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Structures Containing Polyphosphate in *Micrococcus lysodeikticus*[1]

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Granular structures containing inorganic polyphosphate were found in *Micrococcus lysodeikticus*. These structures were isolated by fractionation of the bacterial extract obtained by lysing the organisms with lysozyme. The composition of the fraction which was enriched with these structures was found to be: protein, 24%; lipids, 30%; and polyphosphate, 27%. This fraction also contained small amounts of ribonucleic acids, carbohydrate, and polyvalent cations. The effect of different reagents and enzymes on the integrity of the granules was examined. It was noticed that they accumulate in the bacteria during the logarithmic phase of growth but disappear gradually during the stationary phase.

During our study of the process of lysis of *Micrococcus lysodeikticus* by excess lysozyme (11), electron microscopy revealed the presence of a large number of electron-dense granules in the cell lysate. Although the occurrence of similar particles has been briefly mentioned by other investigators (5, 15), no study of the nature and composition of these particles has been reported.

In this communication, we describe procedures for the isolation of these electron-dense granules and present pertinent data on their chemical and physical properties. It was established that a major constituent of these granular structures is inorganic polyphosphate (poly P), a compound which is known to occur in many microorganisms (12, 17, 29, 31, 34).

MATERIALS AND METHODS

Organism and culture medium. Strains of *M. lysodeikticus* employed were ATCC 4698 (Fleming), ATCC 12698, and J, of unidentified origin (obtained from the Department of Bacteriology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel). Cells were grown on a medium (23) composed of 0.5% Casamino Acids (Difco), 0.1% yeast extract (Difco), 1.0% glucose (autoclaved separately), and 0.1 volume of a salt solution containing 4.2% $NaHCO_3$, 0.4% $MgSO_4 \cdot 7H_2O$, 0.02% $FeSO_4 \cdot 7H_2O$, and 0.016% $MnSO_4$, which was sterilized by passage through an HA filter with 0.45-μ porosity (Millipore Corp., Bedford, Mass.). Growth was carried out in Erlenmeyer flasks incubated on a circulatory shaker (200 rev/min) at 30 C for about 20 hr until turbidity reached 300 units when read in the Klett-Summerson colorimeter, adapted with a no. 54 filter.

Preparation of the particulate fraction. The bacteria were collected by centrifugation and washed twice with 1.0 mM tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, *p*H 7.2, containing 1.0 mM $MgSO_4$. Cells were dispersed in the same buffer in one-tenth the original culture volume and 100 μg of lysozyme per ml was added. After 15 min at 37 C, lysis was complete, as judged by the decrease in turbidity and by phase microscopy. Deoxyribonuclease was added (0.1 μg/ml), and after 5 min at 37 C the lysate was centrifuged at 0 C for 1 hr at 2,000 $\times$ *g*. The sediment was suspended in the same Tris-Mg buffer and centrifuged. Electron microscopic examination revealed that the precipitate was composed mostly of the electron-dense particles and a small amount of membranelike structures. This fraction was dispersed in water, in a salt solution, or in a buffer, according to the particular experiment performed.

Reagents. Purified enzymes were purchased from the Worthington Biochemical Corp., Freehold, N.J., and from Sigma Chemical Co., St. Louis, Mo. Kieselgel DF-5 for chromatography was obtained from CAMAG AG, Muttenz, Switzerland, and activated, acid-washed charcoal, from British Drug House, Ltd., Poole, England. Other chemicals employed were analytical-grade materials obtained from various chemical supply firms.

Dry weight. Samples were heated at 105 C for 24 hr and then slowly cooled in a vacuum desiccator over silica gel.

Ash. Samples were burnt for 16 hr at 600 C, and then cooled in a vacuum desiccator over silica gel.

Spectrographic analysis. Qualitative analysis of metals in ash samples was performed in the ultraviolet (UV) and visual regions of the spectrum, by use of a large Littrow quartz and glass spectrograph (Hilger and Watts, Ltd., London, England). These analyses

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were kindly performed for us by A. Key and J. Ben-Tor at the Israel Institute of Geology, Jerusalem, Israel.

Quantitative determinations of cations. Samples were subjected to the wet ashing procedure (2). Copper was determined with the dithizone (2) and with the diethyldithiocarbamate (30) reagents; iron, with the 1:10-phenanthroline reagent (2, 30); and manganese, with the formaldoxime reagent (4). Calcium and magnesium were assayed by microtitration with ethylenediaminetetraacetate (EDTA; 30). Calcium was also assayed by flame photometry with a Kipp and Zonen (Delft, Holland) flame photometer, and spectrophotometrically with glyoxal bis-(2-hydroxyanil) reagent (26). Sodium and potassium were measured by flame photometry.

Phosphorus determinations. Total orthophosphate was routinely determined according to Fiske and SubbaRow (10) after wet ashing (2) and neutralization. In later steps of the experimental work, an alternative procedure was often employed (1).

Other quantitative determinations. Protein was determined with the phenol reagent (19); total carbohydrate was determined with the phenol-H_2SO_4 reagent (7) with glucose as a standard, and amino sugars were determined colorimetrically (9) with 2-amino-2-deoxy-D-glucose as a standard. Nucleic acids were extracted according to Schneider (25). Ribonucleic acids (RNA) and deoxyribonucleic acid (DNA) were determined colorimetrically with the orcinol and diphenylamine reagents, respectively (25). In several cases, the absorbance at 260 nm was taken as a measure of nucleic acids. Ribonucleotides in alkaline hydrolysates of RNA were detected with the help of a UV lamp after chromatography on Whatman no. 1 filter paper, with ethyl alcohol-1 M ammonium acetate, *p*H 7.5 (7:3, v/v), as the developing solvent.

Metachromasy. Measurements were performed spectrophotometrically with toluidine blue as the indicator (32).

Spectrophotometry. Readings were carried out in a Zeiss model PMQII spectrophotometer with the use of 1.0-cm light path cuvettes.

Lipids. Bacterial fractions were extracted with chloroform-methanol (2:1, v/v) or with ethyl alcohol-diethylether (3:1, v/v) solvents, and were then evaporated to dryness. Lipids were detected by thin-layer chromatography (TLC) on Kieselgel, employing as solvents petrol ether-diethyl ether-formic acid (80:20:1, v/v) for neutral lipids and chloroform-methanol-water-acetic acid (65:25:2:2, v/v) for phospholipids. Spots were developed with iodine vapors, with UV irradiation, or with an H_2SO_4 spray followed by heating to 120 C (21). Esters were determined spectrophotometrically (28). The possible presence of poly-β-hydroxybutyric acid (PHBA) was determined by a selection of several methods (24, 27).

Extraction of polyP. PolyP was extracted according to the method of Harold (13). The procedure involves an extraction with 0.5 N perchloric acid (PCA) at room temperature followed by an extraction with ethyl alcohol diethylether. A second extraction with PCA at 70 C is carried out and the nucleotides are absorbed on activated charcoal.

Determination of polyP. The experimental criteria summarized by Harold (12) were employed to estimate the presence and contents of polyP. These determinations are mainly based on the fact that polyP does not adsorb on charcoal and is totally hydrolyzed to orthophosphate by 15-min hydrolysis in 1 N HCl at 100 C. The phosphate liberated by hydrolysis was determined spectrophotometrically as described above. The orthophosphate liberated forms a complex with molybdate which is soluble in organic solvents (1), whereas polyP does not form this soluble complex. The polyP components could also be precipitated at *p*H 3.0 as the Ba^{++} or Ca^{++} salts, but recoveries in this case were not complete (12).

Electron microscopy. Samples were layered on copper grids (200 mesh) covered with a film of collodion. The dried preparations were shadow-casted with platinum. Samples for ultrathin sections were fixed with glutaraldehyde and OsO_4, dehydrated, and embedded in Epon, according to the procedure described by Pease (22). Thin sections were prepared with a Porter-Blum-type ultramicrotome (Ivan Sorvall Inc., Norwalk, Conn.), layered on copper grids, and stained with uranyl acetate and lead citrate (22). An RCA model EMU-3G electron microscope was used.

RESULTS

Electron microscopy. Precipitates collected by centrifugation of *M. lysodeikticus* suspensions which were obtained by lysis of cells with excess lysozyme (11) showed the presence of electron-dense particles trapped in a membranelike structure (Fig. 1). These particles were also observed with and without shadow casting in bacterial lysates prepared with low concentrations of lysozyme (Fig. 2 and 3). The isolated electron-dense particulate fraction, which was prepared as described in Materials and Methods, contained only small amounts of other contaminating cellular material (Fig. 4). In the cell, the electron-dense particles, which have a diameter of 40 to 80 nm, are organized around a granulated center 150 to 300 nm in diameter. These are seen in ultrathin sections of the bacteria, where the whole structure resembles a rosette (Fig. 5). The two components of the structure, namely, the dense particles and the granulated center, could be detected also in sections of the isolated particulate fraction (Fig. 6). All three strains of *M. lysodeikticus* studied during the present investigation were found to have these particles. The ATCC 4698 strain seemed to be particularly rich in them.

Density. Centrifugation in a sucrose gradient (3) has indicated that the particles sedimented in a sucrose concentration equivalent to a density of 1.23.

Weight. From gravimetric determinations, it was estimated that the particles constituted 9 to

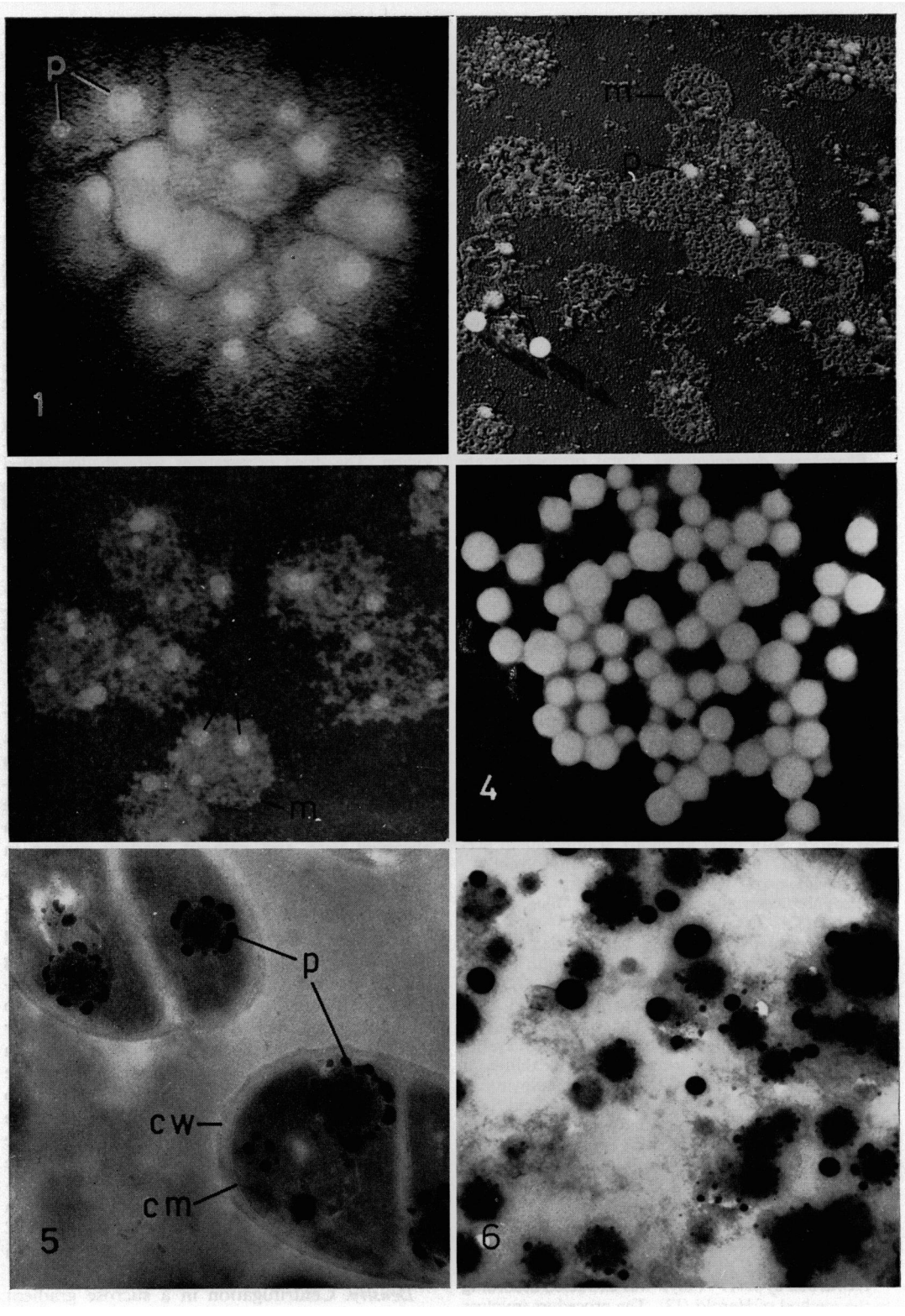

FIG. 1. *Lysozyme-lysate of M. lysodeikticus ATCC 4698, obtained by incubation with high concentration of lysozyme (1 mg/ml for 10 min at room temperature). The letter p indicates particles. × 12,000.*

FIG. 2. *Lysozyme-lysate of M. lysodeikticus ATCC 4698, with low concentration of lysozyme (0.01 mg/ml for 10 min at room temperature). L = latex sphere, 0.269 μ in diameter; m = membrane; p = particles. × 12,000.*

FIG. 3. *Same as Fig. 2, but without shadow casting.*

FIG. 4. *Particulate fraction from lysozyme lysate of M. lysodeikticus ATCC 4698 isolated by centrifugation at 2,000 × g for 60 min at 0 C. No treatment. × 30,000.*

FIG. 5. *Thin section of M. lysodeikticus ATCC 4698. Cell wall (cw), cytoplasmic membrane (cm), and particles (p) are indicated. × 50,000.*

FIG. 6. *Thin section of isolated particulate fraction from M. lysodeikticus ATCC 4698. The granulated center and electron-dense particles are shown. × 80,000.*

12% of the dry weight of cells and that 35 to 40% of the weight of the particles could be accounted for as ash (Table 1).

Components of the particulate fraction. The main components of the particulate fraction were found to be as follows: protein, 24%; lipids, 30%; and polyP, 27%. Carbohydrates, RNA, and metals were also present (Table 2). DNA and amino sugars were not detected in this fraction. PHBA was not detected in the particles or in the whole bacteria.

The particulate fraction contained 42 to 43% of the total phosphate of the cell (Table 3). About 90% of the phosphate in the particles was found in the PCA extracts and could be identified as polyP, whereas the rest was in phospholipids and RNA. About a half of the polyP was soluble in cold PCA ["soluble polyP" (12)]; the remainder was soluble only in hot PCA. The ash contents of the particles (Table 1) can thus be accounted for by 77% $(PO_4^{-})_n$ and 13% cations (Table 2). The residual unidentified ash material is probably composed of other unidentified metal cations, inorganic anions, and some oxygen which was combined to form metal oxides during the process of ashing. Preliminary evidence obtained by high-speed sedimentation velocity and sedimentation equilibrium analyses, as well as by chromatography on paper and Sephadex gels, indicated that the average molecular weight of the sodium polyP in the cold PCA-soluble fraction is about 10^3, whereas that of the hot PCA fraction which was polydisperse, is in the range of 10^5 (experiments performed in collaboration with Z. Eshhar).

Metachromasy. Samples of the particulate fraction added to toluidine blue solution caused a change in the color of the solution from blue to pink (Table 4). This metachromatic reaction indicates the presence of an acidic polymer (32).

Metals. Qualitative spectrographic determinations indicated that the particulate fraction is rich in transition metals but poor in alkali metals. Quantitative chemical analysis confirmed these findings and revealed the presence of a notable concentration of alkali-earth metals (Table 5).

TABLE 1. *Dry weight and contents of ash in fractions from M. lysodeikticus ATCC 4698*

Fraction	Dry wt[a]		Ash[a]		
	Amt (mg)	Per cent	Amt (mg)	Per cent	Percentage of dry weight
Whole bacteria	745.5	100.0	89.9	100.0	12.0
Particles	86.5	11.6	33.0	36.8	38.0
Supernatant fluid	656.5	88.2	55.9	62.2	8.5

[a] Per liter of culture. Cells in the log phase were obtained and fractionated as described in Materials and Methods.

TABLE 2. *Components of the particulate fraction from M. lysodeikticus ATCC 4698*

Component	Amt (μg)/mg of dry wt
Protein	240
Lipids[a]	292
PolyP[b]	268
Carbohydrates[c]	89
RNA	17
Metals[d]	30
Amino sugars	<1

[a] Phospholipids and neutral lipids.
[b] As $(PO_3^{-})_n$.
[c] Analyzed as glucose.
[d] Sum of individual analyses as described in Table 5.

TABLE 3. *Distribution of phosphate in fractions from M. lysodeikticus ATCC 4698[a]*

Fraction	Phosphorus	
	Amt (μg)/mg of dry wt	Per cent
Lysate	26.7	
Supernatant fluid	16.9	
Particles	113.8[b]	100.0
Cold PCA extract[c]	3.5	3.1
Cold PCA extract after hydrolysis[c]	59.3	52.1
Hot PCA extract[c]	22.6	19.7
Hot PCA extract after hydrolysis[c]	45.6	39.8
Lipids	5.1	4.5
RNA	1.7	1.5
Residue	0.15	0.1

[a] Sequential extractions of the particulate fraction were performed with cold PCA, ethyl alcohol-ether and hot PCA (13). Nucleic acids and nucleotides in the PCA extracts were adsorbed on activated charcoal. Absorption spectra of the PCA extracts were read before and after treatment with activated charcoal. Orthophosphate was assayed in the PCA extracts before and after acid hydrolysis (in HCl, 1 N, at 100 C for 15 min). Total phosphate was determined as described in Materials and Methods.
[b] This value is equivalent to 290 μg of $(PO_3^{-})_n$ which constitutes 77% of the ash contents of the particles (Table 1).
[c] Analyzed as orthophosphate.

It is likely that the polyvalent metals are bound to the polyP, thereby contributing to the high electron density of these granules. The calculated cationic content of the fraction (Table 5) was found to be 2.08 μeq/mg (dry weight), of which 2.02 μeq was polyvalent cations, mainly magnesium (1.56 μeq). The polyP content was 3.31 μeq/mg (dry weight). Thus, even if all the cations present are bound to the polyP, 40% of the negative charges of the polyP are still free for other electrostatic interactions.

TABLE 4. *Metachromasy of the particulate fraction from M. lysodeikticus ATCC 4698*[a]

Sample	Particles			Optical density		
	Dry wt/ (μg/ml)	Total P_i (μg of P/ ml)	PolyP (μg of P/ml)	530 nm	630 nm	Ratio, 530/630
I	0.0	0.00	0.00	0.308	1.105	0.278
II	3.3	0.38	0.34	0.364	1.006	0.344
III	16.7	1.90	1.70	0.483	0.377	1.281
IV	50.0	5.70	5.15	0.565	0.307	1.840

[a] Reaction systems contained the particulate fraction at different concentrations and toluidine blue at a final concentration of 1.5 μg/ml. Absorbance was read as described in Materials and Methods.

Lipids. The lipid fraction isolated from the particles was found to contain both neutral lipids and phospholipids. Separation of the phospholipids by TLC yielded two spots. One of these moved as cardiolipin, whereas the other moved as phosphatidic acid. Separation of the neutral lipids by TLC gave only one spot with a mobility similar to that of a cholesterolester. Two spots were detected by TLC analysis of an alkaline hydrolysate of the lipids. One of them moved as palmitic acid and the second moved more slowly. The ratio of ester bonds to phosphorus in the lipid fraction was 1:6.6. Thus, it is apparent that less than half of the lipids in the fraction can be accounted for as phospholipids. A detailed analysis and characterization of the lipid components present in the particles have yet to be performed.

Accumulation and disappearance of the particles. In the course of these studies, it was noted that the electron-dense structures accumulated in the bacteria during the logarithmic phase of growth and tended to disappear gradually during the stationary phase. This phenomenon occurred in the three strains of *M. lysodeikticus* which have been examined and was established either by the technique of shadow casting of lysates or by direct examination of ultrathin sections of the bacteria.

TABLE 5. *Determination of metal constituents in fractions obtained from M. lysodeikticus ATCC 4698*

Metal	Qualitative analysis[a]			Quantitative analysis[b]			Ratio, particles/ supernatant fluid
				Amt (μg) of metal/mg of dry wt			
	Lysate	Supernatant fluid	Particles	Lysate	Supernatant fluid	Particles	
Na	+[c]	+	−	0.67	0.73	0.06	0.09
K	+	+	−	24.40	25.40	2.44	0.1
Mg	+	+	+	—	4.38	18.39	4.2
Ca	+	+	+	—	1.69	7.54	4.5
Mn	+	+	++	0.06	0.04	0.21	5.2
Fe	+	+	+++	—	0.15	1.13	7.4
Cu	+	+	+++	—	0.02	0.18	10.3
Sr	+	+	+				
Ba	+	+	+				
Al	+	+	+				
Si	+	+	+				
Ti	+	+	++				
Cr	+	+	++				
Co	−	−	−				
Ni	+	+	++				
Zn	−	−	−				

[a] Qualitative analysis of the ashed fractions was performed spectrographically.

[b] Quantitative analyses of the cations were performed spectrophotometrically as described in Materials and Methods.

[c] Symbols: +, present; ++, present in excess; +++, present in great excess; −, not detected.

During starvation of washed-cell suspensions, the particles gradually disappeared. Bacteria which were shaken aerobically, in a carbohydrate-free salt solution, lost most of their dense particles after 6 hr of incubation.

Some differences in the amounts of the particles exist among the three *M. lysodeikticus* strains examined. In strains ATCC 12698 and J, the particulate fraction comprised 2 to 3% of the log-phase bacterial dry weight, as compared with 9 to 12% in ATCC 4698 grown under similar conditions. In all three strains, however, the ash content of the particles was 35 to 40% of their total dry weight.

Stability of the particles in different media. The influence of various reagents and enzymes on the integrity of the granules was examined by electron microscopy to obtain information on their structure and composition, and as guidelines for development of procedures for their purification (Table 6). The particles gradually disintegrated during several days of incubation in water at 4 C (Fig. 7 and 8). Freezing and thawing enhanced this process. In a slightly acidic medium, the particles were more stable than in alkaline solution. Incubation with detergents resulted in particle dissolution. A brief exposure to sodium deoxycholate (SDC) (5 mM, 20 min, 37 C) did not cause an apparent change in the morphology of the electron-dense particles. However, it was noted that during this treatment a large quantity of the particles' phosphate had been leached, and a prolonged incubation with SDC caused slow disintegration of the structure. Anionic detergents, such as sodium lauryl sulfate (SLS) and SDC, reduce markedly the turbidity of the particle suspensions. On the other hand, cationic detergents, such as cetyltrimethylammonium bromide (cetavlon), caused an elevation in turbidity. Nonionic detergents, such as Triton X-100, did not change the turbidity of suspensions. Different organic solvents had variable effects on the particles. Shaking with chloroform did not change the shape of the particles, but on shaking with diethyl ether the particles almost completely disintegrated. Incubation with 1.0 mM EDTA solution brought about a rapid dissolution of the particles (Fig. 11). If EDTA was added to the bacterial lysate during the isolation procedure, no

TABLE 6. *Influence of various reagents on the stability of particles*[a]

Reagent	Concn	Incubation		Particles' stability[b]
		Time (min)	Temp (C)	
Phosphate buffer, *p*H 8.0	100 mM	60	30	±
Acetate buffer, *p*H 5.0	100 mM	60	30	+
Sodium deoxycholate	200 mM	5–100	37	±[c,d]
Sodium lauryl sulfate	100 mM	30	37	−[d]
Cetyltrimethylammonium bromide	100 mM	30	37	−[e]
Triton X-100	5%	30	37	−
EDTA	100 mM	15	30	−
NaCl	200 mM	60	37	+
KCl	10 mM	15	37	+
$CaCl_2$	10 mM	15	37	+[f]
$MgSO_4$	10 mM	15	37	+[f]
Trypsin	100 μg/ml	30	37	+
α-Chymotrypsin	200 μg/ml	100	37	+
Phospholipase C	100 μg/ml	30	37	+
Lysozyme	100 μg/ml	30	37	+
Deoxyribonuclease	5 μg/ml	15	37	+
Ribonuclease	5 μg/ml	60	30	−

[a] Reaction system contained 0.5 to 3.0 mg (dry weight) of the particulate fraction per ml. Reagents were added to the suspension and incubated as described in the table. The particles were then precipitated by centrifugation, washed, and examined by electron microscopy after shadow casting. Controls were incubated in distilled water.

[b] Symbols: +, no apparent morphological changes; −, particles completely disintegrated; ±, partial disruption and disintegration of the structure.

[c] Disappearance of particles was enhanced by elevation of detergent concentration and time of incubation.

[d] Turbidity of suspension was reduced.

[e] Turbidity of suspension was elevated.

[f] Particles were more stable in these solutions than in the control suspensions.

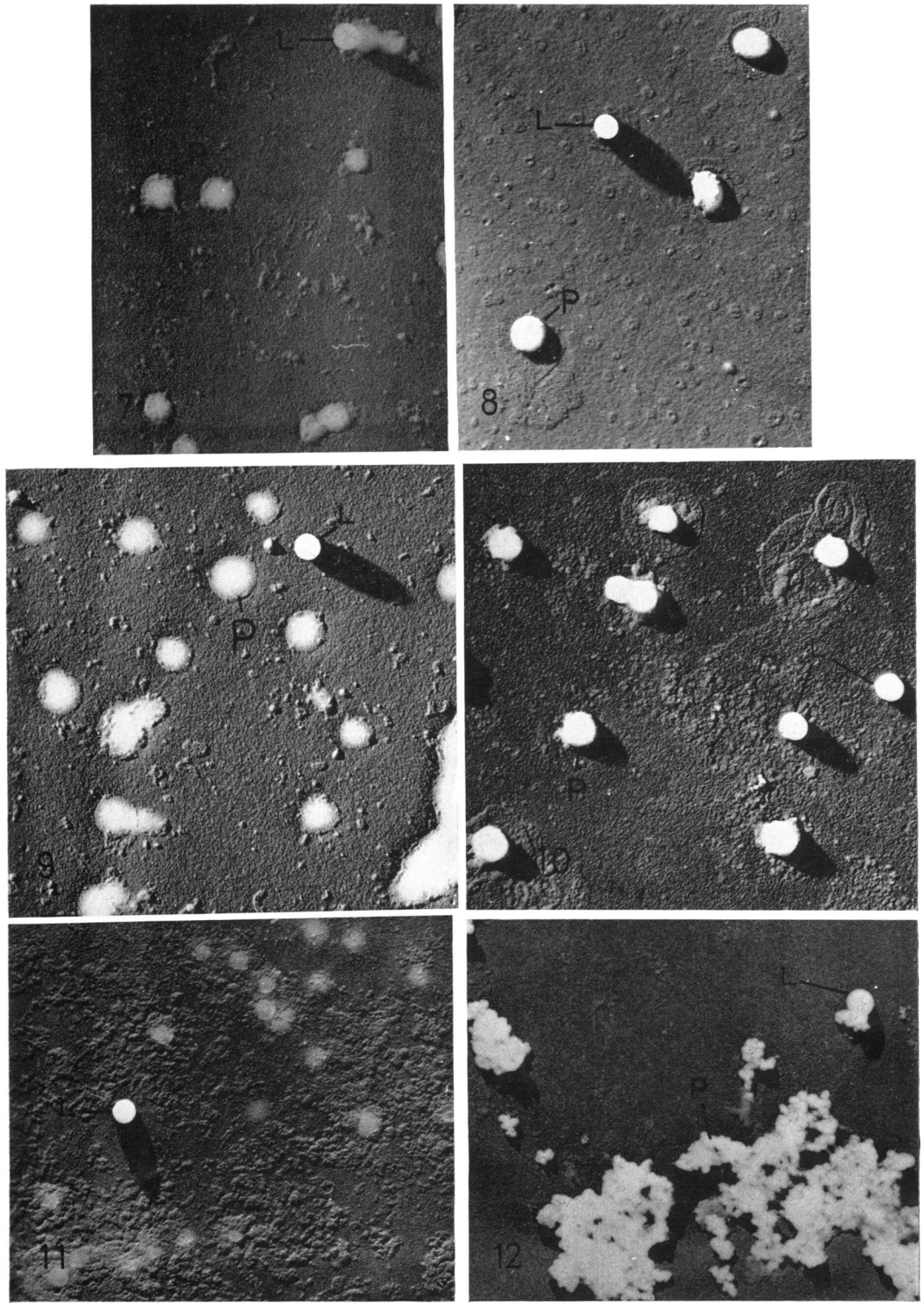

FIG. 7–12. *Shape of isolated particles from M. lysodeikticus ATCC 4698, after different treatments. The bacteria were lysed by lysozyme in presence of water, salts solutions, or EDTA. Particulate fractions were isolated by centrifugation at 2,000 × g for 60 min at 0 C. The EDTA-treated sample was centrifuged at 20,000 × g for 10 min at 0 C. The precipitates were suspended in water, salts solutions, or EDTA solution and incubated for 20 min at 37 C. After being recentrifuged at 0 C and washed with cold water, samples were taken for examination. L = latex spheres, 0.264 μ in diameter; p = particles. × 15,000. (7) Incubation in water, 48 hr at 4 C. (8) Incubation in water for 20 min at 37 C. (9) Incubation in 10 mM NaCl solution for 20 min at 37 C. (10) Incubation in 10 mM $CaCl_2$ solution for 20 min at 37 C. (11) Incubation in 10 mM EDTA solution for 20 min at 37 C. (12) Sonic treatment for 3 min at 0 C.*

precipitate was obtained after centrifugation at 2,000 × *g*. On the other hand, calcium and magnesium salts stabilized the structure of the particles. Thus, if the isolation procedure had been carried out in presence of calcium or magnesium ions, the particles obtained were rigid and compact (Fig. 10). Sodium and potassium ions (up to 200 mM) had no apparent influence on the stability of the particles (Fig. 9). As has been mentioned before, these ions are present in the particulate fraction only in very low concentrations (Table 5). Brief exposures to sonic vibrations (3 min in a 10-kc Raytheon sonic oscillator) brought about disintegration of the granules into smaller fragments (Fig. 12).

DISCUSSION

The electron-dense particulate structures in *M. lysodeikticus* contain about half of the total phosphate content of the cell, even though their weight comprises only about 10% of the cell's dry weight. Most of this phosphate is in the form of polymerized orthophosphate, namely, polyP, of which half could be defined as "soluble" and half as "insoluble" polyP (13). Different ratios of soluble to insoluble polyP have been found in different microorganisms. *Aerobacter aerogenes*, for example, contains only insoluble polyP (13).

Other major components of the particulate fraction were protein, lipids, and small amounts of as yet unidentified carbohydrate, RNA, and metals. This composition is qualitatively similar to that described for metachromatic granules in other microorganisms (12, 29, 33). Widra (33), in a detailed study employing cytochemical and enzymatic treatments of whole cells of *A. aerogenes* and *Corynebacterium xerose*, estimated that the components of volutin granules are polyP, lipoprotein, RNA, and magnesium. The direct chemical analysis of the dense particles of *M. lysodeikticus* presented in this study is in accordance with this description. Also, the metachromatic reaction given by the particles corresponds with reactions of polyP obtained from other biological and also artificial sources (16, 32).

Several points are noteworthy with respect to the isolation procedure and the chemical analysis of the particles. The precipitation of membrane-like structures together with the particles may be the result of mechanical and electrostatic interactions. Such a contamination may indeed account to a certain extent for the rather high content of protein and lipids in the particle fraction. The isolated electron-dense particles have essentially the same form, shape, or dimensions as the dense granules seen in ultrathin sections of whole bacteria when examined by electron microscopy (Fig. 5 and 6). Preliminary evidence, as can be seen in Fig. 5 and 6, indicates that the electron-dense granules are associated with a more complex subcellular structure. This aspect of cell cytology is being examined further.

The following physicochemical interactions among the different components might bring about the formation of the particulate structures. (i) Insoluble salts of polyP may form with polyvalent cations. These cations may also serve as a bridge between polyP and protein, RNA, or phospholipids (33). In the present case, it could be calculated that the cations in the particles neutralize only 60% of the negative charges of the polyP present; thus, sufficient amounts of anions are available for other types of electrostatic interactions. It is obvious that presence of polyvalent cations is obligatory for the particles' stability, since their removal by chelating agents leads to the dissolution of the particles. (ii) A protein-polyP complex might form in an interaction which leads to the formation of insoluble complexes (16, 20). (iii) RNA-polyP complexes may form. Such complexes were isolated from some microorganisms (6, 12, 29), and their detection in whole cells has been reported when Mg^{++} serves as a bridge between the polymers (33). (iv) Formation of nonspecific, electrostatic bonds between the acidic polyP molecules and other basic components in the cytoplasm.

The stability and rigidity of the particles are dependent on the composition of the medium in which they are suspended. It is notable that the particles lost their characteristic shape after treatment with reagents acting on their minor components. Thus, metal chelates and ribonuclease destroyed the structure, whereas proteolytic enzymes or phospholipase C did not bring about any apparent morphological change. It is probable that, in the latter cases, these enzymes could not come into contact with their appropriate substrates, even though they are found in the particles. During incubation in the presence of polyvalent cations, such as Ca^{++} or Mg^{++}, the particles attain a firm and rigid form. Solutions of monovalent cations have no apparent influence on the rigidity of the particles over and above that of water alone (Fig. 7–10), but prolonged incubation results in slow disintegration of the structures.

The polyP-containing particles of *M. lysodeikticus* are markedly different in their shape and organization from the randomly distributed volutin granules described in a limited number of other microorganisms (12). As indicated before, in *M. lysodeikticus* the electron-dense particles (40 to 80 nm in diameter) are part of a more complex structure and they seem to be organized

around a granulated center (150 to 300 nm in diameter) in a rosettelike pattern (Fig. 5). Another interesting difference from other microorganisms is the pattern of accumulation of particles. In most cases hitherto examined, the appearance of volutin granules and accumulation of polyP take place when a situation of unbalanced nutrition prevails. This occurs, for example, when growth ceases for physiological reasons or because of the presence of metabolic inhibitors, even though a sufficient source of energy is still found in the external medium (12, 13). In *M. lysodeikticus*, on the other hand, the polyP particles accumulate during the logarithmic phase of growth and disappear gradually during the stationary phase. Such difference may suggest that the metabolic pathways concerned with polyP biosynthesis, degradation, or aggregation, or the control mechanisms which regulate them, differ in various microorganisms (8, 12, 14, 16). The reactions which are involved with the synthesis and formation of the polyP granules in *M. lysodeikticus* have not yet been fully characterized and are the subject of a further investigation.

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