VOLUTIN ACCUMULATION BY ACTIVATED SLUDGE MICROORGANISMS

by

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I hereby recommend that this dissertation prepared under my direction by Frank Andrew Roinestad

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be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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ABSTRACT

Massive deposits of volutin were found in activated sludge obtained from the Rilling Road Plant at San Antonio, Texas. These volutin deposits occurred in clusters of a coccoid, Gram-negative microorganism designated "plump". The cells of plump were 1 μm in diameter and contained much volutin. Clusters of volutin-filled microorganisms resembling plump were found in activated sludge from the sewage treatment plant at Tucson, Arizona. However, the volutin-containing clusters in Rilling sludge, a sludge with high affinity for phosphate, were considerably more developed than in Tucson sludge, a sludge with low affinity for phosphate. Also, the individual coccoid cells in Rilling sludge showed more inorganic phosphate by Ebel's stain than those in Tucson sludge. Efforts to isolate plump from Rilling sludge resulted in the isolation of a coccoid, Gram-negative bacterium, 1 μm in diameter, that formed clusters and abundant volutin on sewage and sludge media. This organism, designated P 7, formed volutin on all 21 kinds of common media on which it was grown. Isolate P 7 was a strict aerobe, nonmotile, and generally inactive biochemically. In ability to accumulate phosphate during growth, P 7 was exceptional over several other sludge microorganisms with which it was compared. By a modified Schmidt-Thannhauser extraction of P 7 cells, most of the extracted phosphate was in the soluble and insoluble polyphosphate fractions.

CHAPTER 1

INTRODUCTION

The accelerated eutrophication or enrichment of lakes and other waterways has recently become a matter of great public concern. The increased supply of nutrients to these waters promotes massive blooms of blue-green algae that choke lakes and which, upon decay, exhaust the oxygen supply in the bottom waters; the lakes become uninhabitable for fish and unattractive for recreational use. The nutrient usually held responsible for this eutrophication is phosphate. Although carbon or nitrogen may in some instances be the limiting nutrient, phosphate is generally recognized as the element which, depending on its concentration in the water, determines whether an algal bloom can occur. The largest single source of phosphates in most waterways is domestic wastewater. Half of this phosphate in wastewater comes from detergents; the rest comes from human wastes and industrial processes (8).

Many of the laundry detergents in recent use contain between 35 and 40% sodium tripolyphosphate for their builder. The builder's main role is to sequester calcium and magnesium ions present in hard water that otherwise would interfere with the surfactant. Attempts to replace phosphate builders with nonphosphate builders have been largely unsuccessful. The most promising new builder was nitrilotriacetate (NTA), but its use was suspended due to its possible role as a mobilizer of heavy metals across the placental barrier to the fetus, increasing

the probability of birth defects. Polyelectrolytes are potential new builders but have poor biodegradability. Surfactants that will work without a sequestering agent can use precipitating builders, such as carbonates and silicates; these builders provide the alkalinity necessary for soil removal but precipitate with calcium ions in hard water, leaving a deposit on clothes and washing machines (8).

The effective phosphate removal from sewage that occurs at some activated sludge treatment plants has been accounted for by two theories -- a chemical theory and a biological theory. According to the chemical theory, this enhanced phosphate uptake may be explained by slow phosphate precipitation by the calcium already present in the wastewater (7). However, many studies, including several at our laboratory (2, 3, 17, 18), have indicated that enhanced phosphate uptake is essentiallv a biological uptake by the organisms present rather than a chemical precipitation. Enhanced removal of phosphate by activated sludge does not depend on the presence of ions, is optimum within pH and temperature ranges that are optimum for microorganisms, and is inhibited by several antimetabolites that are active against a wide range of metabolic processes (2).

Although most activated sludge treatment plants, such as the one at Tucson, Arizona, have poor removal of phosphate, leaving final effluents with values still above the desired 0.5 mg phosphate per liter, some plants such as the Rilling Plant at San Antonio, Texas, and the Hyperion Plant at Los Angeles, California, have excellent phosphate removal. This superior uptake is due to luxury uptake of phosphate in excess of metabolic requirements. Adenosine triphosphate measurement,

used to quantitate the viable cell mass in sludge, showed that the complete removal of phosphate from sewage by Rilling sludge required no increase in viable cell mass (18).

The capability for enhanced phosphate uptake is inherent in the sludge itself, rather than in the sewage being treated or in the environmental conditions at the plant. Studies (2, 3) showed that whereas phosphate removal from Tucson sewage by Tucson activated sludge was poor, Rilling sludge was able to rapidly remove all phosphate from Tucson sewage; Hyperion sludge gave the same results.

Intracellular surplus phosphate in microorganisms is usually stored in the form of polyphosphate. This substance consists of linear, unbranched chains of orthophosphate groups (PO_4^{2-}) linked together by oxygen bridges; the chain length varies from pyrophosphate, which has two phosphate groups, to chains of ten thousand groups. Polyphosphate is widely distributed in microorganisms, including bacteria, blue-green algae, fungi, protozoa, and algae; it has also been detected in some higher plants and animals (10).

The absorption spectrum of certain basic dyes is shifted when they react with polyphosphate; by such metachromatic staining, intracellular polyphosphate deposits appear as metachromatic (volutin) granules. The polyphosphate content of microbial cells generally correlates with the size and number of volutin granules present (10). In Zoogloea ramigera, a bacterium commonly found in activated sludge, polyphosphate of long-chain length appeared to be the main substance formed during volutin granulation (11). Polyphosphate is not the only cellular component which will stain metachromatically; further staining, such as

precipitation of polyphosphate with lead, followed by conversion to black lead sulfide, is required for cytochemical identification. Ebel found that, at acid pH, polyphosphate of greater than eight phosphate units in length was the only substance in microorganisms that was both metachromatic with toluidine blue and reactive with lead (6).

Polyphosphate may be extracted in two fractions from cells: soluble polyphosphate, extracted by cold 5% trichloroacetic or perchloric acid; and insoluble polyphosphate, extracted with the nucleic acids by hot trichloroacetic or perchloric acid or by 1 N KOH at 35 C. The nucleotides and nucleic acids can be removed from these fractions by adsorption to charcoal; the polyphosphate remains in the supernatant fluid. The orthophosphate content of the supernatant fluid after hydrolysis in 1 N acid at 100 C is essentially a measurement of polyphosphate. Soluble polyphosphate has a smaller chain length than does insoluble polyphosphate; the insoluble form is perhaps complexed with proteins or nucleic acids (10).

CHAPTER 2

STATEMENT OF PROBLEM

The purpose of this study was to find the microbiological basis for differences in phosphate removal by activated sludges. The approach was to examine a sludge having a high affinity for phosphate in search of any organisms that, by unusually high phosphate accumulation, might account for the phosphate activity of the sludge as a whole. For comparison, a sludge having a low affinity for phosphate was examined in the same manner to detect any lack of organisms with high phosphate accumulation. Since volutin, the usual microbial storage form of phosphate, can be stained rapidly and intensely, activated sludges and isolated organisms were screened by metachromatic staining to detect the presence of volutin. The selected organisms that warranted further investigation were isolated and studied in pure culture.

CHAPTER 3

MATERIALS AND METHODS

Activated Sludges

Sludge from the Rilling Road Plant at San Antonio, Texas, was the sludge with high affinity for phosphate used in this study. The Rilling sludge was concentrated at the Rilling Plant by allowing it to settle and pouring off half the volume as supernatant wastewater; this sludge was shipped overnight to Tucson in a plastic container by bus, and was one day old when received at this laboratory where it was stored at 4 C. Sludge from the plant at Tucson, Arizona, was the sludge with low affinity for phosphate used in this study. This sludge was collected in a plastic container and within the hour was in the laboratory and stored at 4 C.

Media

Sewage agar consisted of Tucson primary effluent, filtered through Whatman no. 30 filter paper, plus 2% agar, autoclaved at 121 C for 30 min. Sewage agar with glucose consisted of Tucson primary effluent, filtered through a Gelman filter pad of 0.2 µm pore size, plus 0.1% glucose and 1.5% agar. Coarsely-filtered, autoclaved sewage was prepared by filtering Tucson primary effluent through Whatman no. 30 filter paper, followed by autoclaving at 121 C for 30 min. Autoclaved, membrane-filtered sewage was coarsely-filtered, autoclaved sewage that

was further filtered aseptically through a Millipore pad of 0.2 μm pore size. Activated sludge broth consisted of the supernatant fluid poured off of settled Tucson return activated sludge. Coarsely-filtered, autoclaved sludge broth consisted of activated sludge broth filtered through Whatman no. 30 filter paper, followed by autoclaving for 30 min. Tucson sewage (influent) has a pH of 7.6; and each liter has about 40 mg orthophosphate (13 mg phosphorus), 20 mg magnesium ion, 180 mg sulfate ion, and 100 mg carbon. Tucson final effluent has a pH of 7.7; and each liter has about 30 mg orthophosphate (10 mg phosphorus), 20 mg magnesium ion, 170 mg sulfate ion, and 20 mg carbon.

Peptone broth was a modification of Stokes's medium (13). Peptone broth consisted per liter of 1 g peptone, 100 mg glucose, 200 mg $MgSO_4 \cdot 7H_2O$, 50 mg $CaCl_2$, and 10 mg $FeCl_3 \cdot 6H_2O$ in distilled water; the pH was adjusted to pH 7.1 using 0.1 N NaOH. Peptone agar consisted of peptone broth plus 1.25% agar. Each liter of peptone broth had 2 mg phosphorus, 20 mg magnesium ion, and 96 mg sulfate ion.

Cyanophycean agar (12) contained per liter 5 g KNO_3, 100 mg K_2HPO_4, 50 mg $MgSO_4 \cdot 7H_2O$, 5 mg ferric ammonium citrate, and 15 g agar; the pH was 7.8 without adjustment. Each liter of cyanophycean agar had 18 mg phosphorus, 5 mg magnesium ion, 20 mg sulfate ion, 1 mg carbon, and 693 mg nitrogen.

Three different glucose broths were designated by the amount of phosphorus (P) contained per liter, namely, 1, 4, and 10 mg P. All these broths contained per liter 0.2 g $MgSO_4 \cdot 7H_2O$ for 20 mg magnesium ion. Also, each liter of glucose broth with 1 mg P contained 4 mg KH_2PO_4, 1 g glucose for 400 mg carbon, and 1 g $(NH_4)_2SO_4$; the other two broths

each contained per liter 0.1 g glucose for 40 mg carbon, 0.2 g $(NH_4)_2SO_4$, and 23 or 53 mg K_2HPO_4 for 4 or 10 mg P respectively. These broths were adjusted to pH 7.7 with 0.1 N KOH.

Four different modifications of Crabtree and McCoy's arginine broth (5) were designated by their arginine contents, namely, 0.01, 0.02, 0.03, and 0.05% arginine. Each 0.1 g of arginine per liter supplied 41 mg carbon. Each liter of arginine broths had 0.2 g $MgSO_4 \cdot 7H_2O$ which supplied 20 mg magnesium ion and 78 mg sulfate ion. The variable phosphate content per liter was supplied by 4, 22, or 43 mg KH_2PO_4 for 1, 5, or 10 mg P respectively, or by 0.5 g KH_2PO_4 with 1 g K_2HPO_4 for 300 mg P, or by 1 g KH_2PO_4 with 2 g K_2HPO_4 for 600 mg P. Each liter of broth with 0.5 g arginine received 0.05 mg each of vitamin B_{12} and biotin. Arginine broths were not adjusted for pH and varied within one unit of pH 8.4 during growth of microorganisms.

Microorganisms

Zoogloea ramigera ATCC 19623 and *Aerobacter aerogenes* (departmental culture collection) were the only microorganisms used which were not isolated from activated sludge or sewage in this laboratory. *Sphaerotilus natans* and bacteria designated P 1 through P 7 were isolated from Rilling activated sludge. The protozoan *Monas* and a host bacterium were isolated from Tucson sewage. Stock cultures of *S. natans* were carried on peptone agar slants; the other organisms were carried on nutrient agar slants.

Growth Conditions

Routine broth cultures were prepared with 100 ml of medium plus a small inoculum in a 500 ml Erlenmeyer flask continuously shaken at 200 rev/min on a New Brunswick rotary shaker (model C.S.) at 23 C. All other cultures were incubated at room temperature. Cultures in 125 to 250 ml separatory funnels, one-third filled with medium, were aerated in a hood at a rate of 1.5 liters air per min.

Stains

Volutin was routinely stained using Neisser's stain (4). Solution no. 1 consisted of 1 g methylene blue, 20 ml 95% ethanol, 50 ml glacial acetic acid, and 950 ml distilled water. Solution no. 2 consisted of 1 g gentian violet, 10 ml 95% ethanol, and 300 ml distilled water. Solution no. 3 was prepared by mixing 2 parts of solution no. 1 with 1 part of solution no. 2. Solution no. 4 consisted of 1 g chrysoidin in 300 ml distilled water. The smears were not heat-fixed; they were flooded with solution no. 3 for 2 min, washed off with solution no. 4, flooded with solution no. 4 for 2 min, and tilted for draining. Cells were yellow, and volutin granules were black. Photomicrographs of selected volutin-stained smears were taken with a Leitz Orthomat microscope camera.

Polyphosphate was cytochemically identified by Ebel's method (6). Smears did not require previous fixing. For the metachromatic reaction, smears were flooded with 1% toluidine blue in 10% acetic acid for 2 min and afterwards washed in 10% acetic acid. Volutin deposits were violet, and cells were light blue. For staining inorganic phosphate, smears were

immersed in a 1:1 mixture of 10% lead nitrate with 0.2 M acetate buffer of pH 3.5 for 15 min, washed in distilled water, immersed in 50% $(NH_4)_2S$ for 30 sec, and again washed in distilled water. Smears were counterstained with safranin for 1 min. Inorganic phosphate deposits were black; cells were red.

Lipid granules in bacterial smears were treated and stained by Widra's procedures (15). Free lipid was stained with 0.25% Sudan black B in ethylene glycol at 60 C for 10 min. Lipid masked by protein was stained by a 1:1 mixture of 5% citric acid with 0.25% Sudan black B in ethylene glycol at 60 C for 10 min, followed by rapid passage through 70, 50, 30, and 10% ethanol into distilled water; another treatment for masked lipid was digestion by 1% trypsin in phosphate buffer at pH 8 and 37 C for 1 hr, followed by the stain for free lipid. Extraction of lipid granules was attempted using ether-ethanol (1:1) at 60 C for 1 hr; this was followed by the stain for masked lipid using citric acid and Sudan black B.

Metachromatic granules in bacterial smears were treated and stained by a modification of Widra's procedures (15). These granules were stained by Neisser's volutin stain either without previous treatment or after one of the following treatments: extraction with ether-ethanol (1:1) at 60 C for 1 hr, digestion by 1% trypsin in phosphate buffer (pH 8) at 37 C for 1 hr, immersion in water at 80 C for 10 min, or immersion in 0.2% ribonuclease in distilled water at 37 C for 1 hr. Other smears were immersed in water at 80 C for 10 min, with or without subsequent exposure to 0.2% ribonuclease in distilled water at 37 C for

1 hr, and stained with 0.001% toluidine blue (52% dye content) in distilled water for 10 min, followed by safranin for 30 sec.

Phosphate Measurement

Phosphate was chemically measured by a modification of the stannous chloride method (1). One drop of 0.05% phenolphthalein was added to 10 ml of the sample to be tested; if the sample turned pink, strong-acid, consisting of 28% H_2SO_4 with 0.3% HNO_3, was added until the sample turned colorless. Then 8 drops (0.4 ml) of 2.5% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 28% H_2SO_4 were mixed with the sample; finally 1 drop (0.05 ml) of 2% $SnCl_2$ in glycerol was mixed with the sample; then 10 min was allowed for the formation of molybdenum blue. Percent transmission of the sample was read on a Lumetron colorimeter using a 650 nm filter. A standard curve, prepared using known concentrations of orthophosphate, was used for conversion from %T to mg orthophosphate per liter.

Measurement of the total phosphate content of a sample first required acid hydrolysis. One drop of phenolphthalein was added to 1 ml of the sample; then 3 drops (0.15 ml) strong-acid were added in excess of that required to turn the sample colorless, resulting in 1 N H_2SO_4; the sample was then autoclaved at 121 C for 15 min, diluted to 10 ml with distilled water, neutralized with 3 drops 8 N KOH, and measured for orthophosphate. If the sample contained no organic phosphate compounds, mg total phosphate minus mg original orthophosphate equaled mg polyphosphate present.

Radioactivity from ^{32}P was used as an alternative method for measurement of phosphate content. Carrier free $H_3{}^{32}PO_4$ in 0.02 N HCl, obtained from the New England Nuclear Corp., Boston, Mass., was added to flask cultures before autoclaving. Radioactivity of samples was assayed by adding 0.1 ml of the sample to 10 ml of scintillation counting fluid, consisting of, per liter, 4 g of 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene (BBOT), 80 g of naphthalene, 400 ml of methyl cellosolve, and 600 ml of toluene. The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (model 3320).

Phosphate Uptake

Each shaker flask culture used for phosphate uptake experiments was assayed for phosphate content at inoculation and at harvest. A supernatant sample was obtained at harvest by centrifugation of an aliquot of the culture at 20,200 x g for 15 min at 0 C in a Servall refrigerated centrifuge; the cell button was resuspended in the residue of the culture. The cells were then harvested by filtering the culture through a tared Millipore filter pad of 0.45 μm pore size. The filtrate picked up extraneous phosphate as it passed through the sintered glass filter bed but was saved for assay of ^{32}P activity. The filter pad was dried at 70 C overnight, allowed to cool in a desiccator, and weighed; the pad was then placed in scintillation counting fluid for assay of ^{32}P activity. Phosphate removal per mg dry weight cells was expressed as ^{32}P activity in cells, ^{32}P activity removed from supernatant fluid or filtrate, and mg P removed from supernatant fluid.

Extraction of Cells

Harold's modification of the Schmidt-Thannhauser method (9) was used for the extraction of cells. Cells were harvested from shaker flask cultures by centrifugation at 20,200 x g for 15 min at 0 C. The cell button, consisting of less than 0.1 ml packed cells, was washed once in distilled water, extracted twice with 0.5 N perchloric acid (PCA), 5 ml for 5 min at 20 C, extracted once with 70% ethanol, 5 ml for 30 min at 20 C, and extracted once in ethanol-ether (3:1), 5 ml at boiling for 1 min. Next the cells were suspended in 0.5 N KOH, 4.5 ml for 2 hr at 37 C, and then acidified with 0.5 ml concentrated (60%) PCA. The precipitate, consisting of potassium perchlorate, protein, and DNA was removed by centrifugation. The nucleotides were removed from half the volume of each PCA and KOH fraction by adsorbance with 50 mg of activated charcoal (Norit A), previously washed in 1 N H_2SO_4.

Chromatography

Ascending paper chromatography was performed with Whatman no. 1 chromatography paper cut to 9 by 9 inches. Standards included ^{32}P, uracil-2-^{14}C, intact RNA, RNA hydrolyzed in 0.5 N KOH, orthophosphate, pyrophosphate, and polyphosphates of 3, 6, 15, and 50 phosphate units. Papers were developed in Ebel's solvent (14), consisting of 75 ml isopropanol, 25 ml water, 5 g trichloroacetic acid, and 0.2 ml 58% NH_4OH. When dry, each paper was examined under ultraviolet light for nucleotides; then the paper was cut into strips. Each strip was either placed in a vial of scintillation fluid for assaying radioactivity or else was eluted in acid. Nucleic acids and nucleotides were eluted by immersing

the strip in 3 ml 0.1 N H_2SO_4 for 2 hr at room temperature; after removal of the strip, the optical density of the liquid was measured at 260 nm using a Beckman spectrophotometer. Phosphate was eluted and hydrolyzed by autoclaving the strip at 121 C for 15 min in 1 N H_2SO_4; after removal of the strip, the phosphate in the liquid was measured.

Column chromatography was performed with Sephadex G-50 gel in a column with dimensions of 1.5 x 56 cm, approximately 60 ml in volume. Samples were applied in 1 ml volumes and eluted with distilled water; the void volume was 21 ml. Fractions were collected in 5 ml volumes using a fraction collector equipped with a LKB ultraviolet absorption meter set at 260 nm. Standards used were intact RNA, RNA hydrolyzed in 0.5 N KOH, orthophosphate, pyrophosphate, and polyphosphate of 3, 6, 15, and 1130 phosphate units. Fractions were assayed for ^{32}P activity, orthophosphate, and total phosphate; optical density of the fractions at 260 nm was measured with a Beckman spectrophotometer.

CHAPTER 4

RESULTS

Plump

Fresh Rilling activated sludge was smeared on glass slides and stained for volutin by Neisser's procedure; numerous short plump rods massed in the flocs were completely black while the rest of the sludge had relatively little volutin (Fig. 1A). Other smears were stained for lead reactivity and metachromasy by Ebel's method; the plump rods (designated "plump") stained entirely black with lead and violet with toluidine blue while the rest of the sludge showed comparatively little polyphosphate. Another smear was Gram stained; the center of the flocs resisted decoloration, but at the periphery the plump rods were Gram-negative. Drops of Rilling sludge were crushed between microscope slides to break up the masses of plump, and the resultant smears were stained; plump was seen scattered throughout the smear, singly and in pairs, full of volutin by Neisser's stain (Fig. 1B), full of polyphosphate by Ebel's method, and always Gram-negative.

In a wet mount of Rilling sludge, the clusters of plump resembled bunches of grapes; the bunches were single or multiple, and either in the sludge flocs with other organisms or else separate from the flocs. The clusters varied in size from 15 to 150 µm; each cluster contained hundreds of cells. Clusters were from round to oval in form, and were flattened, resulting in a cushion shape rather than a sphere.

Fig. 1. Photomicrographs of Volutin
Deposits in Activated Sludge Microorganisms

Smears were stained by Neisser's procedure and photographed with 90 X objective. (A) Intact Rilling activated sludge; (B) crushed Rilling activated sludge; (C) intact Tucson activated sludge; (D) sludge bacterium P 7 in coarsely-filtered, autoclaved sludge broth.

In smears of Rilling sludge stained for volutin by Neisser's procedure, plump was usually spherical with an average diameter of 1 μm and a range of 0.3 to 2 μm. Some cells were oval with a length of 0.3 to 2 μm and a width of 0.3 to 1.5 μm. Almost all of the plump in sludge was in the clusters; those cells not in clusters were either single or in pairs. Plump was nonmotile and nonpigmented.

Because of its distinctive morphology and staining reactions, plump was easily distinguished from other organisms in sludge. In the wet mount, the brightly refractile, pure clusters contrasted clearly with the dull, heterogenous population of sludge. With Neisser's volutin stain, the clusters were strikingly black, and sharply outlined even if surrounded in a floc by the other organisms of sludge. With Ebel's metachromatic stain, plump clusters were violet to purple in contrast to the light blue of the surrounding sludge. When activated sludge was Gram stained, plump appeared deep red from safranin while the various other organisms present were pink and pale by comparison. With a Sudan black B stain, using safranin for the counterstain, the cells of plump were yellow, each with a definite outline even in the center of the clusters; other organisms were pink, some with black lipid deposits.

Fresh Tucson sludge was examined for the presence of organisms similar to plump. A wet mount revealed the presence of clusters of plump cells identical to those seen in Rilling sludge. Neisser's volutin stain showed these cells to be full of volutin; Ebel's stains for polyphosphate showed some of the cells to be full of polyphosphate; and with the Gram stain, these cells took the deep red safranin counterstain. By two weeks after collection, the clusters of coccoid cells in both Tucson

and Rilling sludges had lost most of their volutin; smears of the sludges crushed between slides now showed coccoid cells with all amounts of volutin from full to none.

The volutin-containing clusters in Rilling sludge were considerably more developed than those in Tucson sludge. While clusters in Rilling flocs were generally full and large, those in Tucson flocs were often small and scattered (Fig. 1C). Isolated clusters of the coccoid cells were common in Rilling sludge, but were difficult to find in Tucson sludge. While plump appeared to predominate in Rilling sludge, Tucson sludge had a more heterogeneous population of organisms with a predominance of small short rods. Crushed smears of the two sludges stained for inorganic phosphate showed almost all coccoid cells in Rilling sludge to be full of phosphate while most in Tucson sludge were only partly full of phosphate.

In the attempt to isolate plump from Rilling sludge, several isolation techniques were tried and several bacteria were isolated and tested for phosphate uptake. The first six bacteria isolated were not typical of plump and were designated P 1 through P 6. The seventh bacterium isolated was typical of plump and was designated P 7.

P 1

In the first isolation attempt, Rilling sludge was crushed between glass slides to break up the clusters of plump and then streaked out on plates of solid media, including among others sewage agar and sewage agar with glucose. Smears were prepared from each of the resultant colony types for volutin staining. Only one of the many organisms

examined occurred as plump rods in compact clusters; this organism, obtained from the sewage agar with glucose, was designated P 1. This was a nonmotile, oval rod, measuring 1.5 by 2 μm, in compact round clusters up to 20 μm in diameter. A few of these P 1 cells had volutin granules. No volutin granules were formed by P 1 on sewage agar, nutrient agar, peptone agar, or in activated sludge broth or peptone broth; however, granulation did occur in peptone broth supplemented with 0.01% glucose. The granules were confirmed to contain polyphosphate by Ebel's stains for metachromasy and lead reactivity. Isolate P 1 was identified at our laboratory as Aerobacter aerogenes.

The capability of P 1 to form volutin granules at different nutrient conditions was investigated by using glucose broth containing 1 mg P/liter. Results were as follows: at 1% glucose, no volutin accumulated; at 0.1% glucose (the usual concentration for this broth), granules were rare; at 0.01% glucose, most cells had several granules; and at 0% glucose, no growth occurred. If the above glucose broth containing 0.01% glucose was prepared with ammonium chloride and magnesium chloride so that only a trace of sulfate was present in the medium, the majority of the cells of P 1 were nearly full of volutin. No granules were formed if the phosphorus concentration of the glucose broth was increased to 30 mg P/liter or if the magnesium ion concentration was decreased from 20 to 2 mg Mg/liter. The addition of excess phosphate (600 mg P/liter) to a phosphate-starved culture grown in glucose broth with 1 mg P/liter caused P 1 to form round cells of 0.5 μm diameter that usually possessed a single volutin granule that almost filled the cell. In glucose broth with 4 mg P/liter, a broth that approximates the low

sewage concentrations of carbon and nitrogen, P 1 consisted of round cells, single and in irregular clusters; a single small volutin granule was present in some of the cells.

Sphaerotilus

Sphaerotilus natans, isolated from Rilling sludge in our laboratory, was studied singly and in association with P 1. Filamentous bacteria such as S. natans were associated in sludge with the flocs; these filaments had small granules in Rilling sludge but were without volutin in Tucson sludge. In both activated sludge broth and peptone broth, S. natans formed volutin granules.

Some very large, oval, volutin granules, measuring up to 3 x 1 μm in size, were formed in an aged culture of S. natans in peptone broth; smears of this culture were treated with various agents to study the composition of the volutin granules. The granules were not affected by immersing smears in 1% pronase at 37 C for 1 hr or by immersion in ethanol-ether (3:1) at 60 C for 1 hr, but the granules were destroyed by immersion in distilled water at 60 C for 1 hr or by immersion in 0.2% ribonuclease at 37 C for 1 hr.

On a plate of sewage agar with glucose, a single streak of S. natans was crossed with a streak of P 1; in the area of the cross, tight clusters developed which were composed of both P 1 and S. natans. No synergistic increase in granulation by P 1 occurred, and S. natans had no granules.

Zoogloea

Zoogloea ramigera (ATCC 19623), a rod-shaped bacterium that occurs in activated sludge, was studied singly and in association with P 1. Volutin granulation by Z. ramigera did not occur in activated sludge broth or on sewage agar with glucose. However, volutin accumulation can be induced by adding phosphate to a phosphate-starved culture in arginine broth (11). Accordingly, the organism was grown to stationary phase in arginine broth containing 0.05% arginine and 600 mg P/liter. These cells were harvested by centrifugation and resuspended for 16 hr in arginine broth containing 0.05% arginine without phosphorus. Orthophosphate was then added to increase the phosphorus concentration to 20 mg/liter. Four hours were allowed for granule formation. A control culture was treated identically except no phosphate was added to the phosphate-starved cells; no granulation occurred in this culture. The cells were harvested by centrifugation and extracted. The Norit-adsorbed KOH fraction of the control culture contained no phosphate while the corresponding fraction of the culture that received phosphate contained 28% of the total phosphate extracted.

Two shaker flask cultures of Z. ramigera were radioactively labelled to provide markers for paper chromatography. Both cultures were grown in arginine broth with 0.05% arginine and a low concentration of phosphorus (1 mg/liter); the broth in one flask also contained uracil-2-^{14}C. When the cultures reached stationary phase, they were pulsed with additional phosphate for 30 min. The culture grown in labelled uracil received 195 mg P/liter, and the other culture received 0.3 mg P/liter with ^{32}P. After 30 min the cells were harvested, extracted, and analyzed

by paper chromatography. Most of the activity of the Norit-adsorbed KOH fraction from the cells labelled with ^{32}P remained at the origin, the position of polyphosphate of over ten units in length. All the activity of the KOH fraction from the cells labelled with ^{14}C was at the nucleotide position midway to the front; no ^{14}C radioactivity remained at the origin, the position of intact ribonucleic acid.

The polyphosphate extracted in the Norit-adsorbed PCA and Norit-adsorbed KOH fractions was unstable and easily formed orthophosphate by hydrolysis. Some polyphosphate was degraded to orthophosphate in the extraction process itself. This degradation was further increased by additional processes such as separation by column chromatography or storage at 4 C. A freshly extracted Norit-adsorbed KOH fraction, that showed by chemical determination only 8% orthophosphate, was analyzed by column chromatography. Only 24% of the phosphate came out at the void volume, as does long-chain length polyphosphate. Sixty-three percent of the phosphate came out at twice the void volume, as does orthophosphate. Chemical determination of phosphate in a Norit-adsorbed KOH sample held at 4 C for 3 weeks showed 86% of the phosphate in the fraction was orthophosphate.

On a plate of sewage agar with glucose, a single streak of Z. ramigera was crossed with a streak of P 1 and with a streak of S. natans in a pattern that gave each organism alone, each paired with each of the others, and all three together. Volutin granulation occurred only in P 1. Where all three organisms were mixed, S. natans and P 1 formed tight compact clusters together, excluding Z. ramigera; no synergistic increase in granulation by P 1 occurred.

Monas

The next organism isolated from activated sludge and studied for its capacity to accumulate volutin was a protozoan. After 20 days of storage at 4 C, Tucson sewage and sludge had numerous flagellated protozoa typical of the genus Monas; cells were round, 5 to 10 μm in diameter, lacked chromatophores, were biflagellate with one flagellum longer than the other, and occasionally had a stalk. These cells had volutin granules as large as 2 μm in diameter. The 20 day old Tucson sewage and sludge were supplemented with 0.05% glucose, aerated for two days, and stained for volutin; the cells of the flagellates now often had several large volutin granules from 1 to 2 μm in diameter plus many smaller volutin granules. With Neisser's stain, the nucleus of the protozoan cell stained red; these red nuclei could be seen throughout the flocs of both Tucson and Rilling sludge, indicating the presence of the protozoa in these flocs. By wet mount, the outlines of the 5 μm round protozoa could be seen in the two sludges. When a smear of Tucson sewage was boiled in water, the volutin in the protozoa completely disappeared.

The protozoan in association with a rod-shaped bacterium was isolated from Tucson sewage; the bacterium was identified as Alcaligenes faecalis. A culture of Monas in association with P 1 was obtained by inoculating glucose broth having 10 mg P/liter with P 1 and with a culture of Monas associated with A. faecalis; P 1 displaced A. faecalis which could not grow in the glucose broth. A culture of Monas in association with Z. ramigera was similarly obtained by inoculating arginine broth having 0.01% arginine and 10 mg P/liter with both Z. ramigera and Monas associated with A. faecalis.

The contribution to phosphate uptake by the protozoan was ascertained by inoculating ^{32}P-labelled broths with pure bacterial cultures as well as with cultures of the same bacteria in association with the protozoan. Glucose broth with 10 mg P/liter was inoculated with P 1 with and without Monas; arginine broth with 0.01% arginine and 10 mg P/liter was inoculated with Z. ramigera with and without Monas; and coarsely-filtered, autoclaved sewage was inoculated with A. faecalis with and without Monas. After 5 to 6 days the cultures were harvested by Millipore filtration. The presence of the protozoan did not increase the accumulation of ^{32}P per dry weight mass over that accumulated by each bacterium alone (Table 1).

P 2

Phosphate accumulation by P 1 was compared with accumulation by Z. ramigera, A. faecalis (isolate), and A. aerogenes (departmental culture) by growing these organisms in coarsely-filtered, autoclaved sewage, labelled with ^{32}P. After 6 days, the cultures were harvested by Millipore filtration. Accumulation of ^{32}P per mg dry weight mass by P 1 was not greater than accumulation by the other three organisms (Table 2). This clearly indicated that P 1 was not plump.

The second plump rod isolated from Rilling sludge was obtained by streaking nutrient agar plates with the supernatant fluid of fresh sludge. Organisms from 14 types of colonies were studied by wet mount and volutin staining. One of these organisms, designated P 2, was a nonmotile coccoid rod 1 μm in diameter. On sewage agar, P 2 formed compact round clusters and some 0.5 μm volutin granules. This organism was

Table 1. Accumulation of ^{32}P radioactivity from growth media by activated sludge bacteria with and without the protozoan Monas.

Bacterium[a]	Dry weight with Monas (mg)	Dry weight without Monas (mg)	^{32}P uptake with Monas[b]	^{32}P uptake without Monas[b]	Ratio of ^{32}P uptake[c]
P 1	4.0	2.6	9.8	9.1	1.1
Zoogloea	2.2	0.6	8.4	14.1	0.6
Alcaligenes	3.6	3.7	43.3	41.4	1.0

[a] P 1 cultures were in glucose broth with 10 mg P/liter; Zoogloea cultures were in arginine broth with 0.01% arginine and 10 mg P/liter; Alcaligenes cultures were in coarsely-filtered, autoclaved sewage.

[b] Radioactivity of ^{32}P is expressed as 10^5 counts/min per mg dry weight cells; initial total activities were 3×10^7 counts/min for P 1, 3.5×10^7 counts/min for Zoogloea, and 1×10^8 counts/min for Alcaligenes.

[c] Uptake of ^{32}P radioactivity by bacterium with Monas/bacterium without Monas.

Table 2. Accumulation of ^{32}P radioactivity from coarsely-filtered, autoclaved sewage by activated sludge bacterium P 1 compared with other sludge bacteria.

Bacterium	Dry weight (mg)	^{32}P uptake[a]	Ratio of ^{32}P uptake by P 1/ bacterium
P 1	7.8	1.9	
Zoogloea	8.3	1.7	1.1
Alcaligenes	8.3	1.8	1.1
Aerobacter	7.8	1.9	1.0

[a]Radioactivity of ^{32}P is expressed as 10^6 counts/min per mg dry weight cells; initial total activity was 5.8×10^7 counts/min.

compared with P 1 and Z. ramigera for phosphate uptake from membrane-filtered, autoclaved, sewage supplemented with ^{32}P and 30 mg K_2HPO_4/liter. After 3 days, the cultures were harvested by Millipore filtration. Accumulation of ^{32}P per mg dry weight cells by P 2 was no greater than accumulation by P 1 and Z. ramigera (Table 3).

P 3

In the third attempt to isolate plump from Rilling sludge, a cluster of coccoid cells was separated on a slide from a drop of sludge under the microscope (magnification 35 X) by using a probe made from a capillary tube. This cluster was pushed by the probe from the drop of sludge to a drop of water, transferred to a nutrient agar plate with a loop and streaked out. Organisms from eight colony types were examined by wet mount and volutin staining; one of these organisms, a nonmotile coccoid rod of 1 µm diameter, occurring in clusters, was designated P 3. Whereas slants of P 1 and P 2 were white on nutrient agar, slants of P 3 were pale yellow. Volutin granulation by P 3 on sewage agar was limited to only few cells; the granules were up to 0.7 µm in diameter.

P 4 and P 5

Two more isolates were obtained from Rilling sludge by using a modification of the cluster separation technique. Two clusters were pushed on a slide by a probe from the sludge drop to a drop of water for washing; the clusters were then moved out of the water, smeared, and mixed with a second drop of water to get a suspension of cells. Some of these cells were picked up with a loop and streaked out on nutrient agar; the residue of the cell suspension on the slide was allowed to dry,

Table 3. Accumulation of ^{32}P radioactivity from membrane-filtered, autoclaved sewage by activated sludge bacterium P 2 compared with other sludge bacteria.

Bacterium	Dry weight (mg)	^{32}P uptake[a]	Ratio of ^{32}P uptake by P 2/ bacterium
P 2	2.1	9.7	
P 1	1.7	10.8	0.9
Zoogloea	2.0	9.4	1.0

[a]Radioactivity of ^{32}P is expressed as 10^6 counts/min per mg dry weight cells; initial total activity was 1 x 10^8 counts/min; the sewage was supplemented with 30 mg K_2HPO_4/liter.

stained for volutin, and observed to have many plumps full of volutin. Two types of colonies grew on the nutrient agar plate; the two organisms were both nonmotile, long, narrow rods, 2 x 0.5 µm, in clusters, and were designated P 4 and P 5. Neither of these organisms formed volutin when grown in sewage broth. They both formed white growth on nutrient agar slants.

P 6

The cluster separation technique was used to isolate clusters for volutin and Gram staining. Five clusters isolated in a drop of water from 7 day old Rilling sludge were allowed to dry on the slide and stained for volutin; all five clusters were filled with typical plump full of volutin. Other clusters were isolated and Gram stained; the clusters were composed of two distinct kinds of Gram-negative bacteria. One of these bacteria was a narrow rod, 0.3 to 0.5 µm wide by 0.3 to 2 µm in length, that stained pink. The other bacterium was a coccoid rod, 1 µm in width, that stained red; this bacterium was typical plump. The compactly massed plump covered the narrow rods except at the periphery of the cluster and at spaces between plump masses in the cluster. In the volutin stain the narrow rods were usually indistinct and appeared only as amorphous material in the clusters; these narrow rods did not contain volutin.

Isolated clusters were placed intact on the surface of various media, including sewage agar and coarsely-filtered, autoclaved sewage; during incubation the clusters were checked by microscope for enlargement. Generally the clusters either failed to enlarge or were overgrown by cluster contaminants; but on cyanophycean agar a cluster from fresh

(2 day) Rilling sludge enlarged noticeably in 4 days. The cluster was then streaked out with a loop over the plate surface. In another 4 days microscopic colonies, resembling typical clusters in size and shape, appeared on the streaks. Like clusters from sludge, these colonies remained intact when put into water. The colonies were composed of Gram-negative, rod-shaped bacteria, 0.3 to 0.5 μm wide by 0.3 to 2 μm long, with small volutin granules up to 0.5 μm in diameter. This bacterium was designated P 6.

Isolate P 6 was evidently the narrow rod associated with plump in the clusters. The Gram reaction of P 6 was pink rather than red. Its shape on various media was consistently that of a narrow rod rather than coccoid. On sewage agar P 6 formed no volutin. On nutrient agar P 6 gave pale yellow, sticky colonies, 2 mm in diameter. The organism had a single polar flagellum (seen by electron microscopy) and was biochemically inactive. It was identified as Zoogloea.

P 7

Gram and volutin stains of the microscopic colonies of P 6 from cyanophycean agar showed the presence of a few volutin-filled coccoid cells in the P 6 clusters. The coccoid organism was separated from P 6 on a nutrient agar streak plate. The most numerous type of colony on the plate was pale yellow, sticky, 2 mm in diameter, and composed of clustered, narrow rods, 0.3 μm wide by 0.3 to 3 μm long, that stained pink by the Gram stain. Both the colonial and cellular morphologies were typical of P 6. There were a few colonies that were gray, sticky, 2.5 mm in diameter, and composed of clustered, coccoid rods, 0.3 to 1.0 μm

in diameter, that stained red by the Gram stain. This organism, having a morphology typical of plump, was designated P 7.

Volutin granulation by P 7 was temporarily suppressed during its initial isolation. Very few cells had formed volutin on the isolation plate of nutrient agar after 4 days of incubation. Those cells that did have volutin were not randomly distributed when smeared on a slide but rather tended to occur in groups. Some of these cells were full of volutin. After another day of incubation, the groups of volutin-containing cells were more extensive and accounted for a larger proportion of the cells. After transfer of the isolated P 7 to a second plate of nutrient agar, almost all cells of P 7 had volutin deposits after 2 days of incubation.

The morphological and staining characteristics of plump as it occurred naturally in Rilling activated sludge were possessed by P 7 in pure culture. Cells of P 7 occurred singly, in pairs, or massed together in round flat clusters up to 150 μm in diameter. The size range for individual cells in wet mount was 0.3 to 2 μm in diameter, with an average of 1 μm. Cells of P 7 were coccoid on all media except on potato agar; on that medium almost all cells were rod-shaped, 0.3 to 1 μm wide and up to 2 μm long. Gram stains of P 7 grown on various media consistently gave the intensely red stained cells. Isolate P 7 was nonmotile in wet mount, and no flagella were seen on cells of P 7 by electron microscopy.

Abundant volutin was accumulated by P 7 on almost all media used. Each cell with volutin had a single granule, varying in size from small to completely filling the cell. In photomicrographs of

volutin-stained smears, P 7 appeared to be smaller than plump because P 7 cells were usually not completely full of volutin. Practically all cells of P 7 formed volutin granules on sewage agar and in separatory funnel cultures with the following media: coarsely-filtered, autoclaved sewage broth; autoclaved, membrane-filtered sewage broth; and coarsely-filtered, autoclaved sludge broth (Fig. 1D). Also, on all 21 common laboratory media inoculated, P 7 accumulated volutin. Upon prolonged incubation of P 7 on nutrient agar, some of the cells reached 2 μm in diameter, and some of these were completely filled with volutin. The polyphosphate content of the granules in P 7 was confirmed by Ebel's stains for metachromasy and lead reactivity.

Altering nutrient concentrations, except in case of extreme deprivation, had little effect on volutin granulation. In arginine broth with 0.01% arginine and 10 mg P/liter, almost all cells of P 7 had volutin granules; a few cells were full of volutin. Decreasing the phosphorus concentration to 1 mg/liter, or raising it to 300 mg/liter, had little effect on granulation. Decreasing the magnesium concentration from 20 mg/liter to 2 mg/liter while maintaining the sulfate concentration at 79 mg/liter, or decreasing the sulfate concentration to 8 mg/liter at constant magnesium concentration, also had little effect. The deletion of arginine, the carbon source, from the medium while maintaining the nitrogen concentration at 32 mg/liter with $(NH_4)_2SO_4$ resulted in very poor increase in cell numbers; but all cells were full of volutin. This same result also occurred by deleting magnesium or sulfate from the medium. Elevation of arginine to 0.05% with an increase in phosphate to 300 mg P/liter had no apparent effect; neither was

granulation affected by further addition of glucose to a concentration of 0.5%, giving a final carbon concentration of 2200 mg carbon/liter. The addition of phosphate to a phosphate-starved culture grown in broth with 0.01% arginine and 1 mg P/liter showed no evidence of increased granulation from polyphosphate overplus.

The common biochemical tests showed P 7 to be generally inactive, though with several exceptions. Sugar broths, consisting of 0.5% sugar, nutrient broth base, and pH indicator, showed no pH change or gas production during growth of P 7. Sugars tested included: adonitol, arabinose, dulcitol, fructose, galactose, glucose, inositol, inulin, lactose, maltose, mannose, raffinose, rhaminose, salicin, sorbitol, sucrose, and xylose. Indole, methyl red, and Voges Proskauer tests were negative. Nitrites were not produced from nitrates. Hydrogen sulfide was not produced. Oxidase was not present. Gelatin was not liquified. Urea was not hydrolyzed. No growth occurred at 7% salt concentration. Motility medium showed no motility. Growth in thioglycollate was strictly aerobic. A slightly acid reaction in litmus milk required two weeks. Although penicillin (methicillin, 5 µg/disc) was not effective against P 7, streptomycin (10 µg/disc) was effective. A positive test was obtained for catalase. Citrate was used as a sole source of carbon. Potato agar had heavy white growth. Incubation at either 23 C or 37 C gave good growth.

Accumulation of ^{32}P radioactivity from arginine broth by P 7 was compared with P 1 at two carbon to phosphorus ratios and at two growth phases (Table 4). Arginine broth with a C/P ratio of 4.1 had 0.01% arginine and 10 mg P/liter. In this broth P 7 accumulated over four

Table 4. Accumulation of ^{32}P radioactivity from arginine broths by activated sludge bacterium P 7 compared with sludge bacterium P 1.

C / P in the medium[a]	Growth phase	Dry weight of P 7 (mg)	Dry weight of P 1 (mg)	^{32}P uptake of P 7[b]	^{32}P uptake of P 1[b]	Ratio of ^{32}P uptake by P7/P1
4.1	Exponential	0.4	1.3	29.4	6.3	4.7
4.1	Stationary	1.2	2.2	10.9	4.7	2.3
16.4	Exponential	2.9	3.7	1.9	1.0	1.9
16.4	Stationary	4.8	4.6	1.7	0.9	1.9

[a]Ratio of available concentration of carbon to phosphorus in the medium; the 4.1 ratio is for arginine broth with 0.01% arginine (41 mg C /liter) and 10 mg P/liter; the 16.4 ratio is for arginine broth with 0.02% arginine (82 mg C/liter) and 5 mg P/liter.

[b]Radioactivity of ^{32}P is expressed as 10^6 counts/min per mg dry weight cells; initial total activity at C/P ratio of 4.1 was 3×10^8 counts/min, and at C/P ratio of 16.4 was 2×10^7 counts/min.

times as much ^{32}P as did P 1 in the exponential phase, and in the stationary phase, over twice as much. Arginine broth with a C/P ratio of 16.4 had 0.02% arginine and 5 mg P/liter. In this broth P 7 accumulated twice as much ^{32}P as did P 1 in both growth phases. Measurement of disappearance of ^{32}P radioactivity from supernatants and filtrates of the above broths gave somewhat higher P 7 / P 1 removal ratios.

The chemical measurement of phosphorus disappearance from the supernatant fluid of the above arginine broth with a C/P ratio of 16.4 gave slightly higher P 7 / P 1 removal ratios than those obtained by the measurement of ^{32}P accumulation (Table 5). Based on the chemically measured disappearance of phosphorus from supernatant fluid per mg dry weight cells, 6% of the weight of P 7 was phosphorus as compared with 2% for P 1. Volutin stains showed little volutin accumulation by P 1 at either a C/P ratio of 4.1 or 16.4 as compared with P 7.

Heat-fixed smears of P 7 grown in arginine broth with 0.01% arginine and 10 mg P/liter were stained for lipid and metachromatic granules by a modification of procedures used by Widra (15). Almost all the cells of P 7 formed volutin granules. The same results were obtained for P 7 as Widra obtained for A. aerogenes and Corynebacterium xerosis except with P 7 trypsin did not expose lipid deposits, and ether-ethanol did not extract masked lipid (Table 6). Granulation, whether metachromatic or lipid, was characterized by the presence of a single intracellular granule per cell. The results indicated that the volutin granules were composed of polyphosphate and RNA in close association with protein and tightly bound lipid.

Table 5. Disappearance of phosphorus from arginine broth caused by activated sludge bacterium P 7 compared with sludge bacterium P 1.

Growth phase	P removal by P 7[a]	P removal by P 1[a]	Ratio of P removal by P 7 / P 1
Exponential	0.62	0.24	2.6
Stationary	0.57	0.23	2.5

[a]Disappearance of phosphorus is expressed as mg P/liter removed from supernatant fluid per mg dry weight cells; dry weights are recorded in Table 4. The medium was arginine broth with 0.02% arginine (82 mg C/liter) and 5 mg P/liter; these concentrations gave a 16.4 ratio of carbon to phosphorus.

Table 6. Staining reactions of volutin and lipid granules in activated sludge bacterium P '7.

Stain	Granules present	Conclusion
Neisser	+	Polyphosphate present
Hot water + Neisser	-	Polyphosphate labile
Hot water + toluidine blue	+	Acid component other than polyphosphate present
Hot water + ribonuclease + toluidine blue	-	RNA present
Ribonuclease + Neisser	-	Polyphosphate is RNA-based
Trypsin + Neisser	-	Polyphosphate is protein-based
Ether-ethanol + Neisser	+	Polyphosphate is not based in extractible lipid
Sudan black B	-	Free lipid absent
Trypsin + Sudan	-	Lipoprotein absent
Citric-Sudan	+	Masked lipid present
Ether-ethanol + citric-Sudan	+	Lipid tightly bound

Stationary phase cultures of P 1 and P 7 in arginine broth with 0.02% arginine and 5 mg P/liter, labelled with ^{32}P, were extracted by a modification of the Schmidt-Thannhauser method. Phosphate content was determined by measuring both chemical and radioactive phosphate. In both P 1 and P 7 most polyphosphate was insoluble; less than one-third was soluble. The percentage of chemically measured polyphosphate in the total extracted phosphate was 26% for P 1 and 57% for P 7; thus P 7 had more than twice the percentage of polyphosphate as did P 1. By radioactivity measurement of ^{32}P, the percentage of polyphosphate was 17% for P 1 and 52% for P 7.

The extraction fractions of P 7 that contained polyphosphate were analyzed by paper chromatography. These fractions were the PCA, Norit-adsorbed PCA, KOH, and Norit-adsorbed KOH fractions. An additional sample used was a hydrolyzed portion of the Norit-adsorbed KOH fraction, obtained by placing part of that fraction in a boiling water bath for 10 min. After development, the paper was cut into strips. Each strip was tested chemically for phosphate by the molybdate method. Most phosphate in each extraction sample could be accounted for as polyphosphate which remained at the origin or, due to hydrolysis, migrated as orthophosphate near the front. The Norit-adsorbed KOH fraction had 45% of the phosphate at the origin and 40% near the front; the hydrolyzed, Norit-adsorbed KOH sample had 7% at the origin and 69% near the front.

The accumulation of phosphorus by P 7 in arginine broth with 5 mg P/liter and labelled with ^{32}P was compared at three concentrations of arginine, namely, 0.1, 0.2, and 0.3 g/liter. At the time of harvest,

P 7 was in stationary phase except for the broth with 0.03% arginine in which P 7 was late in the exponential phase. In the broth with 0.01% arginine P 7 incorporated two times more ^{32}P radioactivity than in the broth with 0.03% arginine; incorporation was intermediate in broth with 0.02% arginine (Table 7). Measurement of disappearance of ^{32}P radioactivity from both supernatant fluid and filtrate and of disappearance of chemical orthophosphate from supernatant fluid gave somewhat higher P 7 / P 1 removal ratios.

Table 7. Accumulation of ^{32}P radioactivity from arginine broth by activated sludge bacterium P 7 at various arginine concentrations.

% arginine[a]	Dry weight (mg)	^{32}P uptake[b]	Ratio of ^{32}P uptake[c]
0.01	0.8	3.1	2.1
0.02	2.1	1.9	1.3
0.03	3.9	1.5	1.0

[a]Phosphorus concentration was constant at 5 mg/liter.

[b]Radioactivity of ^{32}P is expressed as 10^6 counts/min per mg dry cells; initial total activity was 2×10^7 counts/min.

[c]Uptake of ^{32}P radioactivity by P 7 at each arginine concentration / at 0.03% arginine.

CHAPTER 5

DISCUSSION

The discovery of large masses of plump cells full of volutin in the flocs of Rilling activated sludge led to the isolation from sludge of a microorganism that had an unusually high capacity for phosphate accumulation. This organism, a Gram-negative coccoid bacterium, designated P 7, stood out alone in capacity for ^{32}P accumulation from other organisms that were tested.

The phosphate accumulation by P 7 was directly compared with P 1, another bacterium isolated from Rilling activated sludge. Isolate P 1 was capable of growing in the simple arginine broths used for growing P 7. Furthermore, the capacity for ^{32}P accumulation by P 1 was representative of the other five microorganisms tested that occur in sludge: Zoogloea ramigera (ATCC), Alcaligenes faecalis (isolated from sewage), Aerobacter aerogenes (departmental strain), isolate P 2, and a protozoan isolate.

The superior accumulation by P 7 over P 1 was greatest (4.7 times better) at a low carbon to phosphorus ratio (4.1) in an actively growing (exponential phase) culture. In broth with a C/P ratio of 16.4, 2% of the dry weight of P 1 was phosphorus as compared with 6% for P 7; greater than 3% phosphorus content is generally considered surplus phosphorus not required for metabolism.

Volutin deposition by P 7 was superior to that of the other organisms studied. A protozoan isolated from sewage formed large volutin granules comparable to those of P 7, but the cells of the protozoan were not full of volutin and were relatively few in numbers in activated sludge as compared with plump. Sphaerotilus natans had the ability to form very large oval granules (3 x 1 μm) in peptone broth; but in activated sludge broth, granules were small (0.8 μm or less in diameter). Bacterial filaments in activated sludge flocs had little or no volutin. The other organisms studied had comparatively little volutin accumulation on media, including sewage agar; massive volutin deposits required a severe nutrient imbalance such as phosphate starvation or depletion of magnesium or sulfate. In contrast, P 7 formed dense volutin granules in every medium; no nutrient imbalance was required by P 7 for massive deposition of volutin.

The nutritional requirements of P 7 for growth and phosphate deposition were minimal. Growth, volutin granulation, and ^{32}P accumulation readily occurred in arginine broths consisting only of low concentrations of arginine, $MgSO_4$, and KH_2PO_4 in distilled water; no supplementary elements or vitamins were required. Furthermore, the lower the concentration of arginine, the greater the ^{32}P accumulation per mg dry weight. Changes in nutrient concentrations, short of extreme deprivation, had little effect on granulation.

The polyphosphate composition of volutin granules was confirmed in several ways. Superior orthophosphate removal and ^{32}P accumulation correlated with volutin granulation. Increased volutin deposition also corresponded with increased phosphate in the polyphosphate fractions

obtained by cell extraction. The polyphosphate composition of these fractions was confirmed by paper and column chromatography. The presence of inorganic phosphate deposits by Ebel's lead stain correlated in numbers, position, and size with volutin deposits. This positive stain for lead reactivity taken together with Ebel's stain for metachromasy, using toluidine blue and an acid wash, constitute a positive cytochemical identification for polyphosphate.

The synthesis of polyphosphate in Aerobacter aerogenes appears to be representative of most microorganisms. Orthophosphate upon entering the cell is picked up by adenosine diphosphate (ADP), forming adenosine triphosphate (ATP); the terminal phosphate of ATP is then available for the synthesis of either nucleic acids or polyphosphate. The enzyme polyphosphate kinase is the sole mediator of the transfer of the phosphate from ATP onto a chain of polyphosphate; ADP is regenerated in the process. Accumulation of polyphosphate occurs during nutrient imbalance or when phosphate is added to a phosphate-starved culture. During nutrient imbalance, such as sulfur starvation, nucleic acid synthesis stops; without the incorporation of phosphate into nucleic acids, the phosphate accumulates as polyphosphate. During phosphate starvation, synthesis of polyphosphate kinase is derepressed; when phosphate is subsequently added, the accumulated polyphosphate kinase causes the rapid, extensive, polyphosphate accumulation called polyphosphate overplus. The primary route of polyphosphate degradation in A. aerogenes is hydrolysis to orthophosphate mediated by the enzyme polyphosphatase (10).

Isolate P 7 was observed to both accumulate and deplete stores of polyphosphate. The initial temporary inhibition of volutin formation on nutrient agar suggested the presence of weak controls on polyphosphate synthesis. Repression of polyphosphate accumulation by orthophosphate was not observed; the biosynthetic enzyme appeared to be constitutively derepressed. Except for extreme deprivation, granulation was not greatly affected by the availability of nutrients. The independence of granulation from the availability of nutrients and from the stage of growth suggested that the synthesis of polyphosphate did not require the cessation of nucleic acid synthesis; the usual preferential incorporation of orthophosphate into nucleic acid instead of into polyphosphate was not evident. However, the increased volutin deposition under extreme nutrient deprivation and the increased uptake of ^{32}P at low arginine concentrations suggested that the extent of volutin accumulation of P 7 was at least under partial control. The depletion of volutin stores in aged cultures indicated the presence of an operative route of polyphosphate degradation in P 7.

Isolate P 7 had many of the characteristics of the genus Zoogloea. Like Zoogloea, P 7 was Gram-negative, coccoid to rod-shaped, strictly aerobic, biochemically inactive, and found in compact masses in activated sludge. Unlike Zoogloea, P 7 was nonmotile. In activated sludge, plump was normally associated in clusters with a narrow rod that was isolated (isolate P 6) and identified as Zoogloea.

The capacity for phosphate uptake by Rilling activated sludge, a sludge noted for its superior removal of phosphate, as compared with Tucson activated sludge, a sludge with poor removal, correlated

directly with the occurrence of volutin-filled clusters and the amount of phosphate accumulated by the coccoid organisms. The volutin-containing clusters in Rilling sludge were large and full compared with the underdeveloped, small scattered clusters of Tucson sludge. Almost all coccoid cells in crushed smears of both sludges appeared full of volutin when stained by the very sensitive Neisser stain; but when stained by the less sensitive Ebel stain for inorganic phosphate, most coccoid cells in Tucson sludge were not filled with phosphate while most in Rilling sludge were filled.

Activated sludge treatment plants with unsatisfactory removal of phosphate from sewage probably could increase their removal by adjusting treatment conditions to favor the development of plump and its volutin accumulation in the sludge. The coccoid organism in Tucson sludge appeared to be the same organism as the plump in Rilling sludge; hence plump is probably widespread in occurrence. If necessary, seeding with plump from another plant is a possibility. A very important condition for the development of plump and its phosphate accumulation in sludge would be the maintenance of a highly aerobic environment. Isolate P 7 was strictly aerobic in its respiration. Also, vigorous aeration would ensure maximum cellular production of ATP, the direct source of phosphate for polyphosphate synthesis. Efforts at activated sludge plants to maximize sludge contact with oxygen, such as high aeration efficiency and minimal detention of sludge in secondary sedimentation tanks prior to return to aerator tanks, have been found to enhance phosphate removal at treatment plants (16).

SUMMARY

1. Massive deposits of volutin were found in the flocs of Rilling activated sludge. These volutin deposits were in clusters of a coccoid, Gram-negative microorganism, 1 μm in diameter, that was designated "plump".

2. The volutin-filled clusters were better developed in a sludge of high phosphate affinity than in a sludge of low phosphate affinity. Also, the coccoid cells stained for inorganic phosphate better in the high affinity sludge.

3. The attempt to isolate plump from Rilling activated sludge ended in the isolation of a microorganism of outstanding propensity for volutin accumulation. This organism, designated P 7, was a coccoid, Gram-negative bacterium, 1 μm in diameter, that formed clusters and abundant volutin on sewage and sludge media.

4. In comparison with several other microorganisms found in activated sludge, P 7 stood out alone in ability to accumulate phosphate during growth. Most of the phosphate accumulated by P 7 was extracted in the soluble and insoluble polyphosphate fractions.

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