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Physiological Factors in the Regulation of Alkaline Phosphatase Synthesis in *Escherichia coli*

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Alkaline phosphatase is induced in excess phosphate media by starvation either for pyrimidines or for guanine. Induction is observed both during starvation, after a lag period, and following a period of starvation. Induction is not caused by a lowering of the internal orthophosphate pool, but is linked to alterations in the levels of the nucleotide pools. Experiments with purine-requiring mutants suggest that phosphatase is induced in wild-type strains by an adenine nucleotide. Mutations in the *phoR* gene can produce differential responses to the different starvation regimes.

There are two major classes of regulatory systems for the control of enzyme synthesis in bacteria. In systems under negative control, such as the lactose operon, a metabolite co-repressor interacts with one or more components specified by regulator genes to inhibit enzyme synthesis (12). In positive control systems, such as the arabinose operon, a metabolite inducer interacts with a protein activator molecule specified by a regulator gene to stimulate enzyme synthesis (2). The control of the synthesis of alkaline phosphatase (EC 3.1.3.1) in *Escherichia coli* appears to have features common to both types of control. Genetic studies have shown that at least two distinct regions, the *phoR* and *phoS* genes, are involved in specifying a cytoplasmic repressor (1). The *phoR* gene, in addition, appears to have some activator function, as demonstrated by the isolation of *phoR* mutants, referred to here as $phoR^c$, defective in the maximal induction of phosphatase (6, 7). The *phoS* region appears to be comprised of two cistrons (8).

The metabolite or metabolites that interact with the phosphatase regulatory system are unknown. Because phosphate starvation produces a large derepression of phosphatase (11, 21), it has been suggested that orthophosphate itself is the co-repressor (6, 7). The evidence presented below demonstrates that this is not the case and indicates that phosphatase synthesis is regulated by one or more nucleotide species. In this report, an increase in the differential rate of phosphatase synthesis will be referred to as "induction" rather than "derepression."

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains employed are listed in Table 1.

The basic minimal medium used consists of (in grams per liter): Trizma base (Sigma), 6.05; $Na_3C_6H_5O_7 \cdot 2H_2O$, 0.42; $MgSO_4$, 0.1; $(NH_4)_2SO_4$, 1.0; K_2HPO_4, 0.27; $FeCl_3 \cdot 6H_2O$, 1.8×10^{-3}; glucose, 2.0. Minimal medium lacking a carbon source and other nutrients is designated as TXP. All amino acids were added to a final concentration of 20 μg/ml, except for arginine and proline (each at 100 μg/ml) and threonine (200 μg/ml). Thymine and uracil were at 10 μg/ml, where required, and guanine and adenine sulfate were employed at 20 and 40 μg/ml, respectively. CA medium denotes supplemented minimal medium containing Casamino Acids (Difco) at a concentration of 1 mg/ml.

Unless otherwise noted, starvation experiments were performed by the following procedure. Cultures were grown overnight at 37 C in limiting glucose (100 μg/ml) minimal medium to an optical density at 720 nm (OD_{720}) of 0.10. Aeration was provided by bubbling. Excess glucose (2 mg/ml) and amino acids, where required, were then added and two or more generations of exponential growth were permitted. Each culture was then centrifuged, washed twice in TXP medium, and suspended in prewarmed medium lacking glucose and the indicated supplement. To a control sample of this culture, the supplement was then added, and the experiment was begun with the addition of glucose to both subcultures.

Assay of alkaline phosphatase. Culture samples were centrifuged, suspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.4, and shaken

TABLE 1. *List of strains*

Designation	Characteristics	Derivation or source
B3L1	*thy lac*	B3; J. Gallant
BD1	*ura thy his*	J. Gallant
BD1L	*ura thy his pro lac*	Nitrosoguanidine mutant of BD1
BD1L-P6	*phoR^c ura thy his pro lac*	Nitrosoguanidine mutant of BD1L
15TAU	*thy arg ura*	J. Gallant
Cs101	Hfr Cavalli *met*	J. Gallant
540	*phoR^c purE thy ura lac*	D. Berg; *phoR* mutation from strain C8 (1)
542	*purE thy ura*	*phoR$^+$ lac$^+$* recombinant of Cs101 × 540 cross
AT2465	Hfr Hayes, *guaA thi*	P. de Haan
R257	*guaB*	P. de Haan
AT2682	*arg his purF guaA thi*	P. de Haan

with 0.05 volume of toluene at 37 C for 60 min. One-half milliliter of toluene-treated extract or Tris-diluted extract was then mixed with 2.0 ml of solution containing 1 mg of *p*-nitrophenol phosphate per ml in 1.0 M Tris, *p*H 8.0. The activity was measured as units of OD_{410} per minute per milliliter at 25 C. Where noted, extracts were heated at 70 C for 15 min to destroy residual acid phosphatase (EC 3.1.3.2) activity (present in *E. coli* B strain extracts). Such treatment does not affect alkaline phosphatase activity.

Measurement of protein synthesis. Protein synthesis during exponential growth was followed by reading OD_{720}. The proportionality of protein synthesis to OD_{720} has been previously verified (5). Alternatively, where indicated, protein synthesis was measured by uptake of ^{14}C-leucine (0.1 μCi per nmole per ml) into trichloroacetic acid-precipitable material. Samples were first precipitated in a chilled solution containing 10% trichloroacetic acid-1% CA for 30 to 60 min and then collected on 0.45 μm-membrane filters (Schleicher & Schuell Co.). The filters were dried for 30 to 60 min at 60 C and then immersed in toluene scintillation fluid (Liquofluor) for counting in a Packard Tri-Carb scintillation counter.

Measurement of the internal orthophosphate pool. The procedure was based on that of Sugino and Miyoshi (20). Cells were first grown in the presence of $^{32}PO_4$ under the desired cultural conditions for a suitable time to allow equilibration of the label (ca. 30 to 50 min). During subsequent growth, 0.70-ml samples were removed, filtered on membrane filters, and washed with three successive 1-ml amounts of iso-osmotic, 0.17 M Tris, *p*H 7.4. The filtered cells were suspended in 0.5 N perchloric acid and chilled at 0 C for 1 hr. Samples were then centrifuged for 15 min at 5,000 rev/min in a Sorvall centrifuge, and the supernatant solution was collected. To 1 ml of this solution, containing 10 nmoles of cold phosphate as carrier, was added 0.35 ml of reaction mixture. The reaction mixture consisted of one part 0.2 M triethylamine, *p*H 5.0, two parts 0.08 M ammonium molybdate, and four parts water. Samples were maintained at 0 C for 20 min to allow formation of the phosphomolybdate precipitate and then were filtered. Filtration and counting of samples were as described above.

RESULTS

Induction of alkaline phosphatase by thymine starvation and by uracil starvation. The standard method for induction of alkaline phosphatase in *E. coli* is to starve bacterial cultures for phosphate (11, 21). However, removal of orthophosphate is not required for induction. Prolonged starvation of a thymine auxotroph for thymine was found to induce phosphatase, even though excess phosphate (2×10^{-3} M) was present at all times (Fig. 1a). Accelerated synthesis began after a lag of 90 min. Similarly, phosphatase was induced in excess phosphate medium by starvation for uracil of a uracil auxotroph, strain 15TAU (Fig. 1b). In this experiment, induction began 1 hr after exhaustion of a limiting amount of exogenous uracil (0.4 μg/ml). Although the extent of induction under both starvation conditions was significant, neither treatment yielded the fully induced rate of phosphatase synthesis obtained during phosphate starvation. In strain 15TAU, for instance, the differential rate of synthesis during phosphate starvation is approximately five-fold greater than the maximal rate during uracil starvation (data not shown).

Induction of phosphatase during thymine starvation or uracil starvation takes place in cells whose growth is inhibited. However, growth inhibition is not necessary to produce the effect. Growing cultures also showed induction when they had been previously starved for thymine (Fig. 1c) or for uracil (Fig. 1d). Furthermore, an increase in the external phosphate concentration by an additional 10-fold (to 2×10^{-2} M) did not diminish the extent of induction (Fig. 1c). The extent of induction following thymine starvation was correlated with the extent of cell death during the starvation (Wilkins, *unpublished experiments*). There was a rapid restoration of repression in cells previously starved for uracil, whereas induction continued sometimes for as long as two generations in cells previously starved for thymine. This difference may be an indirect consequence of the slower growth of the latter cultures.

A functional *phoR* gene is required for the induction of phosphatase in excess phosphate

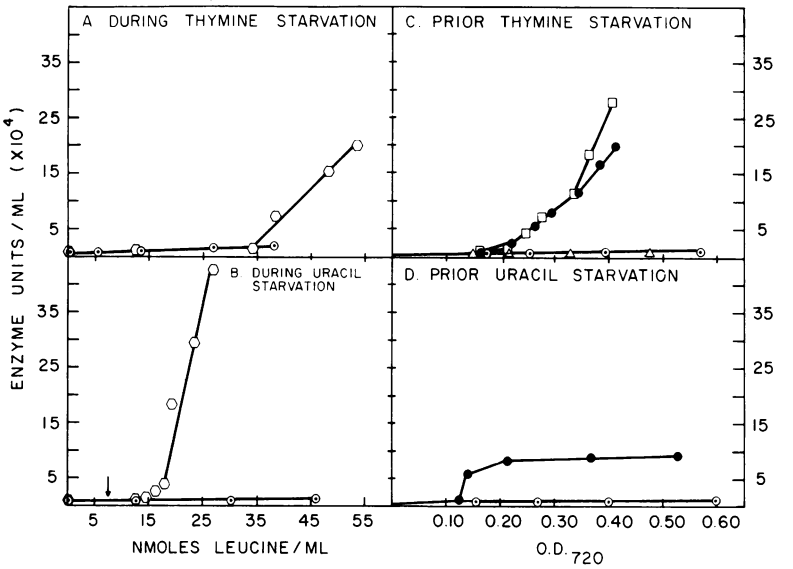

FIG. 1. *Induction of phosphatase synthesis by thymine starvation and by uracil starvation. Procedure of experiments as described in Materials and Methods. (A) Synthesis of phosphatase during thymine starvation of strain B3L1 in CA medium. (B) Synthesis of phosphatase in strain 15TAU following depletion of uracil (initially at 0.4 μg/ml) from the medium. Arrow indicates the break in the optical density curve of the limiting uracil culture. (C) Effect of prior thymine starvation (85 min) on phosphatase synthesis in strain B3L1 in CA medium. (D) Effect of prior uracil starvation (60 min) on phosphatase synthesis in strain BD1 in CA medium. Symbols: ⊙, enzyme synthesis during exponential growth of the indicated strain; hexagons, enzyme synthesis during the course of starvation; ●, enzyme synthesis following the period of starvation; △, enzyme synthesis in culture grown in 2 × 10⁻² M phosphate; □, enzyme synthesis following 85 min of thymine deprivation, grown in the presence of 2 × 10⁻² M phosphate.*

medium. Figure 2a shows that a *phoR*^{c} mutant did not exhibit induction following a period of thymine starvation that was sufficient to induce the wild-type; a similar result was obtained for uracil starvation (Fig. 2b). (The *phoR*^{c} mutant shows only a threefold increase in the differential rate of phosphatase synthesis during phosphate starvation; *phoR*$^+$ strains are induced approximately 1,000-fold.)

Measurement of the internal phosphate pool during uracil starvation. It was of interest to determine whether induction by pyrimidine starvation was a consequence of a lowered intracellular orthophosphate pool. To test this possibility, this pool was measured during uracil starvation of strain 15TAU. No decrease in the internal orthophosphate pool, or in total soluble organic phosphate, occurred during starvation prior to the onset of induction (Fig. 3). The data suggest therefore that orthophosphate is not the co-repressor and that the effector molecule is another metabolite.

Purine metabolism and the regulation of phosphatase. The starvation regimes that induce phosphatase are known to affect nucleotide metabolism. During thymine starvation, deoxyadenosine-5'-triphosphate (dATP) and deoxycytidine-5'-triphosphate accumulate (14–16); during uracil starvation, pyrimidine triphosphate pools decrease and purine triphosphate pools are expanded (4, 13). If there is a causal relationship between alterations of certain nucleotide pools and the induction of phosphatase, then other treatments that alter these pools might be expected to induce the enzyme. Accordingly, the effects of starvation for purines on phosphatase regulation were tested.

The purine biosynthetic pathway is branched, with the synthesis of adenine and guanine nucleotides proceeding separately from the common precursor inosine-5'-monophosphate (IMP). It is possible to diminish both the adenine and guanine nucleotide pools by starving auxotrophs blocked at steps prior to the synthesis of IMP, or to decrease these pools separately by starvation of mutants blocked after the branch point (4).

Figure 4a shows that a period of prior starvation for guanine of a *guaA* mutant, blocked in the synthesis of guanosine-5'-monophosphate (GMP) from xanthosine-5'-monophosphate (XMP), induced phosphatase. A similar result

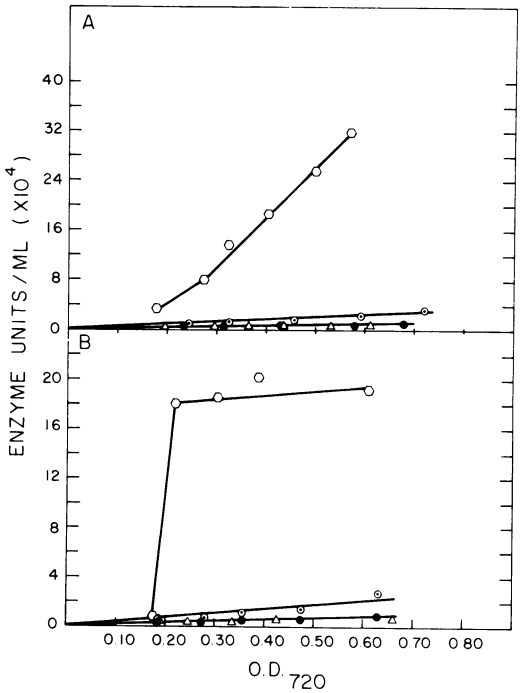

FIG. 2. *Effects of prior thymine starvation and of prior uracil starvation on phosphatase synthesis in strains BD1L (phoR⁺) and BD1L-P6 (phoR^c). Procedure as described in Materials and Methods. The strains were grown in CA medium. (A) Synthesis of phosphatase following 120 min of thymine deprivation. (B) Synthesis of phosphatase following 60 min of uracil deprivation. Extracts were heated to destroy residual acid phosphatase activity. Symbols: ⊙, enzyme synthesis during exponential growth of strain BD1L; hexagons, enzyme synthesis in strain BD1L following the period of starvation; ●, enzyme synthesis during exponential growth of strain BD1L-P6; Δ, enzyme synthesis in strain BD1L-P6 following the period of starvation.*

was found for starvation of strain R257, a *guaB* mutant blocked in the synthesis of XMP from IMP (data not shown). As expected, starvation for guanine of both strains causes a decrease in the guanosine-5'-triphosphate (GTP) pool (4). In contrast to guanine starvation, phosphatase was not induced following a period of purine starvation of a *purE* mutant, a strain deficient in an early step of the purine pathway, the conversion of 5-aminoimidazole to 5-aminoimidazole-4-carboxylic acid ribonucleotide (Fig. 4b). Starvation of *purE* mutants leads to decreases in both the adenosine-5'-triphosphate (ATP) and GTP pools (4). The absence of induction following purine starvation in strain 542 is not the result of some defect in phosphatase regulation, since a period of prior uracil starvation caused induction (Fig. 4b).

The different effects of the two types of purine starvation on phosphatase induction were confirmed in experiments with a *purF guaA* double mutant. Strains with lesions in *purF* are defective in the conversion of 5-phosphoribosyl-1-pyrophosphate to 5-phosphoribosyl-1-amine, and thus, like *purE* mutants, blocked prior to the synthesis of IMP. Figure 5 shows that when this strain was deprived of both adenine and guanine, phosphatase was not induced. However, when starved for guanine in the presence of exogenous adenine, phosphatase was induced, after a lag. This result strongly implies that a derivative of adenine is required for the induction of phosphatase. As would be predicted from these experiments, starvation of *purA* mutants, strains defective in the synthesis of adenosine-5'-monophosphate (AMP) from IMP, did not induce phosphatase (A. Wilkins, Ph.D. thesis, Univ. of Washington, 1969).

One explanation of these data is that there is some accumulation of adenine nucleotides during guanine starvation, unlike general purine starvation, and that phosphatase is induced by an adenine nucleotide. Under this interpretation, the extent of induction reflects the extent of accumulation of the regulatory adenine nucleotide. Adenine nucleotides have been found to accumulate during uracil starvation (4, 13) and during thymine starvation (14-16), two treatments that cause induction.

However, attempts to identify the presumed inducer were unsuccessful. One method involved measurements of the AMP, ADP, and ATP pools by thin-layer chromatographic separation, following labeling with ^{14}C-adenine. Although these pools increased during starvation for either guanine or uracil, all three nucleotide pools decreased during phosphate starvation (A. Wilkins, Ph.D. thesis, Univ. of Washington, 1969). Separate measurement of the dATP pool during phosphate starvation has shown that it decreases in parallel to the ATP pool (J. Gallant, *personal communication*).

Furthermore, direct addition of several adenine nucleotides, each to a concentration of 2×10^{-3} M, to cells made permeable to nucleotides did not induce phosphatase (Table 2). The nucleotides tested were ATP, AMP, dATP, deoxyadenosine-5'-monophosphate, and cyclic adenosine-3',5'-monophosphate (cAMP). The failure of cAMP to induce phosphatase has been previously shown (18). The plasmolyzed cells were capable of induction, however, as shown by the effect of limiting phosphate. (As a check on the altered permeability of the cells, the effect of actinomycin on ^{3}H-GTP

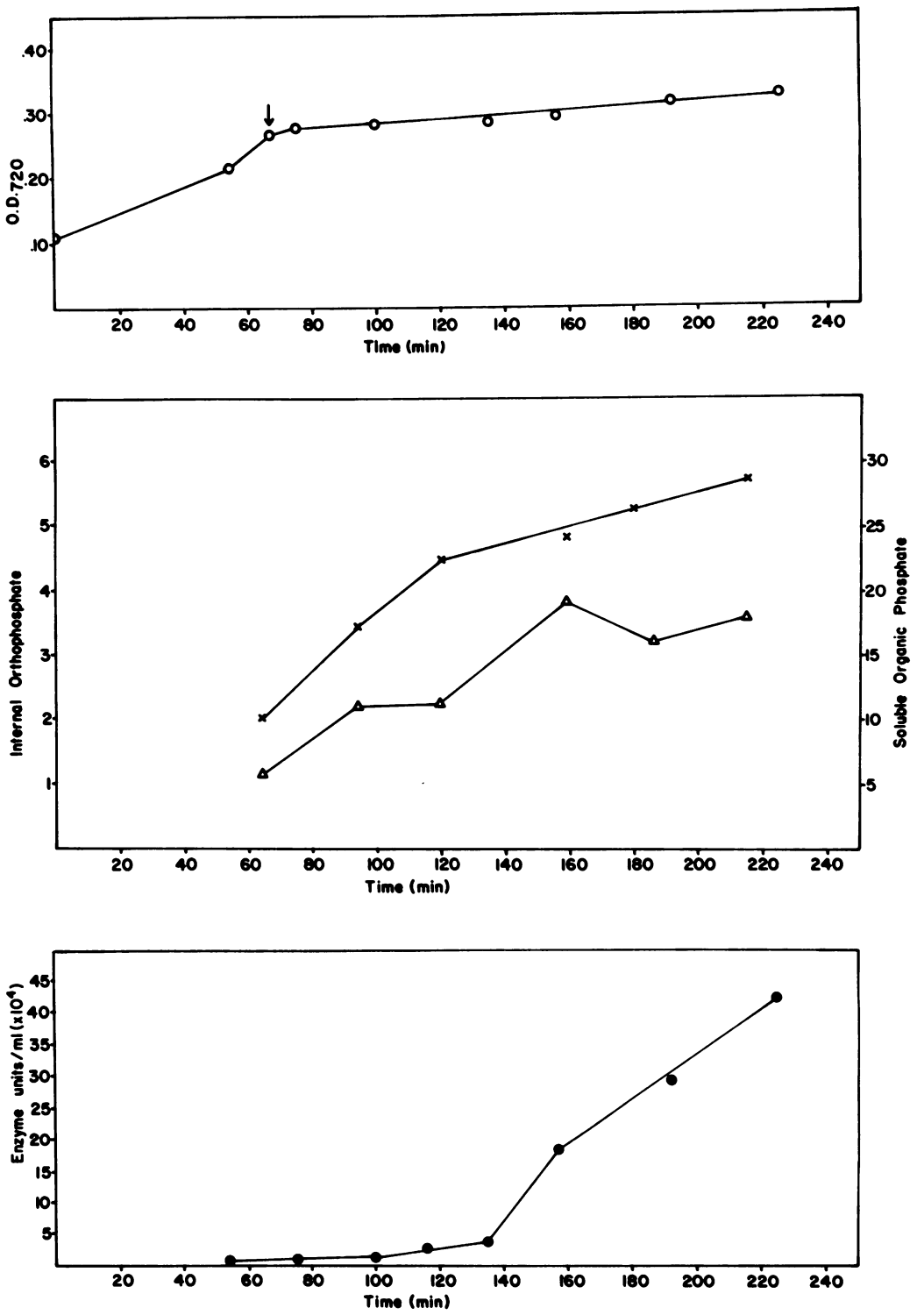

FIG. 3. *Internal orthosphosphate and soluble organic phosphate during uracil starvation of strain 15TAU. In a subculture of the limiting uracil culture described in the legend to Fig. 1b,* $^{32}PO_4$ *was added to a specific activity of 20 μCi/nmole. The measurement of the internal orthophosphate pool was carried out as described in Materials and Methods. Intracellular concentrations are expressed in nanomoles per milliliter. (It is assumed that the average internal volume of a cell is a cubic micrometer.) Total soluble phosphate was measured directly by counting 10λ samples of the perchloric acid-soluble phosphate pool. The soluble organic phosphate values were calculated by subtracting the orthophosphate pool values from total intracellular soluble phosphate. Internal orthophosphate pool, Δ; total soluble organic phosphate, ×——×.*

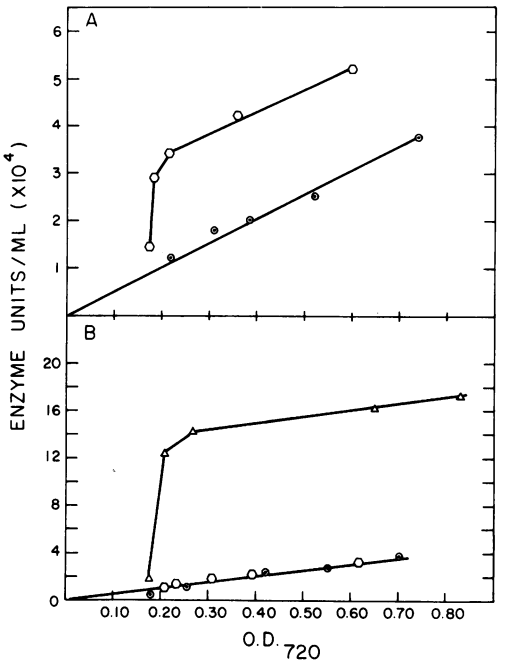

FIG. 4. *Comparison of effects of guanine starvation and general purine starvation on phosphatase synthesis. Experimental procedure as described in Materials and Methods. (A) Induction by prior guanine starvation (60 min) in strain AT2465. (B) Absence of induction following 60 min of adenine starvation (compared to positive effect of 60 min of uracil starvation) in strain 542. Cultures grown in CA medium. Symbols: O, enzyme synthesis during exponential growth; hexagons, enzyme synthesis following period of purine starvation; Δ, enzyme synthesis following period of uracil starvation.*

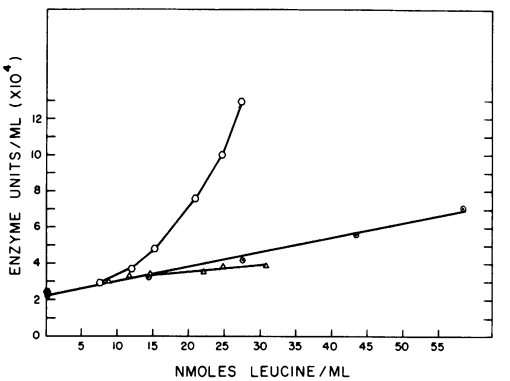

FIG. 5. *Comparison of the effects of adenine and guanine starvation on phosphatase synthesis in strain AT2682. Procedure as described in Materials and Methods. Symbols: ⊙, enzyme synthesis during exponential growth; hexagons, enzyme synthesis during guanine starvation; Δ, enzyme synthesis during joint adenine-guanine deprivation.*

incorporation was tested; the antibiotic inhibited incorporation 91%.)

These results indicate that the inducer is not one of the adenine nucleotides tested, but may be a minor species. (The pool measurements would not have detected any adenine nucleotide present at an intracellular concentration of less than 5×10^{-5} M). The question of the involvement of an adenine nucleotide in phosphatase induction still requires further resolution.

Effect of a phoR partial constitutive mutation on regulatory responses. As shown in Fig. 2, a *phoR*^{c} mutation that lowers the basal rate of phosphatase synthesis and prevents induction by starvation for phosphate also eliminates the potentiality for induction following starvation for pyrimidines. However, the absence of induction by phosphate starvation in *phoR* mutants does not always indicate noninducibility under other conditions. Figure 6 shows the effects of several starvation regimes on strain 540, a partial constitutive

TABLE 2. *Effect of adenine nucleotides on phosphatase synthesis in plasmolyzed cells*[a]

Nucleotide[b]	Phosphate	Specific activity (× 10⁴)	
		0 Min	55 Min
	Lim. P.	11.8	825
	XP	4.1	3.9
dATP	XP	3.9	2.9
cAMP	XP	3.4	3.6
dAMP	XP	3.7	4.7
AMP	XP	3.7	2.6
ATP	XP	3.2	1.6

[a] The method of plasmolysis is that of Gros et al. (10). An 80-ml amount of an exponentially growing culture of BD1L at 5×10^8/ml was centrifuged, washed once with 0.01 M tris(hydroxymethyl)aminomethane (Tris; *p*H 8.0), and then suspended in 4.0 ml of 2.0 M sucrose (in 0.01 M Tris, *p*H 8.0). After 20 min at room temperature, the cells were suspended in 100 ml of CA medium (supplemented minimal medium containing Casamino Acids at 1 mg/ml), lacking glucose and phosphate. A 12-ml amount was put in a separate bubbler, as the limiting phosphate (Lim. P.) culture. To 80 ml of the remainder, phosphate was added to 2×10^{-3} M. Six 12-ml samples were removed, one an excess phosphate (XP) control (without further additions), the others adenine nucleotide test cultures. Each nucleotide was added to 2×10^{-3} M. In separate 1-ml samples, guanosine-5'-triphosphate (GTP) incorporation and inhibition of GTP incorporation by actinomycin were followed. Growth was initiated by the addition of glucose.

[b] Abbreviations: AMP, adenosine-5'-monophosphate; dAMP, deoxyAMP; cAMP, cyclic adenosine-3,5'-monophosphate; ATP, adenosine-5'-triphosphate; dATP, deoxyATP.

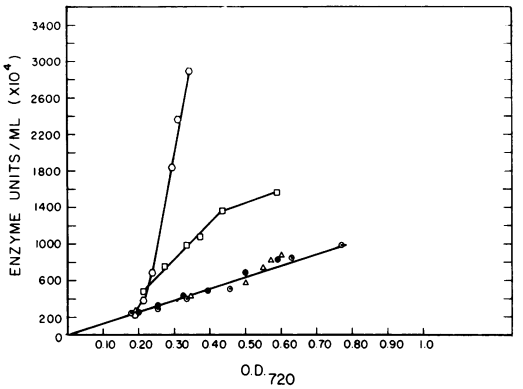

FIG. 6. *Effects of phosphate, uracil, adenine, and thymine starvation on phosphatase synthesis in strain 540. Procedure as outlined in Materials and Methods. Growth was carried out in CA medium. Symbols: ⊙, enzyme synthesis during exponential growth; △, enzyme synthesis during growth in limiting phosphate (5×10^{-5} M); ●, enzyme synthesis following 75 min of uracil starvation; □, enzyme synthesis following 150 min of thymine starvation; hexagons, enzyme synthesis during adenine starvation.*

phoR mutant that synthesizes phosphatase in excess phosphate media at approximately 10% the rate of a phosphate-starved wild-type strain. This strain was not induced by phosphate starvation or by starvation for uracil, but it was induced by a period of prior thymine starvation. Furthermore, in marked contrast to wild-type strain, starvation for purines induced the enzyme (and to the full constitutive rate).

These results show that alteration of the *phoR* gene product by mutation can lead to induction by different metabolites. This conclusion further implies that induction in *phoR*$^+$ strains by different starvation regimes may be produced by different metabolites.

DISCUSSION

The results show that alkaline phosphatase is induced in cells grown in excess phosphate media by three starvation treatments that affect nucleotide metabolism. Apparently, the regulation of phosphatase synthesis by phosphate is indirect, and the regulatory system (consisting of the gene products of *phoR* and *phoS*) interacts with one or more nucleotide species. Although these metabolites have yet to be identified, the kinetics of phosphatase synthesis following the various starvation regimes should provide clues. In particular, enzyme synthesis following a period of thymine starvation is prolonged, indicating that the pool of the regulatory metabolite does not return quickly to the pre-starvation level.

The purine starvation experiments suggest that phosphatase is induced in wild-type strains by the accumulation of an adenine nucleotide. Such an inducer could act in either of two ways: (i) it might activate the *phoR* gene product to stimulate enzyme synthesis, or (ii) it might function as the antagonist of the synthesis or activity of a co-repressor. The data permit some discrimination between these alternatives. If a co-repressor is the only metabolite to react directly with the regulatory system, then the activator role of the *phoR* gene product is to stimulate phosphatase synthesis following depletion or inactivation of the co-repressor. Presumably, under this hypothesis, phosphate starvation leads to the greatest reduction in co-repressor since it is the most effective inducing regime in wild-type strains. Phosphate starvation should therefore always produce the most induction in all strains capable of any induction. However, as Fig. 6 demonstrates, alteration of the *phoR* gene product by mutation leads to insensitivity to phosphate starvation while conferring inducibility under two other conditions (one of which does not induce wild-type cells).

This result favors the interpretation that the *phoR* gene product interacts directly with metabolites, probably nucleotides, to stimulate the synthesis of phosphatase. The constitutivity of the partial constitutive *phoR* mutant might be the result of increased sensitivity to a small molecule normally present in the cells during growth. Induction by starvation for either thymine or adenine in this strain would be a response to nucleotides accumulated during these treatments; failure of uracil starvation and of phosphate starvation to cause induction is the result of insensitivity to the particular effector metabolite or metabolites that normally induce phosphatase in *phoR*$^+$ strains.

A similar explanation of constitutivity for arabinose enzyme synthesis in *araC*^{c} mutants has been proposed (9). Because the regulator protein of these mutants retains some sensitivity to arabinose both in vivo and in vitro, Greenblatt and Schleif suggest that partial constitutive synthesis results from interaction between the mutant regulator protein and an intracellular metabolite. They report, in addition, that D-fucose, a sugar that inhibits arabinose enzyme induction in wild-type strains, can induce ribulokinase synthesis in the mutant strains. This effect appears analogous to the opposite effects of adenine starvation on

phosphatase synthesis in *phoR*$^+$ strains and the *phoR* partial constitutive.

In summary, the data strongly suggest the involvement of nucleotide metabolism in the regulation of alkaline phosphatase synthesis. The *phoR* gene product determines both the extent and specificity of the response to alterations in the nucleotide pools. The role of *phoS* was not examined in this investigation. However, the protein or proteins specified by this region might regulate phosphatase through some participation in nucleotide metabolism.

Control of enzyme synthesis in many bacterial regulatory systems is mediated by an end product of metabolism (3). The regulation of phosphatase synthesis by nucleotides, a major product of phosphatase metabolism, appears to be another example of such control. Although the physiological function of phosphatase is not known, the complexity of its regulation may be a reflection of the complexity of phosphate metabolism.

It is of interest that regulation by adenine nucleotides has been reported in other bacterial systems. For instance, cAMP is known to antagonize catabolite repression of β-galactosidase and other enzymes (18, 19, 22). In addition, maximal induction of the *gua* operon requires an adenine nucleotide (17). These results imply that stimulation of enzyme synthesis by nucleotides may not be an uncommon form of regulation in bacteria.

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