Induced Rapid Release and Uptake of Phosphate by Microorganisms

Abstract. *Cultures of bacteria and of mixed microorganisms are able to release actively a large proportion of their phosphorus to the medium in a matter of hours when kept under anoxic conditions. Only phosphorus is lost, probably as orthophosphate and apparently mostly from the acid-soluble fraction of the cells. The process, which is completely reversible upon aeration, has a change in rate (for a change of 10°C) of 2 to 2.5.*

During a study of the uptake of phosphate by microorganisms in activated sludge sewage treatment (*1*), it was discovered that the organisms took up phosphate during the aeration step, but lost it to the medium very rapidly during the step in which they were concentrated by settling. This behavior is significant for sewage treatment plants and for prevention of algal problems in surface waters.

The organisms making up the so-called activated sludge include such floc-forming bacteria as *Zooglea ramigera, Escherichia intermedium, Bacillus cereus, Flavobacterium*, and *Pseudomonas*; filamentous organisms, such as *Sphaerotilus natans*; and protozoa, mainly stalked ciliates, such as *Vorticella, Opercularia, Epistylis*. Together these organisms are generally present in a concentration of 1000 to 2000 mg (dry weight) per liter in the mixed liquor of the aeration stage of activated sludge treatment. After this stage which lasts 2 to 8 hours, the organisms are concentrated five- to tenfold by settling. During settling the high respiration rate rapidly depletes the oxygen in the mass of organisms.

To study the phenomenon of the release of the phosphate, fresh samples of mixed liquor were settled in large jars for 10 to 15 minutes, the supernatant was siphoned off and discarded, and the concentrated sludge of microorganisms was transferred to 1-liter aspirator bottles in which the sludge was kept under the desired conditions. The bottle contents were stirred and kept from further contact with air by nitrogen bubbling. Periodically, samples were withdrawn, centrifuged briefly, and filtered through Millipore, type HA (0.45 μ) filters. Orthophosphate phosphorus in the filtrate was determined (after dilution) by the stannous chloride modification of the ammonium molybdate method (*2*).

The result of such an experiment is shown in the uppermost curve in Fig. 1. Release of phosphate commenced rapidly, the concentration of dissolved phosphate phosphorus rising from an initial value of 4 to 70 mg per liter in 3½ hours. The phosphorus released represents a significant proportion of that initially present. In a similar experiment it was calculated that 40 percent of the total phosphorus (determined after wet-ashing with perchloric acid) had been released, and the release was continuing.

Further experiments at different temperatures (Fig. 1) showed the phenomenon to be temperature-dependent, as expected. The values for change in rate from 10° to 20°, 15° to 25°, and 20° to 30°C were 2.5, 2.6, and 2.1, respectively, for the period 60 to 180 minutes. Once release began, it became essentially linear with time.

In another experiment the rates of release were compared for four different concentrations of sludge ranging from 1700 to 15,800 mg/liter. Although the concentration of phosphate released into solution was much higher in the case of the more concentrated sludge, the amount of phosphate released per unit of sludge was essentially the same in each case; the higher concentration of phosphate in solution did not inhibit release.

To determine the nature of the release, I investigated its reversibility. After a 3-hour anoxic period for release, air was again bubbled through a culture (Fig. 2). Thus the soluble phosphate phosphorus was taken up from solution at virtually the same rate (55 mg/liter in 178 minutes) at which it had been released (55 mg/liter in 182 minutes). Thus the release was apparently not due to decomposition of the culture under anoxic conditions. This was further substantiated as shown by analyses of the dried organisms at different stages of another similar experiment. The only systematic change was in the phosphorus content; the phosphorus (percentage dry weight) fell from 3.99 to 2.56 (a drop of 36 percent) and then rose upon aeration to 4.31 (an increase over the original value of 8 percent). Percentages of C, H, and N remained essentially unchanged. Consequently the ratio of C to P changed by a factor of almost 2. The indication is that the only substance being released in quantity is phosphorus.

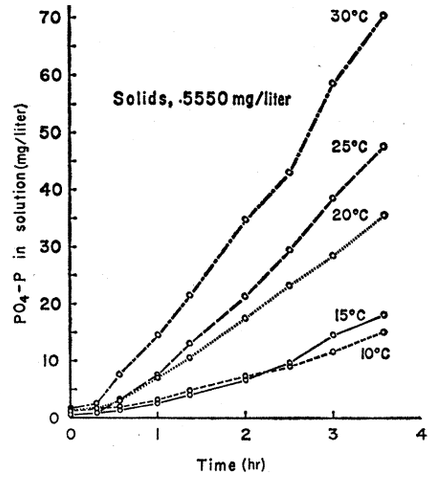

Fig. 1. Effect of temperature on anoxic release of phosphate from sludge microorganisms. PO_4-P, phosphate phosphorus.

Table 1 shows the results of analysis of the filtrate in an experiment in which only release was allowed to occur. Aside from the increase in dissolved phosphate phosphorus, the only systematic change was a slight increase in Kjeldahl nitrogen. This was later shown to be almost entirely ammonia, and it may have resulted from some decomposition or denitrification. The fact that phosphate phosphorus concentrations were essentially the same as the total phosphorus (disregarding experimental error) shows that the phosphorus was released either as orthophosphate itself or as a very easily hydrolyzed compound. In view of the lack of increase in the dissolved carbohy-

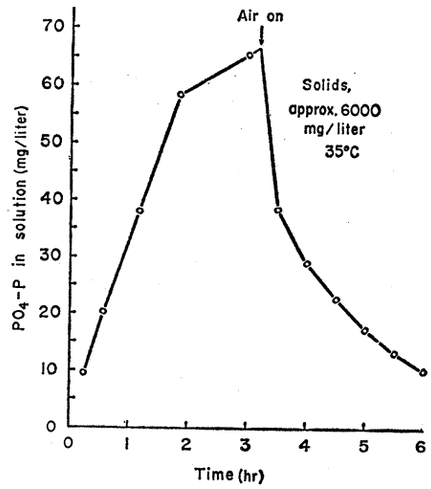

Fig. 2. Reversibility of anoxic release of phosphate. PO_4-P, phosphate phosphorus.

drate, the phosphorus was probably released as orthophosphate. Thus the phenomenon seems to be one in which pure orthophosphate is actively released by the microorganisms under anoxic conditions and reabsorbed under aeration.

To determine the source of the dissolved phosphate, a portion of the culture was centrifuged at intervals during the release, and after the filtered supernatant was set aside for a determination of dissolved phosphate, the organisms were washed, and their phosphorus was partitioned into four categories by the method of Schneider as modified by Krause (*4*). Apparently the phosphorus came initially and mostly from the acid-extractable portion, and later from the nucleic acids (Fig. 3). The phospholipids and phosphoproteins did not lose a significant percentage of their phosphorus. For unknown reasons (possibly because of evaporation) the sum of all fractions at the end of the experiment was 25 percent greater than at the beginning. Thus these results, while suggestive, are not conclusive.

The results indicate that the cellular acid-extractable portion can be rebuilt during aeration as rapidly as it is lost during anoxia. However, to strengthen this conclusion it is necessary to show that alternate explanations are untenable. Specifically, the results could be explained through reduction of a substance such as ferric phosphate during anoxia, followed by its re-formation during aeration. In experiments in which release was measured as a function of dissolved oxygen and redox potential (calomel and platinum electrodes), rapid release occurred only when the redox potential, E_7 had fallen to 150 mv—approximately the voltage at which ferric iron would be reduced to the soluble ferrous state. To check this, I performed experiments in which the Millipore filtrate containing the high phosphate concentration was aerated after release and filtered again to determine whether an insoluble phosphate compound had been formed. While a portion of the phosphate could be "taken up" in this manner, it represented, at most, about one-third of the uptake occurring in the presence of the microorganisms. Because nitrogen bubbling of the filtrate was as effective as aeration (indeed more effective in one case) and because analysis of the original filtrates showed insignificant amounts of iron, oxidation does not seem to be the answer. The main sites of uptake are located on or in the microorganisms. The "uptake" in these aeration experiments was probably due to adsorption of the phosphate to the vessels or to the formation of particulate matter by adsorption of organic substances onto bubbles (*5*).

Table 1. Substances found in solution at various times during anoxic release of phosphate from sludge. Abbreviations: BOD, biochemical oxygen demand, a standard test for dissolved organic substances (*2*); CHO, carbohydrate in glucose equivalents, as measured by the method of Gilles *et al.* (*3*); PO_4-P, phosphate phosphorus. Total P is after perchloric acid digestion.

Time elapsed (min)	Dissolved substances (mg/liter)				
	PO_4-P	Total P	BOD	CHO	Kjeldahl N
8	5.5	4.3	175	8.8	19
24	16.6	16.6	190	6.8	19
61	34.5	36.9	150	5.6	23
103	50.5	48.5	180	6.2	31
123	65.5	61.1	175	7.4	37

Experiments have been done with various poisons to elucidate the mechanism of uptake and release. Uptake is virtually completely inhibited by mercuric chloride and by 2,4-dinitrophenol, the latter in as low a concentration as $2 \times 10^{-3}M$. The results clearly indicate a biological uptake rather than a physical process such as adsorption. Although release is largely inhibited by high concentration of formaldehyde, it is stimulated by poisons such as KCN and $HgCl_2$. Thus, in one release experiment the soluble phosphate phosphorus concentration rose from 19.8 to 34.0 mg per liter in the absence of poisons, but to 40.8 and 44.2 mg per liter in the presence of $10^{-2}M$ KCN and $10^{-3}M$ $HgCl_2$, respectively. The effect of the mercury is rapid. When added to the previously unpoisoned sample in a concentration of $10^{-3}M$, it caused an increase in the dissolved phosphate from 34.0 to 51.0 mg per liter in 20 minutes. Strangely, a lower concentration of KCN ($10^{-3}M$) inhibited release of phosphate phosphorus to an eventual 26.8 mg per liter.

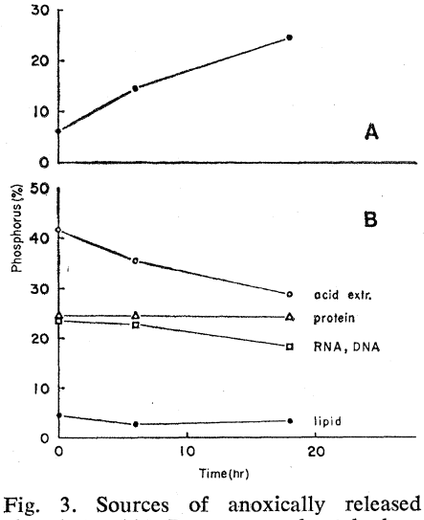

Fig. 3. Sources of anoxically released phosphate. (A) Percentage of total phosphorus which the sludge has lost to solution as orthophosphate. (B) Disposition of phosphorus remaining in the solids as a percentage of the total. Each point is the average of two analyses.

All these experiments were done with sewage organisms. However, some experiments with dense cultures (860 mg/liter, dry weight) of *Escherichia coli* yielded similar results. In such cultures kept at 35°C under anoxic conditions, the dissolved phosphate phosphorus rose from 2 to 6 mg per liter in 3½ hours. Similar cultures with $10^{-2}M$ KCN added lost phosphate to solution at a much greater rate, the dissolved phosphate phosphorus reaching a concentration of 14 mg per liter in the same period. Cultures under continuous aeration reduced the dissolved phosphate concentration but began to lose phosphate rapidly when allowed to become anoxic. For a low initial concentration of dissolved phosphate, the bacteria were grown in hydrolyzed protein-$MgSO_4$ medium, centrifuged, and washed with deionized water before use.

To my knowledge the phenomenon has not been observed before. There are numerous examples of the uptake and release of phosphate by microorganisms, but none are dependent upon the oxygen concentration or the redox potential. For example, Kamen and Gest (*6*) found that washing of *Rhodospirillum rubrum* suspensions with saline could result in loss of up to 87 percent of the acid-soluble phosphate of the cells without affecting their metabolic activity. When saline that contained inorganic phosphate was used as wash, the acid-soluble phosphate of the cells could be increased 50 percent. Similarly, Goldberg (*7*) found that *Asterionella formosa* (a diatom) grown in a medium with a high phosphate concentration would lose up to 50 percent of its phosphorus if rinsed with phosphate-free water. Rautanen and Miikkulainen (*8*) found a similar form of disposable phosphate in *Torulopsis utilis*. However, only Robertson (*9*) has described a similar phenomenon.

He pointed out that in higher plants, the absorption and accumulation of phosphate from dilute solution requires active respiration, and that phosphate accumulation is markedly affected if metabolic activity is inhibited by reduction of oxygen tension in the medium, by low temperature, or by poisons of the cytochrome system.

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Functional Chloroplast Polyribosomes from Tobacco Leaves

Abstract. *Incubation of isolated tobacco chloroplasts with ingredients required for protein synthesis resulted in liberation of 70S ribosomes and polyribosomes that no longer sedimented with the chloroplasts. With increasing time of incubation, polyribosomes broke down to 70S monosomes. Similarly, microgram quantities of ribonuclease caused chloroplast polyribosomes to break down into monosomes. Both polyribosomes and 70S ribosomes that were isolated on sucrose density gradients and tested separately in cell-free systems were capable of protein synthesis; however, polyribosomes formed more protein per unit of RNA than monosomes did.*

Whether protein synthesis by isolated chloroplasts occurs on polyribosomes is a question that has not been answered satisfactorily. Using analytical centrifugation, Clark *et al.* (*1*) demonstrated polyribosomal aggregates, by Schlieren optics, when Chinese cabbage leaves were homogenized in the presence of polyvinyl sulfate, a nuclease inhibitor. Boardman *et al.* (*2*) confirmed this observation for extracts of tobacco leaves but showed that polyvinyl sulfate strongly inhibited protein synthesis by the isolated chloroplasts. In another study (*3*), these investigators concluded that monosomes were mainly responsible for the protein-synthesizing activity in a chloroplast supernatant. The procedure they utilized was first to obtain a chloroplast supernatant by resuspending intact chloroplasts in a buffer of low molarity and then to centrifuge it at 17,000*g*. In their experiments, they measured the protein-synthesizing activity in the supernatant.

In contrast to their procedure, we first incubated intact chloroplasts and then fractionated each incubated mixture into a 17,000*g* pellet and supernatant. By subjecting these supernatants to sucrose density gradient centrifugation, it was possible to fractionate the materials that had synthesized protein and, simultaneously, to locate their positions in the gradient by optical-density measurements. Further investigation showed that functional chloroplast polyribosomes could be identified in rapidly growing leaves of young tobacco plants.

Chloroplasts were prepared from leaves of young tobacco plants by methods already described (*4*). The chloroplast pellet from 5 g of leaves was resuspended in 0.35 ml of a medium containing 0.025*M* tris-HCl (*p*H 7.8), 0.015*M* magnesium acetate, and 0.004*M* 2-mercaptoethanol. To each suspension of chloroplasts was added 0.20 ml of a combined reagent mixture that contained tris, (*p*H 7.8), 4.6 μmole; magnesium acetate, 4.6 μmole; KCl, 27.6 μmole; mercaptoethanol, 2.6 μmole; adenosine triphosphate, 0.4 μmole; phosphoenolpyruvate, 2.5 μmole; pyruvate kinase, 50 μg; and a mixture of 0.02 μmole each of uridine, guanosine, and cytidine triphosphates. Next, 2 μc (20 μl) of an amino acid mixture (uniformly labeled with C^{14} and having a specific activity of 0.1 mc/0.067 mg) was added and incubation was carried out for 1 minute (or longer, where stated) at 28°C. The incubated mixture was immediately chilled in an ice bath and then centrifuged at 17,000*g* for 15 minutes. All operations before and after incubation were conducted at a temperature close to 0°C. Portions (0.3 to 0.5 ml) of the 17,000*g* supernatants were layered directly on linear 5 to 20 percent sucrose density gradients that contained 0.015*M* magnesium acetate and 0.025*M* tris-HCl, *p*H 7.8. Gradients were centrifuged for 2 hours in the Spinco model L-2 centrifuge at 24,000 rev/min with the rotor coasting to a stop without braking. Contents of the gradients were pushed up by a 30 percent sucrose solution that was pumped into the bottom of the tube at a constant speed with a motor-driven syringe; they were then passed through a Vanguard automatic ultraviolet spectrophotometer and the optical density, at 260 mμ, was monitored continuously with a graphic recorder. The flow rate was 0.7 ml/min. Radioactivity incorporated into protein in each fraction was precipitated by hot 5 percent trichloroacetic acid (TCA) (for 20 minutes at 80°C) and collected on glass fiber filters. Precipitates were first washed four times with 5-ml portions of 5 percent TCA; this was followed by three 5-ml washes with 80 percent acetone. They were then dried under an infrared lamp before being placed in a vial with 5 ml of scintillation fluid; radioactivity was counted with a liquid scintillation counter.

When intact chloroplasts were incubated with a mixture of uniformly labeled amino acids in the presence of the mixture of combined reagents, as radioactivity was incorporated into protein, about 50 percent of this radioactivity would no longer sediment at 17,000*g*, whereas the other 50 percent did sediment with the chloroplasts. This ratio of incorporated radioactivity which did not sediment at 17,000*g* to that which did sediment remained about the same whether incubation was for 5 or 30 minutes. The nonsedimentable incorporated radioactivity present in 17,000*g* supernatants that resulted from incubation periods of 1, 5, and 15 minutes was fractionated on sucrose density gradients. Measurements of optical density (OD) revealed a broad peak of ribosomes that increased in amount without significant change in the OD profile as the time of incubation was increased. However, there was a marked change of the incorporated radioactivity from the heavy ribosome regions to the monosome region as the incubation period lengthened.

The possibility was suggested to us that a polyribosome OD profile was being concealed and that it might be revealed by washing the chloroplasts before incubation for protein synthesis. Isolated chloroplasts were therefore