Metabolic Factors Affecting Enhanced Phosphorus Uptake by Activated Sludge

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Activated sludges obtained from the Rilling Road plant located at San Antonio, Tex., and from the Hyperion treatment plant located at Los Angeles, Calif., have the ability to remove all of the orthophosphate normally present in Tucson sewage within 3 hr after being added to the waste water. Phosphorus removal was independent of externally supplied sources of energy and ions, since orthophosphate and 32P radioactivity were readily removed from tap water, glass-distilled water, and deionized water. Phosphorus uptake by Rilling sludge in the laboratory appears to be wholly biological, as it has an optimum pH range (7.7 to 9.7) and an optimum temperature range (24 to 37 C). It was inhibited by HgCl2, iodoacetic acid, p-chloromercuribenzoic acid, NaCl, and 2,4-dinitrophenol (compounds that affect bacterial membrane permeability, sulfhydryl enzymes, and adenosine triphosphate synthesis). Uptake was inhibited by 1% NaCl but was not affected by 10-4 M ethylenediaminetetraacetic acid disodium salt (a chelating agent for many metallic ions).

The presence of phosphorus-containing compounds in waste waters is thought to be partially responsible for nuisance growth of algae now common in lakes and waterways in many parts of the United States (3). One ultimate objective of waste water purification is to reduce the phosphorus levels below 0.5 mg of PO4 per liter, which should control algal growth (2). Activated sludge treatment of waste water, the most common method, usually is unable to remove sufficient phosphorus to control algal blooms when phosphorus is the limiting nutrient. However, sludges from several plants in the United States have been reported to have high affinities for phosphorus and to remove this element rapidly and completely when it occurs in normal amounts in their natural waste waters. One of these sludges occurs at the Rilling Road plant, San Antonio, Tex. (13), and another at the Hyperion treatment plant at Los Angeles, Calif. (1).

The mechanisms by which high-affinity sludges remove phosphorus have not been fully elucidated. Waste waters usually are relatively low in sources of carbon; therefore, microbial growth is relatively limited and slow. Rapid biological uptake of phosphorus in excess of the metabolic needs of the sludge microorganisms is termed enhanced or luxury uptake and implies that the cells have the ability to store the element in some form. Menar and Jenkins (9) 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 Ca2+ in the waste water. Under the proper conditions of pH, a precipitate of calcium phosphate would form and be enmeshed into the activated sludge floc. Subsequent settling of the sludge would result in apparent disappearance of the phosphate from the supernatant fluid. Recent studies by Bargman, Betz, and Garber (1) suggest that removal by Hyperion sludge is largely biological.

Yall et al. (16) reported a study concerned with the uptake of phosphorus by activated sludge from the plant at Tucson, Ariz. This sludge, of relatively low phosphorus affinity, under normal conditions will remove about 20% of the phosphate normally found in its sewage in 6 hr. By means of starvation techniques, the removal was increased to more than 50%. Phosphorus removal by this sludge could not be related to calcium phosphate precipitation. The results of inhibition studies with 2,4-dinitrophenol (DNP) led the authors to conclude that phosphorus uptake by this sludge was largely biological in nature.

The experiments described in this report represent efforts to discover whether various
metabolic factors that affect bacterial metabolism will also affect enhanced phosphorus uptake by activated sludge. These experiments were conducted with Rilling sludge, and a few were duplicated with Hyperion sludge.

**MATERIALS AND METHODS**

*Activated sludge.* Sludge from the Rilling Road plant at San Antonio, Tex., was concentrated by filtration at the plant and shipped to Tucson overnight by surface carrier. The sludge was stored at 4°C until needed, usually within 1 week after collection. It was diluted with tap water to the desired concentration immediately before use. Several experiments were conducted with sludge from the Hyperion treatment plant at Los Angeles, Calif. The material was shipped by air in a frozen state, stored at 4°C, and used within 1 week after collection. The freezing process did not seem to alter the phosphorus removal abilities of the sludge.

*Experimental conditions.* In the general procedure, 33 ml of sludge in the desired concentration (as determined by dry weights) was added to 66 ml of liquid contained in Kimax tubes (38 by 200 mm). The desired amount of $^{32}$P-radioactivity and any additional phosphate (as KH$_2$PO$_4$ and K$_2$HPO$_4$), inhibitor, salt, or ethylenediaminetetraacetic acid (EDTA) disodium salt were placed in the tube before the sludge was added. 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 before 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 × g for 10 min. The supernatant fractions were separated from the pellets and assayed for $^{32}$P-radioactivity with the aid of a Tri-Carb liquid scintillation counting system (model 314 EX-2, Packard Instrument Co., Downers Grove, Ill.; reference 12) and chemically for orthophosphate (16). All counts were corrected for decay. Determinations of pH were made with a pH meter (Leeds and Northrup Co., Philadelphia, Pa.). The usual experimental run consisted of a block of 12 tubes.

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

For manometric experiments, 0.8 ml of sludge was added to 1.2 ml of tap water or sewage containing $^{32}$P-radioactivity (and DNP when required) in the main portion of a 16-ml double side-arm reaction vessel with a center well containing 0.2 ml of 20% KOH. The flasks were placed on a resiprome-ter (model GR-14; Gilson Medical Electronics, Middleton, Wis.) and equilibrated for 15 min at 25°C with the vessels open to air. Then the vessels were closed, and readings were taken over a 1-hr period. When preculture with DNP was required, the powdered inhibitor was added to the sludge in an amount sufficient to give the desired final concentration (10$^{-3}$ M) when diluted in the reaction vessel. The sludge-inhibitor mixture was incubated with shaking at 25°C for 1 hr before addition to the vessel.

For studies concerned with the effects of varying inoculation 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 were conducted as described above in incubators set at the required temperatures.

For the constant time-varying temperature experiments, sludge was incubated for 30 min at the desired temperature and then cooled rapidly to room temperature (25°C). The experiments were conducted as described above.

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 described above.

**Table 1. Effect of suspending medium on removal of orthophosphate and $^{32}$P radioactivity by Rilling activated sludge**

| Medium             | Orthophosphate | $^{32}$P radioactivity in medium |
|--------------------|----------------|---------------------------------|
|                    | Initial (mg/liter) | Final | Per cent removed | Initial | Final | Per cent removed |
| Tucson sewage      | 77              | ND$^c$  | 100            | 102,480 | BKD$^d$ | 100 |
| Tucson effluent    | 110             | ND$^e$     | 100           | 177,700 | BKD | 100 |
| Distilled water    | 110             | ND$^e$     | 100           | 166,100 | BKD | 100 |
| Tap water          | 110             | ND$^e$     | 100           | 167,160 | BKD | 100 |
| Deionized water    | 110             | ND$^e$     | 100           | 176,800 | BKD | 100 |

$^a$ Approximately 250 mg (dry weight) of sludge contained in a final volume of 100 ml was used in each experiment. All experiments were aerated at 24°C for 3 hr.

$^b$ Values expressed as counts per minute per milliliter.

$^c$ None detectable.

$^d$ Background (43 counts/min).
TABLE 2. Effect of various salt concentrations on uptake of $^{32}$P radioactivity by Rilling activated sludge

| Salt     | Final conc (%) | $^{32}$P radioactivity in medium | pH final | Final | Uptake (%) |
|----------|----------------|---------------------------------|----------|-------|------------|
| NaCl     | 0.01           | BDKa                           |          | 100   | 8.2        |
| NaCl     | 0.10           | BDK                           |          | 100   | 7.9        |
| NaCl     | 1.00           | 80,570                          |          | 3     | 7.7        |
| NaHCO₃   | 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 the 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 per min per ml; the initial radioactivity in the other experiments was about 113,220 counts per min per ml.

b Values expressed as counts per minute per milliliter.

c Background (43 counts/min).

For prelabeled conditions, 134 ml of sludge and 266 ml of tap water containing $^{32}$P-radioactivity were placed in a 1-liter glass graduate cylinder and aerated for 3 hr at 25 °C. After aeration, 5-ml samples were removed and assayed for $^{32}$P-radioactivity and orthophosphate in the supernatant fractions and for total uptake of radioactivity in the cells (16). Samples (100 ml) were transferred from the large aerator to tubes (38 by 20 mm) 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 was placed in some of the tubes, and aeration was started. The experiments were conducted as described above, except that 5-ml samples were taken for analysis.

For studies involving optimum pH, the water used to dilute the sludge was titrated with 10% HCl to establish acid ranges, with concentrated NH₄OH for pH 7.0 to 10.0 and with 10% KOH for more extreme alkalinity. The experiments were conducted as described above.

Chemicals. Carrier-free $^{32}$P (orthophosphoric acid in 0.2 N HCl) was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y. NaN₅ (Fishier), HgCl₂ (Matheson, Coleman, and Bell), and DNP (Mallinckrodt) were obtained from local vendors. All other antimonitobites and EDTA were obtained from Sigma Chemical Co., St. Louis, Mo. All chemicals were of the highest purity commercially available.

RESULTS

Rilling sludge was able to remove completely orthophosphate and $^{32}$P radioactivity from Tucson sewage within 3 hr, and no external sources of energy or addition of specific ions was necessary for phosphorus removal (Table 1). Various ions were detected in sewage and tap water, but no Ca$^{2+}$ or Mg$^{2+}$ was detected in the distilled waters by the analytical methods employed (16). Hyperion sludge (data not shown) gave identical results.

The ability of Rilling sludge to remove 100% of the phosphate from tap water (Table 1) was found to persist for at least 11 days after collection. After 16 days of storage, the sludge removed 63% of the orthophosphate in 3 hr. After 36 days, it was able to remove 34%.

Table 2 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 was almost totally inhibitory. The inhibitory ion apparently was Na⁺, as NaHCO₃ was quite effective against uptake and KCl was relatively ineffective. Similar results were obtained when orthophosphate uptake was measured (data not shown).

Tucson sewage usually contains about 30 mg of orthophosphate per liter. When sludge is subjected to periods of storage under somewhat anaerobic conditions, such as those found in a clarifier in a treatment plant, it dumps phosphate into its suspending medium. Under our experi-

TABLE 3. Uptake of radioactivity and orthophosphate released from $^{32}$P-labeled Rilling sludge

| Time (hr) | Sample | Radioactivity in liquid phase | Orthophosphate in liquid phase |
|-----------|--------|-------------------------------|--------------------------------|
|           |        | Counts per min per ml | Per cent removed | Mg/liter | Per cent removed |
| 0         | 1      | 94,780                        | 105 |
|           | 2      | 103,300                       | 205 |
|           | 3      | 102,440                       | 350 |
| 0.5       | 1      | 7,330                         | 92   | 5    | 94   |
|           | 2      | 37,460                        | 64   | 84   | 59   |
|           | 3      | 51,130                        | 50   | 166  | 53   |
| 1         | 1      | 730                           | 99   | ND*  | 100  |
|           | 2      | 13,780                        | 87   | 25   | 88   |
|           | 3      | 34,820                        | 66   | 103  | 71   |
| 3         | 1      | BDKd                          | 100  | ND*  | 100  |
|           | 2      | 3,500                         | 97   | 4    | 98   |
|           | 3      | 18,280                        | 82   | 42   | 88   |

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

b Sample 1 represents orthophosphate dumped from sludge after 12 hr, and samples 2 and 3 represent dumped orthophosphate plus additional orthophosphate.

c None detectable.

d Background (43 counts/min).
menthal conditions, this phosphate represented an addition to the amount already present in the waste water before addition of the sludge. Table 3 shows the results of an experiment designed to determine how much phosphate Rilling sludge contributes to its suspending medium after overnight storage and whether this phosphate is preferentially removed by the sludge. Approximately 29% of the $^{32}$P radioactivity appeared in the liquid phase after 12 hr of storage (Table 3). This radioactivity and orthophosphate were almost completely removed within 1 hr after

**Fig. 1.** Effect of incubation temperature on 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 was aerated for 3 hr at the indicated temperatures in the presence of approximately 130,000 counts of $^{32}$P radioactivity per min per ml.

**Fig. 2.** Effect of various 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 of $^{32}$P radioactivity per min per ml was subjected to the indicated conditions and then aerated at 24°C for 3 hr.

**Fig. 3.** 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 was aerated at 24°C for 3 hr in the presence of approximately 123,000 counts of $^{32}$P radioactivity per min per ml.

| Experiment | Antimetabolite | $^{32}$P radioactivity in medium | Final uptake (%) | Final pH |
|------------|---------------|---------------------------------|-----------------|----------|
| 1          | None          | 0                               | 100             | 8.4      |
| 2          | PCMB<sup>b</sup> | $10^{-3}$                      | 103,480         | 52       | 8.2     |
| 3          | PCMB          | $10^{-1}$                       | 41,100          | 81       | 8.3     |
| 4          | Gramicidin    | $10^{-3}$                       | BKD             | 100      | 8.5     |
| 5          | Rotenone      | $10^{-1}$                       | 160             | 99       | 8.4     |
| 6          | Oligomycin    | $10^{-4}$                       | BKD             | 100      | 8.2     |
| 7          | Antibiotic A  | $10^{-3}$                       | BKD             | 100      | 8.4     |
| 8          | HgCl<sub>2</sub> | $10^{-4}$                      | 180,220         | 16       | 8.1     |
| 9          | PCMB          | $10^{-3}$                       | 108,800         | 49       | 8.2     |

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

<sup>b</sup> p-Chloromercuribenzoic acid.

<sup>c</sup> Values expressed as counts per min per milliliter.

<sup>d</sup> Background (43 counts/min).
PHOSPHORUS UPTAKE BY ACTIVATED SLUDGE

FIG. 4. Effect of various concentrations of metabolic inhibitors on the percentage of uptake of 32P radioactivity by Rilling activated sludge. A, iodoacetic acid, initial radioactivity approximately 83,460 counts per min per ml; B, HgCl₂, initial radioactivity approximately 213,200 counts per min per ml; C, 2,4-dinitrophenol, initial radioactivity approximately 318,900 counts per min per ml; D, NaN₃, initial radioactivity approximately 180,000 counts per min per ml. Approximately 250 mg (dry weight) of sludge in a final volume of 100 ml with tap water was aerated at 24°C for 3 hr in the presence of inhibitor and 32P.

Aeration was resumed (Table 3, sample 1). Samples 2 and 3 in Table 3 represent experiments in which the dumped orthophosphate was supplemented with additional K₂HPO₄·K₃HPO₄. A comparison of the rates at which the radioactivity and chemical orthophosphate were removed by 3 hr indicates that the 32P radioactivity derived from the sludge cells was not removed preferentially to added orthophosphate. Table 3 also shows that this quantity of Rilling sludge was able to remove approximately 100 mg of added orthophosphate per liter from tap water 3 hr after periods of storage, if the sludge was not removed from its suspending medium. Hyperion sludge was found to have the same capability.

Figure 1 shows the effect of incubation temperature on the uptake of 32P radioactivity by Rilling sludge. The optimum temperature appears to be in the range of 24 to 37°C, which would be characteristic of a biological, rather than a chemical, phenomenon.

Figure 2A shows the effect of exposing Rilling sludge to 100°C for different periods of time, up to 20 min. An exposure time of only 2 min resulted in more than a 50% loss in ability to remove 32P radioactivity. Figure 2B shows the effect of varying the temperature for a constant
TABLE 5. Effect of 2,4-dinitrophenol (DNP) on respiration and uptake of $^{32}$P radioactivity by Rilling sludge

| Flask contents | Uptake of $^{32}$P radioactivity (%) |
|----------------|-----------------------------------|
| Sludge, 0.8 ml; water, 1.2 ml | 13.4 | 93 |
| Sludge, 0.8 ml; sewage, 1.2 ml | 15.0 | 87 |
| Sewage, 1.2 ml; water, 0.8 ml | None | None |
| Sludge, 0.8 ml; sewage, 1.2 ml + DNP | 15.6 | 66 |
| Sludge, 0.8 ml; water, 1.2 ml + DNP | 11.4 | 35 |
| Sludge, 0.8 ml; water, 1.2 ml + DNP | 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 plus 0.2 ml of 20% KOH in center well. Sludge used, 5.75 mg/ml, dry weight. Radioactivity introduced, 18,000 counts per min per ml of $H_2^{32}$PO$_4$. DNP used, $10^{-3}$ M final concentration.
c Values expressed as microliters of $O_2$ per hr per milligram (dry weight) of sludge.
d Sludge preincubated with DNP for 1 hr before adding $^{32}$P radioactivity.

was observed when $10^{-3}$ M EDTA was added to the diluting fluid (data not shown).

Figure 4 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 adenosine triphosphate 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.

Oxygen was utilized by the Rilling sludge in the presence of either Tucson sewage or tap water (Table 5). Some increase in Q($O_2$) values occurred in the presence of sewage, however. When DNP was added to the sludge immediately before 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 before 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 was used for most of the experiments in Table 5.

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 and an optimum pH range and is inhibited by several anti-metabolites that are active against a wide range of metabolic processes.

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

Calcium phosphate precipitation as advocated by Menar and Jenkins (9) 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 (10). A pH of 11 is approximately 90% inhibitory for the uptake of $^{32}$P radioactivity by Rilling sludge (Fig. 3). In addition, the glass-distilled water contained little if any Ca$^{2+}$, and yet the uptake of phosphate from it was not affected (Table 1). 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 antimetabolite experiments (Table 4, Fig. 4) may indicate the participation of biological membranes and energy synthetic mechanisms in enhanced phosphate uptake. DNP and NaNO₃ are well known uncouplers of oxidative phosphorylation. Levin and Shapiro (7) and Yall et al. (16) have reported that DNP inhibits phosphorus uptake and retention by activated sludges. Uptake of ³²P radioactivity by Rilling sludge is totally inhibited by heavy concentrations of DNP (Fig. 4C) and 90% inhibited by NaNO₃ (Fig. 4D).

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 4) at the concentrations tested. With the exception of gramicidin, the other compounds are claimed to have little effect on bacteria. Both oligomycin (8) and antimycin (6) are effective against fungi. Rotenone seems to affect phosphorylation in higher plants (4). The mercurials (p-chloromercuribenzoic acid and HgCl₂) are effective against a variety of organisms. They are reported to interfere with membrane function and respiration primarily by combining with sulfhydryl enzymes (11). They are effective against Rilling sludge in tap water and Tucson sewage (Table 4, Fig. 4B). Iodoacetate effectively inhibits uptake of ³²P radioactivity (Fig. 4A). This compound affects some sulfhydryl enzymes but seems to inhibit phosphorylation mainly by acting against the Embden-Meyerhof pathway (14). Hotchkiss (5) reported that a related compound (iodoacetamide), and DNP, HgCl₂, and gramicidin were inhibitors of phosphorus uptake by *Staphylococcus aureus*.

Sludges of high phosphorus affinities are reported to attain total phosphate compositions of 6 to 8% P on a dry weight basis (15). Unpublished studies in our laboratory indicate that most of the phosphorus taken up in the cells remains as orthophosphate and that relatively small amounts are incorporated into organic compounds and polyphosphates. Why the sludge cells accumulate so much phosphorus remains to be explained.

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