VOLUTIN GRANULES IN
ZOOGLOEA RAMIGERA

by
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STATEMENT BY AUTHOR

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Zoogloea ramigera, a Gram-negative bacillus found in activated sludge, formed volutin granules when excess orthophosphate was added to a phosphate-starved culture: Zoogloea ramigera grown in broth, containing 500 mg arginine, 200 mg MgSO₄·7H₂O, 0.005 mg biotin, 0.005 mg vitamin B₁₂, 10 g glucose, and 4 mg KH₂PO₄ per liter of double distilled water, formed few or no granules; but when 1.8 g/liter orthophosphate were subsequently added to a culture grown for 120 hr, abundant volutin granules appeared within 1 hr. These volutin granules were stainable by hydrogen sulfide after lead acetate treatment; they were extracted by 1 N perchloric acid along with the nucleic acids but were not adsorbed by activated charcoal; so apparently they were composed of inorganic polyphosphate. Optimum granule formation in the arginine broth required 10 g/liter glucose, 3 mg/liter initial phosphate ion, and 1 mg to 20 mg/liter magnesium ion; at 1 mg/liter magnesium ion very large granules appeared which often seemed to fill the cell. An excess amount of glucose, initial orthophosphate, or magnesium ion reduced granule formation. In the absence of sulfate, moderate granulation occurred in arginine broth before the addition of excess orthophosphate; granulation did not increase following the phosphate addition.
Zoogloea ramigera is a Gram-negative, rod-shaped bacterium that is strictly aerobic and has an optimum temperature between 28 C and 30 C and an optimum pH between 7.0 and 7.4. Zoogloea ramigera has been reported as being common in the flocs of activated sludge used for the purification of sewage; when activated sludge is aerated with sewage, Z. ramigera rapidly oxidizes the pollutional material present in the sewage (17).

This study was undertaken to find the conditions under which Z. ramigera will form volutin granules. More particularly this study was concerned with volutin granule production at optimum nutrient conditions and at chemical levels which occur in sewage! Also the composition of the volutin granules was studied to confirm the presence of accumulated inorganic phosphate.

Inorganic phosphate may occur as single phosphate groups called orthophosphate or as condensed phosphates in which various numbers of phosphate groups are linked together by oxygen bridges. There are two biologically occurring condensed inorganic phosphates; one kind has a ring structure and is called metaphosphate; the other kind
has an unbranched linear structure and is called polyphosphate. Polyphosphate chains vary from two phosphate units in pyrophosphate to hundreds and thousands of phosphate units (9).

Volutin granules are generally thought to contain inorganic polyphosphate because of the correlation between the amount of inorganic polyphosphate present with the size and number of volutin granules in microorganisms (9). Besides polyphosphate, volutin granules may also contain ribonucleic acid and lipoprotein (18). Some organisms rapidly accumulate large amounts of inorganic polyphosphate when given orthophosphate after phosphate starvation; this phenomenon is called polyphosphate overplus and has been described for Aerobacter aerogenes (8). During polyphosphate overplus, A. aerogenes forms volutin granules; mutants of A. aerogenes which cannot form volutin granules do not accumulate polyphosphate when phosphate is added to a phosphate-starved culture (10).

It is desirable to remove the phosphate from sewage effluent so that this wastewater when added to a lake or stream will not cause excessive growths of aquatic plants. An economical method is needed which will reduce the phosphate level in wastewater effluent to below 0.5 mg/liter in order to control the growth of algae (12). Up to 96% removal of total phosphate from sewage at a sewage plant has been reported with the use of activated sludge (16). One
purpose of this present study is to determine if phosphate uptake by volutin granule formation in *Z. ramigera* of activated sludge can occur in sewage.
CHAPTER 2

MATERIALS AND METHODS

The presence of volutin granules was detected by microscopic examination of stained smears of the organism grown in various media.

Chemicals

All chemicals used were of reagent or analytical grade. The $^{32}\text{P}$ source was carrier free $\text{H}_3^{32}\text{PO}_4$ in 0.02 N HCl, having a radiometric purity above 99%, acquired from the New England Nuclear Corp., Boston, Mass. Each liter of the scintillation counting fluid contained 4 g BBOT, 80 g naphthalene, 400 ml methyl cellosolve, and 600 ml toluene.

Equipment

Photographs of smears were taken with a Leitz Orthomat Microscope Camera. Turbidity of cultures was measured at 540 nm with a Baush and Lomb Spectronic-20 Colorimeter. Cell suspensions were centrifuged at 20,200 x g in a Servall RC-2 refrigerated centrifuge at 0 C for 20 min. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counting system, Model 314 EX-2.
Organism

Zoogloea ramigera was acquired from the American Type Culture Collection (ATCC), Rockville, Maryland, which classifies this organism as ATCC 19623; it was confirmed to be _Z. ramigera_ by the characteristics listed in the seventh edition of _Bergey's Manual of Determinative Bacteriology_ (17).

Media

Trypticase soy broth (Baltimore Biological Laboratory, Inc., Baltimore, Maryland) contains per liter: 17.0 g trypticase, 3.0 g phytone, 5.0 g sodium chloride, and 2.5 g dipotassium phosphate in 1000 ml distilled water. Trypticase soy agar slants were prepared by adding 20 g agar per liter to trypticase soy broth which contained 2.5 g glucose/liter.

Brain heart infusion (BHI) broth (Difco Laboratories, Inc., Detroit, Michigan) contains per liter: 200 g infusion from calf brains, 250 g infusion from beef heart, 10 g proteose peptone, 2 g glucose, 5 g sodium chloride, and 2.5 g disodium phosphate in 1000 ml distilled water. Slants of BHI agar were prepared by adding 20 g agar per liter of BHI broth.

Crabtree's arginine broth (2) contains per liter: 0.5 g arginine, 2 g K$_2$HPO$_4$, 1 g KH$_2$PO$_4$, 0.2 g MgSO$_4$·7H$_2$O, 0.002 mg vitamin B$_{12}$, and 0.002 mg biotin in distilled water. For better growth the vitamin B$_{12}$ and biotin contents were each raised to 0.005 mg/liter; and to insure that the
water contained no interfering ions, double distilled water was used.

The inoculating broth consisted of Crabtree's arginine broth except the amounts of $K_2HPO_4$ and $KH_2PO_4$ were reduced to one-tenth; hereafter this medium is designated as "inoculating broth." The pH of inoculating broth was 8.0 during growth of _Z. ramigera_. The standard inoculum for liquid media consisted of 0.01 ml of a stationary phase culture of _Z. ramigera_ growing in inoculating broth. The phosphate content of 100 ml of receiving liquid medium was raised only 0.02 mg/liter when inoculated with 0.01 ml of inoculating broth; this added amount of phosphate was insignificant compared with the amount already present.

The arginine broth used for experiments consisted of Crabtree's arginine broth with 4 mg/liter $KH_2PO_4$ instead of 2 g/liter $K_2HPO_4$ and 1 g/liter $KH_2PO_4$. Thus this arginine broth contained 3 mg/liter initial phosphate ion, 20 mg/liter magnesium ion, and 1 mg/liter potassium ion; this arginine broth also contained 10 g/liter glucose. Hereafter this medium is designated as "arginine broth." The pH of arginine broth and its modifications was 8.8 initially and 8.0 after 120 hr of growth of _Z. ramigera_.

Hall's medium (5), a synthetic sewage medium, contains per liter: 2.4 g glucose, 0.6 g yeast extract, 0.6 g $NH_4Cl$, 0.25 g $MgSO_4\cdot7H_2O$, 0.01 g $FeSO_4\cdot7H_2O$, 0.01 g $MnSO_4\cdotH_2O$, 0.01 g $CaCl_2\cdot2H_2O$, 0.068 g $KH_2PO_4$, 0.134 g
\text{Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O}, \text{ and } 2.65 \text{ g } \text{Na}_2\text{CO}_3 \text{ in } 100 \text{ ml tap water and enough distilled water to make 1 liter.}

Phosphate-deficient Hall's medium consisted of Hall's medium without the potassium and sodium phosphates; the 10 ml tap water per 100 ml medium furnished sufficient phosphate for growth.

**Growth Conditions**

Broth cultures of \textit{Z. ramigera} consisted of 100 ml of medium in 500 ml Erlemeyer flasks continuously shaken at 200 rpm on a New Brunswick model C. S. rotary shaker. Both the broth cultures and slant cultures were grown at 24 C.

**Measurements of Mass**

Mass measurements were either by turbidity or dry weight. Turbidity measurements were used for uniform culture suspensions. The dry weights of cultures containing flocs were determined by harvesting the whole cultures. Cells were collected on pre-weighed millipore filters with a pore size of 0.45 microns and dried at 70 C overnight before weighing.

**Preparation of Smears**

Smears of \textit{Z. ramigera} were made to enable granule counting. Cells from slants were mixed directly with water and spread on slides; cells in broths were centrifuged, washed in water, and then spread on slides; for broth cultures
containing flocs, several flocs were removed from the broth, washed twice in water, broken up with an inoculating loop, suspended in water, and spread on slides. Smears were air-dried, but they were not heat-fixed.

**Staining Procedures**

Neisser's stain was used to stain volutin granules. The methylene blue solution is made by dissolving 1 g methylene blue in 20 ml 95% ethanol, adding 50 ml glacial acetic acid, and mixing with 950 ml distilled water; the gentian violet solution is made by dissolving 1 g gentian violet in 10 ml 95% ethanol and mixing with 300 ml distilled water. The composite solution is made by mixing two parts of the methylene blue solution with one part of the gentian violet solution. The chrysoidin solution is made by dissolving 1 g chrysoidin in 300 ml distilled water. In the staining procedure the smear is covered with the composite solution for 2 min; the composite solution is washed off with the chrysoidin solution; the smear is covered with the chrysoidin solution for 2 min; the chrysoidin solution is poured off; then the slide is allowed to drain and air-dry. The cells are yellow, and the volutin granules are deep blue (1).

Tandler's inorganic phosphate stain was used to show the presence of inorganic phosphate in volutin granules. The staining procedure is as follows: the smears are fixed in 5% neutral lead acetate for 12 hr, immersed in 5% lead
acetate in 10% formalin for 24 hr, immersed in 5% lead acetate in glacial acetic acid for 15 min, and washed in distilled water; then the smears are immersed in several changes of pyridine, boiled in pyridine for 2 hr, allowed to cool, washed well in distilled water, immersed in 2% lead acetate in 20% ammonium acetate for 24 hr, immersed in 5% lead acetate in glacial acetic acid for 5 min, washed in distilled water, and stained in freshly prepared hydrogen sulfide water for 15 min (15).

When the smears are fixed in the lead acetate, the intracellular inorganic phosphate is converted to lead phosphate. Afterwards the glacial acetic acid removes the lead carbonates, tartrates, malates, and glycerophosphates; the boiling pyridine removes the lead soaps; and the ammonium acetate removes the lead sulfates and oxalates. The only stainable material left after the removal of those lead precipitates is the lead phosphate which is then converted by the hydrogen sulfide to black lead sulfide (15). The smears were counterstained with a 0.5% aqueous solution of safranin. The sites of the inorganic phosphate granules were black, and the cells were red.

Granule Counting

Volutin formation was quantified by counting the number of volutin granules formed in the cells. Smears stained for volutin were observed under a bright field
microscope, using a 10X ocular and a 97X oil immersion objective. An area near the top of the slide was chosen for granule counting where the yellow counterstain had thoroughly drained off. The number of volutin granules in each of 30 cells was recorded. The distribution of \( x \), the number of granules per cell, approximated a Poisson distribution; so the square root of \( (x + \frac{1}{2}) \) was substituted for each \( x \); and the 95% confidence limits were computed for each mean to detect significant differences between the granule counts of different smears. The transformations of \( x \) to the square root of \( (x + \frac{1}{2}) \) were: 0 became 0.707, 1 became 1.225, 2 became 1.582, 3 became 1.871, 4 became 2.121, 5 became 2.345, 6 became 2.550, and 7 became 2.739.

**Extraction Procedure**

The disposition of the orthophosphate taken up by a phosphate-starved culture of *Z. ramigera* was determined by radioactive labeling and subsequent chemical extraction. Carrier free \( H_3^{32}P_4 \) was added to a culture of *Z. ramigera* grown for 120 hr in arginine broth; after 30 min 0.2 g \( K_2HPO_4 \) and 0.1 g \( KH_2PO_4 \) were added to dilute the activity and provide chromatographic markers. After 24 hr approximately 0.1 ml of packed cells from the culture was washed in distilled water and extracted. Duplicate 0.1 ml aliquots of each extract were added to 10 ml of scintillation counting fluid for assay of radioactivity.
The extraction of the cells was by the Ogur-Rosen procedure (13), partially modified. Each reagent was used in succession to extract a different fraction of the cellular components. For each of these fractions, the cells were extracted with 2 ml of the reagent, centrifuged at 20,200 x g for 20 min, washed with 1 ml of the reagent, and again centrifuged; the combined supernatant fluids from the two centrifugations constituted the fraction.

The reagents in their order of use were: 70% ethanol for 30 min at 4 °C for the soluble fraction; ethanol-ether (3:1) for 30 min at 45 °C for the lipid and phospholipid fraction; 0.1 N perchloric acid (PCA) for 1 hr at 4 °C for the cold, acid-soluble nucleoside and nucleotide fraction; 1 N PCA for 18 hr at 4 °C for the RNA fraction; 1 N PCA for 30 min at 70 °C for the DNA fraction; and 0.1 N KOH for 30 min at 70 °C for the alkaline soluble protein fraction. The 1 N PCA fractions were adsorbed with Norit A (3); adsorption curves were plotted before and after adsorption to confirm the disappearance of the nucleic acid peak at 260 nm as measured with a Beckman DU spectrophotometer.

**Chromatography**

The components of the extraction fractions were separated and identified by paper chromatography, using Whatman no. 4 paper cut to 9 inches by 9 inches, spotted
with the fractions and with standards, consisting of $\text{H}_3\text{PO}_4$ and $\text{Na}_4\text{P}_2\text{O}_7$. Wyatt's solvent (19), consisting of isopropanol, concentrated HCl, and water, 170:41:39 v/v/v, was used. After 6 hr of migration at room temperature, the paper was sprayed with a solution containing 60% PCA, 1 N HCl, 4% ammonium molybdate, and water, 5:10:25:60, v/v/v/v (6). The paper was then air-dried and exposed to ultraviolet light at 260 mn for 10 min to develop a blue color at the positions of inorganic phosphate.
CHAPTER 3

RESULTS

Volutin granule formation by \textit{Z. ramigera} in arginine broth at different levels of various ingredients was studied.

\textbf{Growth Characteristics}

The growth characteristics of \textit{Z. ramigera} were investigated. The organism was in the stationary phase in both arginine broth and inoculating broth approximately from 72 hr until 120 hr after inoculation (Fig. 1). The time required to double cell mass and optical density of suspensions of \textit{Z. ramigera} was $5\frac{1}{2}$ hr in trypticase soy broth, 6 hr in arginine broth, 7 hr in Crabtree's arginine broth, and 8 hr in inoculating broth. If 1.8 g/liter phosphate were added to a culture grown for 120 hr in arginine broth, the cell mass tripled in 24 hr. If the glucose concentration of arginine broth was raised, there was no change in cell mass; but if glucose was deleted from the medium, cell mass was reduced to one-fourth. If the initial phosphate concentration of arginine broth was increased, there was no change in cell mass; but if initial phosphate was deleted from the medium, there was no growth. If the magnesium ion concentration in arginine broth was decreased to 2 mg/liter,
Fig. 1. Growth of Zoogloea ramigera in Arginine Broth and Inoculating Broth

Growth was at 24°C on a rotary shaker. The symbols are: ○, optical density (OD) of growth in arginine broth; O, dry weight of growth in arginine broth; ●, OD of growth in inoculating broth.
there was no change in cell mass; but if magnesium was deleted from the medium, cell mass was reduced to one-tenth.

**Granule Formation**

Volutin granules were formed by *Z. ramigera* with several types of media. Moderate granulation occurred in trypticase soy agar, and on brain heart infusion agar. Arginine broth contained only 3 mg/liter phosphate ion; therefore *Z. ramigera* grown in arginine broth normally gave a phosphate-starved culture. Abundant volutin granules were rapidly formed following the addition of 1.8 g/liter phosphate ion (0.2 g K₂HPO₄ plus 0.1 g KH₂PO₄) to a culture of *Z. ramigera* grown for 120 hr in arginine broth (Fig. 2A). No granules formed in unmodified Hall's medium upon addition of excess orthophosphate, but abundant granules were formed when 1.8 g/liter phosphate ion were added to a culture grown for 120 hr in phosphate-deficient Hall's medium. Zoogloea *ramigera* grown for 120 hr in coarsely filtered, autoclaved, activated sludge plus 2 g/liter glucose gave moderate granulation after addition of excess phosphate; no granules formed in the absence of glucose or at 0.1 g/liter glucose.

The pH of arginine broth during granule formation depended on the amount of excess orthophosphate added. When 1.8 g/liter phosphate were added to a culture grown for 120 hr in arginine broth, the pH was maintained at 7.0; this concentration of phosphate was also sufficient to maintain
Fig. 2. Photomicrographs of Volutin Granules in Zoogloea ramigera

Smears, prepared 24 hr after adding 1.8 g/liter orthophosphate to phosphate-starved cultures grown for 120 hr in arginine broth, were stained by Neisser's procedure. Magnification is 1175X. (A) Unmodified arginine broth; (B) arginine broth with 1 mg/liter magnesium ion; (C) arginine broth with 80 mg/liter magnesium ion.
pH 7.0 in all the modifications of arginine broth used during this study. If the added phosphate was 0.18 g/liter or 0.018 g/liter, the pH of unmodified arginine broth was maintained for 24 hr at pH 7.4 or pH 7.7 respectively; there was no change in granule yield.

The number of granules formed per cell varied from none to seven. If only one granule was present in a cell, it was always polar; if two granules were present, they were usually both polar at opposite ends of the cell; if three granules were present, usually two were polar with the third in the center; if four or more granules were present, usually two were polar at opposite ends with the others randomly distributed between them.

These granules apparently contained inorganic phosphate. They stained with Tandler's inorganic phosphate stain; however, they were very soluble in ammonium acetate and dissolved in that reagent despite the presence of lead acetate and despite the previous formalin treatment. If the addition of excess orthophosphate was withheld from a culture in arginine broth, granulation never occurred.

**Extractions**

In the modified Ogur-Rosen extraction (13) of cells of *Z. ramigera*, labeled by adding carrier free $\text{H}_3^{32}\text{PO}_4$ to a phosphate-starved culture in arginine broth, 11% of the activity was extracted in ethanol, 4% in ethanol-ether, 3%
in 0.1 N PCA, 51% in cold 1 N PCA, 30% in hot 1 N PCA, and 1% in KOH. Adsorption of the 1 N PCA fractions by Norit A removed the nucleic acids and left most of the activity with the unadsorbed inorganic phosphate. Likewise, on the chromatograph most activity of the 1 N PCA fractions was in the orthophosphate and pyrophosphate position at the solvent front rather than in the position of the nucleotides behind the front. Microscopically the granules remained distinct until treatment with cold 1 N PCA which caused them to disappear.

**Time Variation**

Volutin granulation in arginine broth was measured at different times. Volutin granules began to form immediately upon addition of 1.8 g/liter orthophosphate to a phosphate-starved culture grown for 120 hr; the maximum number of 2.5 granules per cell was reached in 4 hr. The ability to form granules continued with succeeding generations, maintaining the maximum number for 3 days, during which the dry weight of the cell mass increased fourfold; then the number of granules gradually decreased until 2 weeks after phosphate addition there were only 0.3 granules per cell. The age of the culture at the time of the phosphate addition could be anywhere from 4 days to 8 days without changing the number of granules formed in 24 hr.
Glucose Variation

Granulation in arginine broth was measured at different initial concentrations of glucose. In the absence of glucose essentially no granules were formed. The presence of 0.1 g/liter glucose gave abundant granules which were too faint to count. The presence of 2 g/liter glucose gave abundant dark granules. A further increase in glucose gave darker and more numerous granules; the optimum was reached at 10 g/liter glucose after which increased glucose concentration caused a gradual decrease in count (Fig. 3).

Granule counts at 0 g and 10 g/liter glucose were correlated by assaying media for loss of radioactivity. Carrier free $H_3^{32}PO_4$ was added to cultures of Z. ramigera grown for 120 hr in arginine broth of 0 g and 10 g/liter glucose; in 30 min the cells in 10 g/liter glucose removed fivefold more $^{32}P$ from the medium per mg dry weight cells than did the cells in 0 g/liter glucose.

Initial Phosphate Variation

Granulation in arginine broth was measured at different initial concentrations of orthophosphate. With only 0.6 mg/liter initial phosphate the granule yield was low; but the yield rapidly increased to abundant granulation by 3 mg/liter; then the yield rapidly decreased to a low level by 12 mg/liter (Fig. 4).
Fig. 3. Granulation in Zoogloea ramigera at Different Glucose Concentrations

Granule counts were made 24 hr after adding 1.8 g/liter orthophosphate to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.
Fig. 4. Granulation in Zoogloea ramigera at Different Initial Phosphate Concentrations

Granule counts were made 24 hr after adding 1.8 g/liter orthophosphate to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.
Magnesium Variation

Granulation in arginine broth was measured at different initial concentrations of magnesium ion. Whether or not the sulfate level was held constant had no effect on granulation. No granules were formed in the absence of magnesium ion; but 1 mg/liter magnesium gave large, dark, abundant granules which often appeared to fill the cell (Fig. 2B). The maximum number of granules occurred from 1 mg to 20 mg/liter, but at 20 mg/liter the extra large granules associated with 1 mg/liter were absent. Further increase in magnesium gave a decreased yield (Fig. 5). At 80 mg/liter magnesium the granules were faint (Fig. 2C).

Interactions

Interactions among glucose, initial phosphate, and magnesium in arginine broth were revealed by a factorial design in the analysis. The concentrations of glucose used were 2 g/liter and 10 g/liter; the concentrations of initial phosphate ion used were 3 mg/liter and 18 mg/liter; and the concentrations of magnesium ion used were 2 mg/liter and 20 mg/liter. Zoogloea ramigera was grown in arginine broth with the eight possible combinations of the above three ingredients. As usual, 1.8 g/liter phosphate were added at 120 hr, and the smears were made 24 hr later.

The results were illustrated with three-dimensional co-ordinates by drawing two planes, one representing 2 g/liter
Fig. 5. Granulation in *Zoogloea ramigera* at Different Magnesium Concentrations

Granule counts were made 24 hr after adding 1.8 g/liter orthophosphate to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.
glucose and the other 10 g/liter glucose (Fig. 6). No significant difference was found between the two planes at three of the pairs of corners, but one corner of the 2 g/liter glucose plane was irregularly elevated at 18 mg/liter initial phosphate and 20 mg/liter magnesium. This represents increased granule production due to interaction between low glucose, high phosphate, and high magnesium concentrations.

Other Variations

Granulation in arginine broth was measured at different concentrations of various other ingredients. Normally arginine broth contained 0.5 g/liter arginine; if the arginine concentration was dropped to 0.05 g/liter or raised to 1 g/liter, there was no change in granule count. Arginine broth contained 0.005 mg/liter of both vitamin B₁₂ and biotin; if the concentration of each vitamin was dropped to 0.0005 mg/liter or raised to 0.025 mg/liter, there was no change in granule count. When the potassium ion concentration in arginine broth was raised from the usual 1 mg/liter to 63 mg/liter, there was no change in count. Normally arginine broth contained no calcium; the addition of 50 mg/liter calcium ion did not affect granule formation. If magnesium chloride was substituted for magnesium sulfate in arginine broth so that no sulfate was present, moderate granule production occurred before the addition of excess orthophosphate; the number of granules did not increase
following the phosphate addition and remained lower than if the broth had contained sulfate.
Fig. 6. Granulation in Zoogloea ramigera at Different Concentrations of Glucose, Initial Phosphate, and Magnesium

Granule counts were made 24 hr after adding 1.8 g/liter orthophosphate to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.
CHAPTER 4

DISCUSSION

The immediate formation of volutin granules in _Z. ramigera_ following the addition of excess orthophosphate to a phosphate-starved culture indicated that these granules were composed for some kind of phosphate compound. The metachromatic reaction of these granules with the Neisser's stain indicated the presence of long chain polyphosphate; polyphosphates of eight or more phosphate units in length are metachromatic but not orthophosphate, pyrophosphate, metaphosphate, or polyphosphate with less than eight phosphate units (4). Nucleic acids are also metachromatic; however, they do not stain by Tandler's technique which uses hydrogen sulfide after treatment with lead acetate to give a chemically specific stain for inorganic phosphate (15).

The granules in _Z. ramigera_ were stained by Tandler's technique; however, they readily dissolved in the ammonium acetate reagent even in the presence of lead acetate. This ammonium acetate reagent is used to remove any sulfates and oxalates that may be present. Pyrophosphate and polyphosphate are less resistant to this reagent than orthophosphate. Many yeast granules after conversion to lead phosphate dissolve in this reagent unless lead
acetate is present, so apparently these yeast granules contain pyrophosphate or polyphosphate rather than orthophosphate (15). Likewise, in _Z. ramigera_ the granules did not behave as orthophosphate but were even more soluble in ammonium acetate than were yeast granules.

Polyphosphate is generally characterized as soluble polyphosphate if it is extracted in the nucleotide fraction and as insoluble polyphosphate if it is extracted in the nucleic acid fraction; the insoluble polyphosphate has a considerably higher chain length than does the soluble polyphosphate (9). In the Ogur-Rosen extraction of cells of _Z. ramigera_, labeled by adding H$_3$PO$_4$ to a phosphate-starved culture, most of the label was found in the nucleic acid fractions; and most of the label in those fractions was not removed by adsorption to charcoal. Chromatographically this label was mostly in the orthophosphate or pyrophosphate position. Thus the extraction results indicated the presence of insoluble polyphosphate in these cells of _Z. ramigera_; and from the chromatographic results it appeared that this polyphosphate was degraded considerably by the 1 N PCA used in the extraction, giving orthophosphate, pyrophosphate, and perhaps other light inorganic phosphates. The microscopic examination of the extracted cells showed that the granules remained distinct until extraction with cold 1 N (10%) PCA; apparently enough polyphosphate was either extracted or
broken down below octapolyphosphate at this point to destroy the metachromatic reaction.

If the compound which accumulated when excess orthophosphate was added to a phosphate-starved culture of *Z. ramigera* was polyphosphate as the staining and extraction procedures suggested, then this accumulation was similar to the polyphosphate overplus phenomenon described for *A. aerogenes* (8). The enzyme involved in the granulation in *Z. ramigera* was probably the same as polyphosphate kinase found in *Escherichia coli* which takes the terminal phosphate from adenosine triphosphate (ATP) and builds the polyphosphate polymer (11). In *A. aerogenes* this enzyme mediates the only route of polyphosphate synthesis; mutants of *A. aerogenes* which lack the enzyme cannot accumulate polyphosphate under any conditions (8).

The presence of glucose in arginine broth was necessary for granulation. This may be explained in terms of the polyphosphate kinase route; an energy source such as glucose provides a ready supply of ATP by the phosphorylation of ADP; this ATP is then the source of phosphate for the construction of polyphosphate by polyphosphate kinase. The decrease in granulation at higher concentrations of glucose is typical of catabolic repression.

A low initial phosphate concentration in arginine broth was required for granulation; too much initial phosphate gave a rapid decrease in granulation. In *A. aerogenes*
the synthesis of polyphosphate kinase is apparently subject to repression by exogenous orthophosphate; during phosphate starvation the specific activity of polyphosphate kinase increases 5 to 10 times (8). This same repression could account for the decreased granulation of Z. ramigera at increased concentrations of initial phosphate.

The presence of magnesium in arginine broth was required for granulation in Z. ramigera. Similarly magnesium ion has been found to be required for activity by purified polyphosphate kinase from E. coli (11). The decrease in granulation at higher magnesium concentrations is typical of cationic inhibition.

Sulfur starvation caused granulation to occur in Z. ramigera in arginine broth before the addition of excess orthophosphate. Sulfur starvation stops nucleic acid synthesis which is in competition with polyphosphate synthesis for the available ATP; when nucleic acid synthesis in A. aerogenes was stopped by sulfur starvation, polyphosphate accumulated until exogenous sulfate was added (7). Similarly the sulfur-starved Z. ramigera formed granules even though the culture was also phosphate-starved because in the absence of nucleic acid synthesis the low phosphate content was nevertheless an excess.

Chemical levels in raw sewage vary, depending on location and time. The average levels for Tucson raw sewage are 34 mg/liter orthophosphate, 2 mg/liter polyphosphate,
19 mg/liter magnesium ion, 94 mg/liter calcium ion, and 50 mg/liter combined potassium and sodium ions (personal communication). The carbohydrate level of whole sewage according to one study was found to be about 44 mg/liter carbon; most of this was glucose and sucrose (14). In terms of glucose this carbohydrate level represents about 110 mg/liter glucose.

Granulation in arginine broth showed an unexpected rise when high concentrations of phosphate and magnesium were combined with a low concentration of glucose. The levels were 18 mg/liter initial phosphate, 20 mg/liter magnesium, and 2 g/liter glucose. These phosphate and magnesium levels were close to sewage levels; however, the glucose concentration was 18 times that found in sewage. Likewise, in activated sludge granulation occurred in the presence of 2 g/liter glucose; but granulation did not occur at 100 mg/liter glucose, the level found in sewage. These results suggest that phosphate uptake from sewage by volutin production might occur only if activated sludge containing Z. ramigera were supplied with more carbohydrate during aeration.
SUMMARY

1. Zoogloea ramigera formed volutin granules when excess orthophosphate was added to a phosphate-starved culture in arginine broth which contained glucose and magnesium.

2. These volutin granules contained an inorganic phosphate compound which was extracted by 1 N PCA along with the nucleic acid but was not adsorbed by activated charcoal; this compound apparently was insoluble polyphosphate.

3. Optimum conditions for granule formation in arginine broth were 3 mg/liter initial phosphate, 10 g/liter glucose, and 1 mg to 20 mg/liter magnesium ion. At 1 mg/liter magnesium ion very large granules appeared which often seemed to fill the cell.

4. Granule formation was reduced by excess glucose, excess initial orthophosphate, and excess magnesium ion. Granulation occurred during phosphate starvation in the absence of sulfur.
REFERENCES


