

Regulation of Two Phosphatases and a Cyclic Phosphodiesterase of *Salmonella typhimurium*

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The regulation of three *Salmonella typhimurium* phosphatases in response to different nutritional limitations has been studied. Two enzymes, an acid hexose phosphatase (EC 3.1.3.2) and a cyclic phosphodiesterase (EC 3.1.4.d), appear to be regulated by the cyclic adenosine 3',5'-monophosphate (AMP) catabolite repression system. Levels of these enzymes increased in cells grown on poor carbon sources but not in cells grown on poor nitrogen or phosphorus sources. Mutants lacking adenyl cyclase did not produce elevated levels of these enzymes in response to carbon limitation unless cyclic AMP was supplied. Mutants lacking the cyclic AMP receptor protein did not produce elevated levels of these enzymes in response to carbon limitation regardless of the presence of cyclic AMP. Since no specific induction of either enzyme could be demonstrated, these enzymes appear to be controlled solely by the cyclic AMP system. Nonspecific acid phosphatase activity (EC 3.1.3.2) increased in response to carbon, nitrogen, phosphorus, or sulfur limitation. The extent of the increase depended on growth rate, with slower growth rates favoring greater increases, and on the type of limitation. Limitation for either carbon or phosphorus resulted in maximum increases, whereas severe limitation for Mg^{2+} caused only a slight increase. The increase in nonspecific acid phosphatase during carbon limitation was apparently not mediated by the catabolite repression system since mutants lacking adenyl cyclase or the cyclic AMP receptor protein still produced elevated levels of this enzyme during carbon starvation. Nor did the increase during phosphorus limitation appear to be mediated by the alkaline phosphatase regulatory system. A strain of *Salmonella* bearing a chromosomal mutation, which caused constitutive production of alkaline phosphatase (introduced by an episome from *Escherichia coli*), did not have constitutive levels of nonspecific acid phosphatase.

The preceding papers described the purification and properties of three periplasmic phosphatases from *Salmonella typhimurium*: a cyclic phosphodiesterase with 3'-nucleotidase activity, an acid hexose phosphatase, and a nonspecific acid phosphatase (11, 27). The role of these enzymes could be catabolic. Since all three phosphatases are located outside the cytoplasmic membrane, they can hydrolyze non-transportable phosphate esters to components that the cell can transport and utilize. Phosphatase activity could supply many types of nutrients, depending on the esters' organic moiety, in addition to phosphate. It was expected that periplasmic phosphatase levels would increase during nutrient limitations, which their activity could alleviate. To test this possibility, we investigated the expression of these peri-

plasmic phosphatases in cells grown under a variety of nutrient limitations.

Two well-characterized regulatory systems were candidates for the regulation of one or more of the three periplasmic phosphatases of *Salmonella*. Schlesinger and Olsen introduced the structural gene for *Escherichia coli* alkaline phosphatase into *Salmonella* via an episome and found that this enzyme was regulated by phosphorus availability, as in *E. coli* (22). This suggested that at least some of the alkaline phosphatase regulatory loci were present in *Salmonella* even though *Salmonella* lacks the alkaline phosphatase structural gene. This regulatory system is apparently pleiotropic, since in *E. coli* it has been shown to regulate the levels of a phosphate-binding protein (6, 7) and at least two other periplasmic proteins (16).

A second regulatory system that could regulate one or more of the phosphatases is the cyclic adenosine 3',5'-monophosphate (AMP)

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catabolite repression system. Studies have shown that acid hexose phosphatase levels increase when *E. coli* is grown on poor carbon sources, a pattern usually indicative of regulation by the cyclic AMP system (5, 26). Carillo-Castañeda and Ortega reported that acid phosphatase activity in *Salmonella* was higher when lactate was the carbon source than when glucose was the carbon source (2). The *Salmonella* activity was measured as hydrolysis of *p*-nitrophenyl phosphate and presumably reflected the combined activity of acid hexose phosphatase and nonspecific acid phosphatase.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and genotypes are listed in Table 1. Selection of *Salmonella* mutants that produced alkaline phosphatase constitutively was performed by the procedure of Torriani and Rothman (24). An *E. coli* episome containing genes for lactose utilization and alkaline phosphatase (KLF 254) was introduced into *S. typhimurium* LT-2 by selecting for growth on lactose as a carbon source. The episome-containing *Salmonella* strain was then mutagenized with diethyl sulfate by the procedure of Straus and Wyche (23) and plated on medium containing 1 mM inorganic phosphate and 0.4% (wt/vol) β -glycerophosphate as carbon source. Mutants able to grow on β -glycerophosphate as carbon source in the presence of inorganic phosphate were isolated. Constitutive alkaline phosphatase production was determined by the staining procedure described by Miller (15). Segregants lacking the episome were obtained by screening for strains unable to use lactose as sole carbon source. A new KLF 254 episome was introduced into each of these strains, and the new strains were tested for alkaline phosphatase constitutivity by the alkaline phosphatase staining procedure. One mutant (TA2365) possessed constitutive alkaline phosphatase activity when a new episome was inserted, indicating that the mutation responsible for constitutive alkaline phosphatase production was located on the *Salmonella* chromosome.

Chemicals. Phosphate esters used as substrates and cyclic AMP were purchased from Sigma (St. Louis, Mo.). Yeast ribonucleic acid (RNA) (BDH, distributed by Gallard-Schlesinger) was prepared for use as a phosphate source by suspending the RNA to a concentration of 10 mg/ml in 10 mM tris(hydroxymethyl)aminomethane-hydrochloride

buffer. The RNA was dissolved by adding sufficient NaOH to adjust the pH to 7.3. The RNA solution was applied to a Sephadex G-50 column to separate high-molecular-weight RNA from inorganic phosphate and small molecules. RNA that eluted in the void volume was collected. Samples of this RNA preparation released 140 nmol of phosphate per mg of RNA when treated with *E. coli* alkaline phosphatase and contained less than 1 nmol of inorganic phosphate per mg.

Culture media and growth conditions. Carbon- and nitrogen-free basal salts medium (N^-C^-) and carbon-, nitrogen-, and phosphate-free medium ($N^-C^-P^-$) have been described in the accompanying paper (11). Nitrogen-, carbon-, and sulfur-free medium ($N^-C^-S^-$) is N^-C^- medium with $MgSO_4 \cdot 7H_2O$ replaced by 0.08 g of $MgCl_2 \cdot 6H_2O$ per liter. $N^-C^-Mg^-$ medium (lacking magnesium) is N^-C^- medium with $MgSO_4 \cdot 7H_2O$ replaced by 0.06 g of Na_2SO_4 per liter. Nutrient broth contains 0.8% (wt/vol) nutrient broth (Difco) and 5 g of NaCl per liter. Minimal glucose medium (VBCG) is minimal medium E of Vogel and Bonner (25) supplemented with 0.4% (wt/vol) glucose. A synthetic pools mixture was prepared to enrich basal salts media. This mixture contained the following components: 1 mg each of the 20 amino acids per ml; 1 mg each of adenine, guanine, cytosine, uracil, and thymine per ml; and 0.1 mg each of pantothenate, biotin, nicotinamide, pyridoxine, thiamine, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid per ml. The mixture was adjusted to pH 6.5 with NaOH and sterilized by filtration through a 0.2- μ m-pore-size Nalgene filter unit. Some insoluble materials present in the solution were removed by the filtration procedure. Enriched medium was prepared by diluting the synthetic pools mixture 1:20 into basal salts medium. Solid media contained 1.5% agar (Difco). All cultures were grown at 37°C.

Enzyme assays. Procedures for assaying the *Salmonella* phosphatases in whole cells are described in the preceding papers (11, 27). One unit of enzyme activity is defined as the amount of enzyme that releases 1 nmol of inorganic phosphate per min from the standard substrates. Acid hexose phosphatase activity was calculated by subtracting 1.2 times the units of nonspecific acid phosphatase from the total units of glucose 6-phosphate hydrolysis measured at pH 6.0 (27). Alkaline phosphatase was assayed by the same procedure used for the nonspecific acid phosphatase, except that 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, was used as the assay buffer. The substrate for alkaline phosphatase assays was 5'-AMP. One unit of alkaline phosphatase activity is defined as that amount of enzyme that releases 1 nmol of inorganic phosphate per min from 5'-AMP. The contribution of alkaline phosphatase to the hydrolysis of 5'-AMP at pH 5.5 and of nonspecific acid phosphatase to hydrolysis of 5'-AMP at pH 8.0 was assessed by using 2 mM sodium fluoride to specifically inhibit the nonspecific acid phosphatase.

Limited exponential growth. Growth of *Salmonella* was limited by the use of a limited exponential growth apparatus similar in principle to the appara-

TABLE 1. *Strains used in this study*

Strain	Genotype
LT-2	Wild type
TA2363	LT-2/F'254 (<i>lac</i> ⁺ <i>phoA</i> ⁺)
TA2365	<i>pho-25/F'254</i> (<i>lac</i> ⁺ <i>phoA</i> ⁺) (constitutive alkaline phosphatase production)
TA2366	<i>pho-25</i>
TA3301	<i>cya-408</i>
TA3302	<i>crp-403</i>

tus described by Martin and Felsenfeld for limiting growth of histidine auxotrophs (14). An ISCO model 190 programmable gradient pump (Instrument Specialties Co., Lincoln, Neb.) was modified so that the piston originally used to operate a diaphragm pump instead operated the barrel of a plastic disposable syringe. Pall double-check valves (Pall Corp., Glen Cove, N.Y.) placed on the end of the syringe gave a unidirectional flow of liquid. A later version of this modification used a piston connected to four syringes so that four cultures could be grown with the apparatus at the same time. Vinyl plastic tubing with Luer fittings (Pall Corp.) provided connections between the solution to be pumped and the inlet port of the double-check valve. The same tubing was used to connect the outlet port of the double-check valve to the culture flask. Cultures were grown in suitable size reagent bottles fitted with an autoclavable plastic cap into which holes were drilled for a fritted gas dispersion tube, a disposable syringe needle through which nutrient solutions were dripped into the flask, and a sampling tube. An aeration unit consisted of a vacuum flask containing distilled water, which was attached to a compressed air outlet fitted with a filter unit filled with glass wool. Air was injected into the vacuum flask through a fritted gas dispersion tube and then to the culture through tubing connected to the side arm of the vacuum flask. The premoistening of air in the vacuum flask prior to culture aeration was necessary to avoid evaporation of the cultures. Before use, the culture apparatus and aeration unit were sterilized by autoclaving, allowed to cool, and placed in a 37°C water bath. Nutrient solutions to be added were heated to boiling, and the boiling solutions were used to flush and sterilize the pump lines, which were then connected to the syringe needles of the culture flasks.

After inoculation of the culture flasks, the limited growth operation was begun. The ISCO pump was programmed to deliver exponentially increasing amounts of nutrient into the culture flask. The rate of exponential increase determined the doubling time of the cultures. By using low flow rates and high concentrations of nutrients in the nutrient reservoir, the final dilution of the culture by added nutrient solution was usually less than 10%. Mixing action provided by aeration was sufficient to distribute inoculating cells uniformly within 2 min. Cultures for inoculation were grown overnight on the appropriate media for starvation, containing 0.04% glucose and excess amounts of other nutrients. The amount of glucose used permits growth to an optical absorbance at 650 nm of 0.05 (measured in a Zeiss PMQ II spectrophotometer in cuvettes of 1-cm path length). Growth resumed without any appreciable lag on exposure of the cells to glucose. Prior to inoculation, cells were washed twice by centrifugation and resuspended in 0.9% saline. Cultures were grown for four generations in the limited exponential growth apparatus after inoculation.

Protein determination. The protein content of whole-cell samples was assessed by digesting cell samples, suspended in 0.2 N NaOH, in a microwave oven (Radarange, Amana) for about 2 min until

clearing of the cell suspension was noted. Samples were removed and protein was determined by the method of Lowry et al. (12). Protein levels determined in this manner gave results comparable (within 5%) to those obtained with sonically treated whole-cell preparations. Bovine serum albumin was used as the protein standard.

RESULTS

Effects of growth media on phosphatase levels. When the different basal media used in this study were supplemented with sources of carbon (glucose), nitrogen (NH_4^+), phosphorus (phosphate), or sulfur (SO_4^{2-}), which permitted optimal growth rates, the specific activities of the three phosphatases were as shown in Table 2. Supplementation with nutrient broth or synthetic pools decreases the specific activities of all three enzymes.

The specific activities of the three enzymes in cells grown on N-C-P⁻ media supplemented with different sources of carbon, nitrogen, and phosphorus are shown in Table 3. Acid hexose phosphatase and cyclic phosphodiesterase behaved like catabolite-repressible enzymes. Higher specific activities were observed on poor carbon sources like succinate or pyruvate than on good carbon sources like glucose or glycerol. On poor sources of phosphorus or nitrogen the activities of these two enzymes decreased, presumably because the pools of carbon catabolites increased under these conditions and catabolite repression became more severe (13). In contrast, nonspecific acid phosphatase activity increased on all three types of limitation.

Attempts to demonstrate specific induction of the three phosphatases. Exogenous glucose

TABLE 2. Effects of various media on phosphatase enzyme levels

Basal medium ^b	Doubling time (h)	Sp act (U/mg of protein ^a)		
		Nonspecific acid phosphatase	Acid hexose phosphatase	Cyclic phosphodiesterase
VBCG	0.9	10.0	23.4	27.2
N-C ⁻	1.0	11.7	36.0	25.9
N-C-P ⁻	1.2	8.4	42.2	36.0
N-C-S ⁻	1.0	14.6	38.9	
N-C-P ⁻ + 0.8% nutrient broth	0.5	4.2	19.7	14.2
N-C-P ⁻ + synthetic pools	0.6	4.2	29.2	31.4

^a Enzymes were assayed after growth of LT-2 to midexponential phase.

^b Basal salts were supplemented with 0.4% glucose (all media), 10 mM NH_4Cl (N-C⁻, N-C-P⁻, N-C-S⁻), and enriched N-C-P⁻ media, 1 mM sodium phosphate (N-C-P⁻ and enriched N-C-P⁻ media), and 0.2 mM Na_2SO_4 (N-C-S⁻).

6-phosphate is a specific inducer of the hexose phosphate transport system in *E. coli* (4) and might be a specific inducer of acid hexose phosphatase in *Salmonella*. However, the data in Table 4 indicate that this compound failed to induce acid hexose phosphatase when it was supplied as either a carbon or phosphorus source. Table 4 also shows that several substrates of cyclic phosphodiesterase failed to induce this enzyme when they were supplied as phosphate sources. Glycerol was used as the carbon source for these experiments to decrease catabolite repression. Because nonspecific acid phosphatase lacks substrate specificity, selection of putative inducers is not obvious. However, none of the carbon, nitrogen, or phospho-

rus sources shown in Table 3 resulted in specific induction of this enzyme.

Kinetics of derepression. The regulation of the phosphatases was also examined when exponentially growing cells exhausted their supply of a particular nutrient. Preliminary experiments indicated that maximum derepression of nonspecific acid phosphatase after depletion of glucose or phosphate occurred when N-C-P-medium was supplemented with amino acids, vitamins, and bases. This supplemented medium gave lower specific activities for all phosphatases during exponential growth than did unsupplemented media (Table 2) and also permitted greater increases in specific activity after exhaustion of the major carbon or phos-

TABLE 3. Effect of carbon, nitrogen, and phosphate sources on phosphatase and phosphodiesterase specific activities

Carbon source ^b	Nitrogen source ^b	Phosphate source ^b	Doubling time (h)	Sp act (U/mg of protein) ^a		
				Nonspecific acid phosphatase	Acid hexose phosphatase	Cyclic phosphodiesterase
Glucose	NH ₄ Cl	Sodium phosphate	1.2	8.4	42.3	31.4
Glycerol	NH ₄ Cl	Sodium phosphate	1.6	12.6	53.6	42.0
Pyruvate	NH ₄ Cl	Sodium phosphate	2.6	15.1	105	87.9
Succinate	NH ₄ Cl	Sodium phosphate	3.6	23.4	318	109
Glucose	L-Serine	Sodium phosphate	1.1	8.4	42.6	33.0
Glucose	L-Alanine	Sodium phosphate	1.9	7.9	31.8	33.0
Glucose	L-Arginine	Sodium phosphate	3.1	15.9	17.6	18.0
Glucose	L-Proline	Sodium phosphate	11.0	38.5	5.9	11.7
Glucose	NH ₄ Cl	3'-AMP	1.6	8.4	34.3	32.6
Glucose	NH ₄ Cl	Cyclic 2',3'-AMP	1.7	12.6	50.2	26.4
Glucose	NH ₄ Cl	5'-AMP	1.9	11.3	37.2	29.3
Glucose	NH ₄ Cl	β-Glycerophosphate	2.9	24.3	37.6	19.2
Glucose	NH ₄ Cl	2'-AMP	3.5	18.8	29.3	27.1
Glucose	NH ₄ Cl	RNA ^c	52	48.1	23.4	16.7

^a Enzymes were assayed after LT-2 was grown to midexponential phase.

^b N-C-P- medium was supplemented with 0.4% (wt/vol) carbon source, 10 mM nitrogen source, and 1 mM phosphate source.

^c RNA was added to make 0.15 mg/ml.

TABLE 4. Effects of putative inducers of acid hexose phosphatase and cyclic phosphodiesterase

Enzyme measured	Growth medium ^a	Addition	Sp act (U/mg of protein) ^b
Cyclic phosphodiesterase	N-C- + glycerol/NH ₄ Cl	1 mM 3'-AMP	41.0
	N-C- + glycerol/NH ₄ Cl	1 mM cyclic 2',3'-AMP	40.6
	N-C- + glycerol/NH ₄ Cl	1 mg of RNA ^c per ml	41.8
Acid hexose phosphatase	N-C- + NH ₄ Cl	0.4% glucose	36.0
	N-C- + NH ₄ Cl	0.4% glucose 6-phosphate	37.6
	N-C-P- + glycerol/NH ₄ Cl	1 mM glucose 6-phosphate	26.3
	N-C-P- + glycerol/NH ₄ Cl	1 mM sodium phosphate	42.2

^a Carbon sources were 0.4% (wt/vol); nitrogen sources at 10 mM.

^b LT-2 was grown for four generations to midexponential phase prior to assay.

^c Yeast RNA was not desalted by passage over Sephadex G-50.

phorus source. The effects of glucose depletion on acid hexose phosphatase and nonspecific acid phosphatase activities are shown in Fig. 1. At about the time the culture entered stationary phase, both enzymes were derepressed. Acid hexose phosphatase activity continued to increase for several hours and eventually reached a specific activity eightfold greater than that during exponential growth. The activity then began to decrease and continued to decrease after re-addition of glucose. Cyclic phosphodiesterase activity, which is not shown, behaved similarly to acid hexose phosphatase activity. Nonspecific acid phosphatase activity continued to increase throughout stationary phase and attained a specific activity fivefold greater than that of exponentially growing cells. When glucose was added, nonspecific acid phosphatase activity increased dramatically.

Since only nonspecific acid phosphatase increased during phosphorus limitation (Table 3), only this enzyme was measured during phosphate depletion. The results (Fig. 2) show that the specific activity of this enzyme increased 9.7-fold during the 25 h after phosphate depletion. After phosphate had been added, activity increased for 1.5 h and then declined. Nonspecific acid phosphatase was found to behave in a similar fashion when ammonium ion was exhausted from the culture and then re-added (not shown).

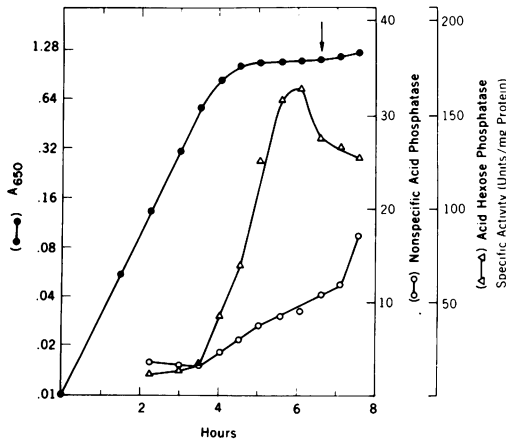


FIG. 1. Kinetics of derepression of nonspecific acid phosphatase and acid hexose phosphatase during glucose starvation. An overnight culture of LT-2 was used to inoculate 250 ml of $N-C-P^-$ medium supplemented with 0.05% glucose (limiting), 10 mM NH_4Cl , 1 mM sodium phosphate, and synthetic pools. At various times samples were removed and assayed for the phosphatase activities. Growth was monitored by measuring optical absorbance at 650 nm. At the time indicated by the arrow, 2.5 ml of 40% glucose was added to the culture.

The observation that nonspecific acid phosphatase activity increased at a dramatic rate after re-addition of the depleted nutrients suggests that its regulatory system does not respond rapidly to nutritional changes. Thus, when the starved cells resumed growth, they directed part of their increased metabolic activity to the synthesis of nonspecific acid phosphatase, even though the starvation conditions had ended.

The differential rates of expression of these enzymes during exponential growth and during stationary phase were calculated from the data of Fig. 1 and 2 and are presented in Table 5. The

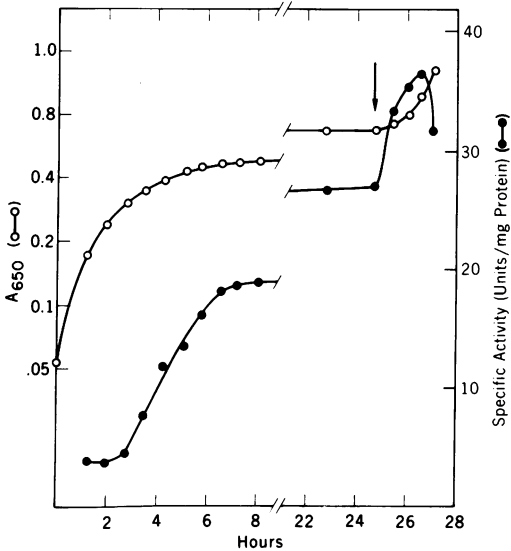


FIG. 2. Kinetics of derepression of nonspecific acid phosphatase activity during phosphate starvation. An overnight culture of LT-2 was used to inoculate 250 ml of $N-C-P^-$ medium supplemented with 0.4% glucose, 10 mM NH_4Cl , 0.075 mM sodium phosphate (limiting), and synthetic pools. At the time indicated by the arrow, 0.25 ml of 1 M sodium phosphate was added to the culture.

TABLE 5. Relative rates of phosphatase synthesis before and after nutrient deprivation^a

Limiting nutrient	Growth state	Enzyme production (U/mg of protein produced)	
		Non-specific acid phosphatase	Acid hexose phosphatase
Glucose	Nonlimited	2.9	43.5
Glucose	Starved	69.0	2,760
Phosphate	Nonlimited	2.1	
Phosphate	Starved	56.9	

^a Calculated from the data shown in Fig. 1 and 2.

high differential rates observed during stationary phase were due to the negligible increases in protein after nutrient depletion as well as to increases in enzyme activity.

Behavior of phosphatases during limited exponential growth. A technique was devised by which cultures could be grown exponentially with any desired doubling time by limiting the supply of an essential nutrient. In this technique the limiting nutrient was added to the culture at a rate that increased exponentially with a specific doubling time. Since cells in culture increase in mass and consume nutrient at an exponential rate, they grow with this doubling time.

Growth in the limited exponential growth apparatus was exponential under all starvation conditions and within 10% of the programmed doubling time. In the case of sulfur and phosphorus starvation, cells that had been extensively washed prior to inoculation were still able to grow at an initial rate that was faster than the programmed rate. This suggests that *Salmonella* contains endogenous reserves of sulfur and phosphorus that can be mobilized under starvation conditions. Similar observations have been made in the case of *E. coli* (20). Growth subsequent to utilization of endogenous reserves of sulfur or phosphorus occurred at the programmed rate. These effects were not observed under conditions of carbon or nitrogen limitation.

Specific activities of the phosphatases were examined after various times under limited growth conditions. Maximal or minimal levels of the phosphatases were obtained after two to three generations of growth at the programmed growth rate. The patterns of specific activity as functions of severity and type of nutrient deprivation (Fig. 3) confirm and extend the results from other studies. Acid hexose phosphatase and cyclic phosphodiesterase behaved as catabolite-repressible enzymes. Specific activities of these enzymes increased under conditions of carbon limitation but not in response to other types of nutrient limitations. The specific activity of nonspecific acid phosphatase increased under a variety of different nutrient deprivation conditions, although the pattern of increase was dependent on the type of nutrient deprivation. In an additional experiment, the effects of starvation for Mg^{2+} were assessed by growing *Salmonella* on $N-C-Mg^-$ medium in the limited-exponential-growth apparatus. One culture contained excess Mg^{2+} , and Mg^{2+} was added to a second culture at a rate such that a doubling time of 6 h was obtained. The nonlimited culture possessed a nonspecific acid phosphatase activity of 14.6 U/mg of protein, and

the Mg^{2+} -limited culture had a specific activity of 21.3 U/mg of protein, only a 1.5-fold increase in specific activity.

Maximal increases in specific activity of acid hexose phosphatase and nonspecific acid phosphatase observed during limited exponential

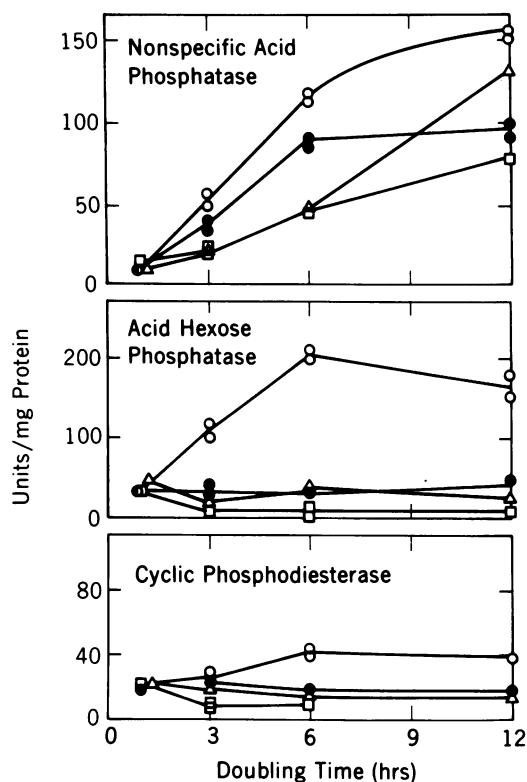


FIG. 3. Specific activities of phosphatases in cultures starved for carbon, nitrogen, sulfur, and phosphate in the limited-exponential-growth apparatus. Cultures of LT-2 were grown in the limited-exponential-growth apparatus as described in the text, with the rate of nutrient addition adjusted such that doubling times of 3.6 and 12 h were obtained. Levels of the phosphatases were also measured in cultures of LT-2 grown in the limited-exponential-growth apparatus in the same medium used for limited growth containing excess required nutrient (at least four times the amount required to reach normal stationary phase). All values of specific activities of the phosphatases represent equilibrium values of phosphatase levels, which were reached after two to three generations of growth in the apparatus. Symbols: ○, $N-C^-$ medium, supplemented with 10 mM NH_4Cl with limiting or excess amounts of glucose added; ●, $N-C^-$ medium supplemented with 0.4% glucose and limiting or excess amounts of NH_4Cl added; Δ, $N-C-P^-$ medium supplemented with 0.4% glucose, 10 mM NH_4Cl , and limiting or excess amounts of sodium phosphate added; □, $N-C-S^-$ medium supplemented with 0.4% glucose, 10 mM NH_4Cl , and limiting or excess amounts of sodium sulfate added.

growth conditions agree with results obtained in kinetics of derepression experiments. When maximal levels of nonspecific acid phosphatase under conditions of carbon and phosphorus limitation (Fig. 3) were compared with the level of nonspecific acid phosphatase of *Salmonella* grown on N⁻C⁻P⁻ medium supplemented with synthetic pools, the observed increases were 37- and 32-fold, respectively. The increase in rate of enzyme production in the kinetics experiments were 24-fold during carbon starvation and 27-fold during phosphate starvation (Table 5). Similarly, acid hexose phosphatase exhibited a 6.9-fold increase in specific activity during carbon limitation (using specific activity on N⁻C⁻P⁻ medium supplemented with synthetic pools as a basal value). The rate of acid hexose phosphatase production increased 6.3-fold under carbon starvation conditions (Table 5).

Role of cyclic AMP in regulation of phosphatase activity. One explanation for the increases in specific activity observed for the three phosphatases under conditions of carbon and energy limitation is that these enzymes are regulated by intracellular cyclic AMP levels (catabolite repression). This hypothesis was tested with mutants that are missing adenyl cyclase and unable to make cyclic AMP and with mutants that are missing the cyclic AMP receptor protein (10). Because these mutants are unable to grow on poor carbon sources, they were subjected to carbon-limiting conditions by adding glucose to cultures in the limited-exponential-growth apparatus. The frequency of reversion to wild type was monitored during the experiments by plating samples of the cultures on McConkey agar plates supplemented with

ribose and glycerol. The *cya*⁻ and *crp*⁻ mutants were unable to ferment either sugar and appeared as white colonies. Revertants to wild type appeared as red colonies, which grew faster than the parent strains. During the course of the starvation experiments, the frequency of wild-type revertants of TA3301 (*cya*⁻) rose from 10⁻⁸ to 10⁻³ after three generations of growth in the absence of cyclic AMP. When cyclic AMP was present in the growth medium, the frequency of revertants remained at about 10⁻⁸. TA3302 (*crp*⁻) revertant frequencies rose from about 10⁻⁸ to 10⁻² during three generations of growth under carbon-limited conditions regardless of the presence of cyclic AMP in the medium. These results suggest that wild-type revertants have some selective advantage under the growth conditions used. Revertants at these frequencies contribute from 1 to 10% to the measured phosphatase levels.

Results of carbon limitation experiments are shown in Table 6. Acid hexose phosphatase and cyclic phosphodiesterase levels were those predicted for enzymes sensitive to catabolite repression. The *cya*⁻ mutant had low levels of these enzymes unless cyclic AMP was present in the medium. The *crp*⁻ mutant failed to produce elevated levels of these enzymes in either the presence or absence of cyclic AMP. In contrast, nonspecific acid phosphatase was produced in high levels by both mutants in response to carbon starvation regardless of the presence or absence of exogenous cyclic AMP.

Isolation and properties of an alkaline phosphatase constitutive mutant in *Salmonella*. The strain possessing constitutive alkaline phosphatase levels, TA2365, and the iso-

TABLE 6. Phosphatase and phosphodiesterase activities of TA3301, TA3302, and LT-2^a

Strain	Culture conditions	Sp act (U/mg of protein)					
		No addition			+ 1 mM cyclic 3',5'-AMP		
		Nonspecific acid phosphatase	Acid hexose phosphatase	Cyclic phosphodiesterase	Nonspecific acid phosphatase	Acid hexose phosphatase	Cyclic phosphodiesterase
LT-2	Nonlimited	10.5	29.3	25.9	8.4	31.4	26.4
TA3301 (<i>cya</i> ⁻)	Nonlimited	16.7	10.4	13.0	8.8	16.7	15.5
TA3302 (<i>crp</i> ⁻)	Nonlimited	15.5	15.9	18.8	15.5	12.6	21.3
LT-2	Glucose limited	116	205	54.8	112	230	63.6
TA3301	Glucose limited	93.7	21.3	17.1	90.8	165	69.5
TA3302	Glucose limited	90.8	15.1	25.5	88.3	22.2	32.2

^a Glucose-limited cultures were grown on N⁻C⁻ medium + 10 mM NH₄Cl in the limited-exponential-growth apparatus with glucose added at a rate such that the cells had a doubling time of 6 h. Nonlimited cultures were grown on N⁻C⁻ medium supplemented with 0.4% glucose and 1 mM NH₄Cl in the limited-exponential-growth apparatus. Enzymes were measured after three to four generations of exponential growth. Results for TA3301 and TA3302 limited for glucose are the average of three separate experiments each. Individual values for these experiments differed by less than 10% from the average of the three experiments.

genic strain lacking the alkaline phosphatase episome, TA2366, were assayed for nonspecific acid phosphatase and alkaline phosphatase activity under conditions that should depress the specific activity of both enzymes (growth on enriched medium containing excess phosphate) and under conditions of phosphate starvation, which should elevate the specific activities of both enzymes. Results of these experiments are presented in Table 7. The results show that TA2365 does contain a mutation that results in constitutive production of alkaline phosphatase, but this mutation has not great effect on the levels on nonspecific acid phosphatase. The results obtained with TA2263 under conditions of phosphate starvation verify that alkaline phosphatase is subject to regulation in *Salmonella*.

DISCUSSION

The data of Von Hofsten (26) and Dvorak et al. (5) suggest that *E. coli* acid hexose phosphatase is regulated by catabolite repression. Our studies on the levels of acid hexose phosphatase during carbon deprivation have extended this observation to *S. typhimurium*. Definitive experiments with *cya*⁻ and *crp*⁻ mutants verified that this enzyme is controlled by cyclic AMP levels. Cyclic phosphodiesterase was also responsive to the cyclic AMP regulatory system. Control of the two enzymes by the cyclic AMP system is not dependent on the presence of exogenous inducing compounds. We were unable to demonstrate specific induction of either enzyme. This type of direct regulation by a

pleiotropic control system would be suitable for enzymes with broad substrate specificity and a general scavenging capacity. Other examples of systems regulated solely by the cyclic AMP system are the aromatic permease system (M. Alper and B. N. Ames, submitted for publication) and chloramphenicol transacetylase (3). The control of acid hexose phosphatase and cyclic phosphodiesterase by cyclic AMP levels indicates that one role of these enzymes is to supply carbon sources to the cell under conditions of limited carbon availability.

Levels of nonspecific acid phosphatase have been shown to increase in response to several types of nutrient deprivation. The extent of derepression is not strictly an inverse function of growth rate, since cultures limited for a carbon source have much higher enzyme levels than cultures limited for Mg²⁺ growing with the same doubling time.

An examination of two known regulatory systems failed to provide evidence that nonspecific acid phosphatase is regulated by either of these systems. The cyclic AMP catabolite repression system is apparently not involved, since nonspecific acid phosphatase levels of *cya*⁻ and *crp*⁻ mutants were regulated in a manner similar to wild-type cells in response to limiting carbon conditions. A mutation causing derepression of alkaline phosphatase had no significant effect on nonspecific acid phosphatase levels. However, this observation does not totally preclude a role of *Salmonella* alkaline phosphatase regulatory genes in controlling nonspecific acid phosphatase, since the complete regulatory system probably involves several genes and we have tested only one mutant. Morris et al. (16) have reported that levels of a periplasmic protein of *E. coli* increased during phosphate starvation, but increased amounts of this protein were not observed in *phosS* or *phoT* mutants, which produce alkaline phosphatase constitutively. This indicates that some *E. coli* proteins can increase during phosphate starvation conditions but are apparently not regulated by the alkaline phosphatase regulatory system.

Regulation of nonspecific acid phosphatase by several types of nutrient deprivation could be controlled by a single regulatory system capable of responding to each of the nutrient starvations or to several regulatory systems, each of which responds to a particular nutrient deprivation. Other examples are known of bacterial enzyme systems that respond to more than one type of nutrient deprivation. Bolton and Dean demonstrated increases in acid phosphatase levels in *Klebsiella* under conditions of carbon, nitrogen, potassium, magnesium, and

TABLE 7. Effect of alkaline phosphatase constitutive mutation on nonspecific acid and alkaline phosphatase levels^a

Strain	Growth state	Phosphatase sp act (U/mg of protein)	
		Non-specific acid	Alkaline
LT-2	Nonlimited ^b	4.2	<0.4
TA2366	Nonlimited ^b	4.6	<0.4
TA2363	Nonlimited ^b	3.3	1.2
TA2365	Nonlimited ^b	3.8	58.2
LT-2	Phosphate limited ^c	36.4	<0.4
TA2366	Phosphate limited ^c	46.4	<0.4
TA2363	Phosphate limited ^c	44.3	72.0
TA2365	Phosphate limited ^c	41.0	93.7

^a Cultures were grown on N-C-P⁻ medium supplemented with synthetic pools, 0.4% glucose, 10 mM NH₄Cl, and a limiting amount of phosphate (0.075 mM).

^b Samples were assayed during exponential growth.

^c Samples were assayed 4 h after growth was limited by phosphate availability.

sulfur limitation (1). The acid phosphatase activity was apparently due to more than one enzyme. Protein turnover in *E. coli* had been observed to increase during limitation for carbon, nitrogen, phosphorus, amino acids, adenine, and uracil (17, 19). It is interesting that protein turnover in *E. coli* increased during carbon starvation of a *cya*⁻ mutant (8), as we have observed for nonspecific acid phosphatase. Glycogen synthetase levels increase in *Salmonella* limited for carbon or nitrogen (S. B. Govons, Ph.D. thesis, University of California, Davis, 1970). In the eucaryotic organism *Neurospora crassa*, levels of an extracellular protease were found to increase under conditions of carbon, nitrogen, or sulfur limitation (9). In this case, regulation of a single structural gene is apparently controlled by three separate regulatory systems, each capable of responding to a particular type of limitation. It is possible that nonspecific acid phosphatase of *Salmonella* represents one of a number of enzymes whose levels are elevated in response to environmental stress conditions, and that these enzymes are regulated by an as yet uncharacterized pleiotropic system.

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