Biological Uptake of Phosphorus by Activated Sludge

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The ability of activated sludge to remove phosphates was studied by adding carrier-free $^{32}$P to raw sewage and measuring incorporation of the radioactivity into the cells over a period of time. Radioisotope determinations indicated that 48% of the $^{32}$P radioactivity was removed by 12 hr. However, chemical methods indicated that only 30% of the orthophosphate apparently disappeared from the sewage during this period. Experiments with sludge prelabeled with $^{32}$P indicated that considerable phosphate turnover occurred. The cells released large amounts of radioactivity as they were incorporating fresh phosphates. Starvation in isotonic saline for 18 hr caused the sludge to dump phosphate. When introduced into fresh sewage containing $^{32}$P, the starved sludge removed about 60% of the radioactivity in 6 hr with little phosphate turnover. The ability of sludge to remove $^{32}$P was inhibited approximately 83% by 10$^{-3}$ M 2,4-dinitrophenol. This inhibition was at the expense of the cell fraction that contained ribonucleic acid and deoxyribonucleic acid. The sludge cells released orthophosphate when exposed to the chemical agent. Experiments using $^{45}$Ca indicated that calcium phosphate precipitation plays a minor role in phosphate removal under our experimental conditions.

The increased presence of phosphorus and nitrates in wastewaters due to use of fertilizers (and detergents) has led to an acceleration of the process of eutrophication of lakes and other surface waters caused by algal blooms (4). These algal blooms degrade the quality of a lake by giving the water an offensive appearance, odor, and taste. They consume oxygen from the lower waters, forcing fish to leave. The prolific algal growth renders boating and fishing difficult and discourages swimming (3). It has been estimated that phosphorus levels below 0.5 mg of PO$_4$ per liter would control nuisance growth of algae and levels below 0.05 mg of PO$_4$ per liter would almost stop algal growth (2). Present methods of treating wastewaters, through the use of activated sludge, are very effective in removing organic matter and pathogenic bacteria, but they do relatively poorly in removing phosphorus. Because wastewaters usually are relatively low in sources of carbons which results in limitations in microbial growth, any amount of biological phosphorus removal would have to be of the enhanced or "luxury" type. Enhanced uptake implies the ability of the sludge microorganisms to remove and store phosphorus in excess of that actually required for their metabolic needs during the growth process. Such uptake has been reported at the Rilling Road Plant located at San Antonio, Tex. (7).

Two schools of thought exist as to the possibility of enhanced biological phosphorus uptake. Levin and Shapiro (5) reported a series of experiments which in their view indicated that enhanced phosphorus uptake by the microorganisms of activated sludge did occur under the proper conditions of aeration. However, many of their laboratory experiments included the addition of succinate and glucose which could act as extra sources of carbon.

Menar and Jenkins (6) presented data which in their opinion indicated that the high removal of phosphorus from wastewaters reported to occur at the Rilling Road Plant was not the result of enhanced biological phosphorus uptake. They concluded that the sludge microorganisms removed no more than what required for cell synthesis. The remaining phosphorus disappeared from the wastewater as a result of a reaction with calcium present in the water to form a fine precipitate of calcium phosphate followed by an enmeshing of the precipitate into the activated sludge floc. The degree of precipitation would be controlled by the pH conditions existing during the normal course of treatment.

Experiments reported in this paper are the results of efforts to reproduce in the laboratory conditions that might exist in an optimally
aerated, plug flow-activated sludge unit. $^{32}$P or $^{45}$Ca was added to settled sewage in an attempt to discover whether these radioisotopes would become associated with the sludge flocs in a ratio that could suggest a calcium phosphate precipitate or whether sludge has the potential of enhanced biological phosphorus uptake.

MATERIALS AND METHODS

Experimental conditions. (i) For normal conditions, experiments were conducted with 1-liter glass graduate cylinders containing 120 ml of settled, fresh return-activated sludge and 280 ml of fresh raw sewage taken from the primary clarifier at the sewage treatment plant located in Tucson, Ariz. The desired amount of $^{32}$P or $^{45}$Ca radioactivity was placed in the sewage contained in the cylinder prior to the addition of the sludge. The mixtures were aerated from the bottom of the cylinders at the rate of 3 liters of prewet air per min and incubated at 25 C. Any sludge adhering to the sides of the vessels 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 were removed before the sludge settled. The samples were centrifuged in the cold at 27,000 $\times$ g for 10 min and washed once with centrifugation in equal volumes of cold 0.85% saline. 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 were assayed for radioactivity.

(ii) For starved conditions, 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 for normal conditions.

(iii) For prelabeled conditions, sludge was prepared by incubating it in sewage and $^{32}$P for 12 hr under the conditions described for normal conditions. After this time, the aeration was stopped and the sludge was centrifuged at 1,200 $\times$ g for 10 min, washed once with an equal volume of cold raw sewage followed by centrifugation, and resuspended in 70% raw sewage in the cylinders; aeration was accomplished as described for normal conditions.

(v) For inhibitory conditions, the effect of respiratory inhibitors on phosphate uptake was studied by preincubating 120-ml portions of the settled sludge, contained in 500-ml Erlenmeyer flasks, with 10$^{-4}$ M (final concentration) of 2,4-dinitrophenol (2,4-DNP) for 30 min with shaking at 25 C. The mixture was added then to the aeration cylinders containing 180 ml of sewage and the desired amount of radioisotope. The experiments proceeded as described for normal conditions.

(vii) To determine total 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.

Extraction procedure. The sludge components were extracted by the procedure of Wiame (9) as modified by Boughton (Ph.D. Dissertation, University of Arizona, Tucson, 1969). To the pelleted and washed sludge material, 10 ml of freshly prepared 10% trichloroacetic acid was added. The mixture was incubated for 30 min at 4 C. The sample then was centrifuged for 20 min at 17,300 $\times$ 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, but not removed by the initial washing procedure, would 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% trichloroacetic acid for 30 min at 100 C. The sample was centrifuged as described above. The supernatant fraction should contain hydrolyzed ribonucleic acid, deoxyribonucleic acid, long-chain polyphosphates, and acid-soluble protein. It was designated the “hot acid fraction.”

Residual material was extracted with 10 ml of 0.1 n KOH for 30 min at 70 C. The sample was centrifuged as described above. The supernatant fraction should contain alkaline-soluble materials.

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

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 methods 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 hardness in sewage was determined by the ethylenediaminetetraacetate titration method of Hach. Biochemical oxygen demand (BOD) determinations were performed according to standard methods (1). 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 made 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, III.), using techniques that have been described (10). All counts were corrected for decay.

**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.

**RESULTS**

Figure 1 shows the per cent uptake of radioactivity from $^{32}$P or $^{45}$Ca from the sewage as well as the disappearance of orthophosphate, as determined chemically, under our 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 figures represent 5% of the total $^{32}$P radioactivity and 67% of the total $^{45}$Ca radioactivity removed from the sewage in 12 hr. The orthophosphate removed, as indicated by chemical methods (Table 1), 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

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**TABLE 1. Removal of orthophosphate from sewage by activated sludge**

| Sludge condition | Time (hr) | Per cent removal |
|------------------|-----------|------------------|
| Normal           | 3         | 19               |
|                  | 6         | 20               |
|                  | 12        | 30               |
| Starved          | 3         | 42               |
|                  | 6         | 53               |
|                  | 12        | 56               |

* 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.

**Fig. 1. Uptake of $^{32}$P, $^{45}$Ca, and orthophosphate from sewage by activated sludge under normal conditions. Approximately 9,359,430 counts/min of $^{32}$P radioactivity and approximately 8,852,000 counts/min of $^{45}$Ca radioactivity were used per 10 ml of mixture.**

**Fig. 2. Uptake of $^{32}$P, $^{45}$Ca, and orthophosphate from sewage by starved activated 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.**
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 \(^{32}\text{P}\) or \(^{45}\text{Ca}\) 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 (5, 8). 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.

At zero time, approximately 4% of the \(^{32}\text{P}\) radioactivity and 18% of the \(^{45}\text{Ca}\) 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 \(^{32}\text{P}\) 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 1). Starvation enhanced somewhat the uptake of \(^{45}\text{Ca}\), 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 \(^{32}\text{P}\) or \(^{45}\text{Ca}\) in various fractions. At zero time, only 33% of the \(^{32}\text{P}\) 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 \(^{32}\text{P}\) radioactivity in the soluble fraction and 42% in the nucleic acid-polyphosphate fraction. The normal sludge had 92% of its \(^{45}\text{Ca}\) 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 \(^{45}\text{Ca}\) radioactivity associated with the soluble fraction and more, 14%, associated with its nucleic acid fraction. This distribution was unchanged by 12 hr.

Sludge prelabeled with \(^{32}\text{P}\) was placed in fresh raw sewage to examine the possibility that the apparent discrepancy observed when the data obtained by measuring \(^{32}\text{P}\) uptake into normal cells was compared to chemical orthophosphate remaining in the liquid might be due to phosphate turnover (Table 2). 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.

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

a Approximately 3,671,500 counts/min of \(^{32}\text{P}\) 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.
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.

**DISCUSSION**

Phosphate utilization by activated sludge seems to be a dynamic phenomenon requiring a source of energy but not necessarily increases in total cell mass. The differences between the amount of $^{32}$P taken up by the sludge and chemical orthophosphate disappearing from the sewage, observed in the normal experiments (Fig. 1 and Table 1), can be explained by postulating that as the cells take up fresh phosphate they release some that had previously been acquired back into the medium. This supposition is strengthened by the experiment with $^{32}$P-prelabeled sludge (Table 2) which indicates release of $^{32}$P radioactivity while the cells are removing phosphate under normal conditions. Sludge subjected to starvation under nonaeration conditions released phosphate while in physiological saline in a similar manner to that reported by Wells (8), but its ability to remove phosphate was greatly enhanced when placed in sewage under aeration conditions (Fig. 2). These results under our laboratory conditions may indicate some merit to the proposal by Levin and Shapiro (5) that a phosphate stripping procedure on a full plant scale would improve the phosphate uptake from wastewater during sludge treatment.

The experiments with 2,4-DNP (Fig. 4, 5) confirm that most of the uptake of $^{32}$P is biological in nature. This chemical uncouples electron transport and phosphorylation in many microorganisms. Our results indicate that not only is $^{32}$P uptake inhibited but also the ability of sludge to retain phosphate is affected. It would seem that much of the phosphate taken up by the sludge is associated with a mechanism involving the synthesis of adenosine triphosphate. Levin and Shapiro (5) reported that orthophosphate uptake by sludge was inhibited by 2,4-DNP. However, the sewage used in their experiments contained added glucose and succinate.

Our experimental results with $^{45}$Ca show that whereas some of this isotope becomes associated
with the sludge floc, there is little relationship between this and the $^{32}$P taken up. 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. This assumes that no other types of soluble phosphates are present. The remaining $^{32}$P was rapidly disseminated among the various cell fractions. These fractions are being subjected to analytical procedures in an effort to discover the identities of the individual compounds and the amounts of phosphorus present in each throughout the duration of the experiments.

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