	1.0	3.0	61.2	1.0	11.2	10.0	5.7	4.7
PL-grown	trace	–								
l-PG										
Control	Not detected	–								
PL-grown	8.5	13.9	1.0	2.0	43.3	1.0	26.1	19.6	4.4	2.0
PI										
Control	13.6	35.8	2.0	4.0	56.3	1.0	8.2	17.1	5.5	4.8
PL-grown	11.5	18.8	1.0	2.0	30.6	1.0	17.1	37.9	6.2	2.9

[a] Figures are reported as % of total fatty acid present in each phospholipid (fatty acids in trace amounts are not included). Pantoyl lactone was added at 12 hr, and cells were harvested 6 hr later. Figures are the mean of 3 determinations, and designations are given in Table 1. *Abbreviations:* DPG, diphosphatidylglycerol; *l*-DPG, lysodiphosphatidylglycerol; PG, phosphatidylglycerol; *l*-PG, lysophosphatidylglycerol; PI, phosphatidylinositol. Free fatty acid and carotenoids are not given and account for 7% of total lipid in control cells and 8.1% of total lipid in PL-grown cells.

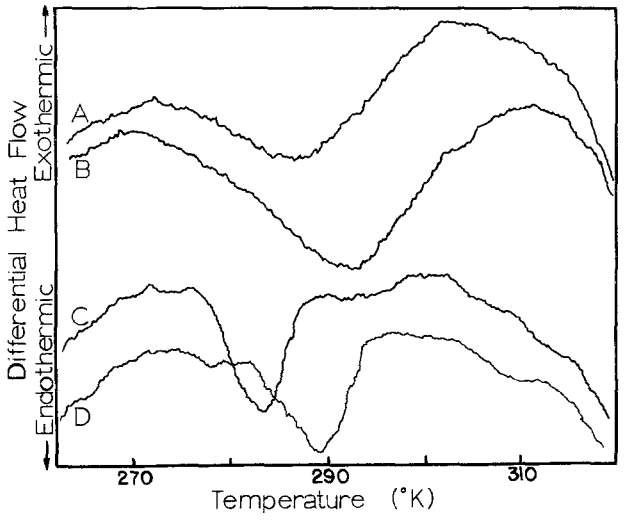

Fig. 1. DSC of whole cell and membranes of (*A*) whole cells grown 24 hr (melting range, 287.2±2.2 °K); (*B*) whole cells grown for 12 hr in pantoyl lactone (melting range, 291.6±1.5 °K; (*C*) membrane preparation of *A* (melting range, 283.4±0.8 °K); (*D*) membrane preparation of *B* (melting range, 289.0±0.6 °K)

Table 4. Transport of D-alanine, L-glutamic acid, and L-aspartic acid following growth in the presence of pantoyl lactone for 12 hr

	nmol/min/mg/cell dry wt	
Substrate	Control	+ Pantoyl lactone (30 sec)
D-Alanine		
Control cells	133.5	69.4
Pantoyl lactone-grown	133.0	70.2
L-Aspartic acid		
Control cells	41.0	12.1
Pantoyl lactone-grown	39.5	13.4
L-Glutamic acid		
Control cells	315	206
Pantoyl lactone-grown	323	200

Cells were grown with and without pantoyl lactone (0.15 M) in the growth medium for 12 hr. Cells were then harvested, washed three times in buffer salts solution and resuspended. After a 20-min incubation with aeration, the reaction was initiated by adding the appropriate labeled compound and terminated by collecting 1 ml of cell suspension on a 0.22 μm Millipore filter. The filter was washed twice with 1 ml ice cold salts solution and counted. Effects of pantoyl lactone were determined by adding pantoyl lactone to cell suspensions 30 sec prior to the addition of label.

Differential Scanning Calorimetry

To ascertain that true differences exist in the physical characteristics of the different cell membranes, differential scanning calorimetry of whole cells and cell membranes was accomplished. This procedure was chosen because pantoyl lactone induces significant changes in membrane fatty acid content and these components are important in maintaining fluidity of the structure. Data given in Fig. 1 reveal that, regardless of which type material is analyzed, cells or cell membranes from cells grown in the presence of pantoyl lactone melt at higher temperatures. The difference of about 8.2 °K is best seen in curves *C* and *D* since sharper melts are obtained using cell membranes.

Uptake Ability of Cells Grown in the Presence of Pantoyl Lactone

One of the more common tests of the functional integrity of the cell membrane is the measure of transport ability. Pantoyl lactone has been shown to inhibit the uptake of glycerol, pyruvate, malate, 2-deoxy-D-glucose, uracil, and several amino acids within 30 sec after addition to the uptake medium (Grula & King, 1971; Staerkel, 1973). In every case tested, this rapid inhibition of uptake can be completely negated by simply removing the cells from the medium containing pantoyl lactone. We therefore grew cells in the presence of pantoyl lactone for 12 hr to alter the membrane lipids and studied the effect of such lipid alterations on uptake ability.

Rate determinations for the uptake of D-alanine, L-aspartic acid, and L-glutamic acid by *M. lysodeikticus* disIIp$^+$ grown in media with and without pantoyl lactone are given in Table 4. It is significant that even after extensive qualitative and quantitative changes have occurred in cell membrane lipids, such alterations do not affect the transport of these amino acids. However, when pantoyl lactone was added to the uptake medium of such altered cells, an immediate inhibition of transport was observed (Table 4). This inhibition was very similar to that seen when pantoyl lactone was added to control cells.

Discussion

Along with other effects attributed to pantoyl lactone (Grula and King, 1971; King and Grula, 1972; Grula and Hopfer, 1972), modification of membrane lipids can now be added. These alterations are quantitative and qualitative and involve both phospholipid head groups and fatty acid moieties.

The extent to which the lipids of *M. lysodeikticus* are altered by growth in the presence of pantoyl lactone is surprising. Yet, although the membranes of such cells contain large amounts of lysophospholipids (up to 20% after 12 hr growth in the presence of pantoyl lactone), an important membrane function

such as transport proceeds at normal rates after removal of pantoyl lactone (Table 4). Also, although cell growth rate is slowed by pantoyl lactone, normal sized cells are produced even in the presence of division inhibiting agents (Grula & King, 1970; King & Grula, 1972).

It is difficult to relate all of the lipid alterations to the positive effect that pantoyl lactone exerts on cell division primarily because these alterations are not evident until cells have grown in the presence of the compound for several hours. Stimulation of cell division by pantoyl lactone is apparent within the first generation time period (about 1 hr) after addition to D-cycloserine-containing media. Further, the changes induced by pantoyl lactone result in a cell membrane lipid composition that is significantly different from that which exists in control cells wherein division activity must be considered normal. Therefore, it can be concluded that instead of the changes being necessary for division, the cells are able to carry out the events involved in this process in spite of the alterations.

Although more organisms should be examined, alteration of cell membrane lipids by growth in the presence of pantoyl lactone may be a general phenomenon. Large decreases occur in the major fatty acid (16:1) of *Erwinia carotovora* during growth in the presence of pantoyl lactone (0.077 M) and are accompanied by similar effects on transport, growth, and cell division (E.A. Grula, T.W. Rice and M.M. Grula, *unpublished*).

It should be noted that unsaturated fatty acids are equivalent to branched-chain fatty acids in that both have a melt temperature lower than their corresponding saturated or unbranched forms. That cell membranes from cells grown in the presence of pantoyl lactone have a higher melt temperature is clearly evident (data given in Fig. 1). Thus, a major effect of growth in the presence of pantoyl lactone is to bring about a decrease in lower melting fatty acids in the cell membrane. Whether such decrease is due to effects of pantoyl lactone on synthesis, incorporation, or removal of unsaturated and/or branched-chain fatty acids remains to be clarified. Preliminary experiments reveal that pantoyl lactone does not stimulate removal of any phospholipid fatty acids from isolated cell membranes of *M. lysodeikticus*.

We wish to express our sincere appreciation to Drs. George Odell and George Waller, Department of Biochemistry, Oklahoma State University, for their generous advice and use of gas chromatographic and mass spectroscopic instrumentation. This research was supported in part by an Oklahoma State University Presidential Challenge Grant Award to E.A.G. and a Research Career Development Award from the National Institutes of Health to B.M.F.

References

Adler, H.I., Hardigree, A.A. 1965. Growth and division of filamentous forms of *Escherichia coli*. *J. Bacteriol.* **90**:223

Butler, T.F., Smith, G.L., Grula, E.A. 1967. Bacterial Cell Membranes. I. Reaggregation of membrane subunits from *Micrococcus lysodeikticus*. *Can. J. Microbiol.* **13**:1471

Folch, J., Lees, M., Sloane-Stanley, G.M. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**:497

Grula, E.A. 1960. Cell Division in a species of *Erwinia*. II. Inhibition of division by D-amino acids. *J. Bacteriol.* **80**:375

Grula, E.A., Grula, M.M. 1962. Cell Division in a species of *Erwinia*. III. Reversal of inhibition of cell division caused by D-amino acids, penicillin, and ultraviolet light. *J. Bacteriol.* **83**:981

Grula, E.A., Grula, M.M. 1964. Cell Division in a species of *Erwinia*. VII. Amino sugar content of dividing and nondividing cells. *Biochem. Biophys. Res. Commun.* **17**:341

Grula, E.A., Hopfer, R.L. 1972. Cell Division in a species of *Erwinia*. XIII. Leakage of proteins from the peripheral area of filamentous cells. *Biochim. Biophys. Acta* **255**:822

Grula, E.A., King, R.D. 1970. Inhibition of cell division in *Micrococcus lysodeikticus* dis-II. *Can. J. Microbiol.* **16**:317

Grula, E.A., King, R.D. 1971. Changes in cell membranes of dividing and nondividing cells of *Micrococcus lysodeikticus* disIIp$^+$. *Biochem. Biophys. Res. Commun.* **44**:1356

Johnson, J.H., Grula, E.A., Berlin, K.D., Fung, B.M. 1978. Interaction of pantoyl lactone with membrane components. *In:* Biomolecular Structure and Function, A Symposium of Biophysical Approaches to Biological Problems. P.F. Agris, editor. pp. 115–121. Academic Press, New York

King, R.D., Grula, E.A. 1972. Condition of cell-wall mucopeptide in dividing and nondividing cells of *Micrococcus lysodeikticus* disIIp$^+$. *Can. J. Microbiol.* **18**:519

Nelson, G.J., Freeman, N.K. 1960. The phospholipid and phospholipid fatty acid composition of human serum lipoprotein fractions. *J. Biol. Chem.* **235**:578

Rhyage, R., Stenhagen, E. 1960. Mass spectrometry in lipid research. *J. Lipid Res.* **1**:361

Shaw, N. 1968. The detection of lipids on thin-layer chromatograms with periodate-Schiff reagents. *Biochim. Biophys. Acta* **164**:435

Staerkel, R.E. 1973. Effects of pantoyl lactone on transport and fatty acid composition in *Micrococcus lysodeikticus*. M.S. Thesis, Oklahoma State University, Stillwater

Wade, H.E., Morgan, D.E. 1953. Detection of phosphate esters on paper chromatograms. *Nature (London)* **171**:529

Received 18 December 1979; revised 22 April 1980