Factors Affecting Phosphate Uptake by *Aerobacter aerogenes* in a System Relating Cell Numbers to $^{32}$P Uptake

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The uptake of phosphate, from media limited in this ion, by resting cells of *Aerobacter aerogenes* has been investigated and shown to be dependent upon several factors. An incubation medium composed of $10^{-2}$ M K$_3$, $5 \times 10^{-3}$ M Mg$_2^+$, 1 mg of glucose per ml, and 1 $\mu$Ci of $^{32}$PO$_4^-$ per ml, buffered at pH 6.55 with 0.05 M 2-hydroxyethyl-piperazine-$N'$-2-ethanesulfonic acid (HEPES), was found to stimulate optimum accumulation of $^{32}$P-orthophosphate. The temperature of incubation, incubation time, the concentration of unlabeled orthophosphate, as well as arsenate, and several metabolic inhibitors were found to affect the accumulation. The labeled cells were collected on a membrane filter, which had been previously boiled in glass-distilled water, for measurement of the radioactivity accumulated. Under optimum conditions, as few as 20,000 cells were capable of accumulating detectable amounts of $^{32}$P-orthophosphate in 1 hr of incubation.

The uptake of phosphate by microorganisms has received much less study than has that of organic nutrients. Only in the cases of *Staphylococcus aureus* (17–20), *Streptococcus faecalis* (8, 9) bakers' yeast (2, 11, 13, 22, 23), and red blood cells (1, 21) has the accumulation of this anion been investigated in depth. *Escherichia coli* (16, 24, 25) and *Aerobacter aerogenes* (5, 6, 14, 15) have also been studied, but in lesser detail. These previous studies, however, all concerned factors affecting uptake in the presence of excess phosphate. In this study, we have attempted to delineate the major factors affecting the accumulation of phosphate by *A. aerogenes* from media severely limited in this ion, with a view to investigating the possibilities of employing the ability of cells to accumulate phosphate as a means of estimating cell populations in a short time period.

**MATERIALS AND METHODS**

**Culture.** *A. aerogenes* Macdonald College Culture Collection no. 112 was employed in these studies. Freeze-dried samples were prepared and were employed routinely as inocula for broth cultures.

**Growth and harvesting of cells.** Cells were grown in Trypticase Soy Broth for 16 hr at 30 C on a rotary shaker. The culture was harvested by centrifugation (13,000 X g at 4 C), and the pellet was washed three times by suspension in, and recentrifugation from, sterile glass-distilled water (GDW) followed by re-suspension in filter-sterilized GDW to a level of 10$^9$ cells per ml.

**Preparation of glassware.** All glassware employed in these experiments was suspended for a period of not less than 3 hr in sulfuric acid-dichromate cleaning solution. The glassware was then rinsed 10 times in distilled water, followed by three rinses in GDW.

**Preparation of filters.** Membrane filters were sterilized by autoclaving at 121 C for 15 min. To reduce the nonspecific retention of the radioactive phosphate, it was necessary to boil the filters in sterile GDW (approximately 15 ml per filter) for a period of 1 hr. After boiling, the water was decanted and the filters were suspended in sterile GDW.

**Preparation of incubation media.** The compositions of the incubation media employed in these studies are as follows. Medium 1 contained 5 X $10^{-3}$ M KCl, 2 X $10^{-3}$ MgSO$_4$, 10 mg of glucose per ml, 5 X $10^{-3}$ M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, 1 $\mu$Ci of $^{32}$PO$_4^-$ per ml (pH 7.2). Medium 2 contained $10^{-3}$ M KCl, 5 X $10^{-3}$ MgCl$_2$, 1 mg of glucose per ml, 5 X $10^{-3}$ M Na - 2 - hydroxyethylpiperazine - $N'$ - 2 - ethanesulfonic acid (HEPES) buffer, 1 $\mu$Ci of $^{32}$PO$_4^-$ per ml (pH 6.55). In general, a double strength basal medium, containing the buffer and $^{32}$P solution in all cases, was prepared, and the pH was adjusted by the addition of KOH (to a maximum of 2 X $10^{-3}$ M) or 0.5 M Tris buffer or HCl. (Carrier-free $^{32}$P-orthophos-
Phosphate was obtained from Atomic Energy of Canada Limited, Ottawa, Ontario. Upon receipt, the solution was diluted to 1 mCi/ml and sterilized by membrane filtration (HA; Millipore Corp., Bedford, Mass.). This basal medium was then sterilized by passage through a membrane filter (HA; Millipore Corp.) and 5-ml samples of the medium were dispensed into a series of 50-ml Erlenmeyer flasks. To achieve the final concentrations of incubation medium components as shown above, portions of washed cell suspensions to give the desired final cell levels and of filter-sterilized solutions of components (the effect of which was being investigated) or filter-sterilized GDW, or of the appropriate combination of the aforementioned, were added to the various flasks. Control flasks, in which the cell suspensions were replaced by equal portions of filter-sterilized GDW, were also prepared for each experiment.

Labeling procedure and the detection of \(^{32}\)P accumulated by the cells. Flasks were incubated at 37°C in a rotary water bath shaker for 1 hr, unless otherwise indicated. Five 1-ml samples of the suspension were each filtered through a membrane filter, and the filter was washed with a 5-ml portion of sterile GDW to remove radioactive incubation medium trapped in the filter. The filters were then glued, face down, to planchets and air dried. The radioactivity retained was determined by the use of a Geiger-Müller tube attached to a digital scaler (Bio-Planchette Counter, model 4338, Nuclear Chicago Corp., Des Plaines, Ill.). All results, unless otherwise noted, are recorded as counts per minute per milliliter of incubation medium filtered. All deviations recorded are average deviations from the mean.

Variability of the blank. As mentioned previously, control flasks were prepared in which the cell suspensions were replaced by equal portions of filter-sterilized GDW. When 1-ml samples of these control solutions were passed through membrane filters, the radioactivity retained was found to vary from experiment to experiment and, in some cases, was extremely high. This variability was traced to the filter base used to support the membrane filter employed for the prefiltration of the basal medium. By preselecting a sintered-glass filter base which would produce a basal medium giving a low count when portions of it were filtered, or by using a stainless-steel filter support to hold the filter used for prefiltration, high counts on filters derived from the control flasks could be avoided. (We acknowledge the assistance of Jared Fein and Graham Tolfree of this department in providing a solution for this aspect of the problem.)

Enumeration of viable cells inoculated. The number of viable cells added to each test flask was determined by a surface plate method. After appropriate dilution, 0.2-ml portions were distributed from a pipette, with the aid of a plating wheel, to each of five dried plates of Trypticase Soy Agar (TSA), made 2% with respect to agar. The plates were incubated for 24 hr at 30°C and the total number of colonies on the surface of the five plates was recorded. The total number of colonies counted was usually in excess of 400, and, under these conditions, the coefficient of variation was less than 5%.

RESULTS

Effect of pH and buffer on phosphate accumulation. Preliminary studies, in which incubation medium 1 was employed, indicated that the pH of the incubation medium had a marked effect on phosphate accumulation. Maximum accumulation was observed to occur when the pH was in the range of 6.5 to 6.7. As the pH was varied from this range, the cells accumulated decreasing amounts. Since 7.2 is the lowest pH at which Tris (the buffer employed in the preliminary studies) can be employed as an effective buffer, it was obvious that an alternate buffering system was required. As it was essential that the buffer be nonmetabolizable, effective at pH 6.5 to 6.7, and not contain phosphate, the choice of buffer was limited to one of the zwitterionic buffers developed by Good et al. (7). Two of these, HEPES (pKₐ at 20°C, 7.55) and N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES; pK₂ at 20°C, 7.5), were compared with Tris-hydrochloride to determine whether they were antagonistic to phosphorus accumulation. It was found that neither of these buffers reduced the uptake capacity of the cells.

To determine more definitively the optimum pH for phosphate accumulation, various flasks of incubation medium 2, containing 0.025 M HEPES and 0.025 M Tris buffers, were adjusted with HCl to various pH values between 6.0 and 7.4. The two buffers were used in combination to ensure pH stability during incubation of the higher pH levels examined. All flasks were then inoculated to the same cell level (10⁶ cells/ml), and the initial pH of each flask was recorded. After 1 hr of incubation at 37°C, the \(^{32}\)PO₄³⁻ accumulated by the cells was determined, and the final pH was measured and recorded. (In no case did the initial and final pH values vary by more than 0.5 units.) The results (Fig. 1) reveal that the optimum pH for the uptake of inorganic orthophosphate by this organism was approximately 6.6.

Effect of composition of the incubation medium on the extent of labeling of the cells. Various compounds were added to suspensions of cells in an incubation medium containing 1 μCi of \(^{32}\)PO₄³⁻ per ml and buffered in GDW at pH 6.55 with 0.05 M HEPES to determine their effect on the rate and extent of labeling of the cells. Several cations, K⁺, Na⁺, NH₄⁺, and Mg²⁺, as well as an energy source, glucose, were first examined for their effect, singly, on phosphate uptake. Those which caused a stimulation were then examined in combination to determine whether a synergistic
stimulated uptake to the level observed in the presence of \(5 \times 10^{-3} \text{ M} \text{Mg}^{2+}\). Potassium ion, at an optimum level of \(10^{-2} \text{ M}\), produced a stimulation in phosphate uptake from a medium devoid

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**Fig. 1.** Effect of pH on the accumulation of \(^{32}\text{P}-\text{orthophosphate} by A. aerogenes. The study was conducted in incubation medium 2 with 0.025 M HEPES and 0.025 M Tris employed as the buffering system. Flasks were adjusted to the desired pH by the addition of HCl. The radioactivity values recorded represent the radioactivity retained by a membrane filter when 1-ml samples of the incubation medium, containing \(10^6\) cells/ml, were filtered after 1 hr of incubation at 37°C.

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**Fig. 2.** Effect of magnesium concentration on the accumulation of \(^{32}\text{P}-\text{orthophosphate} by A. aerogenes. The basal incubation medium employed was medium 2 prepared without added MgCl\(_2\) and containing \(10^6\) cells per ml. Cell samples were incubated for 1 hr at 37°C. Results from two separate experiments are recorded.

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**Fig. 3.** Effect of the addition of glucose, in the presence and absence of magnesium ion, on the accumulation of \(^{32}\text{P}-\text{orthophosphate} by washed cells of A. aerogenes. The basal incubation medium employed was medium 2 prepared without MgCl\(_2\) or glucose and containing \(10^6\) cells per ml. All samples were incubated for 1 hr at 37°C. Curve 1, \(5 \times 10^{-3} \text{ M} \text{Mg}^{2+}\); curve 2, \(5 \times 10^{-2} \text{ M} \text{Mg}^{2+}\); curve 3, no added \(\text{Mg}^{2+}\).
of Mg$^{2+}$ and glucose (Fig. 4); in the presence of optimum levels of these two factors, however, no added stimulation was observed.

**Determination of optimum temperature.** The uptake of phosphate by *A. aerogenes* was shown to be highly dependent on the temperature of incubation. Optimum uptake occurred at 37°C (Fig. 5). The response to temperature was linear from 20 to 30°C (slope; 1.81) and was followed by a leveling off.

**Relationship of time of incubation to accumulation of phosphate.** Under optimum conditions, a linear response (slope; 1.0) between time of incubation and phosphate uptake was observed up to 75 min, at which time the rate began to decrease (Fig. 6). This decrease appeared to be due to the fact that approximately 80% of the radioactivity originally present in the solution had been removed in 75 min.

**Effect of $^{32}$P-orthophosphate level.** The effect of increasing levels of external $^{32}$PO$_4^{3-}$ on its uptake was studied over a range of 0.1 to 5 μCi/ml. The response was linear (slope; 1.70) up to the point where the scaler could no longer differentiate between samples because of excessive coincidence loss (Fig. 7). On the basis of these results, 1 μCi of $^{32}$P-orthophosphate per ml was demonstrated to be a workable level for these studies.

**Effect of unlabeled phosphate and arsenate.** Since the studies on the effects of the period of incubation (Fig. 6) and the level of $^{32}$P-orthophosphate (Fig. 7) on the accumulation of phosphate had indicated that a level of 1 μCi of $^{32}$PO$_4^{3-}$ (10$^{-10}$ M) per ml was far below the uptake capacity of the cells, it was decided to investigate the effect of increasing levels of unlabeled orthophosphate.

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**Fig. 4.** Effect of potassium concentration on the accumulation of $^{32}$P-orthophosphate by *A. aerogenes* in the absence of Mg$^{2+}$ and glucose. The basal medium employed consisted of 1 μCi of $^{32}$P-orthophosphate per ml, buffered at pH 6.55 with 0.025 M HEPES and 0.025 M Tris, and contained 10$^8$ cells per ml. Samples were incubated at 37°C for 1 hr.

**Fig. 5.** Effect of incubation temperature on the accumulation of $^{32}$P-orthophosphate by *A. aerogenes*. Incubation medium 2 containing 9 X 10$^8$ cells per ml was used. Incubation time, 1 hr.

**Fig. 6.** Relationship of incubation time to the extent of accumulation of $^{32}$P-orthophosphate by *A. aerogenes*. Incubation medium 2 containing 8 X 10$^8$ cells per ml was used. Incubation temperature, 37°C. The medium originally contained 150,000 counts per min per ml.
phosphate on the uptake of the labeled compound. From the results (Table 1), it is obvious that the accumulation system was capable of assimilating much higher levels of phosphate. In fact, it was necessary to increase the level of unlabeled phosphate to $10^{-6}$ M before any appreciable reduction in the amount of radiolabel accumulated was evident. In addition, the effect of increasing levels of arsenate [a competitive inhibitor of the phosphate transport system of S. aureus (18), S. faecalis (8), and yeast (11, 22)] on the accumulation of phosphate was investigated. One of the criteria for the identification of a permease- and carrier-mediated transport process, according to Cirillo (4), is that structural analogues are capable of reducing the accumulation of the substance under study. The results of the present study indicate that arsenate may well be a competitive inhibitor of the phosphate transport system. Equivalent levels of arsenate, as of unlabeled orthophosphate, were required to cause an appreciable reduction in the amount of $^{32}$P accumulated.

**Effect of inhibitors on the uptake of $^{32}$P-orthophosphate.** The effect of certain metabolic inhibitors on the accumulation of $^{32}$P-orthophosphate by *A. aerogenes* was investigated. Cyanide (KCN), azide (NaN$_3$), or 2,4-dinitrophenol (DNP), all of which are inhibitors of some stage of energy generation (3, 29), each effected a considerable reduction in the amounts of $^{32}$P accumulated (Table 2) but did not cause a loss of cell viability. p-Chloromercuribenzoate (p-CMB), which combines with sulfhydryl groups (28), almost completely inhibited uptake and, in addition, caused the death of the cells during the incubation period. Since cells which had previously been destroyed by heat treatment were also able to accumulate a similarly small degree of radioactivity, it was concluded that the radioactivity retained in the latter two cases was probably due to nonspecific binding by the cells. When cells were treated with formaldehyde, which has been reported to effectively “seal” the cell membrane against solute passage (12), only a small amount of phosphate was accumulated. Since these cells remained viable upon dilution of the formaldehyde, the phosphate accumulated was considered to be bound to specific sites in the cell envelope, perhaps similar to those isolated from *Escherichia coli* by Medveczky and Rosenberg (16).

**Limits of detectability of cells by means of $^{32}$P labeling.** The relation between numbers of cells in suspension to radioactivity retained by membrane filters was determined when cells were incubated for 1 hr at 37 C in either buffered GDW

![Fig. 7. Effect of increasing levels of $^{32}$P-orthophosphate on the amount of radioactivity accumulated by *A. aerogenes*. The basal incubation medium used was medium 2 prepared without added $^{32}$PO$_4$- and containing $2 \times 10^8$ cells per ml. All samples were incubated at 37 C for 1 hr.](http://aem.asm.org/)

**TABLE 1. Effect of unlabeled orthophosphate and arsenate on the uptake of $^{32}$PO$_4^-$ by *A. aerogenes* a**

| Conc of $^{32}$PO$_4^-$ (M) | Counts/min$^b$ | Conc of AsO$_4^-$ (M) | Counts/min |
|-----------------------------|-----------------|------------------------|------------|
| + Cells                     | - Cells         | + Cells                | - Cells    |
| 0                           |                 | 0                      |            |
| $10^{-10}$                   | 87,004 ± 3,871  | 2,479 ± 460            |            |
| $10^{-8}$                    | 76,029 ± 1,922  | 2,356 ± 327            |            |
| $10^{-6}$                    | 69,901 ± 1,771  | 2,160 ± 202            |            |
| $10^{-4}$                    | 24,364 ± 1,494  | 1,344 ± 128            |            |

a Unlabeled orthophosphate was added as Na$_2$HPO$_4$. Arsenate was added as Na$_2$HAsO$_4$. The uptake in 1 hr at 37 C from incubation medium 2 containing $1.1 \times 10^8$ cells per ml.

b Results recorded represent the mean and average deviation of the mean of five determinations.
plus $10^{-2}\text{M} K^+$, medium 1, or medium 2. The results (Fig. 8) indicate that $^{32}$P uptake by the cells was appreciable even in buffered GDW and that detection began at a cell concentration of about

| Treatment                  | Counts/min $^b$ | Reduction (%) |
|----------------------------|-----------------|---------------|
| Cyanide ($10^{-2}\text{M}$) | 69,057 ± 1,565  | 70            |
| 2,4-Dinitrophenol (5 × 10^{-4}\text{M}) | 20,800 ± 581    | 80            |
| Azide (3 × 10^{-3}\text{M}) | 40,851 ± 711    | 41            |
| $p$-Chloromercuribenzoate (10^{-3}\text{M}) | 8,395 ± 463     | 88            |
| Formaldehyde (1%)          | 10,709 ± 624    | 86            |
| Heat-killed cells $^c$     | 12,953 ± 781    | 83            |

$^a$ Accumulation in 1 hr from incubation medium 2 containing $1.9 \times 10^6$ cells per ml.

$^b$ Results recorded represent the mean and average deviation of the mean of five determinations.

$^c$ Cells were heated at 100°C for 5 min.

2 × $10^4$ cells per ml when a 1-ml sample of the suspension was filtered. Between 2 × $10^4$ and $10^5$ cells on the filter, the relation between cell numbers and radioactivity was linear. Above $10^5$ cells/ml, the $^{32}$P in the incubation medium was exhausted before the end of the incubation period. When medium 1 was used, the lower limit of detectability was slightly improved, whereas with medium 2, which contained the various components at their optimum concentrations (as determined in this study), a 10-fold increase in the sensitivity of the method was achieved. As few as 2 × $10^4$ cells could be detected on the filter after incubation of the cells in this medium for 1 hr.

**DISCUSSION**

Phosphate uptake by A. aerogenes, from a medium limited in this ion, was demonstrated to be strongly influenced by both the pH of the medium and the temperature of incubation. At the optimum pH, 6.6, cells accumulated twice as much phosphate as at pH 6.0 or 7.4. In addition, the cations Mg$^2+$ and $K^+$ each stimulated phosphate uptake from medium which was devoid of the other. In the presence of Mg$^2+$, no additional effect due to $K^+$ could be demonstrated. These responses to $K^+$ and Mg$^2+$ were not surprising in light of the results of Tempest and his colleagues (see reference 26 for a review) wherein they reported that Mg$^{2+}$, $K^+$, phosphate, and ribonucleic acid (RNA) levels in another strain of A. aerogenes were interrelated; high intracellular levels of the former three ions were always accompanied by high levels of RNA and vice versa. Cells which had been grown in $K^+$- or Mg$^{2+}$-limited media exhibited lowered levels of RNA (27). In addition, Rothstein et al. (23) have shown that the transport of phosphate is strongly influenced by Mg$^{2+}$ ion in bakers’ yeast. Furthermore, Borst Pauwels (2) has demonstrated that the rate of glycolysis, phosphate uptake, and the availability of Mg$^{2+}$ are interrelated in a strain of *Saccharomyces cerevisiae*. Potassium ion has also been shown by Solomon (24, 25) and Eddy and his co-workers (5, 6) to stimulate phosphate uptake by *E. coli* and *A. aerogenes*, respectively.

Although studies with metabolic inhibitors strongly suggested that phosphate accumulation was energy-dependent, a consistent stimulation by added glucose could not be demonstrated. Invariably, glucose, or another energy source, has been demonstrated to be essential for phosphate uptake by microorganisms in the presence of an excess of phosphate (2, 9, 13, 18, 22). It thus appeared that the energy required for the accumulation of phosphate was being generated by the normal endogenous metabolism of the cell. Glu-
cose, however, did appear to serve a role in overcoming the inconsistency of the response to added Mg$^{2+}$. This fact, in addition to the apparent energy dependency of the accumulation process, led to the inclusion of glucose at a level of 1 mg/ml in the optimum incubation medium.

It was determined that as few as 20,000 viable resting cells of *A. aerogenes* are capable of accumulating detectable amounts of $^{32}$P-orthophosphate after 1 hr of incubation under optimum conditions. A previous report (14) that considerably fewer cells could be detected by this procedure has since been found to be unreliable (15). In suggesting a $^{32}$P-incorporation technique as a rapid presumptive test for bacterial populations, it is realized that a number of potential problem areas must be investigated. First, for such a technique to be applied to mixed populations of bacterial cells, the relative capacity of different cells to accumulate $^{32}$P-orthophosphate under standard conditions will have to be established. Second, the effect of the nutritional state of the organism will also have to be elucidated. These two considerations are, however, the main ones afflicting any other rapid presumptive technique which is based on either a metabolic capacity of cells or on the total amount of, or activity of, a constitutive biochemical character. From our studies, it would appear that a rapid presumptive detection technique based on accumulation of $^{32}$P-orthophosphate offers a potentially fruitful approach to solving the problem of rapid detection of bacterial contamination or pollution. Holm-Hansen and Booth (10) have proposed a method of estimating viable cells which relates the number of cells to the adenosine triphosphate content of samples. The minimum number of cells reported to be detectable by this technique was 35,000 which is approximately twice the number of *A. aerogenes* detectable uptake of $^{32}$P-orthophosphate. Separate experiments (to be published) have established that similar numbers of cells of *E. coli*, *S. marcescens*, and *A. aerogenes* are capable of producing a measurable phosphate accumulation by the procedure reported here.

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