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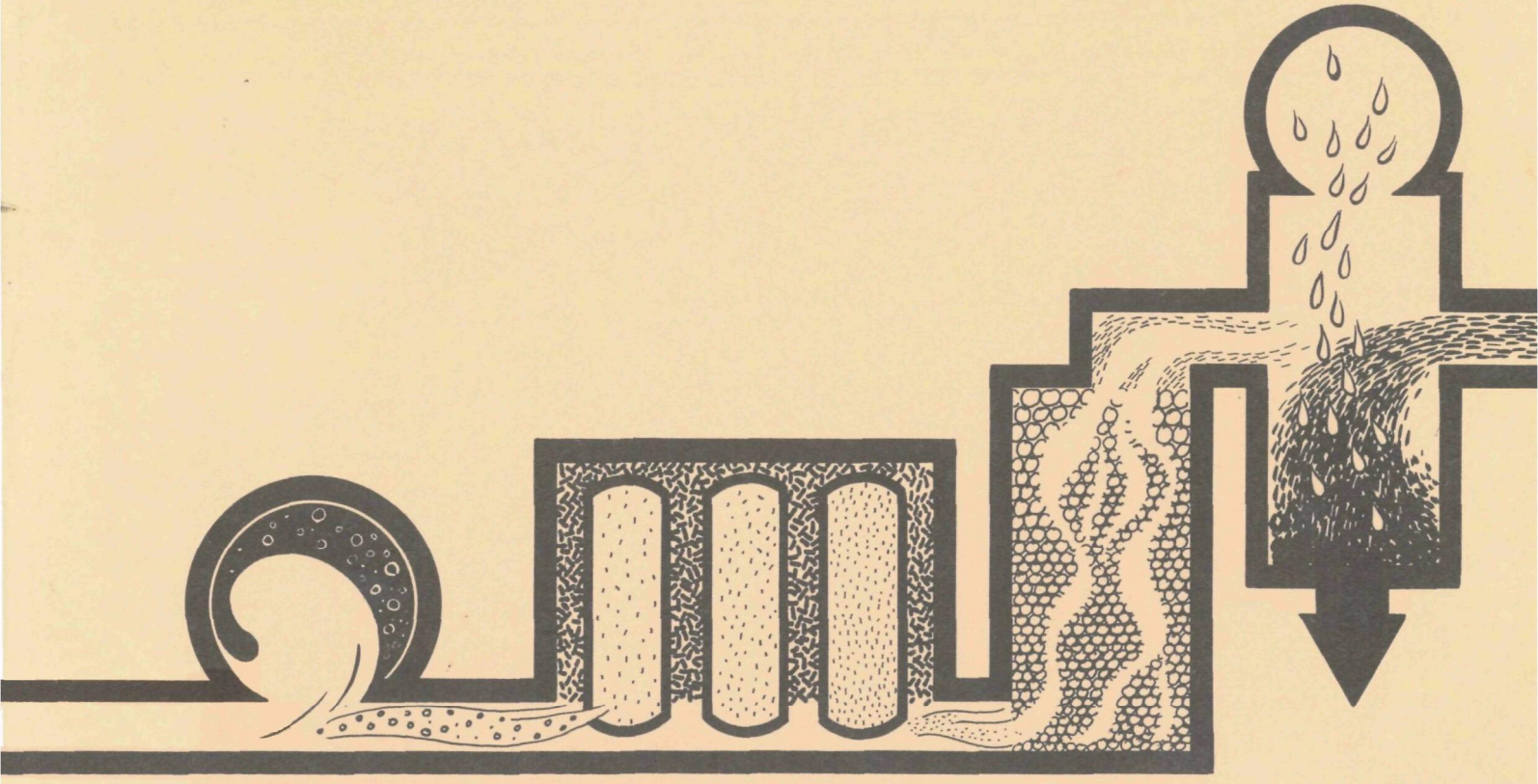
MECHANISMS OF BIOLOGICAL LUXURY PHOSPHATE UPTAKE



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MECHANISMS OF BIOLOGICAL LUXURY PHOSPHATE UPTAKE

by

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for the

ENVIRONMENTAL PROTECTION AGENCY

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ABSTRACT

Activated sludges obtained from the Rilling Road plant located at San Antonio, Texas and from the Hyperion treatment plant located at Los Angeles, California have the ability to remove large amounts of phosphorus from Tucson sewage and other liquors by means of biological mechanisms. Most of the phosphorus seems to accumulate within the sludge cells as orthophosphate. Tucson sludge seems to take up phosphorus by biological mechanisms but removes considerably less from its medium than does Rilling sludge. However, phosphorus uptake by Tucson sludge is improved if the sludge is starved prior to the addition of sewage.

The bacteria isolated from Rilling sludge do not individually seem to account for a high phosphorus affinity when compared to those from Tucson sludge. A culture of Sphaerotilus natans was isolated from Rilling but not from Tucson sludge. This organism had a higher affinity for phosphorus than others tested but not sufficient to account for the superior removal properties exhibited by the Texas sludge.

A known sludge bacterium, Zooqloea ramiqera formed volutin granules when excess orthophosphate was added to a phosphate starved culture. However, the conditions necessary to produce these granules in this organism probably do not exist in normal sewage.

This report was submitted in fulfillment of Project number 17010 DDQ under the partial sponsorship of the Environmental Protection Agency.

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SECTION I

CONCLUSIONS

1. Activated sludges from the Rilling Road plant at San Antonio, Texas and the Hyperion plant at Los Angeles, Calif. are capable of luxury phosphorus uptake from Tucson sewage during the course of laboratory experiments. Phosphorus removal was independent of externally supplied sources of energy and ions since orthophosphate and ^{32}P radioactivity were readily removed from tap water, glass distilled water, and deionized water. Phosphorus uptake by Rilling sludge seems to be wholly biological since it has an optimum pH range, an optimum temperature range, and is inhibited by various antimetabolites that affect enzymes involved in the synthesis of adenosine triphosphate in bacteria.

2. Tucson sludge also removes phosphorus from sewage by biological mechanisms. However, its net removal is considerably less than that of the high uptake sludges because of a high turnover of phosphorus occurring within the sludge cells. When the sludge is starved of phosphorus before adding sewage, turnover is almost eliminated and net uptake is improved considerably.

3. Rilling and Tucson sludges were extracted and the various cell fractions containing ^{32}P radioactivity were analyzed. Most of the radioactivity appeared to be in orthophosphate within the cells. Little if any net synthesis of nucleic acids occurred during a 6 hr. exposure to sewage. This confirms the luxury nature of the phosphorus uptake. A somewhat larger percentage of radioactive polyphosphate was found in Rilling sludge than in Tucson but not enough to account for the high removal capability of the former sludge.

4. Bacteria were isolated from high uptake sludges from Rilling and a plant located in Houston, Texas and compared to those isolated from Tucson sludge. A total of 229 pure bacterial cultures were screened by a qualitative procedure to determine their affinities for ^{32}P radioactivity. No significant distributions of high affinity bacteria were found for Rilling sludge as compared to Tucson. However, *Sphaerotilus natans* was isolated from Rilling sludge which had a significantly greater phosphorus affinity than did the others. This organism was not isolated from Tucson sludge which also had filamentous bacteria. The amount of phosphorus removed by *S. natans* was about the same as that

removed by Tucson sludge but not sufficient to account for the superior abilities of Rilling sludge.

5. Zooqloea ramigera, a bacterium isolated from sludge, had the ability to remove phosphorus and form volutin granules under certain conditions. These conditions included more glucose than is normally present in most waste waters. Low phosphorus affinity bacteria isolated from sludge became high affinity organisms when small amounts of glucose were added to sewage.

SECTION II

RECOMMENDATIONS

This program was limited to laboratory experimentation although the conditions used were designed to approach those that might exist in the field under optimum circumstances. Further studies should take place first with a pilot plant and then on a larger scale. Such studies might include the effects of sewage from a system of lower phosphorus affinity such as Tucson on a high affinity sludge such as Rilling over a prolonged period of time. The sludge should be monitored for gross changes in microbial population as well as for phosphorus affinity. To successfully accomplish the former, some population markers will have to be established.

The bacterial survey was inconclusive in pin pointing any particular members of the bacterial population of Rilling sludge as being responsible for the high uptake of phosphorus. One organism, Sphaerotilus natans, showed some promise but not sufficient to account for the sludge's superiority. It is possible that no single organism was responsible but that the uptake was the result of synergistic activity on the part of more than one genus, species, or strain. The possibility also exists that the active organism was not isolated. Further experimentation should clarify these points.

Too many experiments written into the literature have used some form of synthetic sewage as a medium for sludge. Such media containing glucose result in changes in the nature of the sludge within a few hours. Coliform organisms which are not usually the primary flora of sludge will proliferate. These organisms in the presence of glucose probably will improve phosphorus uptake but will result in the sludge losing other desirable characteristics.

Phosphorus uptake by Tucson sludge increased with starvation. If provisions were made in the plant for sludge, before being returned, to dump its phosphorus in a tank containing liquid relatively free of the element, uptake might be improved. Also, effluent containing phosphate should not be recirculated through the plant. Similar suggestions have been made in the literature by other workers in the field.

The presence of filamentous organisms such as S. natans has generally been regarded as a nuisance that results in bulking sludge. However, the engineers at Rilling have reported no difficulty in separating sludge from effluent. If in pilot plant experiments the organism still shows some efficiency in phosphorus removal, then conditions in low affinity plants might be altered sufficiently to permit the organism to grow on a limited scale.

SECTION III

INTRODUCTION

The presence of large amounts of phosphorus containing compounds in waste waters due to greater use of detergents containing this element is thought to be partially responsible for the nuisance growth of algae now observed in many lakes and waterways throughout the United States that receive effluents from treatment plants (1). One objective of waste water purification is to reduce the phosphorus levels below 0.5 mg. of PO_4 per liter which should aid in the control of algal growth (2). Activated sludge treatment of waste water, the most common method, usually is unable to lower the amount of phosphorus in the effluent sufficiently to prevent algal blooms when the element is the limiting nutrient. However, a number of plants throughout the country such as the Rilling Road plant at San Antonio, Texas (3) and the Hyperion treatment plant at Los Angeles, California (4) have reported sludges that have high phosphorus affinities and remove this element rapidly and completely when it occurs in their natural waste waters.

The mechanisms by which high affinity sludges remove phosphorus have not been fully elucidated. Waste waters are usually low in utilizable sources of carbon so microbial growth is relatively limited and slow. The rapid uptake of phosphorus by biological mechanisms in excess of the metabolic needs of the sludge cells is termed enhanced or luxury uptake and implies that the microorganisms have the ability to store the element in some form. Menar and Jenkins (5) concluded that the high phosphorus affinity shown by Rilling sludge was not biological in nature. They believed that excess removal, above that required for cell synthesis, was controlled by pH and the presence of Ca^{2+} in the waste water. Under proper conditions of pH, a precipitate of calcium phosphate would form followed by an enmeshing into the activated sludge floc. Subsequent settling of the sludge would result in apparent disappearance of the phosphate from the supernatant fluid. Recent work suggests that removal by Hyperion sludge is largely biological (4).

While it has been established that the microorganisms of activated sludge play an important role in the stabilization of organic waste waters (6), there is only limited

information in the literature concerning the role played by the various microbial components of activated sludge in phosphate uptake. Among these is the work of Srinath et al. (7). These workers investigated removal of radioactive phosphorus (^{32}P) from sewage by activated sludge, mixed bacterial cultures isolated from sludge, Zoogloea, sp., and the protozoan Epistylis sp. Based on their observations the authors concluded that removal of ^{32}P from sewage was due largely to vorticellid protozoa such as Epistylis sp. in sludge. The authors did demonstrate, however, that bacteria were responsible for uptake of a considerable amount of ^{32}P but since the bacteria remained dispersed in the medium it was concluded that bacterial efficiency of removal was poor. Whether the protozoans play a primary role in phosphorus removal or simply serve as a means for concentrating phosphorus taken up by bacteria was unresolved.

In 1969 the FWPCA awarded a contract to The University of Arizona to study the mechanisms of biological luxury phosphate uptake by sludge. The results of the investigation will be reported under three headings: whole sludge experiments, isolation of sludge bacteria, and volutin granules in Z. ramigera.

SECTION IV

MATERIALS AND METHODS

WHOLE SLUDGE EXPERIMENTS

Activated Sludge

Sludge from the Rilling Road plant at San Antonio, Texas was concentrated by filtration at the plant and shipped to Tucson overnight by surface carrier. Upon receipt the sludge was stored at 4°C. until needed, usually within one week after collection although its phosphorus uptake ability was not impaired by 11 days of storage (see Table 1). It was diluted with tap water to the desired concentration just before use. Return sludge from the Tucson plant was collected and allowed to settle before use. Several experiments were conducted with sludge from the Hyperion treatment plant located at Los Angeles, California. The material was frozen and then shipped by air and was used within one week after collection. The freezing process did not seem to alter the sludge's phosphorus removal abilities.

Experimental Conditions

General procedure. Many of the experiments were conducted by mixing 33-ml. of settled sludge in the desired concentration (as determined by dry weights) with 66-ml. of liquid contained in 38 X 200 mm. tubes. The desired amount of ^{32}P or ^{45}Ca radioactivity, additional phosphate (as KH_2PO_4 and K_2HPO_4) when required, and any other inclusions were placed in the tube prior to the addition of the sludge. The mixtures were aerated from the bottom of the tube at the rate of 0.8 liter of prewet air per min. and incubated at 24°C. Any sludge adhering to the sides of the vessel was removed with a spatula and returned to the mixture prior to each sampling. At the desired times, the aeration was stopped for approximately 10 sec. and 10-ml. samples removed before the sludge settled. The samples were centrifuged in the cold at 27,000 X g. for 10 min. The supernatant fractions were assayed for radioactivity (^{32}P or ^{45}Ca) and chemically for orthophosphate and calcium hardness. The pellets were extracted and the fractions assayed for radioactivity. The usual experimental run consisted of a block of 12 tubes.

Liquids used were: fresh raw sewage taken from the primary clarifier at the Tucson plant; Tucson city water taken from the tap; glass distilled water; and deionized water which was distilled water passed through a Barnstead mixed bed demineralizer.

When larger batch experiments were performed, increased amounts of sludge and liquid (in proportions of 1/3 to 2/3) were added to vessels of 1-liter, 2-liter, or 4-liter capacity. The vessels were kept slightly less than half-full. The aeration rate was increased to 3 liters of prewet air per min. All other conditions were the same as above.

Prelabeled conditions. 133-ml. of sludge were placed in a 1-liter glass graduate cylinder with 266-ml. of tap water or sewage and ^{32}P radioactivity and aerated for 3 hr. (for Rilling sludge) or 12 hr. (for Tucson sludge) at 25°C. After aeration, 5-ml. samples were removed and assayed for radioactivity and orthophosphate in the supernatant fractions and total uptake of radioactivity in the cells. Samples (100-ml.) were transferred from the large aerator to 38 X 200 mm. tubes and left undisturbed for 12 hr. After this time, 5-ml. samples were taken to determine the amount of ^{32}P radioactivity and orthophosphate "dumped"; additional orthophosphate placed in some of the tubes, and aeration started. The experiments were conducted as described under general procedure except that 5-ml. samples were taken for analysis.

Starved conditions. Tucson sludge was prepared by adding settled fresh return sludge to an equal volume of 0.85% saline in distilled water and allowing it to stand undisturbed at 25°C. for 18 hr. After standing, the sludge was mixed and allowed to resettle. The aqueous portion was drawn off and the sludge was added to sewage plus ^{32}P or ^{45}Ca contained in cylinders; the experiments were conducted as described under general procedure.

Determination of sludge mass. Dry weights were determined for the normal condition experiments by filtering 100-ml. samples taken from parallel larger batch experiments (4 liters) using predried and weighed 9-cm. circles of Whatman no. 30 filter paper. The filter paper and sludge were dried by heat to constant weight. The larger amounts were used to minimize errors in sampling that occurred due to cohesiveness of the sludge components when 10-ml. amounts

were treated in this fashion.

Temperature effects studies. For studies concerned with the effects of varying incubation temperatures, 33-ml. of sludge and 66-ml. of tap water containing ^{32}P radioactivity were incubated separately for 30 min. at the desired temperature to reach equilibrium. They were mixed together and the experiments conducted as indicated under general procedure in incubators set at the required temperatures.

For the constant temperature-varying time experiments, sludge was heated in a boiling water bath for the desired time and then cooled rapidly to 25°C. The experiments were conducted as indicated under general procedure.

Optimum pH studies. The water used to dilute the sludge for these studies was titrated with 10% hydrochloric acid (HCl) to establish acid ranges, with concentrated ammonium hydroxide for ranges between pH 7.0 to 10.0, and with 10% potassium hydroxide for more extreme alkalinity. The experiments were conducted as described under general procedure.

Manometric experiments. Sludge (0.8-ml.) was added to 1.2-ml. of tap water or sewage containing ^{32}P radioactivity, and 2,4-dinitrophenol (DNP) when required, in the main portion of a double sidearm 16-ml. reaction vessel containing 0.2-ml. of 20% potassium hydroxide. The flasks were placed on a model GR-14 respirometer (Gilson Medical Electronics, Middleton, Wis.) and equilibrated for 15 min. at 25°C. with the vessels open to air. After this time the vessels were closed and readings taken for 1 hr. When it was desired to preincubate with DNP, the powdered inhibitor was added to the sludge in sufficient amount to give the desired concentration (10^{-3}M) when diluted in the reaction vessel. The sludge and inhibitor mixture were incubated with shaking at 25°C. for 1 hr. prior to addition to the vessel.

Extraction Procedure

Three procedures were followed for extracting sludges during the course of the experiments covered in this report. These were: the Wiame method (8) as modified by Boughton (9), the Ogur-Rosen method (10), and the method of Schmidt and Thannhauser (11).

Wiame method. This technique was employed as a rapid means of obtaining the distribution of ^{32}P or ^{45}Ca radioactivity among the various fractions resulting from the extraction of the Tucson sludge. To the pelleted and washed sludge material, 10-ml. of freshly prepared 10% trichloroacetic acid (TCA) was added. The mixture was incubated for 30 min. at 4°C. The sample was centrifuged then for 20 min. at 17,300 X g at 0°C. The supernatant fraction was saved and designated the "cold acid soluble pool." According to work with yeasts, this fraction should contain cellular orthophosphate, free bases, nucleosides, nucleotides, and di-, tri-, and polyphosphates. Any ^{32}P or ^{45}Ca radioactivity that was adhering to the exterior of the sludge mass should register in this fraction.

The residual pellet was extracted with 10-ml. of freshly prepared ethanol-ether (3:1) for 30 min. at 45°C. The sample was centrifuged as described above. The supernatant fraction should contain lipids and phospholipids.

The residual material was extracted with 10-ml. of freshly prepared 5% TCA for 30 min. at 100°C. The sample was centrifuged as described above. The supernatant fraction should contain hydrolyzed RNA, DNA, long-chain polyphosphates, and acid soluble protein. It was designated the "hot acid fraction."

The residual material was extracted with 10-ml. of 0.1 N potassium hydroxide for 30 min. at 70°C. The sample was centrifuged as described above. The supernatant fraction should contain alkaline soluble components.

The sum of the amount of radioactivity found in each of the above fractions, as well as the residue remaining after the potassium hydroxide treatment was taken as the total amount of radioactivity fixed by the sludge. These agreed within 10% with the amount calculated as disappearing from the liquid during the course of the experiments.

Ogur-Rosen method. This method was employed in the latter experiments with Rilling sludge in an effort to obtain intact RNA. The pellets of sludge cells were extracted with 20-ml. of 70% ethanol for 30 min. at 4°C. and then centrifuged in order to obtain alcohol soluble materials. The residue was

then extracted with 20-ml. of 1 N hot perchloric acid (PCA) (70°C. for 45 min.) which should extract hydrolyzed DNA components as well as some polyphosphates.

Schmidt and Thannhauser method. This method was used for measuring the amounts of DNA and RNA from sludge which could not be done satisfactorily by the Ogur-Rosen procedure. (See Results). The main differences in the modified procedure, as compared to Ogur-Rosen, are substitutions of 10% TCA for the cold ethanol and PCA steps in the latter procedure and the use of 0.3 N potassium hydroxide for 60 min. at 37°C. instead of the hot PCA extraction. The use of dilute warm base results in extraction of the nucleic acids. The RNA is hydrolyzed into its component nucleotides. DNA is not affected in the same way. The DNA may be separated from the RNA components by precipitation with 1 N HCl.

Chemical and Radioactive Assays

Orthophosphate and total phosphate were determined by the ammonium molybdate and Stanna Ver method as given in the 6th edition of the manual of Hach Chemical Co. Ames, Iowa. The amount of color developed was read in a Hach model 585 DC-DR colorimeter. This method was found to have sufficient accuracy as judged by the use of our own prepared standards. Calcium and magnesium hardness in sewage was determined by the ethylenediaminetetraacetate (EDTA) titration method of Hach.

Biochemical oxygen demand (BOD) determinations were performed according to standard methods (12). A Fieldlab Oxygen Analyzer (Beckman Instruments, Inc., Fullerton, Calif.) was used to measure the dissolved oxygen expressed in milligrams per liter. Determinations of pH were with a pH meter (Leeds and Northrup Co., Philadelphia, Pa.). Radioactive assays were made in a Tri-Carb liquid scintillation counting system (model 314 EX-2, Packard Instrument Co., Downers Grove, Ill.) using techniques that have been described (13). All counts were corrected for decay. Distribution of the ^{32}P radioactivity among the organic and inorganic phosphorus containing components of the various fractions was determined after adsorption of the former by Norit A (14). Aqueous mixtures of orthophosphate and polyphosphates were resolved in this laboratory by the use of Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N.J.) columns which excluded polyphosphates and retained orthophosphate. Polyphosphate was

measured by heating and then using the ammonium molybdate technique. The chemical amounts of RNA present were determined by an orcinol colorimetric procedure (15). The amounts of DNA were measured by the diphenylamine procedure of Dische (16).

Chemicals

Carrier-free ^{32}P (orthophosphoric acid in 0.2 N HCl) was obtained from Schwarz BioResearch, Orangeburg, N.Y. New England Nuclear Corp., Boston, Mass., was the supplier of $^{45}CaCl_2$ in 0.5 N HCl, which was claimed to have a radiometric purity of 99% and to contain 1.2 mg. of total solids per ml. Chemicals used for determinations were obtained from Hach Chemical Co. Sodium azide (Fisher), mercuric chloride (Matheson, Coleman and Bell), and DNP (Mallinckrodt) were obtained from local vendors. All other antimetabolites and EDTA were obtained from Sigma Chemical Co., St. Louis, Mo. All chemicals were of the highest purity commercially available.

ISOLATION OF SLUDGE BACTERIA

Media and Cultural Conditions

Trypticase soy agar (TSA) (Bioquest, Cockeyville, Maryland), activated sludge extract agar (ASEA), and activated sludge extract agar plus glucose (ASEAG) were used for the primary isolation of bacteria from fresh return activated sludge obtained from the municipal wastewater plant located at Tucson, Arizona. Sludge samples ranged in temperatures from 22° to 24°C. and had a reaction of pH 7.2 to 7.4 at time of sampling. Sewage agar was used as the phosphorus uptake medium.

ASEA was prepared by autoclaving return sludge for 15 min. at 121°C. The suspension was filtered through cheesecloth and Whatman No. 1 filter paper. Agar (1.5%) was added to the filtrate and the mixture sterilized by autoclave. Where required, filter sterilized 0.5% glucose (final concentration) was added aseptically to sterile ASEA. Sewage agar was prepared by filtering effluent from the primary clarifier of the Tucson plant through Gelman membrane filters (pore diameter 0.20u) and collecting in bottles. Agar (1.5%) was added and the mixture sterilized by autoclave. When required the sewage agar was supplemented with 0.1% filter sterilized glucose

added aseptically.

Viable counts were obtained by serially diluting sludge following various treatments in order to disperse flocs. Sonication was done with a Bronwill Biosonic BP-1 sonifier at a power setting of 60 for 3, 10, and 30 sec.; homogenization with a Waring blender at high speed for 3, 10, and 30 sec.; and shaking in dilution bottles filled with Escher-type stoppers were used. Isolated colonies were picked at random from plates at the highest dilutions and subcultured on ASEAG or TSA plates for pure culture. All cultures were incubated aerobically for 96 hr. at 24°C.

In an attempt to enhance outgrowth of dominant sludge bacteria, samples of activated sludge were collected, homogenized for 30 sec., and centrifuged for 5 min. at 500 X g to remove large debris using a Sorvall RC-2B centrifuge set at 4°C.

Forty ml. of supernatant fluid was placed in each of five sterile 150-ml. Erlenmeyer flasks and treated in the following manner: one flask received no additions; a second flask received 0.5% glucose (final concentration); a third received 0.5% glucose and 0.1% yeast extract; a fourth received 0.5% glucose and 0.1% K_2HPO_4; and a fifth received 0.5% glucose, 0.1% yeast extract, and 0.1% K_2HPO_4. All additions were presterilized and added aseptically. Flasks were aerated by shaking at 250 rev./min. for 96 hr. on a New Brunswick gyro-rotary shaker. Following incubation, samples were plated on TSA, ASEA, and ASEAG plates which were then incubated at 24°C. for 96 hr. Colonies were picked at random and pure cultures isolated.

Characterization of Isolates

Isolates were characterized on the basis of their morphology, physiology, and biochemistry. Morphological characteristics included cell size, shape, and arrangement; motility, determined by wet mount observations and reaction in motility medium; type of flagellation using a Philips EM-75 electron microscope; and colony size, form, margin, elevation, luster, density, and pigmentation. Physiological and biochemical tests employed were those described by Shewan et al. (17) for the identification of genera of Gram-negative bacteria and those set forth by the Manual of Microbiological Methods of the Society of American Bacteriologists (18). These included oxidase test; production of fluorescent pigment;

2-ketogluconic acid formation; pigement production on skim milk; gelatin liquefaction; nitrate reduction; and anaerobic and aerobic dissimilation of glucose, lactose, and sucrose. Cultures were incubated at 24°C.

Assay for Phosphorus Uptake

Qualitative Screening Procedure. Isolates were grown for 48 hr. on ASEAG slants at 24°C. The cells were suspended in a small volume, 1 to 2-ml. of double distilled water. Samples (0.5-ml.) were filtered through dried, tared 24 mm. 0.20 μ diam. Gelman membrane filters. The filters were placed on sewage agar plates containing carrier free $H_3{}^{32}PO_4$ (Schwarz Bio-Research) at a final activity of 700,000 counts/min./ml. or approximately 14,000,000 counts/min. per plate. A 64-mm. petri dish accommodated five filters, two organisms in duplicate, and one control membrane. Plates were incubated aerobically at 24°C. for 6 hr. After incubation, filters were removed, dried to constant weight, weighed, placed in vials and radioactivity measured.

Quantitative Measuring Procedure. The bacteria were streaked from stock cultures onto sterile sewage-agar-glucose slants (filter sterilized sewage, 2% agar, and 0.1% glucose). Two slants were made per culture. The organisms were grown for 48 hr. at 24°C. The organisms were washed from each slant using 1-ml. of sterile distilled water into an 8-ml. sterile water blank and resuspended. Two ml. of each suspension were then placed aseptically in each of four 500-ml. Erlenmeyer flasks containing 100-ml. of filter sterilized raw sewage and 0.1% glucose. The flasks were continuously shaken at 200 rev./min. on a gyrorotary shaker at 24°C. for 48 hr. at which time the optical density of the suspension in each flask was approximately 1 when measured with a Bausch and Lomb Spectronic-20 colorimeter at 540 nanometers. The contents of the four flasks were pooled and centrifuged at 4°C. at 27,000 X g for 10 min. The pellets were combined and washed once with distilled water. The organisms were resuspended in filtered sewage to an optical density corresponding to a known dry weight of the bacteria. Fifty ml. portions of the suspension plus ^{32}P radioactivity were added to 38 X 200 mm. Kimax tubes which were aerated and sampled as previously described. Dry weights were determined by collecting the cells on preweighed membrane filters (0.45 μ pore size; Millipore Corp., Bedford, Mass.) and drying at 70°C. overnight.

Experimental Conditions

Zoogloea ramigera ATCC 19623, maintained on Trypticase soy (Bioquest) agar slants supplemented with 0.25% glucose, was employed in this work. Other media used included: activated sludge broth prepared by coarsely filtering autoclaved activated sludge and adding varying amounts of glucose as supplements; inoculating broth, a modification of Crabtree and McCoy's arginine broth (19) containing 0.2 g. of K_2HPO_4 and 0.1 g. of KH_2PO_4 per liter of medium; and arginine broth, which was a modification of Crabtree and McCoy's broth containing 4 mg. of KH_2PO_4 per liter. The standard inoculum for liquid media consisted of 0.01-ml. of stationary phase bacteria. The organisms were grown for 120 hr. in 100-ml. of liquid media contained in 500-ml. Erlenmeyer flasks with shaking at 24 C. prior to studying the effects of various conditions on granule formation.

Staining Procedures

Neisser's stain was used to stain volutin granules. The solution of methylene blue plus gentian violet stains the granules deep blue and the chrysoidin solution stains the cells yellow (20). Tandler's inorganic phosphate stain was used to show the presence of inorganic phosphate in volutin granules (21). The cells were counter-stained red with safranin.

Granule Counting

Smears stained for volutin were observed at 970 X by using bright field microscopy. Photographs of some smears were taken with a Leitz Orthomat Microscope Camera. 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 number of granules per cell approached a Poisson distribution and was treated as such in computing confidence limits of means.

Chromatography

Samples and standards ($H_3{}^{32}PO_4$ and $Na_4P_2O_7$) were applied to Whatman no. 4 chromatography paper cut to 9 by 9 in. (23 by 23 cm.), developed in a mixture of isopropanol, concentrated HCl, and water (170:41:39, v/v) (22), and sprayed with a mixture of 60% PCA, in HCl, 4% ammonium molybdate, and water

(5:10:25:60, v/v) (23). The paper was sprayed, air dried, and exposed to ultraviolet light at 260 nanometers for 10 min., whereupon the inorganic phosphate appeared as blue spots.

SECTION V

RESULTS

WHOLE SLUDGE EXPERIMENTS

TABLE 1. EFFECT OF STORAGE TIME ON THE ABILITY OF RILLING SLUDGE TO REMOVE PO_4-P FROM TUCSON SEWAGE[a]

Storage time[b]	PO_4-P in sewage	
	p.p.m.	% removed
4	0	100
11	0	100
16	3.7	63
23	5.7	43
30	6.0	40

[a] PO_4-P = 10 mg/l

[b] Days after collection

Table 1 shows the effect of storage time at 4°C. on the ability of Rilling sludge to remove PO_4-P from Tucson sewage. This material can be stored for at least 11 days after collection and still remove all of the phosphorus from the waste water.

Figure 1 shows the per cent uptake of radioactivity from ^{32}P or ^{45}Ca from the sewage by Tucson sludge under normal experimental conditions. Zero time represents the interval required to mix the sludge with the sewage, remove a sample and separate the pellet from the liquid fraction by centrifugation. The total amount of radioactivity present was determined prior to additions of the sludge. Chemical orthophosphate could be determined only at zero time because the manipulation of the sludge contributed to total phosphate. At zero time, approximately 2.5% of the ^{32}P radioactivity became associated with the sludge as compared to 12% of the total ^{45}Ca activity. These fiqures represent 5% of the total ^{32}P radioactivity and 67% of the total ^{45}Ca

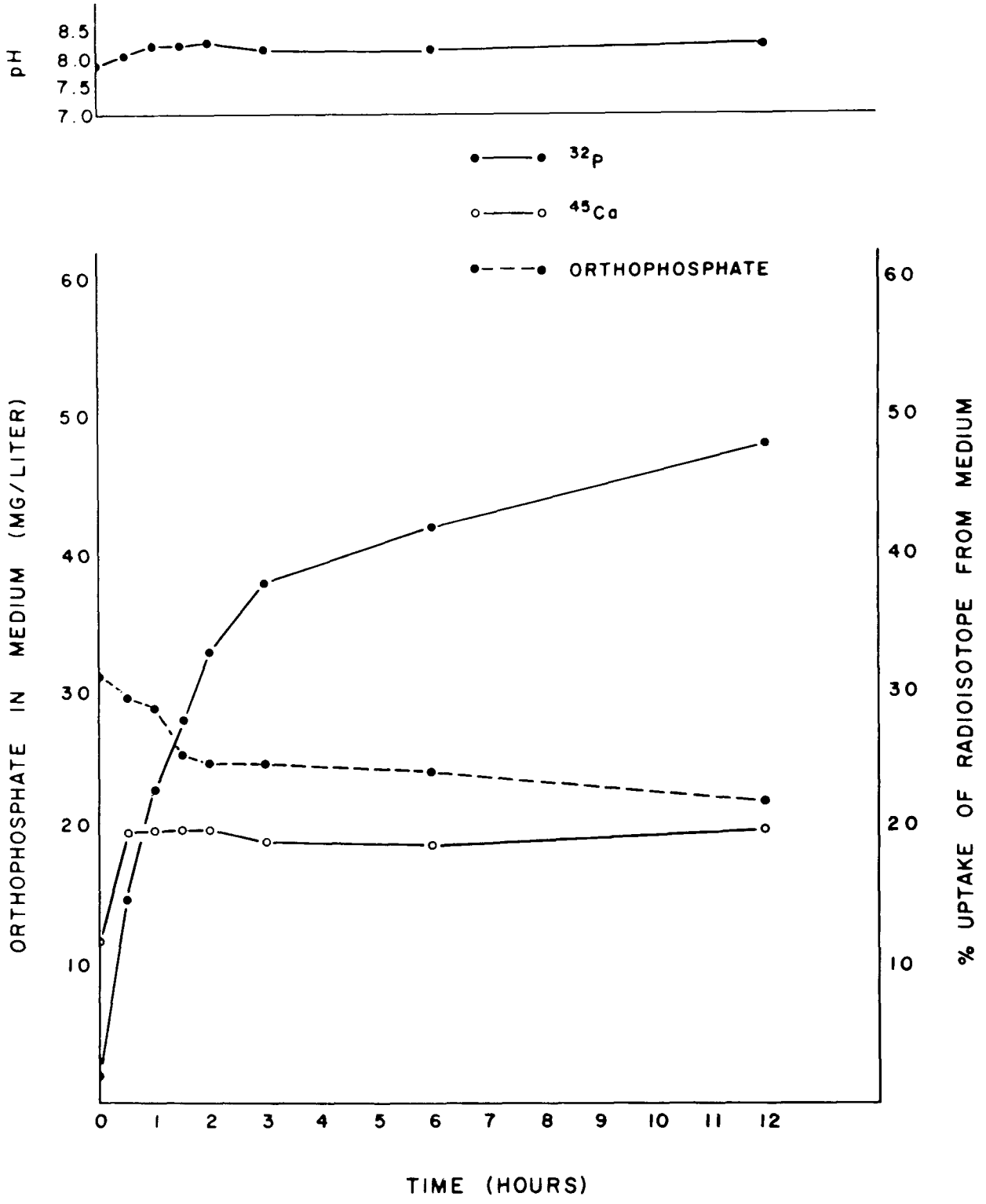

FIG. 1. UPTAKE OF ^{32}P, ^{45}Ca, AND ORTHOPHOSPHATE FROM SEWAGE BY TUCSON SLUDGE UNDER NORMAL CONDITIONS. Approximately 9,584,350 counts/min. of ^{32}P radioactivity and approximately 8,852,000 counts/min. of ^{45}Ca radioactivity were used per 10-ml. of mixture.

TABLE 2. REMOVAL OF ORTHOPHOSPHATE FROM SEWAGE BY TUCSON ACTIVATED SLUDGE[a]

Sludge condition	Time (hr.)	% Removal
Normal	3	19
	6	20
	12	30
Starved	3	42
	6	53
	12	56

[a]Based upon amount (milligrams per liter) present at zero time. See Fig. 1 and Fig. 2 for chemical amounts of orthophosphate and corresponding amounts of ^{32}P.

radioactivity removed from the sewage in 12 hr. The orthophosphate removed, as indicated by chemical methods (Table 2), was 30% in 12 hr. as compared to 48% of ^{32}P radioactivity (Fig. 1).

Dry-weight determinations, using parallel experiments, indicated that a sludge mass of 55.6 mg/100 ml was present at zero time. No increase in dry weight was observed by 3 or 6 hr. Determinations of BOD (not shown) indicated that the sources of carbon were essentially consumed by 3 hr.

Figure 2 indicates the effect of sludge starvation on the uptake of orthophosphate and radioactivity from ^{32}P or ^{45}Ca from sewage. The process of starvation resulted in the dumping or stripping of orthophosphate from the sludge which was observed in this laboratory and by others (24, 25). Much of this phosphate was present in the interstitial spaces of the sludge mass and was not removed when the sludge was added to sewage containing radioisotope. Therefore, the chemical amount of orthophosphate present in the sludge-sewage mixture was considerably higher (92.5 mg./liter) than that from normal conditions.

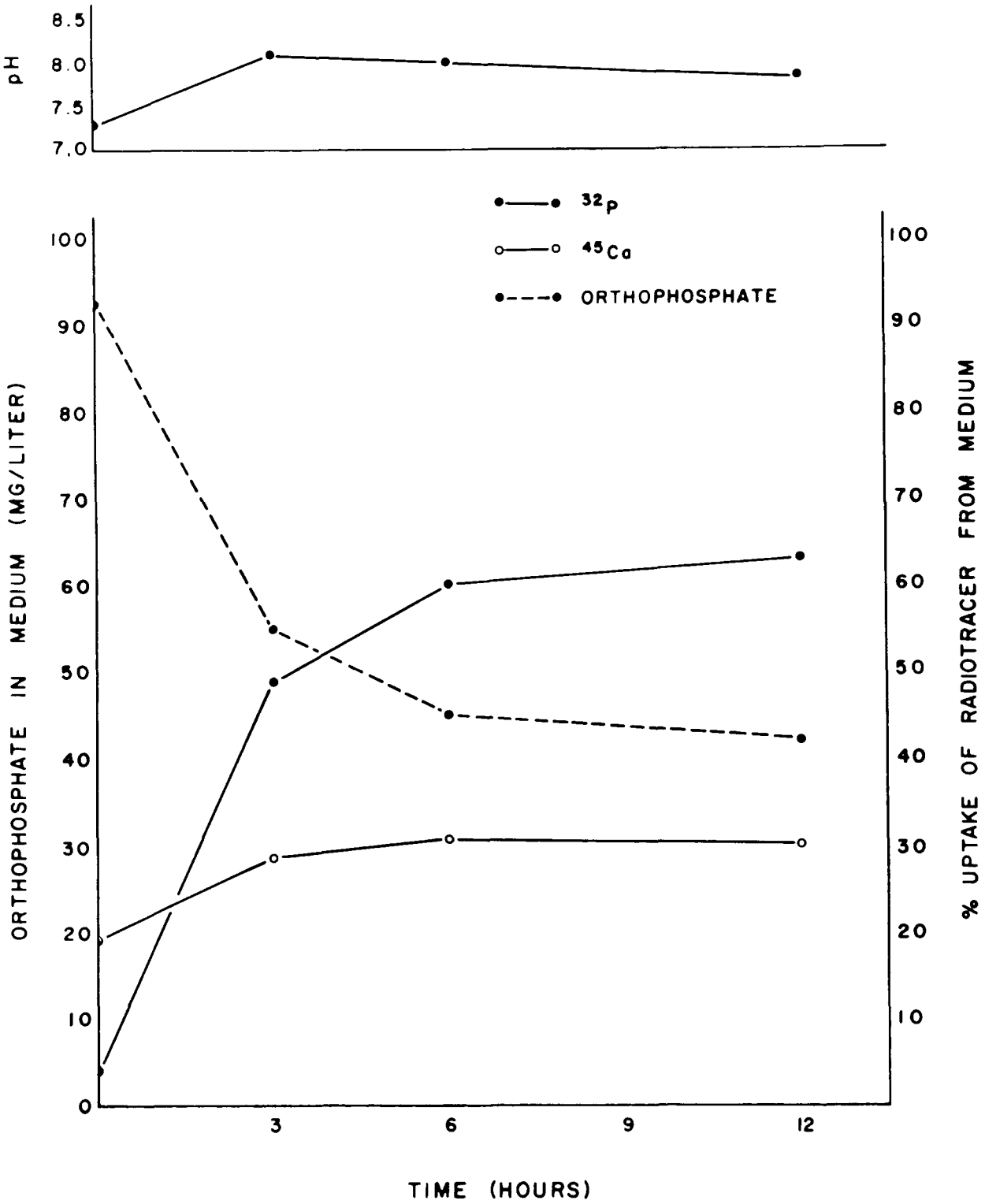

FIG. 2. UPTAKE OF ^{32}P, ^{45}Ca, AND ORTHOPHOSPHATE FROM SEWAGE BY STARVED TUCSON SLUDGE. Approximately 1,133,700 counts/min. of ^{32}P radioactivity and approximately 626,300 counts/min. of ^{45}Ca radioactivity were used per 10-ml. of mixture.

At zero time, approximately 4% of the ^{32}P radioactivity and 18% of the ^{45}Ca radioactivity were found to be associated with the sludge. Despite the higher amount of orthophosphate initially present as compared to the normal-condition experiments (Fig. 1), this sludge was more efficient in removing ^{32}P radioactivity, taking up approximately 63% by 12 hr. Orthophosphate removal from the sewage, as determined chemically, showed better agreement with the tracer results in that about 56% disappeared (Table 2). Starvation enhanced somewhat the uptake of ^{45}Ca with approximately 30% becoming associated with the sludge by about 12 hr. However, about 60% of the total taken up was removed at zero time. The association of calcium with the sludge seemed mainly to be confined to the radioactive ions added just prior to the beginning of the experiment because no loss other than that attributable to error in the method was found when calcium hardness of the sewage (which was found to be approximately 130 mg./liter) was measured chemically during the treatment of sludge.

Figure 3 shows the distribution of radioactivity of cell-fixed ^{32}P or ^{45}Ca in various fractions. At zero time, only 33% of the ^{32}P radioactivity in the normal sludge was associated with the cold acid-soluble pool components or possibly just adhering to the exterior of the sludge mass, perhaps as calcium phosphate. The majority of the radioactivity already was distributed among the various cell components. Most of the radioactivity seemed to be associated with the fraction that would contain nucleic acids and long-chain polyphosphates. The starved sludge had about 35% of its ^{32}P radioactivity in the soluble fraction and 42% in the nucleic acid-polyphosphate fraction. The normal sludge had 92% of its ^{45}Ca radioactivity associated with the soluble fraction and 6% with the nucleic acid fraction. This distribution was unchanged by 12 hr. The starved sludge showed a lesser amount, 81% of its ^{45}Ca radioactivity associated with the soluble fraction and more, 14%, associated with its nucleic acid fraction. This distribution was unchanged by 12 hr.

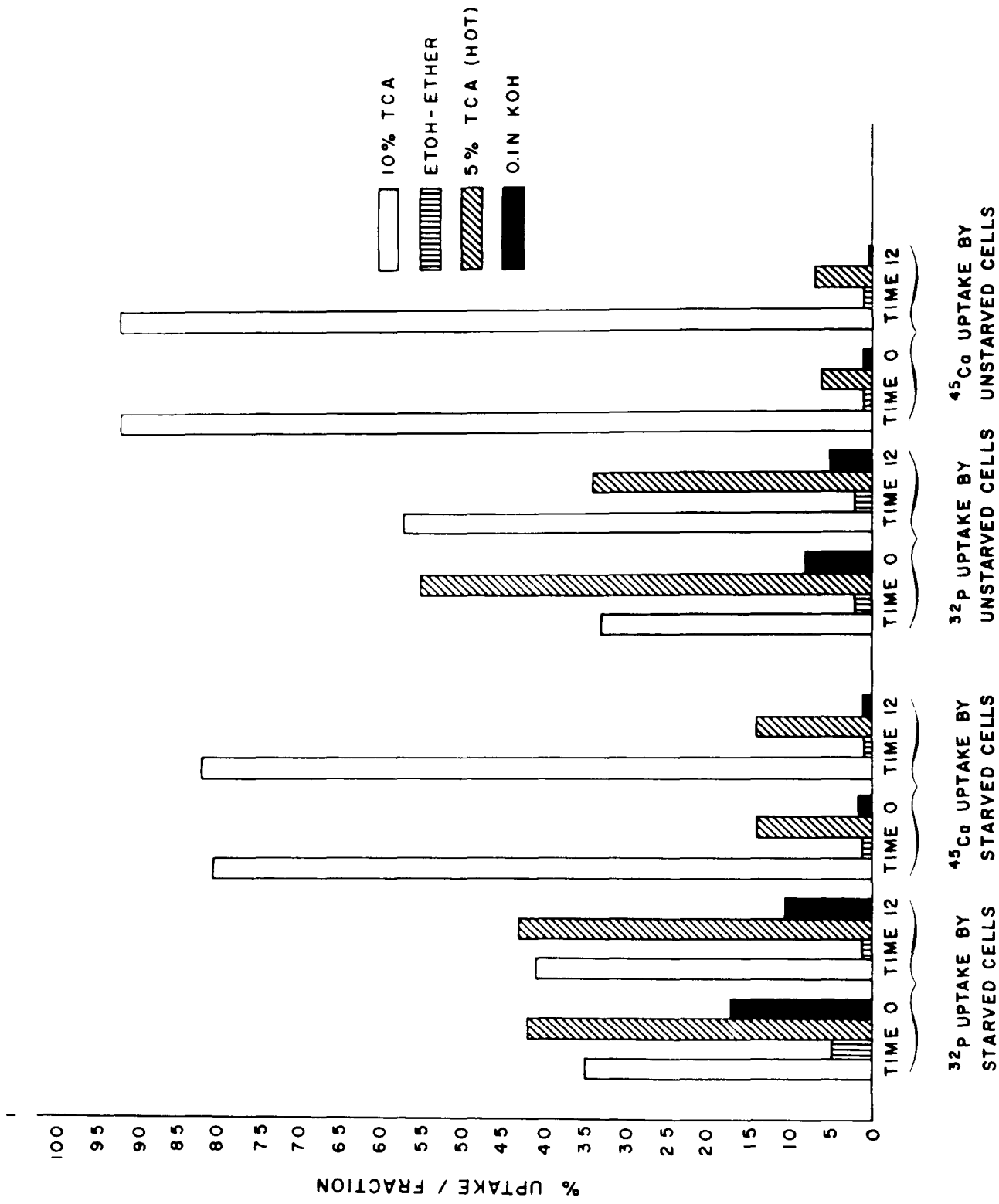

FIG. 3. DISTRIBUTION OF RADIOACTIVITY FROM ^{32}P OR ^{45}Ca IN FRACTIONS OBTAINED FROM STARVED OR NORMAL SLUDGE BY EXTRACTIONS USING MODIFIED WIAME TECHNIQUE. See legends for Fig. 1 and Fig. 2 for amounts of radioactivity.

TABLE 3. RADIOACTIVITY (RA) RELEASED FROM ^{32}P-LABELED TUCSON SLUDGE DURING THE UPTAKE OF ORTHOSPHOSPHATE[a]

Time (hr)	RA in liquid phase[b]		Orthophosphate in liquid phase		pH
	Counts/min	Per cent of total fixed	Mg/ liter	% removed	
0	712,200	19	27.5		8.10
0.5	770,000	21	25.0	9	8.15
3	975,200	27	23.0	16	8.20
6	1,173,600	33	21.6	21	8.20

[a]Approximately 3,671,500 counts/min. of ^{32}P radioactivity were fixed per 10-ml. sample of sludge-sewage mixture.

[b]Amount of radioactivity found in supernatant fraction of 10-ml. sample of mixture.

Sludge prelabeled with ^{32}P was placed in fresh raw sewage to examine the possibility that the apparent discrepancy observed when the data obtained by measuring ^{32}P uptake into normal cells was compared to chemical orthophosphate remaining in the liquid might be due to phosphate turnover (Table 3). A considerable portion of the radioactivity from the sludge was found in the liquid phase at zero time (19%). About 33% of the radioactivity was in the liquid phase by 6 hr. Approximately 21% of the orthophosphate in the mixture, as determined chemically, was removed from the liquid phase. This compares to the figure of 20% observed for the normal experiments (Fig. 1).

Figure 4 shows the effects of DNP on the ability of sludge to take up ^{32}P, ^{45}Ca, and orthophosphate. Under our experimental conditions, ^{32}P uptake was inhibited approximately 83% and ^{45}Ca uptake was inhibited approximately 34%. Some dumping of orthophosphate from the sludge cells into the liquid phase was observed

Figure 5 shows the distribution of ^{32}P or ^{45}Ca radioactivity among the various fractions of the sludge cells subjected to 2,4-DNP treatment for 3 hr. The most striking feature is the inhibition of ^{32}P incorporation into the nucleic acid-polyphosphate fraction. The distribution of ^{45}Ca radioactivity was essentially unchanged from that of normal cells.

The next sludge to be examined was obtained from San Antonio (Rilling). The Rilling sludge removed all ^{32}P added to Tucson sewage and its phosphorus content (about 10 mg. per liter PO_4-P) by 3 hr. (Fig. 6). Tucson sludge removed about 26% of the radioactivity and about 2 mg. per liter of phosphorus (not shown) by 6 hr. The presence of DNP (10^{-3}M) resulted, in this case, in approximately 44% inhibition of uptake by Rilling sludge after 3 hr.

Figure 7 shows the percentage of the ^{32}P radioactivity taken up by the sludges among the fractions obtained by the use of the Ogur-Rosen extraction procedure. Orthophosphate seems to predominate. Although there is some apparent increase in the amount of labeled polyphosphate isolated from the Rilling sludge, this does not seem to be sufficient to be responsible for the high phosphorus affinity shown by this sludge. The presence of DNP inhibited the uptake of ^{32}P radioactivity into organic phosphate compounds. No significant amounts of radioactivity were found in the ethanol and ethanol-ether fractions so they were not included in the

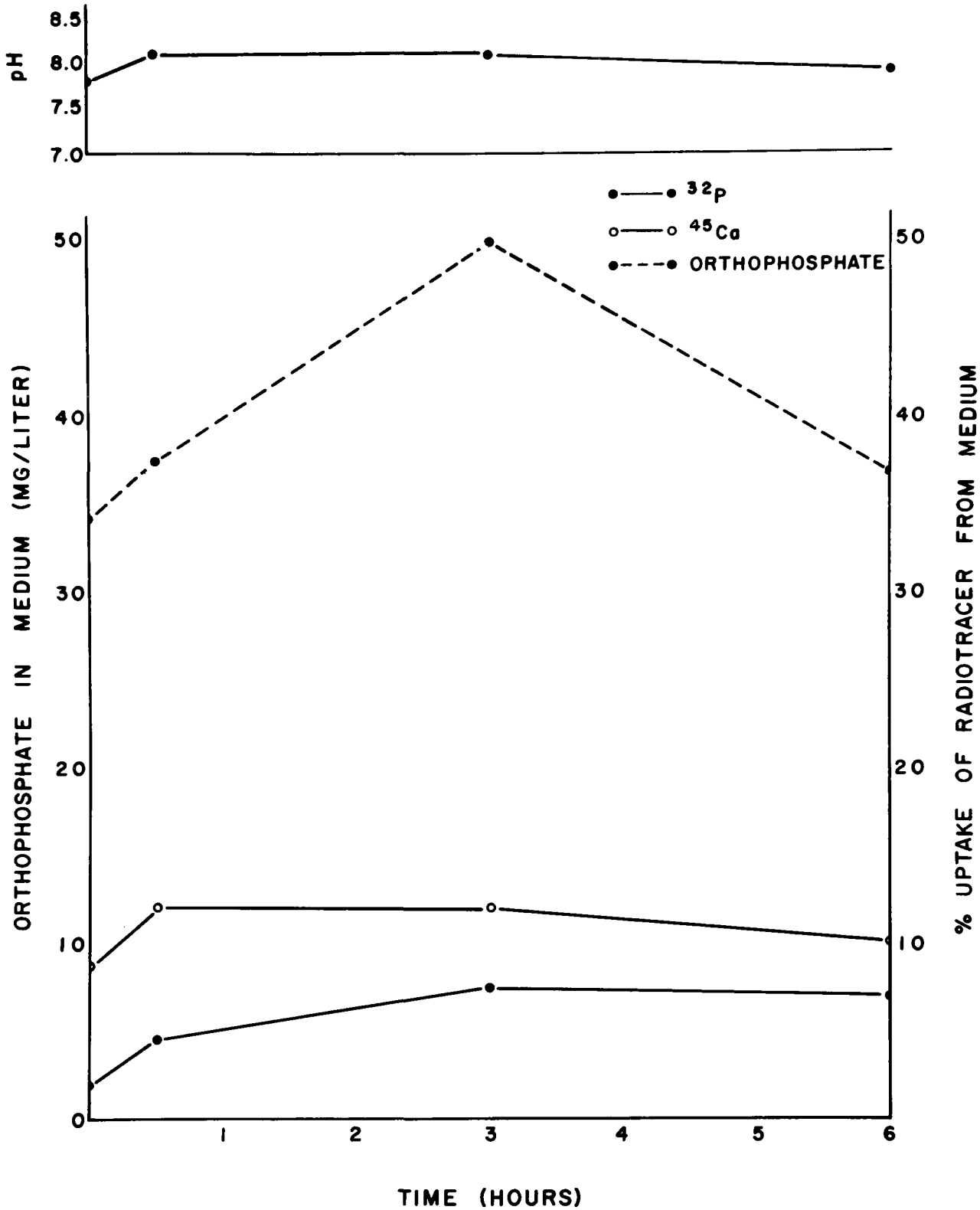

FIG. 4. THE EFFECT OF 2,4-DINITROPHENOL (2,4-DNP) ON THE UPTAKE OF ^{32}P, ^{45}Ca, AND ORTHOPHOSPHATE FROM SEWAGE BY TUCSON SLUDGE. Approximately 5,925,100 counts/min. of ^{32}P radioactivity and about 7,853,200 counts/min. of ^{45}Ca radioactivity were used per 10-ml. of mixture. Approximately 10^{-3} M final concentration of 2,4-DNP was employed.

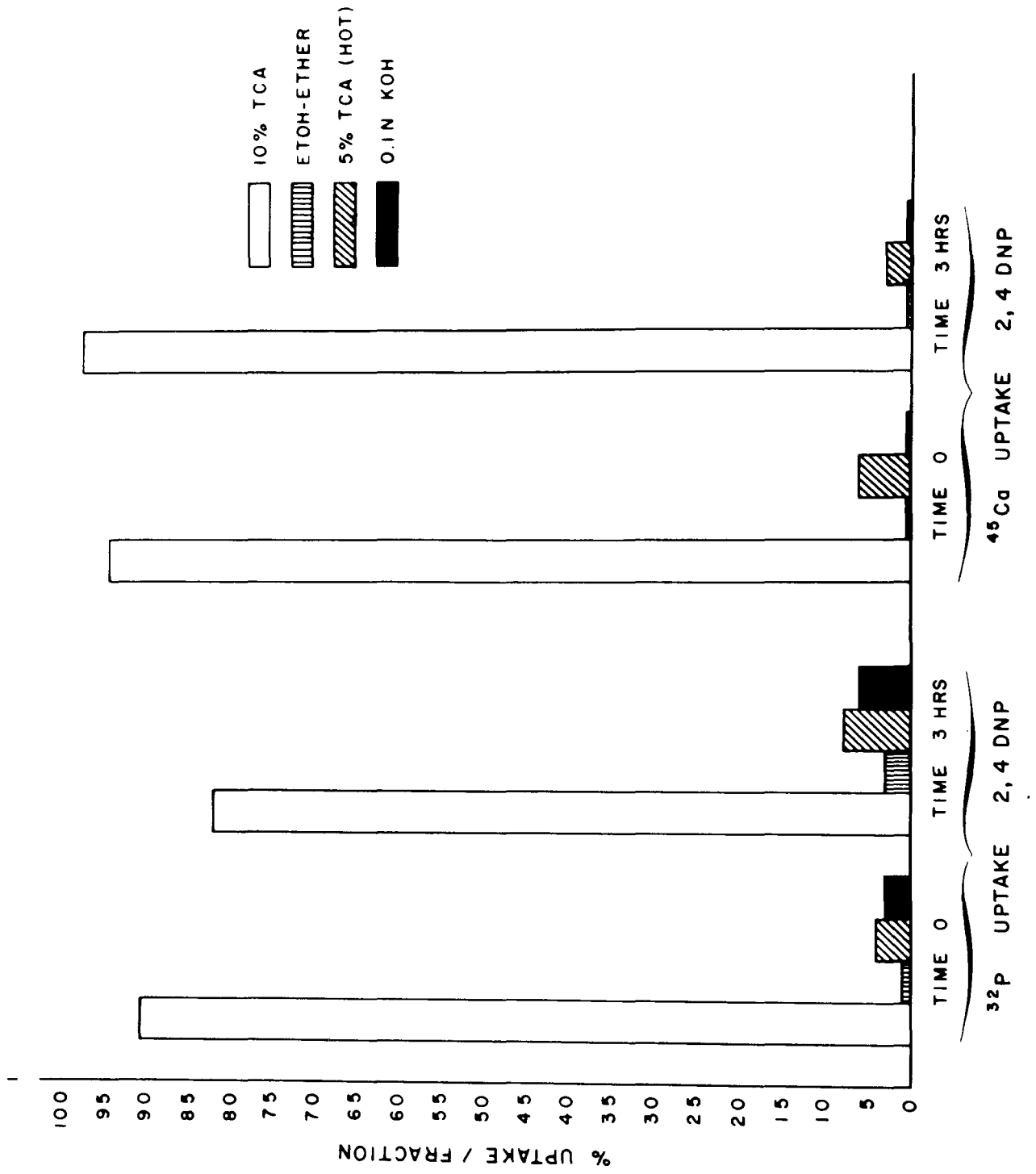

FIG. 5. DISTRIBUTION OF RADIOACTIVITY FROM ^{32}P OR ^{45}Ca IN FRACTIONS OBTAINED FROM 2,4-DINITROPHENOL TREATED SLUDGE BY EXTRACTIONS USING MODIFIED WIAME TECHNIQUE. See Fig. 4 legend for amounts of radioactivity.

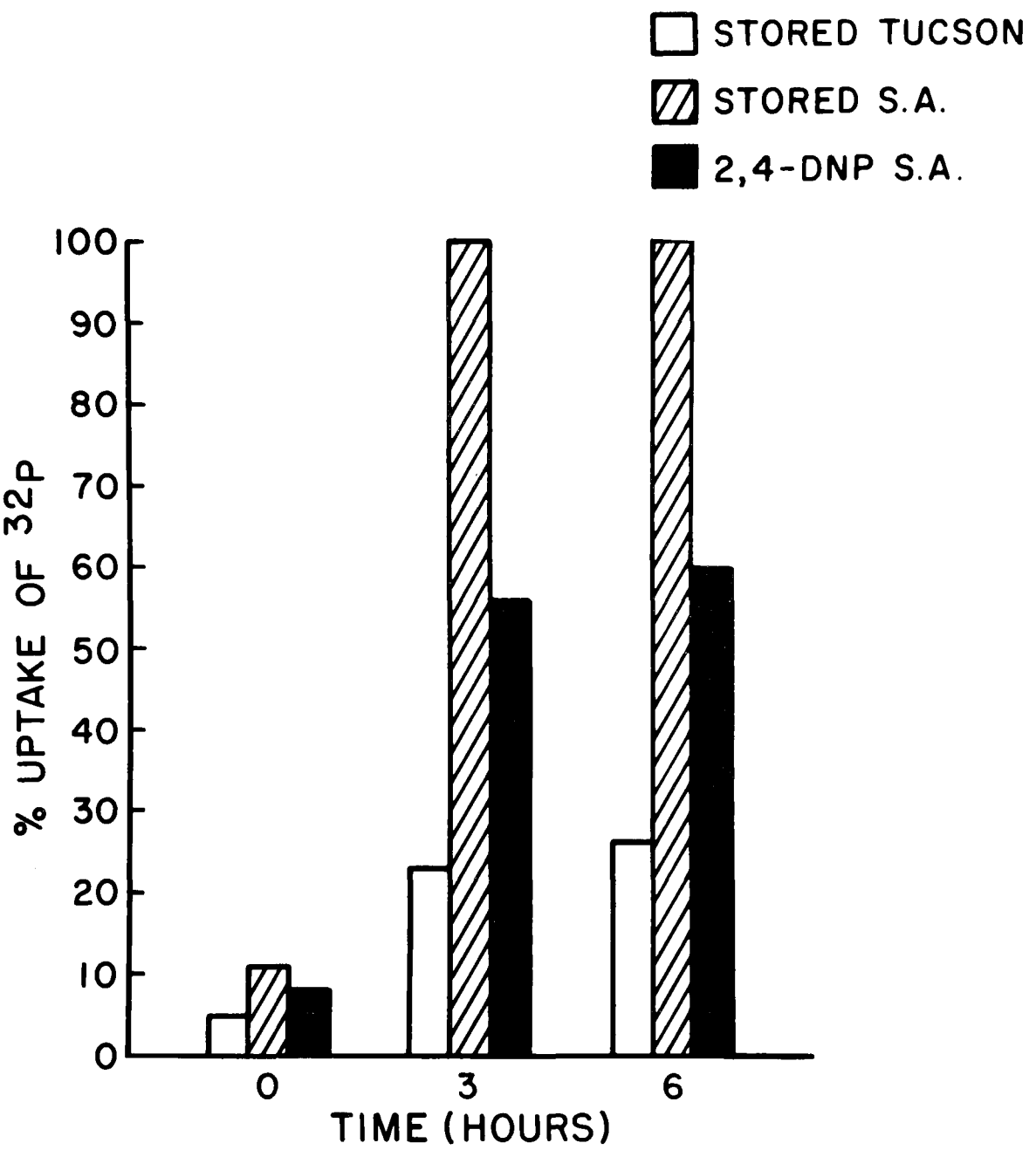

FIG. 6. A COMPARISON OF UPTAKES OF ^{32}P RADIOACTIVITY FROM SEWAGE BY TUCSON AND RILLING SLUDGES. Approximately 130 mg. (dry weight) of Tucson sludge and 265 mg. (dry weight) of Rilling sludge were aerated in Tucson sewage containing approximately 180,000,000 counts per min. per 100-ml. of ^{32}P radioactivity for up to 6 hr.

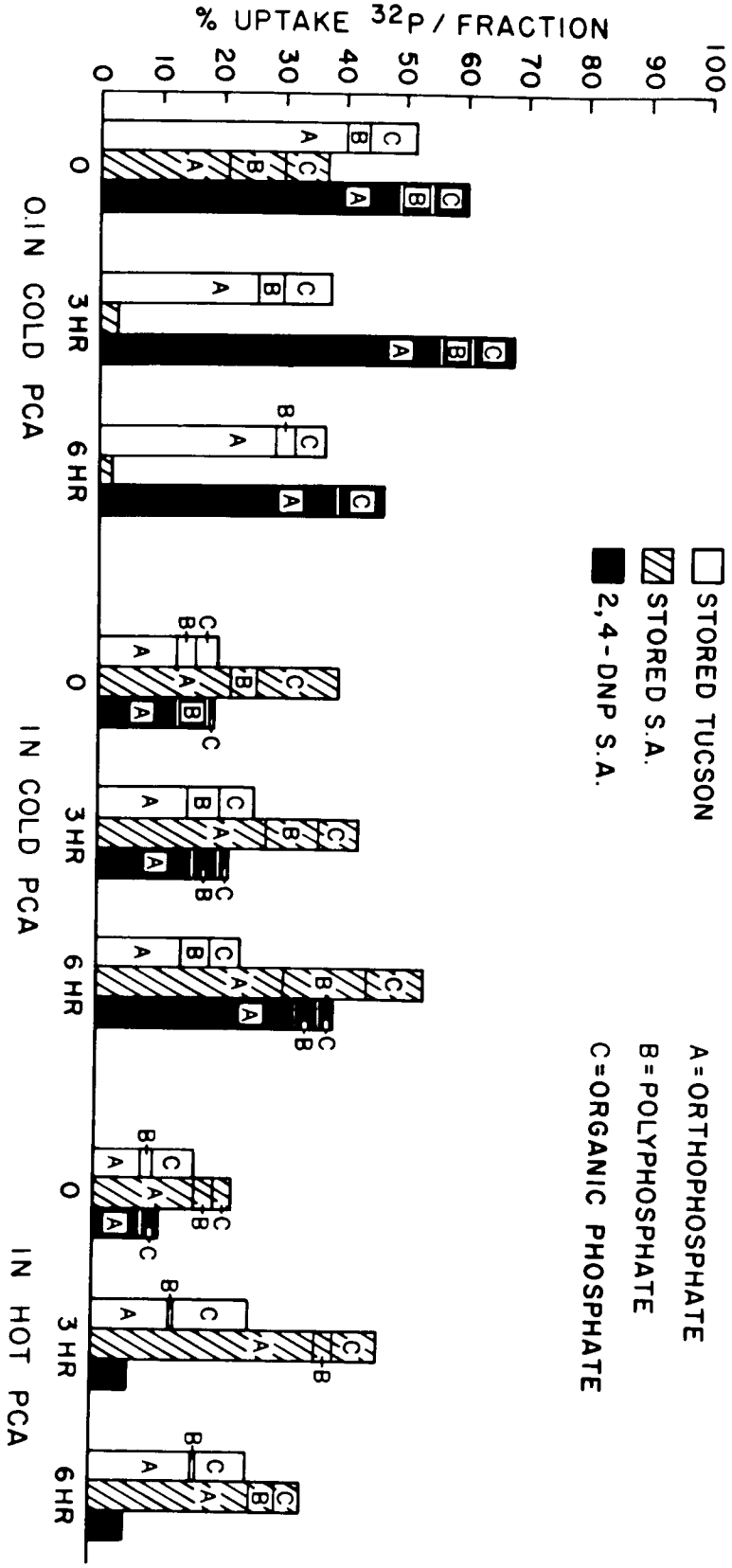

FIG. 7. PER CENT DISTRIBUTION OF ^{32}P RADIOACTIVITY AMONG FRACTIONS EXTRACTED FROM ACTIVATED SLUDGES BY THE OGUR-ROSEN PROCEDURE. For amount of radioactivity taken up, see Fig. 6.

figure.

Table 4 shows that no external sources of energy or addition of specific ions are necessary for phosphorus removal by Rilling sludge. Various ions were detected in sewage and tap water but no Ca^{++} or Mg^{++} were detected in the distilled waters by the analytical methods employed. Hyperion sludge (data not shown) gave identical results.

Table 5 shows the effect of diluting Rilling sludge with various concentrations of salts in distilled water on the uptake of ^{32}P radioactivity. A concentration of 1% NaCl is almost totally inhibitory. The inhibitory ion appears to be Na^{+} as $NaHCO_3$ is quite effective against uptake and KCl is relatively ineffective. Similar results were obtained when orthophosphate uptake was measured (data not shown). The salt effects were reversible as activity was restored when the sludge was washed in tap water (data not shown).

Rilling, like Tucson sludge, will dump phosphate into its suspending medium under conditions of storage. Under our experimental conditions, this phosphate represents an addition to the amount that is already present in the waste water prior to the addition of the sludge. Table 6 shows the results of an experiment designed to discover how much phosphate Rilling sludge will contribute to its suspending medium after overnight storage and whether this phosphate is preferentially removed by the sludge. The data in Table 6 indicate that approximately 29% of the ^{32}P radioactivity appears in the liquid phase after 12 hr. of storage. This radioactivity and orthophosphate is almost completely removed by 1 hr. after aeration is resumed (Sample 1). Samples 2 and 3 represent experiments in which dumped orthophosphate was supplemented with additional KH_2PO_4-K_2HPO_4. A comparison of the rates at which radioactivity and chemical orthophosphate were removed by 3 hr. indicates that the ^{32}P radioactivity which was derived from the sludge cells was not removed preferentially to added orthophosphate. Table 6 also shows that this quantity of Rilling sludge can remove approximately 100 mg. per liter of added orthophosphate from tap water in 3 hr. after periods of storage if the sludge is not removed from its suspending medium. Hyperion sludge was found to have the same capability by similar experiments conducted in this laboratory.

TABLE 4. EFFECT OF SUSPENDING MEDIUM ON REMOVAL OF ORTHOPHOSPHATE AND ^{32}P RADIOACTIVITY (RA) BY RILLING ACTIVATED SLUDGE[a]

	Orthophosphate			RA in Medium[b]		
Medium	Initial (mg/l.)	Final	% Removed	Initial[b]	Final[b]	% Removed
Tucson Sewage	77	ND[c]	100	102,480	BKD[d]	100
Tucson Effluent	110	ND	100	177,700	BKD	100
Distilled H_2O	110	ND	100	166,100	BKD	100
Tap H_2O	110	ND	100	167,160	BKD	100
Deionized H_2O	110	ND	100	176,800	BKD	100

[a]Approximately 250 mg. (dry weight) of sludge contained in a final volume of 100-ml. were used per experiment. All experiments were aerated at 24°C for 3 hr.

[b]Counts/min./ml.

[c]ND = None Detectable

[d]BKD = Background

TABLE 5. EFFECT OF VARIOUS SALT CONCENTRATIONS ON THE UPTAKE OF ^{32}P RADIOACTIVITY (RA) BY RILLING ACTIVATED SLUDGE[a]

Salt	Concentration (%) (Final)	RA in Medium		pH (Final)
		Final[b]	% Uptake	
NaCl	0.01	BKD[c]	100	8.2
NaCl	0.10	BKD	100	7.9
NaCl	1.00	80,570	3	7.7
$NaHCO_3$	1.00	99,350	13	9.1
KCl	1.00	42,270	63	8.2

[a]Approximately 250 mg (Dry Weight) of sludge was diluted to 100-ml. with tap water containing indicated salt concentration and aerated at 24°C for 3 hr. The ^{32}P radioactivity used in the NaCl experiments was approximately 83,460 counts/min/ml.; the initial radioactivity in the other experiments was about 113,220 counts/min/ml.

[b]Counts/min/ml.

[c]BKD = background

TABLE 6. UPTAKE OF RADIOACTIVITY (RA) AND ORTHOPHOSPHATE RELEASED FROM ^{32}P LABELED RILLING SLUDGE[a]

Time (Hr.)	Sample	RA in liquid phase		Orthophosphate in liquid phase[b]	
		Counts/min/ml.	% Removed	mg/liter	% Removed
0	1	94,780	--	105	--
	2	103,300	--	206	--
	3	102,440	--	350	--
0.5	1	7,330	92	6	94
	2	37,460	64	84	59
	3	51,130	50	166	53
1	1	730	99	ND[c]	100
	2	13,780	87	25	88
	3	34,820	66	103	71
3	1	BKD[d]	100	ND	100
	2	3,500	97	4	98
	3	18,280	82	42	88

[a]Approximately 346,990 counts/min. of ^{32}P were fixed per ml. of mixture.

[b]Sample 1 represents orthophosphate dumped from sludge after 12 hr. Samples 2 and 3 represent dumped + additional orthophosphate.

[c]ND = none detectable

[d]BKD = background

Figure 8 shows the effect of incubation temperature on the uptake of ^{32}P radioactivity by Rilling sludge. The optimum temperature appears to be in the range between 24-37°C., which would be characteristic of a biological rather than a chemical phenomenon.

Figure 9A shows the effects of exposing Rilling sludge to 100°C. for varying lengths of time up to 20 min. An exposure time of only 2 min. resulted in a loss of more than 50% in ability to remove ^{32}P radioactivity. Figure 9B shows the effects of varying the temperature for a constant time (30 min.) on the sludge's ability to remove ^{32}P. Rilling sludge can withstand a wide temperature variation (between 5 to 50°C.) for a half-hour under our laboratory conditions without affecting its phosphorus removal capabilities. A drop to 48% in uptake capability resulted when the sludge cells were exposed to 70°C. for 30 min. Autoclaving for 30 min. resulted in complete loss of ability to remove radioactive phosphate.

The effects of varying the pH of the diluting fluid on ^{32}P uptake is seen in Fig. 10. The values represent final pH. A pH range between 7.7 to 9.7 appears to be optimal for phosphorus removal by Rilling sludge in the laboratory.

Table 7 shows the effects of various antimetabolites on the uptake of ^{32}P radioactivity by Rilling sludge. Of those listed, the antimetabolites containing heavy metals such as p-chloromercuribenzoic acid (PCMB) and $HgCl_2$ were effective against phosphorus uptake. Inhibition occurred when the ^{32}P radioactivity was in either tap water or sewage (Table 7, Fig. 11B). No inhibition was observed when 10^{-3}M EDTA was added to the diluting fluid (data not shown).

Figure 11 shows the effects of various concentrations of four antimetabolites on the uptake of ^{32}P radioactivity by Rilling sludge. These compounds, which act on enzymes involved in energy yielding reactions and ATP formation, were quite effective against phosphorus uptake by the sludge. Phosphorus uptake by Hyperion sludge was inhibited by DNP (10^{-3}M). No other antimetabolite experiments were conducted with this sludge.

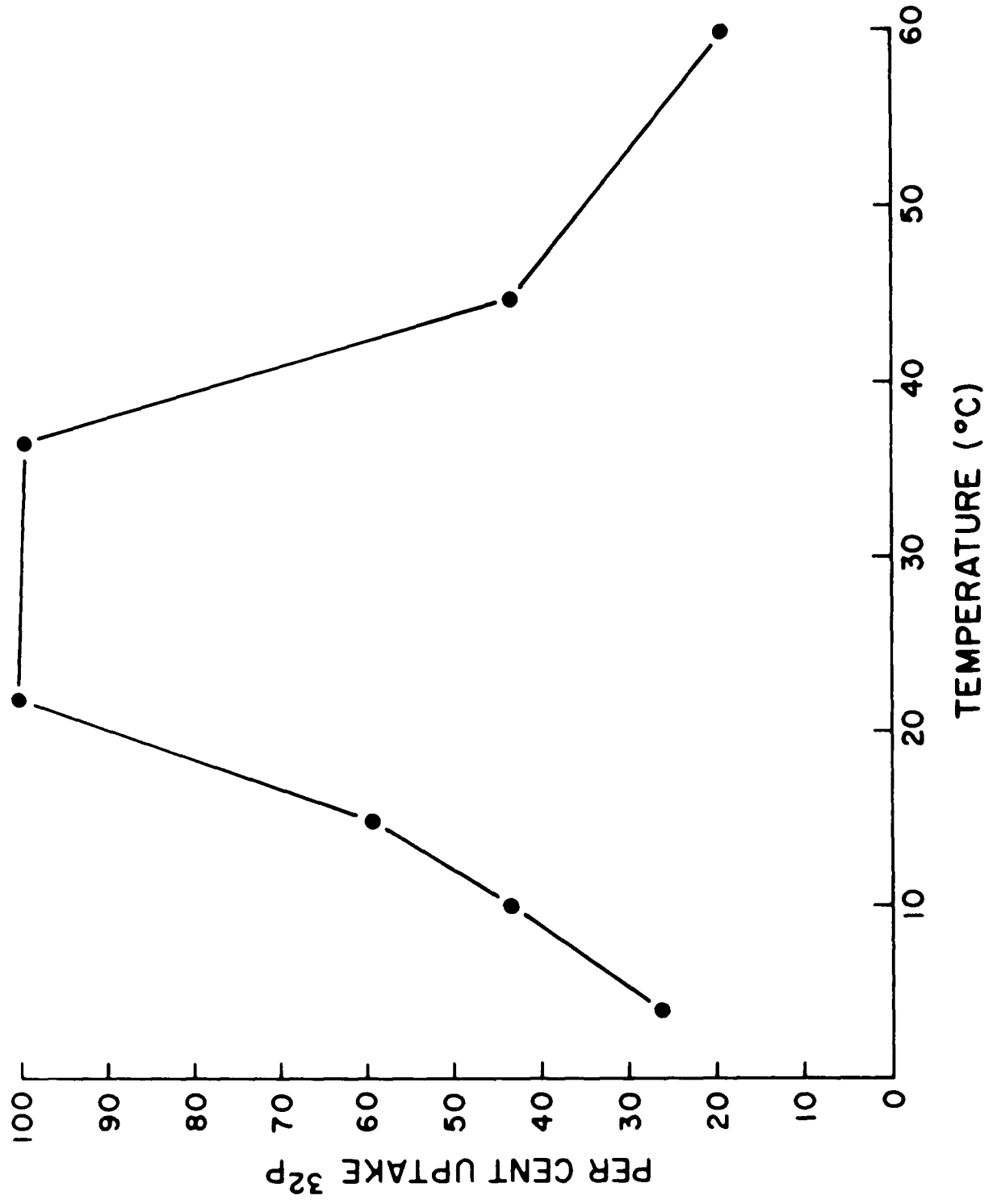

FIG. 8. EFFECT OF INCUBATION TEMPERATURE ON THE UPTAKE OF ^{32}P RADIOACTIVITY BY RILLING ACTIVATED SLUDGE. Approximately 250 mg. (dry weight) of sludge in a final volume of 100-ml. of tap water were aerated for 3 hr. at the indicated temperatures in the presence of approximately 130,000 counts/min/ml. of ^{32}P radioactivity.

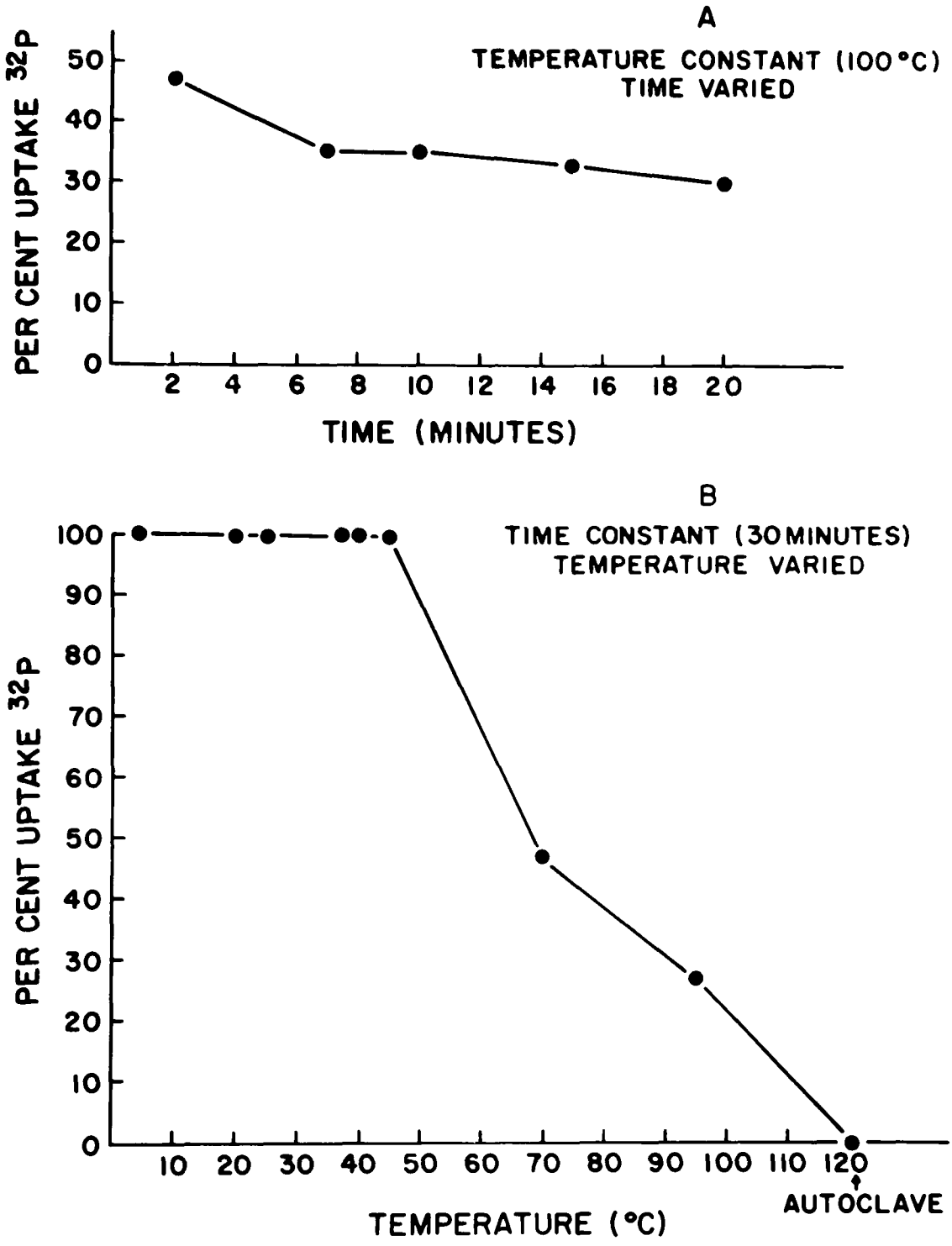

FIG. 9. EFFECT OF VARYING TEMPERATURES AND TIMES ON THE UPTAKE OF ^{32}P RADIOACTIVITY BY RILLING ACTIVATED SLUDGE. (A) temperature constant (100°C.) time varied; (B) time constant (30 min) temperature varied. Approximately 250 mg (dry weight) of sludge in a final volume of 100-ml. with tap water containing approximately 261,000 counts/min./ml. of ^{32}P radioactivity were subjected to the indicated conditions and then aerated at 24°C. for 3 hr.

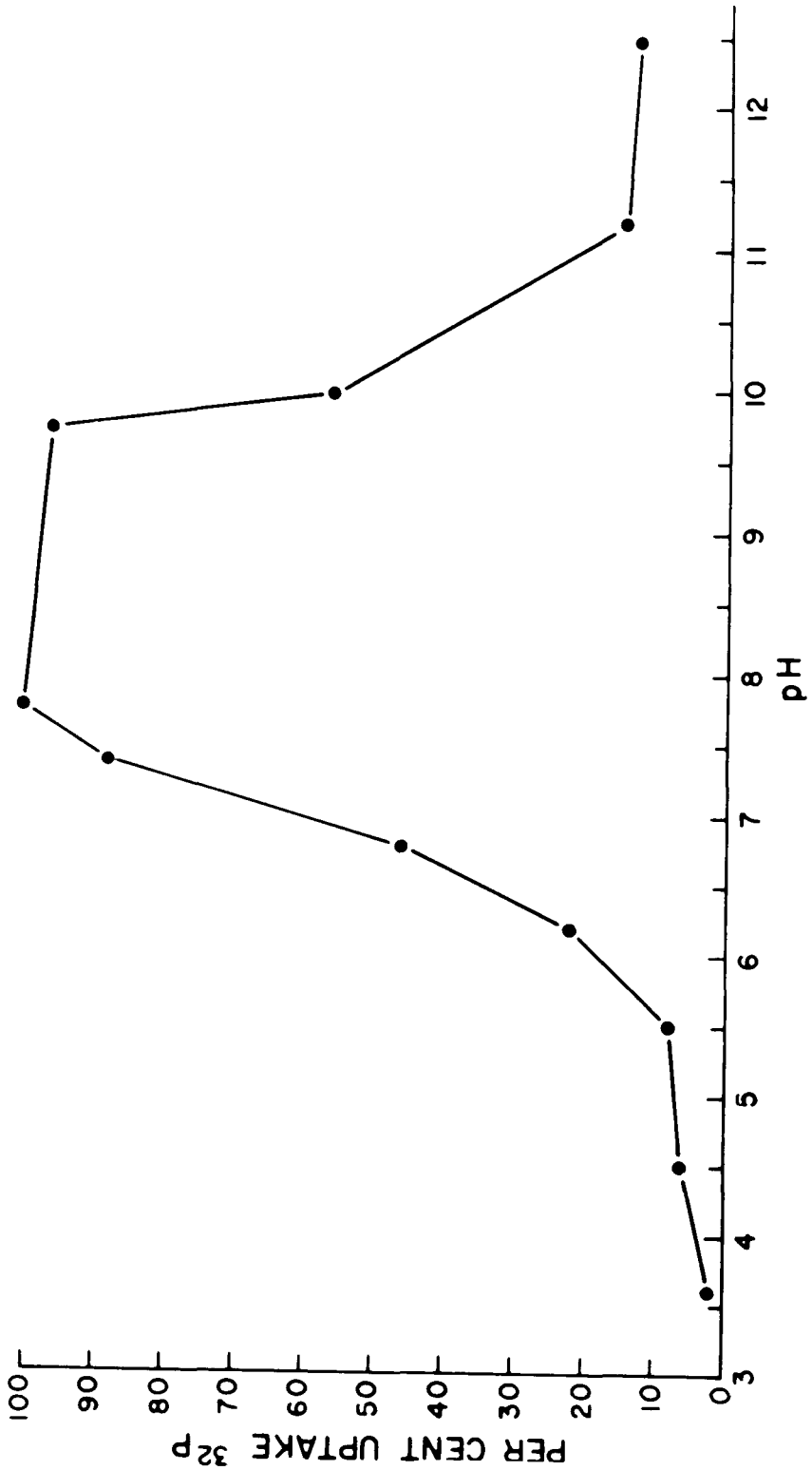

FIG. 10. EFFECT OF VARYING pH ON THE UPTAKE OF ^{32}P RADIOACTIVITY BY RILLING ACTIVATED SLUDGE. Approximately 250 mg. (dry weight) of sludge in a final volume of 100-ml. of tap water adjusted to various pH values were aerated at 24°C. for 3 hr. in the presence of approximately 123,000 counts/min/ml. of ^{32}P radioactivity.

TABLE 7. EFFECT OF VARIOUS ANTIMETABOLITES ON THE UPTAKE OF ^{32}P RADIOACTIVITY (RA) BY RILLING SLUDGE[a]

Experiment Number	Antimetabolite Name	Antimetabolite Final conc.[b]	RA in medium Final[c]	RA in medium % uptake	pH (final)
1	None	0	BKD[d]	100	8.4
2	p-Chloromercuribenzoic acid (PCMB)	10^{-3}	103,480	52	8.2
3	PCMB	10^{-4}	41,100	81	8.3
4	Gramicidin	10^{-3}	BKD	100	8.5
5	Rotenone	10^{-3}	160	99	8.4
6	Oligomycin	10^{-3}	BKD	100	8.2
7	Antimycin A Type III	10^{-3}	BKD	100	8.4
8	$HgCl_2$	10^{-3}	180,220	16	8.1
9	PCMB	10^{-3}	108,800	49	8.2

[a]Approximately 250 mg (dry weight) of sludge contained in a final volume of 100-ml. were used per experiment. All experiments were aerated at 24°C. for 3 hr. Tap water was medium for experiments 1-7 and Tucson sewage was medium for experiments 8 and 9. Approximately 213,320 counts/min/ml. of ^{32}P radioactivity were added initially for all experiments.

[b]moles/liter

[c]counts/min/ml.

[d]BKD = background

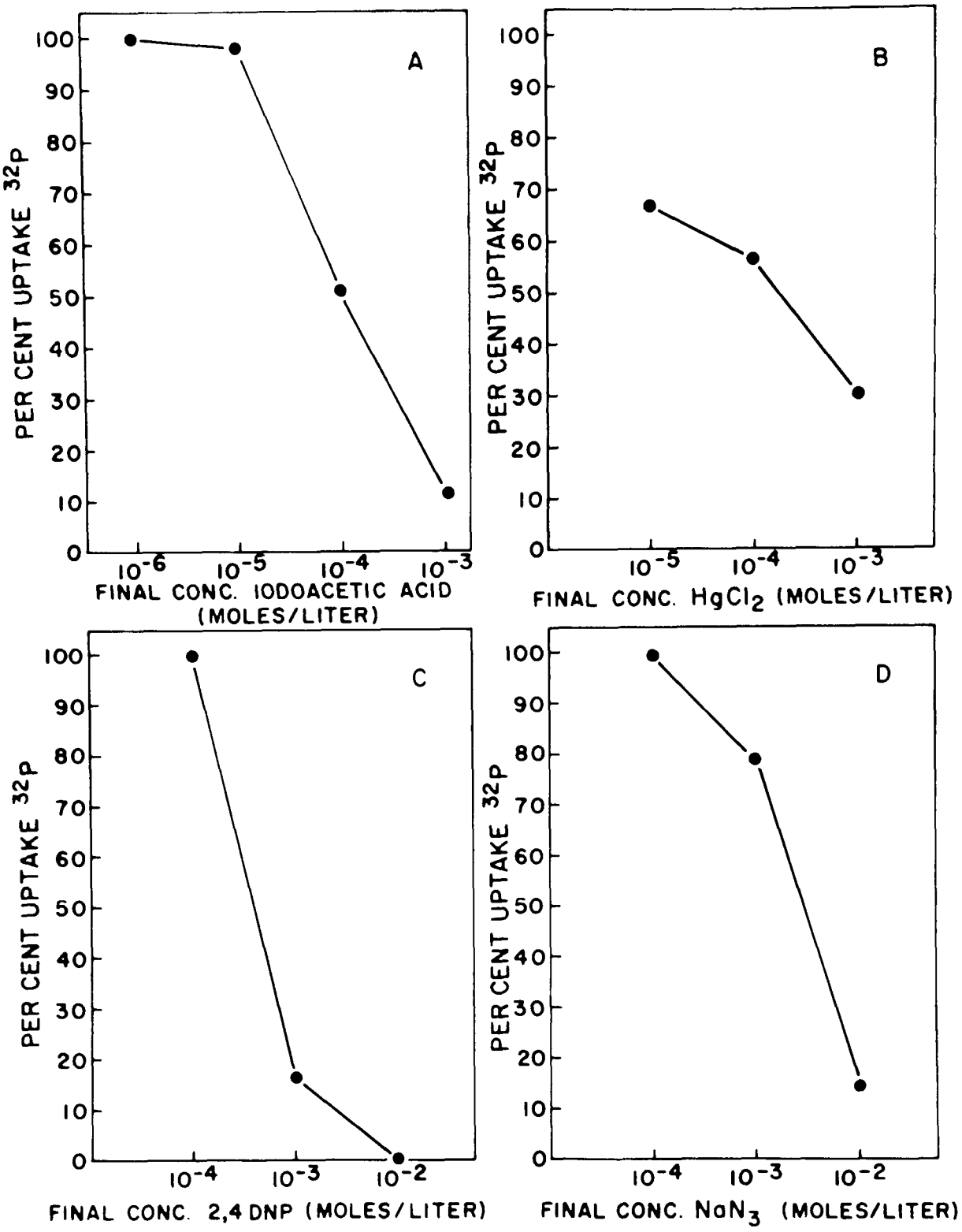

FIG. 11. EFFECT OF VARIOUS CONCENTRATIONS OF METABOLIC INHIBITORS ON THE PER CENT UPTAKE OF ^{32}P RADIOACTIVITY BY RILLING ACTIVATED SLUDGE. (A) iodoacetic acid-initial radioactivity approximately 83,460 counts/min/ml.; (B) $HgCl_2$ - initial radioactivity approximately 213,200 counts/min/ml.; (C) 2,4-dinitrophenol-initial radioactivity approximately 318,900 counts/min/ml.; (D) NaN_3 - initial radioactivity approximately 180,000 counts min/cc. Approximately 250 mg (dry weight) of sludge in a final volume of 100-ml. with tap water were aerated at 24°C, for 3 hr. in the presence of inhibitor and ^{32}P.

Table 8 shows that oxygen is utilized by the Rilling sludge in the presence of either Tucson sewage or tap water. Some increase in Q_{O_2} values occurred in the presence of sewage, however. When DNP was added to the sludge just prior to the start of the experiment, the uptake of ^{32}P radioactivity was inhibited but little effect was seen on the Q_{O_2} values.

When the sludge was preincubated for 1 hr. with DNP prior to the addition of the water containing the ^{32}P radioactivity, considerable inhibition of both oxygen utilization and uptake of radioactivity occurred. This indicates that the Q_{O_2} was affected by a higher initial concentration of DNP than has been used for most of the experiments shown in Table 8.

The nucleic acid content of the sludges were investigated. The orcinol procedure indicated that the 1 N cold PCA extraction (Ogur-Rosen) was not removing all of the RNA from sludge and that a large amount was present in the hot PCA fraction which should contain hydrolyzed DNA. The Schmidt and Thannhauser method was used in order to get a better separation and a more accurate recovery of the nucleic acids. Table 9 gives the per cent dry weight of the nucleic acids extracted from Rilling and Tucson sludges. This table indicates that no net synthesis of DNA and little if any net synthesis of RNA occurs in the sludges by 6 hr. Some RNA must be synthesized as indicated by the action of DNP (see Fig. 7) which inhibits the incorporation of ^{32}P radioactivity into organic phosphate. However, almost equal amounts seem to be broken down.

Table 10 shows the specific activities of the ^{32}P labeled RNA isolated from the sludges. Radioactivity is incorporated into the nucleic acid. However, when this data is examined in conjunction with that shown in Table 9, turnover of the components seems to be the mechanism of incorporation rather than net synthesis.

TABLE 8. EFFECT OF 2,4-DINITROPHENOL (DNP) ON RESPIRATION AND UPTAKE OF ^{32}P RADIOACTIVITY (RA) BY RILLING SLUDGE[a]

Flask Contents[b]	Q_{O_2}[c]	Per Cent Uptake RA
Sludge, 0.8-ml. H_2O, 1.2-ml.	13.4	93
Sludge, 0.8-ml. Sewage, 1.2-ml.	15.0	87
Sewage, 1.2-ml. H_2O, 0.8-ml.	None	None
Sludge, 0.8-ml. Sewage, 1.2-ml. + DNP	15.6	66
Sludge, 0.8-ml. H_2O, 1.2-ml. + DNP	11.4	35
Sludge, 0.8-ml. H_2O, 1.2-ml. + DNP[d]	2.6	21

[a] Experiments represent averages of duplicate flasks on Gilson respirometer incubated with shaking at 25°C. for 1 hr.

[b] Flask contents included the variables listed in the table plus 0.2-ml. of 20% KOH in center well. Sludge used = 5.75 mg./ml. dry wt. Radioactivity introduced = 18,000 counts/min/ml. of $H_3{}^{32}PO_4$. 2,4-Dinitrophenol used = 10^{-3} M final concentration.

[c] Q_{O_2} = ul O_2/hr/mg. dry wt. sludge.

[d] Sludge preincubated with DNP for 1 hr. prior to adding ^{32}P RA.

TABLE 9. NUCLEIC ACID CONTENT OF ACTIVATED SLUDGES

Sludge source	Time (Hr.)	Per cent dry weight		
		Total nucleic acid	RNA	DNA
San Antonio (Rilling)	0	14.6	12.2	2.4
	3	16.5	14.2	2.3
	6	16.1	13.8	2.3
Tucson	0	15.3	11.8	3.5
	3	15.9	12.5	3.4
	6	16.2	12.7	3.5

TABLE 10. SPECIFIC ACTIVITIES (COUNTS/MIN./MG.) OF ^{32}P LABELED RIBONUCLEIC ACID (RNA) ISOLATED FROM ACTIVATED SLUDGES

Sludge source	Time (hr.)	RNA (spec. act.)
San Antonio (Rilling)	0	6,150
	3	90,200
	6	102,700
Tucson	0	2,230
	3	75,800
	6	85,400

ISOLATION OF SLUDGE BACTERIA

Viable bacterial counts of Tucson sludge obtained on successive days were determined using TSA, ASEA, and ASEAG plating media following various treatments to disperse flocs. Results of typical experiments are presented in Table 11. As indicated, viable counts were approximately two- to three-fold higher on ASEAG medium than on TSA irrespective of the nature of the method of dispersal used. In addition, sonication and homogenization proved to be far more effective than conventional shaking for recovery of bacteria from sludge. Four trials were made for each condition except that ASEA was used as a plating medium in only one trial. Approximately 5 to 10 colonies were picked from plates at the highest dilution and streaked for pure culture.

Eighty-five pure cultures have been isolated from Tucson sludge. The majority, 78, were Gram-negative, nonspore-forming, rod-shaped bacteria. Five isolates were Gram-positive rods or cocci including 3 bacilli, 1 micrococcus, and 1 streptococcus. Two isolates proved to be yeasts. The Gram-negative rod shaped bacteria were further characterized according to previously described methods. The results of these tests are summarized in Table 12. All Gram-negative isolates have been tentatively placed in five groups. The Pseudomonas-Xanthomonas (type 1) was the largest comprising 38% of the total Gram-negative bacteria isolated. These organisms were characterized primarily on the basis of polar flagellation, production in some cases of fluorescent pigments, positive cytochrome oxidase test, and their predominantly oxidative metabolism of sugars. Members of the second largest group, *Alcaligenes* (type II), comprising 22% of the Gram-negative isolates were characterized primarily on the basis of lack of pigmentation and motility, positive cytochrome oxidase test, and their inability to metabolize sugars. The *Escherichia-Aerobacter* group (type III), comprising about 17% of the total, were characterized primarily on the basis of peritrichous flagellation and their predominantly fermentative metabolism of sugars particularly lactose. Members of the *Flavobacterium* group (type IV), approximately 12% of the isolates, were so designated owing to the possession of a nondiffusible yellow pigment and a positive cytochrome oxidase reaction. Nine of the isolates, approximately 12% of the total, were placed in the Achromobacter group (type V). This group was characterized primarily on the basis of morphology and their limited biochemical activity. No attempt was made to accurately speciate each individual isolate since we were primarily interested in biological

TABLE 11. EFFECT OF VARIOUS TREATMENTS ON THE RECOVERY OF BACTERIA FROM TUCSON ACTIVATED SLUDGE.

Treatment	Viable counts X 10^7 on:		
	TSA[1]	ASEA[1]	ASEAG[1]
Shaking	1.5[2]	5.3	7.5
Sonication			
3 Sec.	5.5	-	18.5
10 Sec.	9.0	-	25.0
30 Sec.	10.1	-	30.2
Homogenization			
3 Sec.	7.5	-	19.5
10 Sec.	8.5	-	27.2
30 Sec.	11.7	-	29.1

[1] TSA, Trypticase soy agar; ASEA, activated sludge extract agar; ASEAG, activated sludge extract agar + 0.5% glucose.

[2] Mean value derived from counts of three replicate plates.

TABLE 12. PHYSIOLOGICAL PROPERTIES OF BACTERIA ISOLATED FROM TUCSON ACTIVATED SLUDGE.

Type	No. of isolates	Motility	Pigment	Cytochrome oxidase	Nitrite test	Gelatin hydrolysis	Glucose	Lactose	Sucrose	Tentative Group
I[1]	30	+	fluorescent or achromogenic	+	+	variable	A	-	A	Pseudomonas Xanthomonas
II	17	-	-	+	+	-	-	-	-	Alcaligenes
III[1]	13	+	-	-	+	-	AG	AG	AG	Escherichia Aerobacter
IV	9	-	yellow	+	variable	-	-	-	-	Flavobacterium
V	9	-	-	-	-	-	-	-	-	Achromobacter

[1]Type 1 - Polar flagella; type 3-peritrichous flagella

TABLE 13. SOURCE AND DISTRIBUTION OF GRAM-NEGATIVE ISOLATES FROM VIABLE PLATE COUNTS

Plating Medium	Number of Isolates	Type I	II	III	IV	V
TSA[1]	26	12	5	2	2	5
ASEA[1]	14	6	5	1	2	0
ASEAG[1]	8	6	0	0	0	2

[1]TSA, trypticase soy agar; ASEA, activated sludge extract agar; ASEAG, activated sludge extract agar plus 0.5% glucose.

activity such as phosphorus uptake rather than identity. For this reason we relied to a large extent on the determinative scheme for the identification of Gram-negative bacteria proposed by Shewan, et al. (17) and were accordingly able to place our isolates into five major groups based on their gross morphological and physiological properties.

The source of the Gram-negative isolates studied as well as their distribution according to type are shown in Table 13 and Table 14. Thirty-four of 48 isolates obtained from the plating series were types I and II, Pseudomonas-Xanthomonas and Alcaligenes respectively. Moreover, since these isolates were picked from plates at highest dilutions, these data suggest that members of the Pseudomonas-Xanthomonas group are representative of predominant aerobic heterotrophic bacteria of sludge. Source and distribution of isolates from the enrichment series are shown in Table 14. Types of bacteria appear to be randomly distributed and because of limited numbers few generalizations can be made. It is of interest, however, that 10 of 25 isolates obtained from enrichment media containing glucose were of type III, Escherichia-Aerobacter. Thus addition of glucose to activated sludge favors development of lactose fermenting bacteria.

All Gram-negative isolates were assayed for their ability to remove ^{32}P from sewage. A summary of the data obtained

TABLE 14. SOURCE AND DISTRIBUTION OF GRAM-NEGATIVE ISOLATES FROM ENRICHMENT MEDIA[1]

Enrichment Medium	Number of isolates	Type I	II	III	IV	V
ASE[2]	5	2	1	0	1	1
ASEG[2]	7	2	1	4	0	0
ASEGP[2]	5	1	1	3	0	0
ASEGY[2]	8	1	4	2	1	0
ASEGPY[2]	5	0	0	1	3	1

[1]Primary isolation media TSA and ASEG agar.

[2]ASE, activated sludge extract; G, glucose 0.5%; P, K_2HPO_4 0.1%; Y, yeast extract 0.1%.

is shown in Table 15. Zoogloea ramigera, ATCC 19623 and a laboratory strain of E. coli were also assayed as to their ability to remove ^{32}P radioactivity. The former had a specific activity of 150,000 counts per min. per mg. of dry wt. The data indicate that the Pseudomonas-Xanthomonas, Alcaligenes, and Achromobacter take up the largest amounts of radioactivity from phosphorus. Analysis of variance of data indicated that the Pseudomonas-Xanthomonas group, which includes Z. ramigera, take up significantly (within 99.9% confidence limits) more ^{32}P radioactivity than the members of the Escherichia-Aerobacter group which includes E. coli.

Since the addition of glucose to sludge appears to favor the development of a predominantly fermentative microflora, it was of interest to determine whether or not addition of glucose to sewage affected the removal of ^{32}P radioactivity by sludge bacteria. Eight isolates were selected for study including four with high affinity for ^{32}P and four with low affinity. Z. ramigera and E. coli were included for comparative purposes. Results are shown in Table 16. The presence of glucose in sewage markedly enhanced the uptake of ^{32}P radioactivity from sewage. Indeed there was

TABLE 15. REMOVAL OF RADIOACTIVE PHOSPHORUS (^{32}P) FROM SEWAGE BY ACTIVATED SLUDGE BACTERIA.

Group	Number of Isolates	Specific Activity			Log Mean Specific Activity[1]	Standard Error
		Low	High	Mean		
Pseudomonas						
-Xanthomonas	30	9,000	232,000	49,680	4.697	±0.239
Alcaligenes	17	5,000	300,000	35,320	4.548	±0.344
Achromobacter	9	1,000	432,000	57,680	4.761	±0.458
Flavobacterium	9	8,000	83,000	24,380	4.387	±0.458
Escherichia						
-Aerobacter	13	1,000	41,000	10,570	4.024	±0.368

[1] Counts per min. per mg. dry wt. cells; radioactivity used approximately 14,000,000 counts per min. per plate.

TABLE 16. THE EFFECT OF GLUCOSE ON THE UPTAKE OF ^{32}P RADIOACTIVITY FROM SEWAGE BY SELECTED SLUDGE BACTERIA.

Organism		Specific Activity[1]	
		Plus 0.1% Glucose	Minus Glucose
High Group	50	536,000	281,000
	6	256,000	171,000
	93	588,000	141,000
	8	200,000	45,000
	Z. ramigera	306,000	71,000
Low Group	36	616,000	26,000
	46	544,000	17,000
	70	497,000	14,000
	30	541,000	13,000
	E. coli	365,000	11,000

[1]Counts per min. per mg. dry wt. cells; corrected for controls; radioactivity used approximately 14,000,000 counts per min. per plate.

very little difference between representatives of both groups when glucose was present. In the absence of glucose, however, representatives of the high group took up ^{32}P radioactivity in significantly greater quantities. For example in the presence of glucose E. coli and Z. ramigera had specific activities of 365,000 and 306,000 counts per min. per mg. dry wt. respectively. In the absence of glucose Z. ramigera and E. coli had specific activities of 71,000 and 11,000 counts per min. per mg. respectively in the experiment shown in Table 16.

Twelve of these organisms were picked for their "high" or "low" ^{32}P uptake ability and subjected to quantitative assays. Data from some representative experiments are given in Table 17. The amount of radioactivity in the cells was obtained by extracting them with hot 4N HCl and measuring the fraction using a scintillation counting system. In all cases the readings were the same as that obtained for disappearance of the radioactivity from the medium.

Of the organisms listed in Table 17, No. 6 and No. 8 represent the "high" uptake group, as shown by the screening procedure, and were members of the Pseudomonas-Xanthomonas group. Zoogloea ramigera was used as a "high" uptake control. This organism has been found in many sludges although it was not isolated from Tucson material. Organism No. 100 represents the "low" category and is a member of the Escherichia-Aerobacter group. Escherichia-coli served as a control.

The data from all the bacteria tested indicated that the "high" uptake organisms did take up more ^{32}P than did the "low" uptake organisms and that generally there was a corresponding drop in the amount of PO_4-P in the medium.

The next step in the study was a comparison of organisms isolated from high phosphorus affinity sludges and those from Tucson sludge. Cultures were isolated from Rilling sludge and that from a Houston, Texas plant which was reported to be an effective phosphorus remover. The total number of cultures isolated were 151, 98% of which were Gram negative. All of the isolates were subjected to the ^{32}P screening procedure. Table 18 shows a comparison (as per cent) of the ^{32}P affinities of the 229 different cultures isolated from the Houston, Rilling, and Tucson plants.

On the basis of the percentage of isolated organisms falling into each category of ^{32}P affinity, the data in Table 18 does not account for the high uptakes of phosphorus shown by the Rilling sludge as compared to Tucson sludge.

TABLE 17. UPTAKE OF ^{32}P RADIOACTIVITY (RA) AND PO_4-P FROM TUCSON SEWAGE BY BACTERIA ISOLATED FROM TUCSON SLUDGE AND KNOWN ORGANISMS.[a]

Organism	Time (Hr.)	RA in sewage		PO_4-P in sewage	
		Cts./min.	% of total fixed	Mg./liter	% removed
Zooqloea ramigera (ATCC 19623)	0	10,000,000	--	8.0	--
	2	8,200,000	18	7.0	12
	4	6,210,000	38	5.8	27
	6	5,550,000	45	5.2	35
No. 6	0	9,950,000	--	8.0	--
	2	8,457,500	15	7.2	10
	4	6,467,500	35	6.0	25
	6	5,870,000	41	5.4	32
No. 8	0	10,100,000	--	8.0	--
	2	8,585,000	15	7.6	5
	4	6,565,000	35	6.4	20
	6	6,000,000	41	6.0	25
Escherichia coli (lab strain)	0	10,000,000	--	8.0	--
	2	9,050,000	10	7.8	3
	4	8,900,000	11	7.6	5
	6	8,100,000	19	6.4	20
No. 100	0	10,500,000	--	8.0	--
	2	9,500,000	11	7.2	10
	4	8,500,000	19	6.6	18
	6	8,085,000	23	6.4	20

[a]Approximately 20 mg. (dry wt.) of the organism was aerated in 50-ml. of Tucson sewage containing ^{32}P at 24°C.

TABLE 18. A COMPARISON OF ^{32}P AFFINITY RANGES FOR BACTERIAL TYPES ISOLATED FROM VARIOUS ACTIVATED SLUDGES

Sludge source	% of bacterial types isolated	Range[a]
Tucson	17	High
Houston	13	10^5-10^6
San Antonio (Rilling)	22	
Tucson	70	Moderate
Houston	48	10^4-10^5
San Antonio (Rilling)	44	
Tucson	12	Low
Houston	26	10^3-10^4
San Antonio (Rilling)	19	
Tucson	1	Very low
Houston	13	Less than 10^3
San Antonio (Rilling)	15	

[a]Counts/min./mg. of organism

There is a slight increase in the percentage of high ^{32}P affinity organisms isolated from Rilling sludge. However, there is a considerably higher percentage of organisms in the moderate affinity range from Tucson than from Rilling (70% vs 44%). The distribution of organisms isolated from Houston sludge resembles that of Rilling sludge.

A filamentous organism, S. natans, was isolated from Rilling but not from Tucson sludge which appeared superior to the other microorganisms in its ability to remove ^{32}P radioactivity and phosphorus from Tucson sewage. This data may be seen in Table 19. Table 20 compares the uptake of phosphorus by this organism to those listed in Table 17 which confirms the superior phosphorus removing ability of the filamentous organism.

TABLE 19. UPTAKE OF ^{32}P RADIOACTIVITY (RA) AND PO_4-P FROM TUCSON SEWAGE BY SPHAEROTILUS NATANS ISOLATED FROM RILLING SLUDGE[a]

Time (Hr.)	RA in sewage		PO_4-P in sewage	
	counts/min.	% of total fixed	Mg./liter	% removed
0	18,326,000	--	5.8	--
3	6,767,000	63	2.5	57
5	4,364,100	76	1.5	74

[a] 3 mg. (dry wt) of bacteria were aerated in 10-ml. of Tucson sewage containing ^{32}P radioactivity at 24°C.

TABLE 20. AMOUNT OF PO_4-P REMOVED FROM TUCSON SEWAGE PER MG. (DRY WEIGHT) OF ORGANISM

Organism	Amount PO_4-P (mg./l.) removed
Zoogloea ramigera[a]	0.14
No. 6[a]	0.13
No. 8[a]	0.10
Escherichia coli[a]	0.08
No. 100[a]	0.08
Sphaerotilus natans[b]	1.43

[a]For total amounts of PO_4-P removed, see Table 17. Exposure time of organisms to sewage was 6 hr.

[b]For total amounts of PO_4-P removed, see Table 19. Exposure time of organism to sewage was 5 hr.

Figure 12 shows that the organism was in the stationary phase in both arginine broth and the inoculating broth approximately from 72 hr. until 120 hr. after inoculation. Its doubling time was 6 hr. in arginine broth and 8 hr. in the inoculating broth. Cell growth was decreased or stopped if glucose, initial phosphate, or magnesium were deleted from the medium.

Arginine broth contained 3 mg. per liter of phosphate ion; therefore, cultures grown in this medium were phosphate starved by 120 hr. Abundant volutin granules were formed within 4 hr. following the addition of 1.8 g. per liter of phosphate ion to these cultures (Fig. 13A). These granules apparently contained inorganic phosphate as they stained with Tandler's inorganic phosphate stain (21). If the addition of excess orthophosphate was withheld from a culture in arginine broth, granulation never occurred.

When cells labeled with $H_3{}^{32}PO_4$ under phosphate starved conditions in arginine broth were extracted by the Ogur-Rosen procedure, most of the radioactivity appeared in the 1 N cold PCA fraction. Norit A removed the nucleic acids and left most of the activity with the unadsorbed inorganic phosphate. Chromatograms revealed that most of the radioactivity resided in the 1 N PCA fraction as orthophosphate and pyrophosphate rather than as nucleotides. Microscopically the granules remained distinct but completely disappeared when treated with 1 N PCA.

Glucose variation.

In the absence of glucose essentially no granules were formed in arginine broth by the cultures despite the presence of excess phosphate. The presence of 0.1 g. per liter of the monosaccharide gave abundant granules which were too faint to count. The presence of 2 g. per liter of glucose gave abundant dark granules. A further increase in glucose concentration gave darker and more numerous granules; the optimum of 2.5 granules per cell was reached at 10 g. per liter of the sugar after which increased carbohydrate caused a gradual decrease in count (Fig. 14).

Granule counts at 0 g. and 10 g. per liter amounts of the sugar were correlated by assaying media for loss of radioactivity. Carrier free $H_3{}^{32}PO_4$ was added to 120 hr. cultures of Z. ramigera in arginine broth containing either no glucose or 10 g. per liter. In 30 min. the cells in the

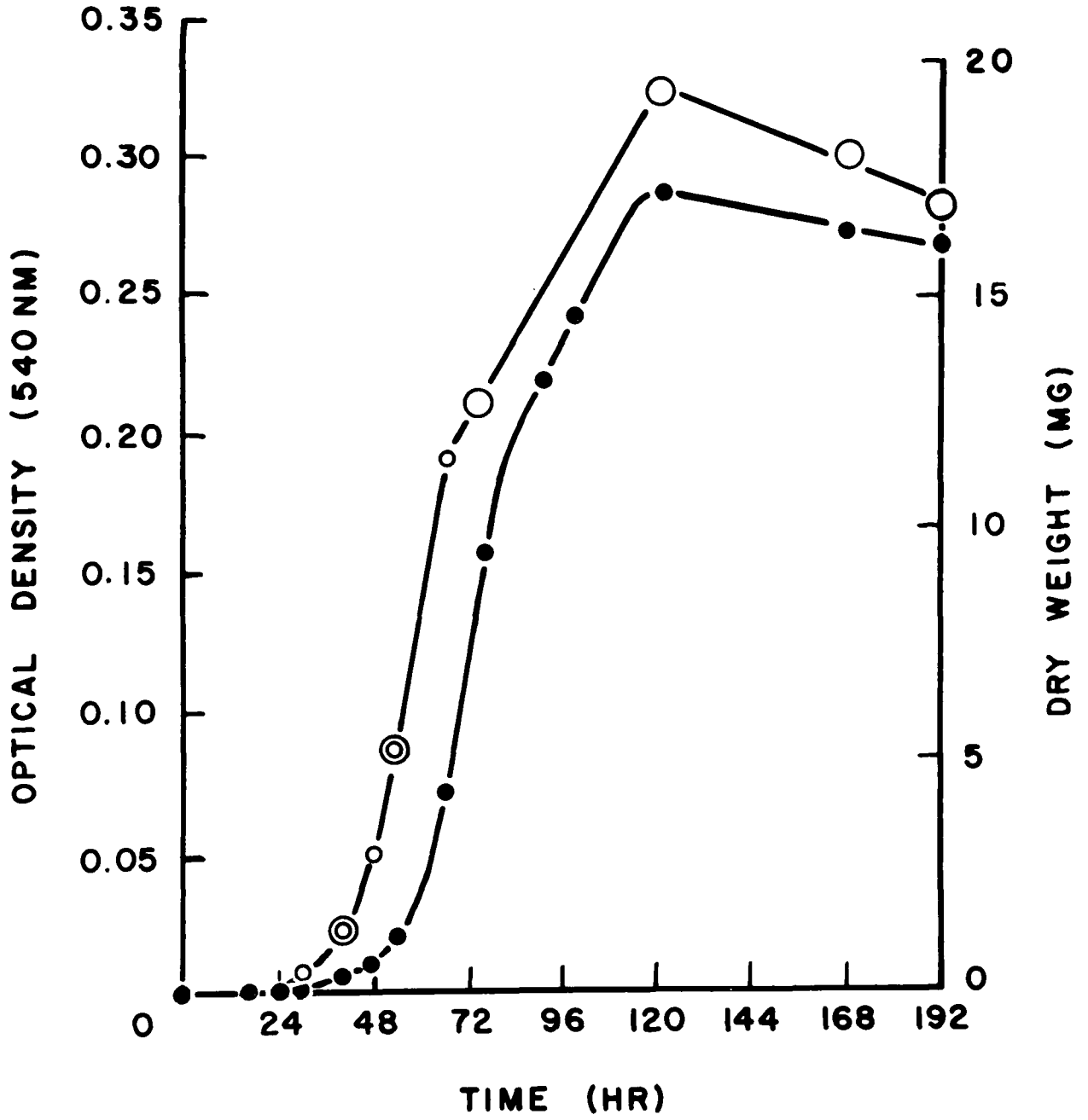

FIG. 12. GROWTH OF ZOOGLOEA RAMIGERA IN ARGININE BROTH AND INOCULATING BROTH. Growth was at 24°C. on a rotary shaker. The symbols are: o, optical density (OD) of growth in arginine broth; O, dry weight of growth in arginine broth; ●, OD of growth in inoculating broth. Ordinate scale is linear in optical density.

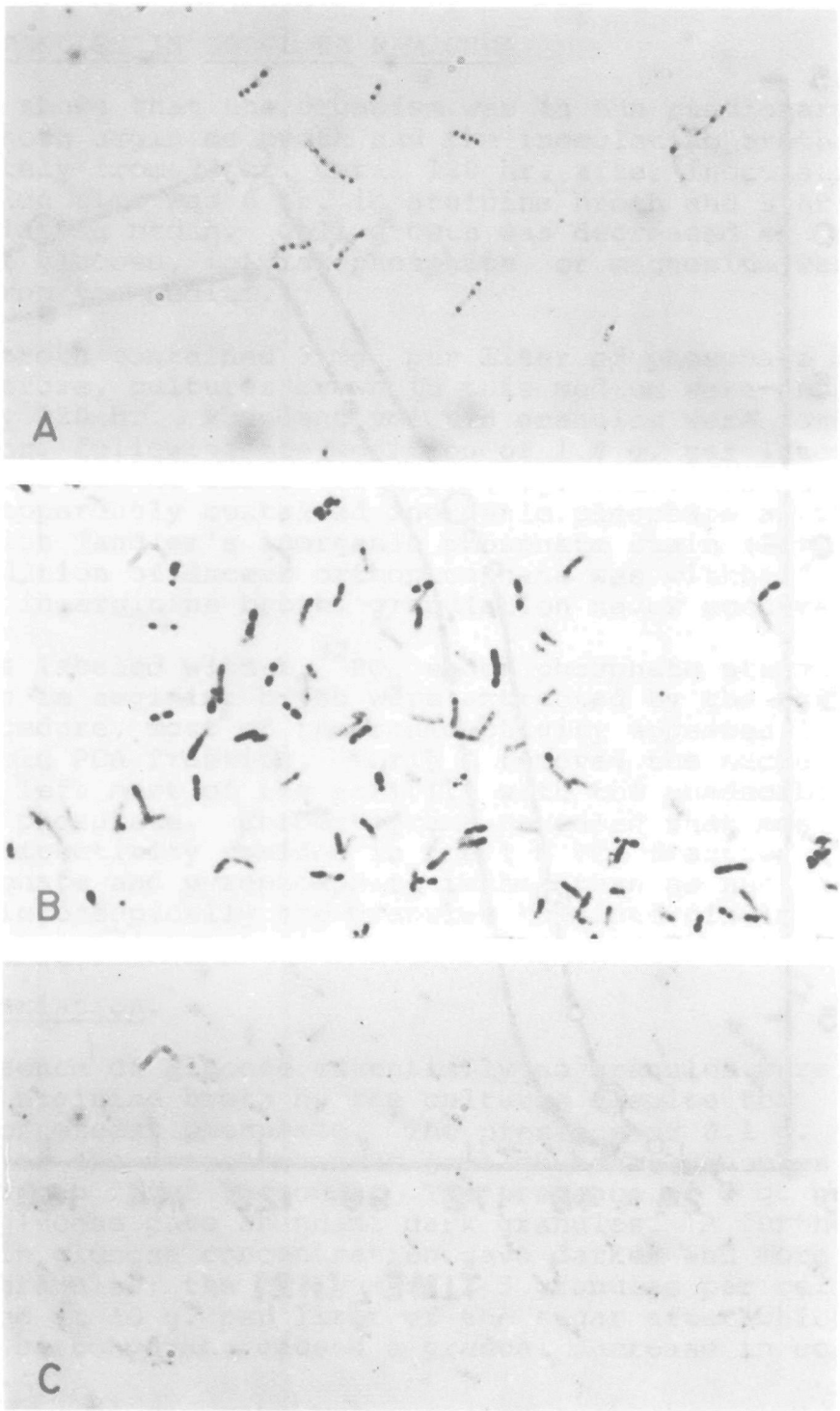

FIG. 13. PHOTOMICROGRAPHS OF VOLUTIN GRANULES IN ZOOGLOEA RAMIGERA. Smears, prepared 24 hr. after adding 1.8 g/liter orthophosphate to phosphate-starved cultures of Z. ramigera grown for 120 hr. in arginine broth, were stained by Neisser's procedure. Magnification is 1250X. (A) Unmodified arginine broth; (B) Arginine broth with 1 mg./liter magnesium ion; (C) Arginine broth with 80 mg./liter magnesium ion.

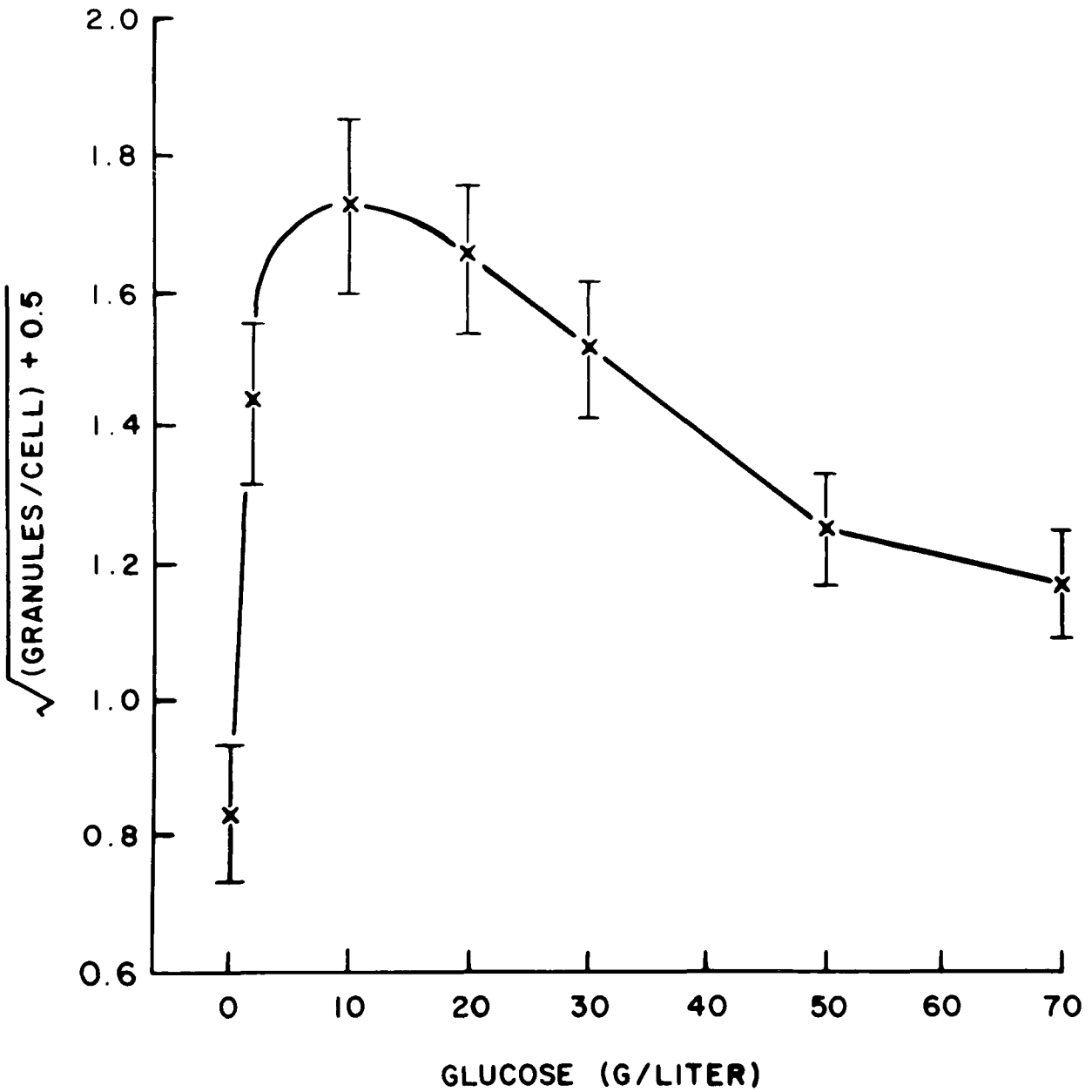

FIG. 14. GRANULATION IN ZOOGLOEA RAMIGERA AT DIFFERENT GLUCOSE CONCENTRATIONS. Granule counts were made 24 hr. after adding 1.8 g./liter orthophosphate to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.

medium containing the sugar removed fivefold more ^{32}P from the medium per mg. dry weight than did the cells in the medium lacking the hexose.

Initial phosphate variation. With only 0.6 mg. per liter of initial phosphate in the arginine broth the granule yield was low. The yield rapidly increased to abundant granulation at a level of 3 mg. per liter; then the level rapidly decreased to a low level by 12 mg. per liter (Fig. 15).

Magnesium variation. No granules were formed in arginine broth in the absence of magnesium ion; but a level of 1 mg. per liter gave large, dark, abundant granules which often filled the whole cell (Fig. 13B). The maximum number of granules occurred when magnesium was present in a range of from 1 mg. to 20 mg. per liter, but at 20 mg. per liter the extra large granules associated with 1 mg. per liter were absent. Further increase in magnesium gave a decreased yield (Fig. 16). At a level of 80 mg. per liter the granules were faint (Fig. 13C).

Interactions. A factorial design was used to find interactions between glucose, initial phosphate, and magnesium in arginine broth. The concentrations of glucose used were 2 g. per liter and 10 g. per liter; the concentrations of initial phosphate ion used were 3 mg. per liter and 18 mg. per liter; and the concentrations of magnesium ion used were 2 mg. per liter and 20 mg. per liter. The organism was grown in arginine broth with the eight possible combinations of the above three nutrients. As usual, 1.8 g. per liter of phosphate were added at 120 hr. and the smears made 24 hr. later.

The results were illustrated with three-dimensional coordinates by drawing two planes, one representing 2 g. per liter of glucose and the other 10 g. per liter of glucose (Fig. 17). No significant difference was found between the two planes at three of the corners, but one corner of the 2 g. per liter of glucose plane was irregularly elevated at 18 mg. per liter of initial phosphate and 20 mg. per liter of magnesium. This represented increased granule production due to interaction between low glucose, high phosphate, and high magnesium concentration.

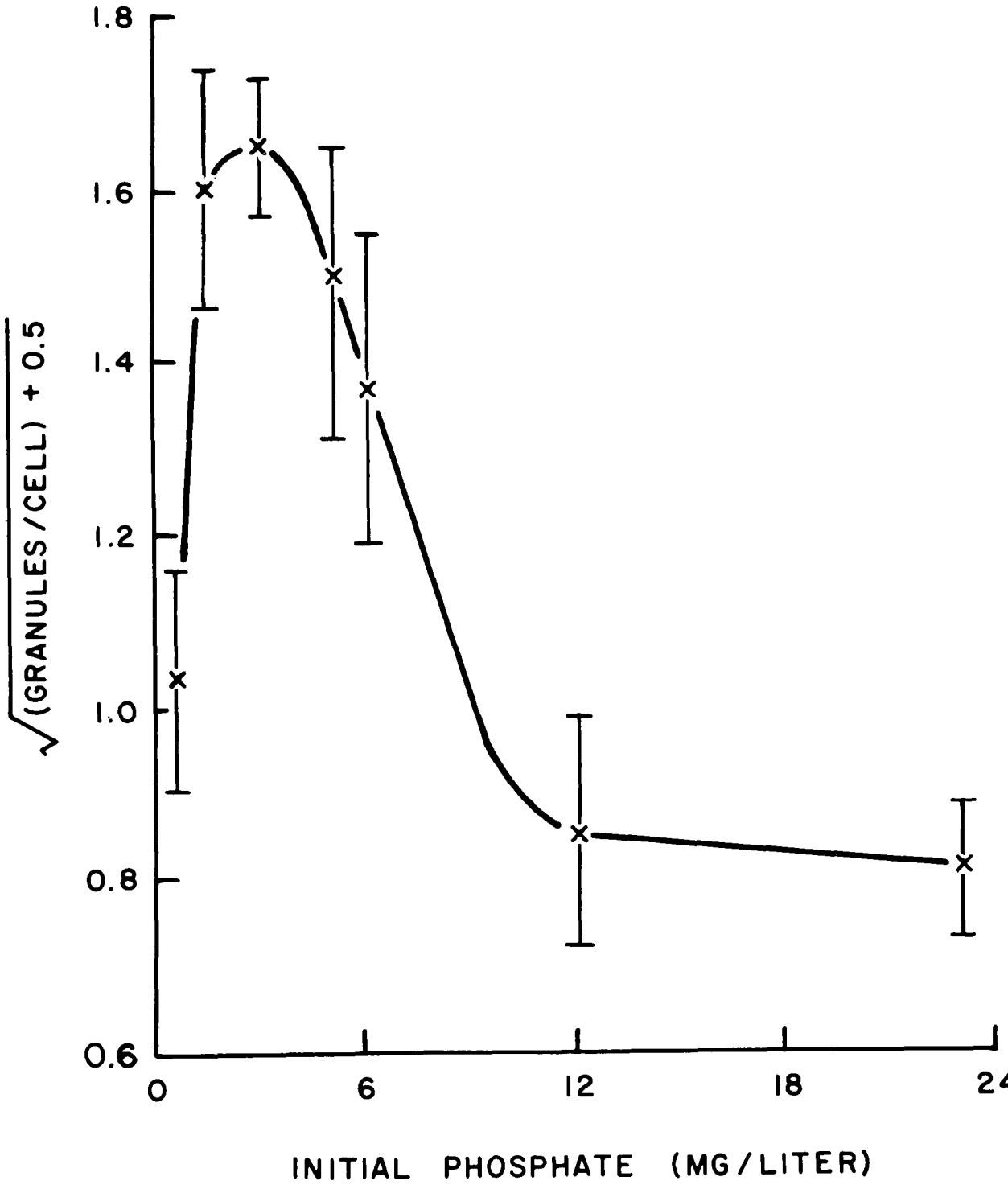

FIG. 15. GRANULATION IN ZOOGLOEA RAMIGERA AT DIFFERENT INITIAL PHOSPHATE CONCENTRATIONS. Granule counts were made 24 hr. after adding 1.8g./liter orthophosphate to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.

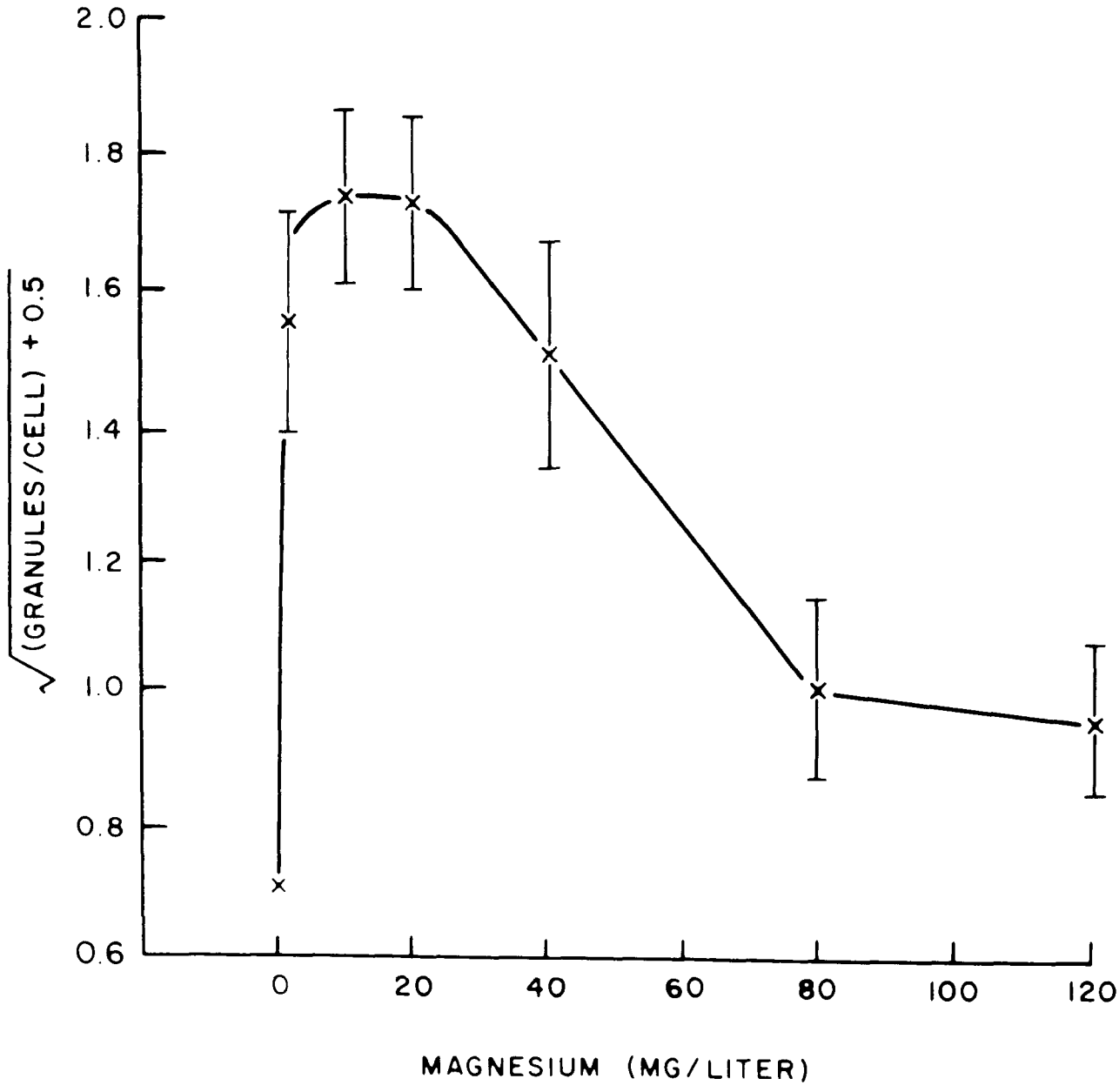

FIG. 16. GRANULATION IN ZOOGLOEA RAMIGERA AT DIFFERENT MAGNESIUM CONCENTRATIONS. Granule counts were made 24 hr. after adding 1.8g./liter orthophosphate to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.

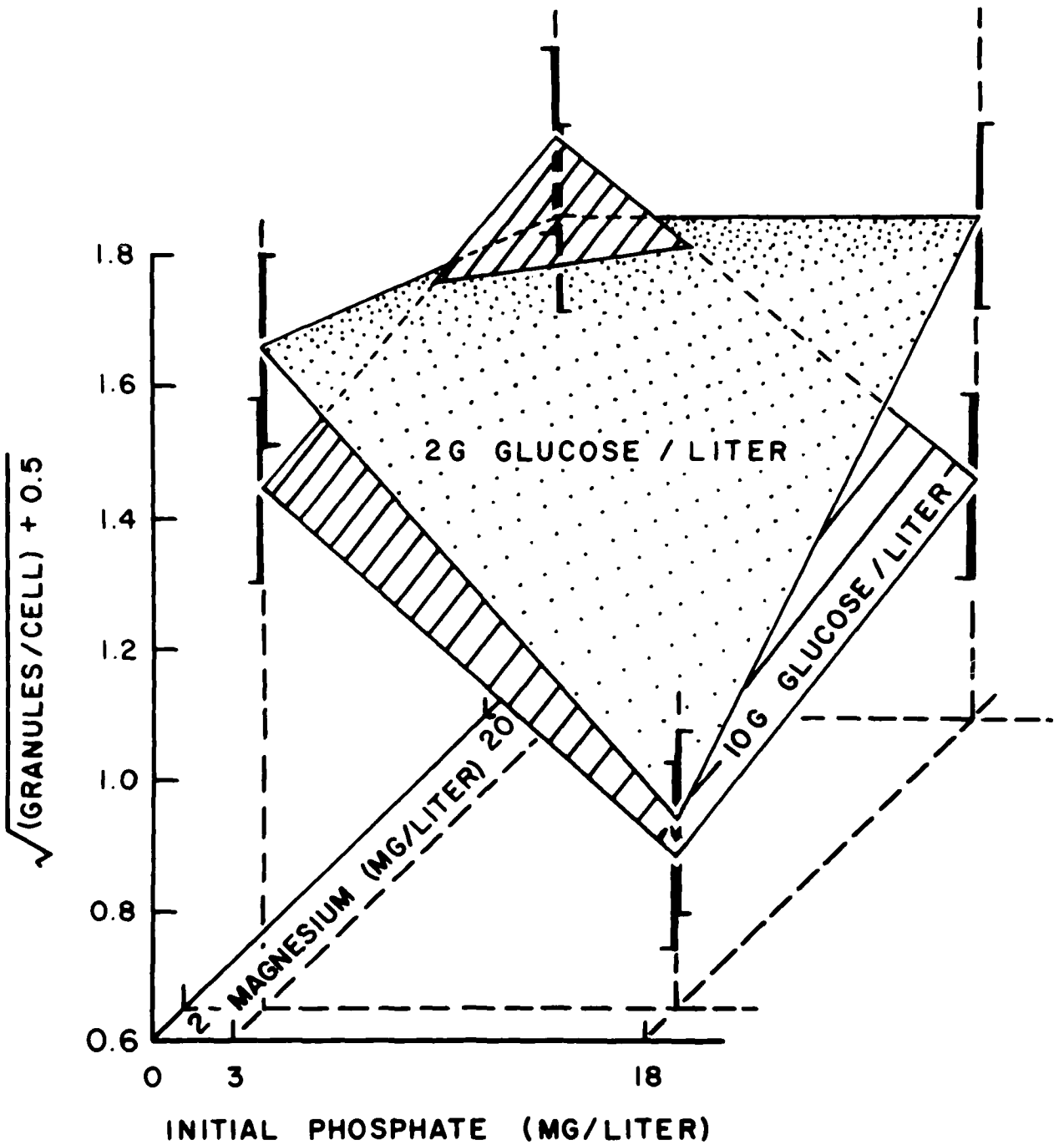

FIG. 17. GRANULATION IN ZOOGLOEA RAMIGERA AT DIFFERENT CONCENTRATIONS OF GLUCOSE, INITIAL PHOSPHATE, AND MAGNESIUM. Granule counts were made 24 hr. after adding 1.8 g./liter orthophosphate to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.

SECTION VI

DISCUSSION

Enhanced phosphorus uptake by Rilling sludge appears to be biological in nature with no specific requirements for exogenous sources of carbon or ions. The uptake is characterized by an optimum temperature range, an optimum pH range, and is inhibited by several antimetabolites that are active against enzymes that result in the ultimate synthesis of adenosine triphosphate.

The experiments shown in Fig. 9 indicate that at least two types of enzyme systems or microbial populations exist that participate in this uptake. One is heat labile and seems to be inactivated by heating at 100°C. for 2 min. (Fig. 9A) or 70°C. for 30 min. (Fig. 9B). The second is very stable and is not fully inactivated until the sludge is autoclaved.

Calcium phosphate precipitation as advocated by Menar and Jenkins (5) seems to play a negligible role under our experimental conditions. The optimum precipitation of phosphates from waste waters by calcium oxide seems to occur at pH 11 (26). According to Fig. 10 pH 11 is approximately 90% inhibitory for the uptake of ^{32}P radioactivity by Rilling sludge. In addition, the glass distilled water contained little if any Ca^{++} and yet the uptake of phosphate from it was not affected (Table 4). The presence of EDTA in tap water did not affect uptake despite the fact that the compound is a chelating agent for calcium (The Merck Index). The uptake of ^{45}Ca radioactivity by the Tucson sludge seemed to have little relationship to the uptake of ^{32}P. Figure 3 shows that a maximum of 33 to 35% of the approximately 4% of the ^{32}P associated with the sludge at zero time could be in the form of an inorganic calcium precipitate.

The uptake of phosphorus by sludge was inhibited by DNP, which is a well known uncoupler of oxidative phosphorylation. Tucson sludge had both uptake and retention of the element affected by the inhibitor (Fig. 4). Uptake of ^{32}P radioactivity by Rilling sludge is totally inhibited by heavy concentrations of DNP (Fig. 11C) and 90% inhibited by NaN_3 (Fig. 11D). This antimetabolite affected the incorporation of ^{32}P into organic compounds (Fig. 7).

The other antimetabolites tested are reported to affect membrane function and in some cases other enzyme systems in various organisms. Gramicidin, Rotenone, Oligomycin, and Antimycin A had no effect on phosphate uptake (Table 7) at

the concentrations tested. With the exception of Gramicidin, the other compounds are claimed to have little effect on bacteria. Both Oligomycin (27) and Antimycin (28) are effective against fungi. Rotenone seems to affect phosphorylation in higher plants (29). The mercurials (PCMB and $HgCl_2$) are effective against a variety of organisms. They are reported to interfere with membrane function and respiration primarily by combining with sulfhydryl enzymes (30). They are effective against Rilling sludge in tap water and Tucson sewage (Table 7, Fig. 11B). Iodoacetate effectively inhibits uptake of ^{32}P radioactivity (Fig. 11A). This compound affects some sulfhydryl enzymes but seems to inhibit phosphorylation mainly by acting against the Embden-Meyerhoff pathway (31). Hotchkiss (32) reported that a related compound (Iodoacetamide) as well as DNP, $HgCl_2$, and Gramicidin were inhibitors of phosphorus uptake by Staphylococcus aureus.

The luxury nature of the phosphorus uptake by Rilling sludge has been confirmed by reports that it can attain total phosphate compositions of 6 to 8% P on a dry weight basis (25). Tables 9 and 10 show that the high phosphorus activity is not related to increased synthesis of nucleic acids. The per cent dry weight of nucleic acids and their specific activities for both Rilling and Tucson sludges are quite similar.

A number of different bacteria were successfully isolated from several sludges by the use of media containing ASE. Similar successes using this type of medium have been reported by other laboratories (33, 34). Activated sludge contains heterogenous populations of bacteria. Species of Pseudomonas-Xanthomonas and Alcaligenes groups appear to predominate when no supplemental sources of carbon such as glucose are added to sewage. The development of a predominantly fermentative microflora from sludge, as well as increased affinity for ^{32}P, is observed in the presence of supplemental glucose.

A filamentous form, S. natans seems to have the best phosphorus affinity of those bacteria isolated (Tables 19 and 20). On the basis of the amount of phosphorus it removes from sewage, it is probably not the primary organism as the intact Rilling sludge can remove about 10 fold as much. It is possible that the primary remover will be this organism in combination with others.

The volutin granules in Z. ramigera appeared to be composed of long chain polyphosphate: They formed immediately after adding excess orthophosphate to a phosphate-starved culture. They were metachromatic, a characteristic of long chain polyphosphate but not of orthophosphate, pyrophosphate,

metaphosphate, or polyphosphate of less than eight phosphate units in length (35). They stained with Tandler's technique which is specific for inorganic phosphate. Most of the radioactivity of ^{32}P labeled orthophosphate taken up by phosphate-starved cells was extracted with the nucleic acids but not subsequently adsorbed by activated charcoal, indicating insoluble polyphosphate, the polyphosphates of high chain length (36). Chromatographically this label was mostly in the orthophosphate and pyrophosphate position, probably due to considerable degradation during extraction. This may be the reason why so much orthophosphate is apparently present in Rilling sludge.

The apparent accumulation of polyphosphate by Z. ramigera when excess orthophosphate was added to a phosphate-starved culture was similar to the polyphosphate overplus phenomenon in A. aerogenes (37). The enzyme involved was probably the same as polyphosphate kinase found in Escherichia coli which takes the terminal phosphate from ATP and builds the polyphosphate polymer (38); in A. aerogenes this enzyme mediates the only route of polyphosphate biosynthesis (37).

Optimum concentrations for glucose, initial phosphate, and magnesium were found for granulation in arginine broth. Too little glucose apparently caused a shortage of ATP, the intracellular phosphate source for polyphosphate biosynthesis; too much glucose caused typical catabolic repression. Too much initial phosphate apparently caused repression of polyphosphate kinase such as occurs in A. aerogenes (37). The requirement for magnesium ion was similar to the magnesium requirement by purified polyphosphate kinase from E. coli (38). Too much magnesium caused typical cationic inhibition.

Granulation occurred in activated sludge in the presence of 2 g. per liter glucose. Granulation in arginine broth showed an unexpected rise in the factorial design with the combination of 18 mg. per liter initial phosphate, 20 mg./liter magnesium, and 2 g. per liter glucose. Tucson raw sewage contains about 34 mg. per liter orthophosphate 19 mg. per liter magnesium ion (personal communication). The carbohydrate level of whole sewage in one study was about 44 mg. per liter carbon: most of this was glucose and sucrose (39). Thus the phosphate and magnesium levels used in the factorial design were close to sewage levels; however, the glucose concentration was 18 times that found in sewage.

SECTION VII

ACKNOWLEDGMENTS

Dr. Irving Yall, Professor of Microbiology and Medical Technology at The University of Arizona was the Project Director and Principal Investigator and Dr. Norval A. Sinclair, Associate Professor was Co-Investigator. Both were the authors of this Report. Dr. William H. Boughton was Research Associate. Richard J. Gottfried, Richard C. Knudsen, William C. Lafferty, and Frank A. Roinestad were Research Assistants.

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SECTION VIII

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SECTION IX

LIST OF PUBLICATIONS

1. Roinestad, F. A., and Yall, I., "Volutin Granules in Zoogloea ramigera," Applied Microbiology, 19, pp 973-979 (1970).

2. Yall, I., Boughton, W. H., Knudsen, R. C., and Sinclair, N. A., "Biological Uptake of Phosphorus by Activated Sludge," Applied Microbiology, 20, pp 145-150 (1970).

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SECTION X

GLOSSARY

Antimetabolite- A substance that prevents metabolism-see Inhibitor.

Adenosine triphosphate-ATP- A compound composed of adenine (a purine base), ribose (a 5-carbon sugar) and three phosphorus atoms as esters on the 5' carbon of the sugar.

Aseptic- Precaution to exclude undesired bacteria.

Deoxyribonucleic acid-DNA- A molecule of high molecular weight composed of subunits of nucleotides containing deoxyribose as the sugar, frequently found in cell nucleus.

Equivalent- That amount of a substance (measured in grams) numerically equal to the formula weight divided by the valence.

Inhibitor- An agent which slows or interferes with growth of bacteria.

Metabolism- The sum of the processes concerned in the building up of protoplasm.

Molarity-M- The number of moles of solute per liter of solution.

Mole- The formula weight of a substance expressed in grams.

Normality-N- The number of equivalences of a substance (solute) per liter of solution.

Nucleotide- 5'-Phosphate ester composed of a purine or pyrimidine base, pentose (5-carbon sugar) and an atom of phosphorus.

Polyphosphates- Inorganic compounds containing more than three atoms of phosphorus (joined in straight chain).

Ribonucleic acid-RNA- Molecule of high molecular weight composed of subunits of nucleotides containing ribose as the sugar, frequently found in cell cytoplasm.

Synergism- The total effect is greater than the sum of two or more effects taken independently.

Volutin- A chromatoid substance, occurring as metachromatic granules in the cytoplasm of various cells.

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16. *Abstract* Activated sludges obtained from the Rilling Road plant located at San Antonio, Texas and from the Hyperion treatment plant located at Los Angeles, California have the ability to remove large amounts of phosphorus from Tucson sewage and other liquors by means of biological mechanisms. Most of the phosphorus seems to accumulate within the sludge cells as orthophosphate. Tucson sludge seems to take up phosphorus by biological mechanisms but removes considerably less from its medium than does Rilling sludge. However, phosphorus uptake by Tucson sludge is improved if the sludge is starved prior to the addition of sewage. The bacteria isolated from Rilling sludge do not individually seem to account for a high phosphorus affinity when compared to those from Tucson sludge. A culture of Sphaerotilus natans was isolated from Rilling but not from Tucson sludge. This organism had a higher affinity for phosphorus than others tested but not sufficient to account for the superior removal properties exhibited by the Texas sludge. A known sludge bacterium, Zoogloea ramigera formed volutin granules when excess orthophosphate was added to a phosphate starved culture. However, the conditions necessary to produce these granules in this organism probably do not exist in normal sewage.

17a. *Descriptors* Phosphates*, Activated Sludge*, Bacteria*, Pseudomonas, Enteric Bacteria

17b. *Identifiers* Tucson, (Ariz.)*, San Antonio, (Texas)*, Los Angeles, (Calif.)* Houston, (Texas), Sewage Treatment Plants, Bacterial Isolation*

17c. *COWRR Field & Group* 05D

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