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PHOSPHATE TRANSPORT IN *BACILLUS CEREUS*

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SUMMARY

1. *Bacillus cereus* accumulates phosphate against a concentration gradient by a process which is energy-dependent and exhibits a high temperature coefficient with an activation energy of over 12 kcal.

2. The rate of uptake is doubled in cells which had been deprived of phosphorus for two hours. In such cells, a 'primary' pool of phosphate must be filled before esterification of phosphate begins, and the fast rate of uptake persists throughout this period. The rate of uptake falls to about one-half the fast value once the primary pool is filled. Isotope displacement experiments show that phosphate, taken up from that point in time, still passes through that pool.

3. Arsenate and pyrophosphate which share the phosphate transport system in *Bacillus cereus*, can also fill the primary pool.

4. The primary pool appears to be closely related to the external phosphate and to its transport, since it does not interact with phosphate liberated within the cell from α-glycerophosphate.

INTRODUCTION

Phosphate (P_i) is an essential metabolite in all cells and its entrance into the cell appears to be controlled at the cell surface[1–4]. Recently, bacterial mutants unable to transport phosphate have been described[3,5]. The conditions under which phosphate enters different cells varies widely, as does the fate of the phosphate immediately following entry.

In the course of a previous study of phosphate transport in *Bacillus cereus*[5], we found that washed cells took up phosphate at a constant rate from the moment they were exposed to this compound. In contrast, cells which had been deprived of phosphorus for at least 2 h, initially took up phosphate at a much faster rate until about 10 nmoles of phosphate had been taken up per $1 \cdot 10^8$ cells. The rate of uptake then fell to that found in non-starved cells. The present paper describes this phenomenon in greater detail and attempts to elucidate the nature of the different intracellular pools in which phosphate is distributed. The transport of phosphate into the first pool is an active process and proceeds against a gradient. This pool can be occupied by any one of the several ions which share the phosphate transport system[5], but once this pool is filled, phosphate is transported only at the second, slower rate.

MATERIALS AND METHODS

The sources of chemicals used, details concerning the microorganism (*B. cereus*, W) and conditions for its growth have been described elsewhere[6]. The bacteria were grown at 30° on a complex medium (PPYG) which contained (w/v) 2% Difco proteose-peptone, 0.5% Difco yeast extract and 1% glucose. A defined, phosphorus-free medium (BXPG)[6] was used for uptake studies. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES) was purchased from CalBiochem. *N*-Ethylmorpholine (Eastman Kodak) was purified by distillation.

Labelled compounds

Radioactive phosphate ($^{32}P_i$) was supplied by the Australian Atomic Energy Commission. The solutions (pH 2) were made 1.0 mM with respect to P_i with sterile carrier phosphate and were stored at 3° to avoid the formation of particle-adsorbed $^{32}P_i$, which we and other workers[7] have encountered. Appropriate dilutions were membrane-filtered before use.

α-Glycero[^{32}P]phosphate was prepared by the method of POSSMAYER AND STRICKLAND[8], but calcium precipitation was avoided. Instead, the reaction products (1 mmole) were added directly to a column (2.5 cm × 36 cm) containing Dowex 1-X8 (HCO_3^-, 100–200 mesh) and eluted with an increasing gradient (0–0.1 M) of $KHCO_3$ (ref. 9). Three emergent peaks were located by their radioactivity and α-glycerophosphate was identified in the second peak by chromatography with authentic material, followed by radioautography. $KHCO_3$ was removed from the solutions by treatment with excess Dowex 50 (H^+) resin. A second passage through Dowex 1 (HCO_3^-) produced radiochemically pure α-glycerophosphate.

Uptake studies

Unless otherwise stated, stationary-phase cells were starved of phosphorus by shaking for 2 h at 30° in a mixture of equal volumes of BXPG medium and 0.1 M buffer (pH 7.5) (usually *N*-ethylmorpholine–HCl, but in later stages imidazole–HCl and Tris–HEPES were used). The buffer did not affect the nature of the uptake, but rates were slightly higher in HEPES.

The labelled substrate was added to appropriately diluted cells ($1 \cdot 10^8$/ml) and samples (0.5 or 1.0 ml) were withdrawn at intervals. These were either filtered immediately onto membrane filters or pipetted rapidly into tubes pre-cooled on ice, where they were kept at 0° until the end of a run (the cells lost no radioactivity during this period). The suspensions were then filtered as usual. The membranes were washed twice with 2 ml of 0.9% saline, fixed to planchettes and dried. They were counted on a Nuclear Chicago gas-flow counter, fitted with a thin mylar window.

Paper electrophoresis

This was carried out in a cooled-plate apparatus[10] on Whatman 3 MM paper, using a buffer at pH 2 (43.5 ml glacial acetic acid and 12.5 ml 98% formic acid made to 1 l with distilled water). The voltage gradient was approx. 100 V/cm and runs were carried out for 10–15 min. Radioautographs were prepared by exposing the dried papers for a suitable interval to Kodak 'Blue Brand' film. Phosphates were detected on chromatograms as described elsewhere[11].

RESULTS

The effect of phosphorus-deprivation on the rate of P_i uptake

When cells grown on PPYG media were centrifuged, washed and suspended in buffered BXPG containing 0.5 mM $^{32}P_i$, they took up P_i at a constant rate of about 1 nmole/min per $1 \cdot 10^8$ cells. However, cells starved for about 2 h in the phosphorus-free (BXPG) medium, took up P_i at twice this rate (Fig. 1) until 10–11 nmoles P_i had been taken up per $1 \cdot 10^8$ cells. At this stage, the rate changed back to that observed in non-starved cells, *i.e.* 1 nmole/min per $1 \cdot 10^8$ cells. (These two phases of uptake, and the phosphorus pools filled during those phases, will be referred to as 'primary' and 'secondary', respectively.) When starved cells were 'pre-loaded' with 10 nmoles of unlabelled P_i 5 min before the addition of $^{32}P_i$, the primary uptake phase was abolished and the cells behaved essentially as non-starved cells. The primary pool could also be pre-loaded by pyrophosphate and arsenate (Fig. 1), which enter *B. cereus* by the phosphate transport system[5].

The primary uptake phase was observed only in cells starved 90 min or more. The relationship between the initial rate of uptake and starvation time (Fig. 2) suggests that the primary pool is depleted somewhere between 30 and 90 min from the time the cells are denied phosphorus.

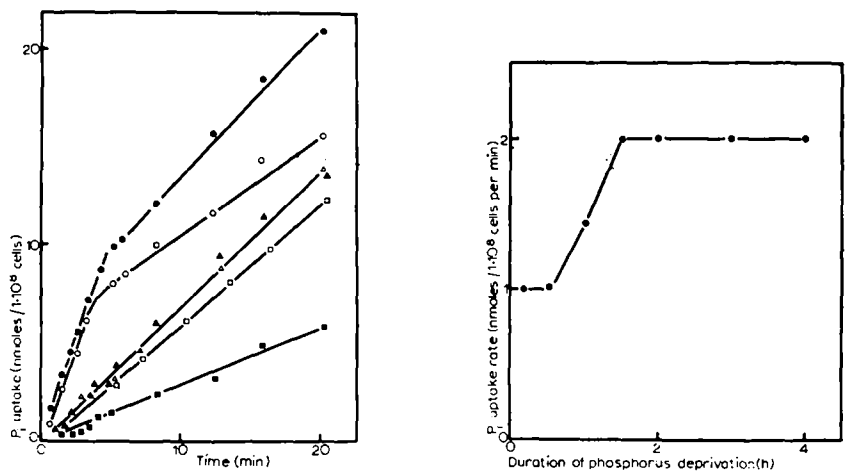

Fig. 1. The increase of the initial rate of phosphate uptake by starvation and its suppression in *B. cereus*. $^{32}P_i$ uptake was measured using washed non-starved cells (▲—▲) and phosphorus-starved cells, either without further additions (●—●) or pre-treated, for 5 min before addition of $^{32}P_i$, with 10 nmoles of one of the following: phosphate (△—△), arsenate (□—□), pyrophosphate (■—■), glycerophosphate (○—○).

Fig. 2. The effect of phosphorus-deprivation on initial rates of phosphate uptake in *B. cereus*. The cells were shaken up to 4 h in the phosphorus-free medium and samples of the suspension were removed at times shown for the assay of initial rates of $^{32}P_i$ uptake.

The effect of temperature and pH on P_i uptake

The uptake of P_i exhibits a high temperature coefficient both for the primary and secondary phase (Fig. 3). The effect of cooling was reversible, since the cells recovered normal uptake rates as soon as they were re-warmed to 30°.

In the temperature range where uptake could be demonstrated (20–37°) the primary phase exhibited a higher temperature sensitivity than the secondary phase; when the data for the primary phase were plotted as an Arrhenius plot, the activation energy for the uptake was calculated to be about 13 kcal/mole P_i. The maximal rate of uptake was at about pH 7.2 (Fig. 4).

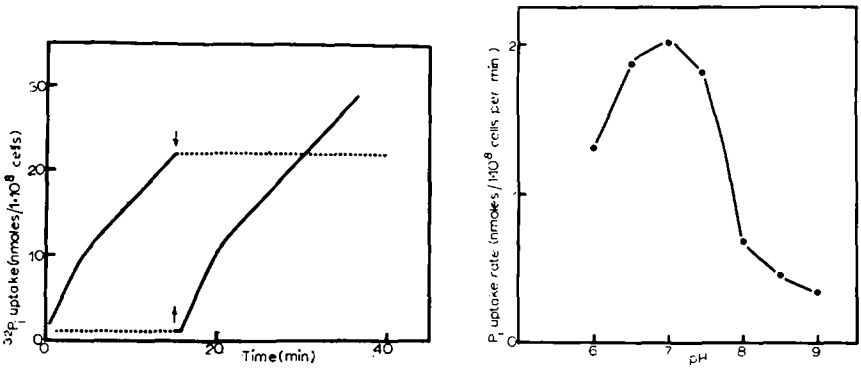

Fig. 3. The effect of temperature on phosphate uptake in *B. cereus*. ———, incubation at 30°; – – – – –, at 0°. Arrows indicate the point at which the cells were cooled to 0° (↓) or warmed to 30° (↑).

Fig. 4. The effect of pH on phosphate uptake in *B. cereus*. The buffer used was K^+–HEPES (50 mM).

Metal ion requirement for P_i uptake

The rate of P_i uptake in the primary phase showed little variation when K^+, Na^+ or triethanolamine were present as the counter ion. Some activation was elicited by the addition of Co^{2+}, Ca^{2+} or Mg^{2+}, but Mn^{2+} and Ni^{2+} were inhibitory. In the secondary phase, a high K^+/Na^+ ratio was required for optimal rates of uptake (Table I).

TABLE I

THE EFFECT OF METAL IONS ON THE RATE AND EXTENT OF PHOSPHATE UPTAKE IN *B. cereus*

Monovalent ion (mM)		*Other additions*		*$^{32}P_i$ uptake rate (nmoles/1·10^8 cells per min)*		*Extent of primary uptake (nmoles/1·10^8 cells)*
K^+	*Na^+*	*Ion*	*Concn. (mM)*	*Primary*	*Secondary*	
—	—	Triethanolamine	20	2.0	0.5	9.5
20	—	—	—	2.0	1.0	9.5
18	2	—	—	2.0	0.85	9.5
10	10	—	—	2.0	0.75	10.5
2	18	—	—	2.0	0.63	9.0
—	20	—	—	2.0	0.40	9.0
—	20	Ca^{2+}	10	2.7	0.50	11.5
—	20	Mg^{2+}	10	2.7	0.60	11.5
20	—	Ca^{2+}	10	2.0	1.0	9.5
20	—	Mg^{2+}	10	2.0	1.0	9.5
20	—	Co^{2+}	10	2.5	1.0	12.5
20	—	Ni^{2+}	10	1.1	0.06	5.0
20	—	Mn^{2+}	10	0.60	0.06	3.6

The effect of energy sources and metabolic inhibitors on P_i uptake

An energy source is required for the maximal uptake of phosphate. Omission of the energy source during the uptake phase alone had little effect; omission during the 2-h phosphorus-starvation period resulted in a decrease of P_i uptake, dependent upon the severity of carbohydrate deprivation (Table II). Glycolytic inhibitors, uncouplers

TABLE II

REQUIREMENT OF AN ENERGY SOURCE FOR MAXIMAL PHOSPHATE UPTAKE IN *B. cereus*

Carbon source	*Final concn. (%) during*		*$^{32}P_i$ uptake rates (nmoles/1·10⁸ cells per min)*		*Extent of primary uptake (nmoles/1·10⁸ cells)*
	Starvation	*Uptake*	*Primary*	*Secondary*	
Glucose	1	1	2.0	1.0	10
	1	0.1	2.0	0.8	10
	1	0	2.0	0.8	9.0
	0	0	0.15	0.15	0*
	0.25	0.25	1.2	0.65	5.3
Succinate	0.1 M	0.1 M	2.0	0.95	10

*One phase only.

TABLE III

THE EFFECT OF METABOLIC INHIBITORS ON PHOSPHATE UPTAKE IN *B. cereus*

Inhibitor	*Concn. (mM)*	*$^{32}P_i$ uptake rates (nmoles/1·10⁸ cells per min)*		*Extent of primary uptake (nmoles/1·10⁸ cells)*
		Primary	*Secondary*	
None	–	2.0	1.0	10
N-Ethylmaleimide	0.1	0.57	0.23	4.0
Azide	0.1	0.74	0.084	3.5
2,4-Dinitrophenol	1	1.7	0.17	6.3
2,4-Dinitrophenol*	1	1.1	0.17	5.0
Iodoacetamide	0.5	1.5	0.60	7.0

*Succinate was substituted for glucose during both phosphorus-starvation and uptake.

of oxidative phosphorylation and sulphydryl reagents all affected the rates of both the primary and secondary uptake, as well as the ultimate level of filling of the primary pool, *i.e.* the level of uptake at the end of the primary phase. To avoid the presentation of a large number of figures, we have collated the results in terms of these three parameters (Table III).

The chemical identity of the phosphorus in the primary pool

The distribution of label during the first 15 min of $^{32}P_i$ uptake by *B. cereus* was examined by direct electrophoresis of whole cells after collection on membrane filters (Fig. 5). This showed that, initially, P_i was the predominant radioactive compound associated with the cells. Quantitative determination of the radioactivity in the various

fractions over the period of time studied, showed (Fig. 6) that the P_i reached a maximum at about 6 min, after which its pool remained relatively constant, while label appeared (and increased) in some organophosphates and in the insoluble cell residue left on the membrane filters.

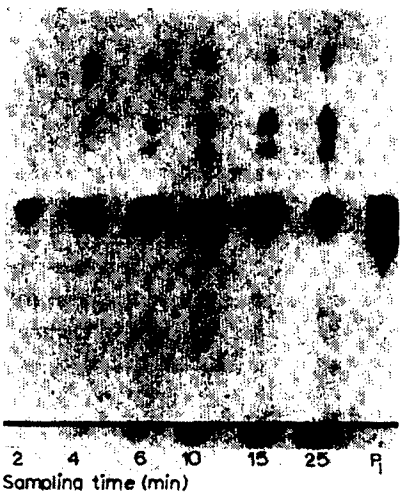

Fig. 5. Direct electrophoresis of *B. cereus* cell contents after filtration on membranes. Suspensions of phosphorus-starved cells were shaken with $^{32}P_i$ and samples were filtered at times shown. The washed membranes were immediately placed on glass plates at $-20°$ until all samples were collected. They were then cut in two and the cell-bearing surfaces folded together. These folded membranes were placed at the origin of the electropherogram and electrophoresis was carried out as usual. The figure shows the radioautograph of the dried paper, with the membranes still fixed to the origin (indicated by a line). $^{32}P_i$, phosphate reference standard.

Displacement of P_i from the primary pool

Using the technique described in the previous section, we followed the distribution of radioactivity in starved *B. cereus* cells under conditions of discontinuous supply of $^{32}P_i$ and $^{31}P_i$. Cells given only sufficient $^{32}P_i$ to fill the primary pool retained all radioactivity in the presence or absence of further additions of $^{31}P_i$ (Fig. 7). From the time when all the available $^{32}P_i$ had been taken up, the radioactivity in the P_i pool fell, while that in 'product' fractions increased. This pattern was accentuated as a result of a 'cold chase'. These results suggest that P_i enters the cells against a concentration gradient and therefore by an active process, and that it fills a primary pool which is drawn upon for the various metabolic reactions.

The nature of the primary pool

In order to determine whether the primary P_i pool was intimately linked with the transport of external phosphate, we followed the uptake and fate of α-glycero-[^{32}P]phosphate, which cannot pre-load the primary pool and thus abolish the primary uptake (Fig. 1). We followed the appearance of $^{32}P_i$ within the cells using the electrophoretic technique described above. We found that (Table IV), by 4 min, 10 nmoles α-glycerophosphate had been taken up per $1 \cdot 10^8$ cells and that it had all been hydrolysed. The cell content of P_i at this stage (about 10 nmoles/$1 \cdot 10^8$ cells) was sufficient to fill the primary pool, yet failed to abolish the primary uptake.

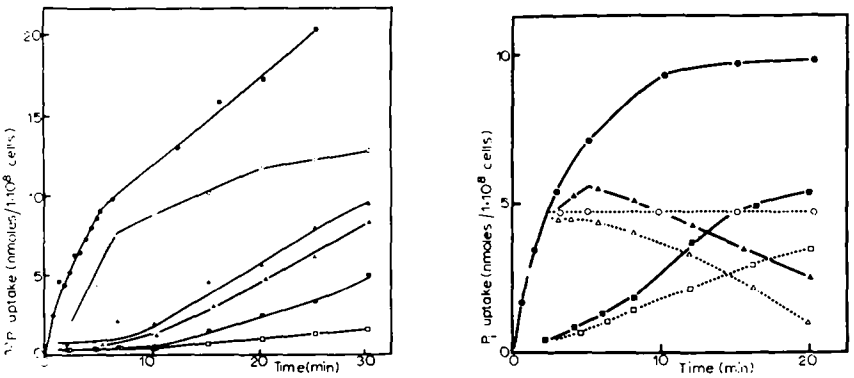

Fig. 6. The distribution of radioactivity from $^{32}P_i$ taken up by phosphorus-starved cell suspensions of *B. cereus*. The data were obtained by cutting out and counting relevant areas from the electropherogram shown in Fig. 5 after location of the spots by radioautography. ●—●, total ^{32}P uptake; ○—○, P_i; ■—■, soluble organophosphates (not identified); ▲—▲, total cell-bound material; △—△, nucleic acids (radioactivity extracted from membranes by hot trichloroacetic acid); □—□, radioactivity not extracted by hot trichloroacteic acid. Note: no measurable radioactivity could be extracted from the cells by mixtures of chloroform and methanol.

Fig. 7. Displacement of radioactivity from the P_i pool. Two suspensions of phosphorus-starved *B. cereus* were each given (at time 0) 10 nmoles $^{32}P_i$ per $1 \cdot 10^8$ cells. One suspension (solid lines, solid symbols) received no further additions; the other (broken lines, open symbols) received excess (0.5 mM) $^{31}P_i$ at 2 min. Total uptake (circles), intracellular $^{32}P_i$ (triangles) and cell-bound ^{32}P (squares) were followed in each suspension by the techniques described in Figs. 5 and 6.

TABLE IV

THE DISTRIBUTION OF ^{32}P AFTER α-GLYCERO[^{32}P]PHOSPHATE UPTAKE BY *B. cereus*

In nmoles/$1 \cdot 10^8$ cells.

Time (min)	*Total uptake*	*Intracellular contents*		
		α-Glycero-phosphate	*P_i*	*Cell-bound phosphate*
5	10.1	0	9.1	1.0
10	14.2	0	9.1	3.4
20	20.0	0	9.1	8.5

DISCUSSION

B. cereus possesses a compartment for P_i which is the first to be rapidly filled when phosphorus-starved cells are presented with P_i. The capacity of this compartment is about 10 nmoles P_i per $1 \cdot 10^8$ cells. Since this corresponds to about $1 \cdot 10^7$ molecules P_i per cell, it could not possibly represent phosphate held on binding sites. This compartment does not empty easily; in fact, its existence can only be demonstrated in cells starved at least 90 min. It can be filled, or pre-loaded, not only by P_i, but also by arsenate and pyrophosphate, both of which share with phosphate the same transport system[5]. Once the pool is filled, phosphate utilization begins, and the rate of uptake falls to the secondary value. On the other hand, 'chase' data clearly indi-

cate that P_i accumulated during the primary phase is passed on into the cellular metabolic system and is replaced by external P_i. It is, however, possible that P_i is present in *B. cereus* independent of the primary pool. This is indicated by the observation (Fig. 1), that at the end of the primary uptake phase when P_i esterification begins, there is a sharp fall in the rate of P_i uptake, but total intracellular P_i continues to rise slightly. This increase may represent the passage of radioactivity from the primary pool to another pool. Furthermore, P_i liberated intracellularly from α-glycerophosphate does not appear to be able to fill the primary pool since phosphorus-starved cells, given sufficient α-glycerophosphate to liberate 10 nmoles P_i per $1 \cdot 10^8$ cells (Table II) still exhibit the primary uptake (Fig. 1). It is thus possible to have, within the cell, P_i in sufficient amounts to fill the primary pool, yet without access to it.

It could be argued that the primary pool is not true phosphate, but a labile compound which releases P_i during electrophoresis. We have, however, demonstrated in an experiment such as shown in Fig. 5, that even electrophoresis of the membrane filters at pH 7.2 produces the same result as that at pH 2.0. We know of no covalently linked phosphate labile under these conditions.

In this connection, it should be mentioned that, when 10 nmoles/$1 \cdot 10^8$ cells of $^{32}P_i$ was presented to the cells, it was all taken up and two-thirds of it was shown to exist as P_i (Fig. 7) within the cell. Phosphate is thus accumulated against a very high gradient, as indeed, is the non-metabolisable phosphite in the same system[5]. This, and the lack of any loss of radioactivity from the cells during the cold chase (Fig. 7) excludes the possibility that the primary uptake is the result of a fast exchange.

A fast initial uptake of P_i in phosphorus-deficient algae has been reported recently[12] and a biphasic uptake of sulphate, depending on sulphate-deprivation and showing many features similar to those discussed here, was demonstrated in Chlorella[13]. The phenomenon is therefore not unique and may, in fact, be widespread. We have recently observed a similar pattern in *Escherichia coli*[14].

Stimulation of phosphate uptake by monovalent ions has been demonstrated in yeast[2], in *Streptococcus faecalis*[3], in a marine fungus[15] and in other systems. In *B. cereus*, as in Euglena[4], these ions had little effect. However, our measurements were made only at the optimal pH for uptake (pH 7.2), where phosphate exists as the monovalent anion, and it has been shown that in yeast[2] K^+ stimulated P_i uptake only below the pH optimum (7.0). We agree with BLUM's suggestion[4] that Tris (or, in our case, triethanolamine) enters the cell as the counter ion, but the entry of H^+ is not excluded.

It should also be noted that, unlike yeast[2,16] and the red blood cells[17], where phosphate has been reported to be esterified on entry, *B. cereus* accumulates intracellular phosphate to a certain level before esterification begins.

The uptake of phosphate requires a source of energy and this requirement can be satisfied by glucose or succinate. The absence of carbohydrate during the uptake phase alone had little effect on P_i uptake, and the requirement could only be demonstrated if carbohydrate were denied during the phosphorus-deprivation period as well. *B. cereus* is known to accumulate poly-β-hydroxybutyrate, especially when an essential nutrient is absent from the medium[18]. It seems possible that the presence of carbohydrate during phosphorus-deprivation might have led to this situation, and that poly-β-hydroxybutyrate could have supplied the energy necessary for uptake once the cells were deprived of carboxyhydrate.

The accumulation of phosphate against a concentration gradient, the effect of

inhibitors on this process and its requirement for an energy source, all support the idea of an energy-linked, active transport system for phosphate in *B. cereus*.

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