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Phosphate Inhibition of Secondary Metabolism in *Serratia marcescens*

FRANK R. WITNEY, MARK L. FAILLA,[1] AND E. D. WEINBERG*

Department of Microbiology and Program in Medical Sciences, Indiana University, Bloomington, Indiana 47401

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The synthesis of prodigiosin by non-proliferating cells of *Serratia marcescens* was examined in the presence of a wide range of concentrations of inorganic phosphate (P_i). A high elevation of pigment formation was obtained at ≤ 0.3 mM, and a broader but much lower elevation was obtained at 10 to 250 mM P_i. The synthesis of two immediate precursors of the pigment also was inhibited by P_i. The mechanism of action of P_i did not involve changes in pH or accumulation of the trace metal nutrient iron or zinc. Inhibition was most pronounced when P_i was added to the induction system before the onset of pigment formation. The inhibitor also diminished the burst of alkaline phosphatase activity that occurred in the period between the start of induction and appearance of prodigiosin.

The synthesis of secondary metabolites has long been known to have a much narrower tolerance for environmental concentrations of inorganic phosphate (P_i) than does growth of the producer cells (1, 14). The range of P_i concentration in culture media that permits good to excellent growth of either procaryotic or eucaryotic cells is approximately 0.3 to 300 mM. In contrast, in 24 systems for which sufficient data have been reported, the mean highest quantity of P_i that permitted maximal yield of secondary metabolites or of differentiated structures was 1.2 mM; the mean lowest quantity that maximally depressed yield was 10.3 mM (14).

However, in three systems in which many concentrations of P_i were examined, two elevations of secondary metabolite production were observed. In cultures of *Streptomyces jamaicensis*, monamycin was formed optimally at 0.2 mM, whereas an antibiotic of a different chemical family was synthesized at 0.6 mM P_i (5). With *Serratia marcescens*, a high elevation of prodigiosin at ≤ 0.3 mM and a broader but much lower elevation at 20 to 260 mM P_i were obtained (E. D. Weinberg, M. Beattie, B. New, C. Peterson, and R. Raelson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, E73, p. 13). Likewise, optimal synthesis of bacitracin by *Bacillus licheniformis* occurred at ≤ 0.3 mM, whereas the lower elevation was seen at 10 to 20 mM P_i (M. A. Beattie, M.S. thesis, Indiana University, Bloomington, 1973). A double elevation of bacitracin formation (at 1.5 and 75 to 150 mM P_i) was also observed independently (4).

In none of the three systems was it clear to what extent the two elevations might have resulted from possible P_i-induced events before onset of secondary metabolism. Therefore, in the present study we used the prodigiosin induction system developed by Williams et al. (15, 16), in which secondary metabolism is distinctly separated from cell growth. We observed that the two-elevation response to P_i is indeed a manifestation of events associated with secondary metabolism. In this system, we also examined possible mechanisms of action of P_i that have been proposed by various investigators (1, 14). These include (i) alteration of pH, (ii) alteration of trace metal metabolism, and (iii) inhibition of alkaline phosphatase activity.

MATERIALS AND METHODS

Organisms. *S. marcescens* Nima was obtained from our departmental culture collection, and *S. marcescens* 9-3-3, a mutant of strain Nima, was kindly supplied by R. P. Williams, Baylor College of Medicine, Houston, Tex. The mutant is unable to synthesize 2-methyl-3-amylpyrrole (MAP), the volatile moiety of prodigiosin. It does produce the nonvolatile moiety, 4-methoxy-2-2'-bipyrrole-5-carboxaldehyde (MBC).

Media and culture conditions. The organisms were grown at 30°C on nutrient agar slants enriched with 0.5% glucose. Cultures were stored at 4°C and transferred biweekly. For experiments with strain Nima, cells were grown on a shaker at 200 rpm in

[1] Present address: Department of Nutrition, Cook College, Rutgers University, New Brunswick, NJ 08903.

the following minimal medium: glycerol, 140 mM; $(NH_4)_2SO_4$, 15 mM; KH_2PO_4, 0.3 mM; $MgSO_4 \cdot 7H_2O$, 3.0 mM; sodium citrate, 1.0 mM; 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (Tris buffer), 40 mM. The medium was adjusted to pH 7.2 with 1.0 N NaOH. Cells that did not contain prodigiosin were obtained by centrifugation of cultures that had been incubated at 37°C and had attained a concentration of 5×10^8 to 6×10^8 cells per ml (late exponential phase). Cells producing prodigiosin were obtained by centrifugation of stationary-phase cultures that had been incubated at 30°C. In each case, centrifugation was carried out at 10,000 × *g* for 10 min at 4°C in a Sorvall RC-2B centrifuge; the sedimented cells were washed twice in distilled deionized water.

Induction of prodigiosin synthesis by non-proliferating cells. Cells of strain Nima that were grown at 37°C were resuspended in 50 mM Tris buffer at pH 7.2 to a concentration of 10^9 cells/ml and incubated at 30°C on a shaker at 200 rpm. To induce synthesis of prodigiosin, L-proline was added, generally to a concentration of 85 mM. Under these conditions, the cells did not proliferate (Fig. 1). In some experiments, L-proline or L-alanine was used as an inducer in a range of quantities. Potassium dihydrogen phosphate was added at amounts and times listed in Results.

Assay of prodigiosin. Cells from the induction system were harvested at various times by centrifugation at 10,000 × *g* for 10 min at 4°C. Pigment was extracted from cell pellets by shaking the latter in acidic methanol (9.0 parts methanol + 0.4 part 1.0 N HCl) for 30 min at 30°C. Debris was removed by centrifugation at 10,000 × *g* for 20 min at 4°C, and absorbance of appropriately diluted samples was measured in an Hitachi Perkin-Elmer 139 spectrophotometer at 535 nm. The quantity of prodigiosin was determined by multiplying the quotient of absorbance divided by the absorptivity value of 51 g/ml by the dilution of the sample.

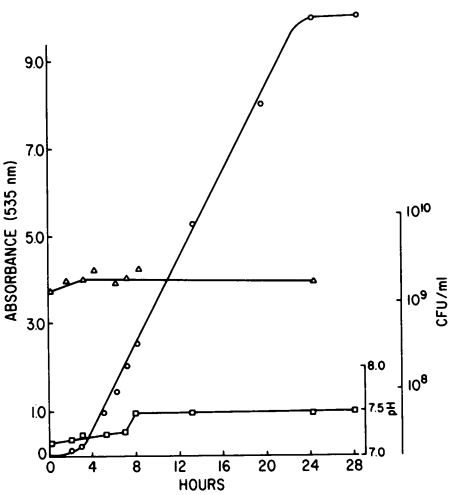

FIG. 1. *Formation of prodigiosin in the induction system. Symbols:* (△) *Viable cell number;* (○) *prodigiosin;* (□) *pH of medium. CFU, Colony-forming units. In this figure and Fig. 3 and 4, samples were diluted to obtain spectrophotometer readings of between 0.1 and 0.6. Absorbance units plotted in these figures were the product of the readings times the dilution.*

Assay of alkaline phosphatase activity, P_i, and polyphosphate. Alkaline phosphatase, a periplasmic enzyme, was released from cells of strain Nima by heat shock, and its activity was measured in terms of its ability to hydrolyze *p*-nitrophenyl phosphate (11). P_i and polyphosphate were determined, respectively, by the method of Fiske and Subbarow (3) and a modification described by Weimberg and Orton (12). Cells were boiled in water for 10 min and then removed by centrifugation at 14,000 × *g* for 10 min. Nucleotides and nucleic acids were removed from the supernatant by absorption to Norite A and subsequent clarification of the liquid phase by centrifugation at 14,000 × *g* for 10 min. The concentration of P_i was determined on a sample of the supernatant. The remaining portion of the latter was then boiled for 7 min in 1.0 N HCl, neutralized with 1.0 N NaOH, and reassayed for P_i. The pellet from the original boiled extract was resuspended in 1.0 N NaOH for 30 min at 25°C; the cells were removed by centrifugation at 14,000 × *g*. The supernatant was treated as described for the boiled extract, and the results obtained for both extracts were combined. The difference between the values obtained before and after acid hydrolysis was considered to represent the quantity of polyphosphate.

Assimilation of iron and zinc and intracellular distribution of iron. Pigmented cells of strain Nima in stationary phase were inoculated into minimal medium that had been autoclaved with 3.0% alumina, filtered, and enriched with either 1.5 μCi of ^{59}Fe or 1.0 μCi of ^{65}Zn. The cultures were incubated at 30°C. At various times, samples were removed and filtered through 0.45-μm membrane filters (Millipore Corp.), washed three times with a total of 9 ml of 50 mM EDTA (pH 7.2; 30°C), and air-dried. Radioactivity was counted in a Beckman Biogamma counter.

Site of P_i suppression of prodigiosin synthesis. Cells of the 9-3-3 and Nima strains were cultured on silica gel plus minimal medium enriched with various quantities of P_i. Because of its low background of P_i, silica gel was used in place of agar as the

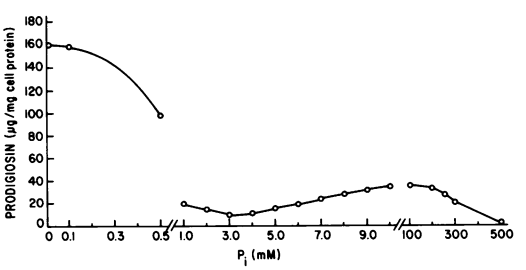

FIG. 2. *Effect of P_i added to induction system at t_0 on yield of prodigiosin at t_{24}.*

solidifying agent. Cross-feeding between the donor Nima strain (which supplied volatile MAP) and the MAP-requiring 9-3-3 strain was then permitted by fastening the inoculated plates together with tape, with the donor strain on the bottom. After 2 days at 30°C, the bacteria were removed from the plates and the amount of protein and of pigment in each strain was determined. In this system, the quantity of prodigiosin in the mutant strain is a function of the amount of volatile MAP supplied by strain Nima.

Miscellaneous procedures. Protein was determined by the method of Lowry et al. (6), cell number was determined by means of a Petroff-Hausser counting chamber, and cell viability was determined by standard pour-plate technique.

RESULTS

Effect of P_i on synthesis of prodigiosin. The kinetics of synthesis of prodigiosin in the induction system are shown in Fig. 1. Note that the cells did not proliferate in this system and that, with the buffer used, the pH remained between 7.2 and 7.5. The pH stability likewise was maintained in subsequent experiments in which various quantities of P_i were added at either the time of induction (t_0) or later.

The effect of P_i on yield of prodigiosin is shown in Fig. 2. The maximal elevation obtained between 0 and 0.5 mM and the lesser elevation between 5.0 and 300 mM were reproducible and very similar to results obtained in batch cultures in which various amounts of P_i had been included in the medium at the time of inoculation. In contrast, the growth rate and total yield of cells in batch culture were minimal at 0.1 to 0.3 mM P_i; they were somewhat depressed between 20 and 500 mM and were maximal at 1.0 to 20 mM.

The effect of inhibitory concentrations of P_i on the kinetics of prodigiosin synthesis in the induction system is illustrated in Fig. 3 and 4. P_i delayed the onset of synthesis and also depressed the rate (Fig. 3). However, if P_i was added at t_3 (i.e., 1 h after prodigiosin synthesis had begun), it caused a delay in further production but, when synthesis was resumed, only a slight depression in rate as compared with the control that lacked P_i (Fig. 4). If added $\geq t_4$, P_i had no inhibitory action. In contrast, when P_i was supplied between t_0 and t_2 (the period in which prodigiosin synthetases presumably were being formed), the kinetics and quantity of prodigiosin assembly were delayed and lowered.

Effect of P_i on alkaline phosphatase activity and on intracellular P_i and polyphosphate. Inasmuch as an increase in alkaline phosphatase activity has been observed before the onset of synthesis of such secondary metabolites as vancomycin (8) and pyocyanine (L. S. Stecher, M.S. thesis, Indiana University, Bloomington, 1974), we monitored the prodigiosin induction system for such activity. A burst of activity did indeed occur at the predicted time (Fig. 5). The activity was partially inhibited by 50 mM P_i and was completely absent when the proline inducer was withheld. The extent to which 50 mM P_i in the induction system enhanced intracellular amounts of P_i and of polyphosphate is shown in Table 1.

Effect of P_i on accumulation of iron and zinc and on intracellular distribution of iron. Inasmuch as (i) the range of iron concentrations that permits the synthesis of such bacterial secondary metabolites as prodigiosin is much narrower than that which permits cell growth

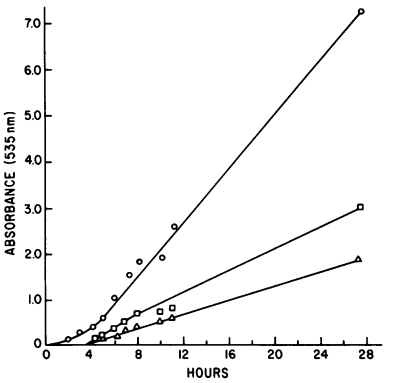

FIG. 3. *Effect of P_i added to induction system at t_0 on kinetics of prodigiosin synthesis. Symbols:* (○) *No P_i;* (△) *1.0 mM P_i;* (□) *50 mM P_i.*

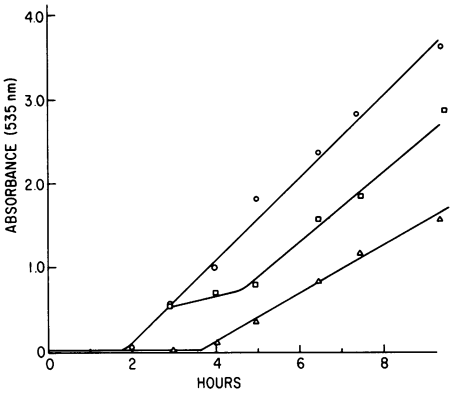

FIG. 4. *Effect of 50 mM P_i added to induction system at various times on kinetics of prodigiosin synthesis. Symbols:* (○) *P_i added at t_4;* (□) *P_i added at t_3;* (△) *P_i added at t_2. Curves for experiments in which P_i was added after t_4 or in which no P_i was added were similar to the curve for t_4. Curves for experiments in which P_i was added before t_2 were similar to the curve for t_2.*

(13) and (ii) alkaline phosphatase is a zinc-dependent enzyme (10), we monitored the effect of P_i on accumulation of each of these metals and on the intracellular distribution of iron. In the induction system, cells did not accumulate either metal. In growing cultures, the uptake systems of ^{59}Fe and of ^{65}Zn, each of which required energy, were not affected by altering the P_i content of the medium between 0.3 and 100 mM. The distribution of iron in the cytosol of cells grown in 0.3 and 50 mM P_i is shown in Fig. 6. The peak at fraction 17 was not associated with polyphosphate; the latter material was eluted in fractions 30 to 40.

Effect of P_i on synthesis of prodigiosin precursors. Results of cross-feeding experiments between the Nima and 9-3-3 strains of *S. marcescens* are listed in Table 2. P_i at 3 mM inhibited synthesis by strain Nima of prodigiosin and also, as demonstrated by the small amount of pigment formed by 9-3-3, of MAP (Table 2A). In Table 2B, it may be seen that 3.0 mM P_i partially inhibited synthesis of prodigiosin by strain 9-3-3. The data in Table 2B do not distinguish between inhibition by P_i of MBC synthesis or of the enzyme that condenses MAP and MBC to yield pigment. In preliminary experiments, we observed that condensation was not altered by 3.0 mM P_i.

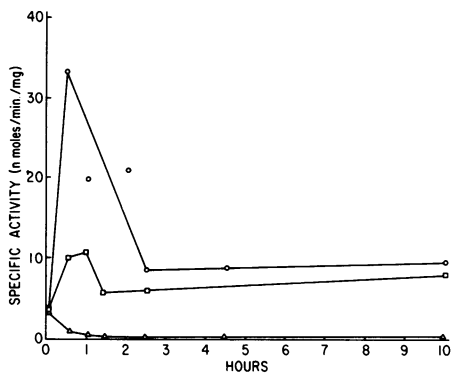

FIG. 5. *Effect of 50 mM P_i and 85 mM L-proline added at t_0 on kinetics of alkaline phosphatase activity in the induction system. Symbols:* (○) *L-Proline;* (□) *L-proline + P_i;* (△) *neither L-proline nor P_i.*

TABLE 1. *Effect of P_i on intracellular accumulation of P_i and polyphosphate*

Time after induction (h)	Intracellular content of:			
	P_i[a]		Polyphosphate[b]	
	None[c]	50 mM	None	50 mM
0	260	260	None	None
3	295	515	45	None
6	285	585	15	165
9	270	705	30	110

[a] Nanomoles per milligram of protein.
[b] Difference between P_i values before and after acid hydrolysis.
[c] P_i added to induction system at t_0.

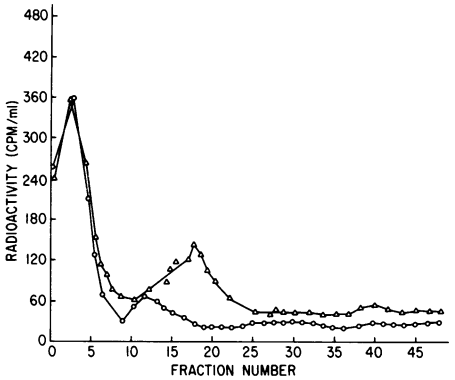

FIG. 6. *Effect of P_i in growth medium on intracytosol distribution of ^{59}Fe. Symbols:* (○) *0.3 mM P_i;* (△) *50 mM P_i. Fraction volume, 3.0 ml; V_V, 67 ml; V_T, 187 ml.*

TABLE 2. *Effect of P_i on synthesis of prodigiosin precursors*

System[a]	P_i (mM) in growth medium		Prodigiosin[b] formed by:	
	Nima	9-3-3	Nima	9-3-3
A	0.3	0.3	0.35	0.23
	3.0	0.3	0.09	0.05
B	0.3	0.3	0.35	0.34
	0.3	3.0	0.35	0.19

[a] In system A, the quantity of volatile 2-methyl-3-amylpyrrole supplied by donor strain Nima to recipient strain 9-3-3 was limited by 3.0 mM P_i; in system B, the quantity of nonvolatile 4-methoxy-2-2'-bypyrrole-5-carboxaldehyde synthesized by strain 9-3-3 was limited by 3.0 mM P_i.
[b] Absorbance (535 nm) per milligram of cell protein.

Effect of inducers on inhibitory action of P_i. Inasmuch as the inhibitory effect of P_i on ergot synthesis in *Claviceps* sp. is neutralized by the in vivo inducer (tryptophan) of that system (9), we monitored the action of P_i in the presence of various amounts of each of two inducers (proline and alanine) of the prodigiosin system. With 20 to 100 mM proline, 100 mM P_i inhibited prodigiosin formation by 75 ± 5%. With 20 to 60 mM L-alanine, P_i inhibition was 89.5 ± 1.5%. However, at 80 and 100 mM L-alanine, the inhibitory action of 100 mM P_i was lowered to 79 and 65%, respectively.

DISCUSSION

The present study confirmed that two elevated levels of prodigiosin synthesis occur as a function of P_i concentration and demonstrated that the effect is independent of events associated with cell proliferation. Moreover, the range of P_i that permitted maximal growth was approximately similar to that which depressed formation of the secondary metabolite between the two elevations. The study also established that P_i acts early in the induction period, i.e., during the presumed initial phase of synthetase formation.

The mechanism of action of P_i was found not to involve changes in pH or in accumulation of iron or zinc. High amounts of P_i apparently caused a redistribution of iron in the cytosol, as well as an increase in intracellular P_i and polyphosphate. However, the location of the latter was not altered; evidently this polymer is not acting as an iron reservoir. Additionally, this study demonstrated that the P_i block could not be alleviated by increasing the quantity of the proline inducer; however, it could be partially neutralized by high concentrations of the alanine inducer.

When P_i was added to the induction system at t_0, it had no effect on the increased rate of ribonucleic acid and protein synthesis that occurs before the appearance of prodigiosin (16) (experiments performed by F.R.W. in the laboratory of R. P. Williams). It did cause a 30-min lag in onset of oxygen uptake; the latter then proceeded at a rate identical to that in induced cultures that contained no added P_i. The observation that P_i does not inhibit ribonucleic acid and protein synthesis has also been made with candicidin-producing cells (7).

The syntheses of alkaline phosphatases are derepressed in low-P_i environments (11). Possible roles of alkaline phosphatases and of dephosphorylation have been suggested in the formation of such secondary metabolites as streptomycin, lincomycin, viomycin, vancomycin, and pyocyanine (2, 8, 14). With the latter two, alkaline phosphatase activity was observed to increase just before the onset of synthesis of the secondary material; in this study, we made the same observation with prodigiosin and also showed that the increased activity failed to occur in noninduced cells. The inhibitory action of P_i on activity of the enzyme was roughly proportional to its inhibitory effect on prodigiosin synthesis. Although no specific role for phosphatases in the formation of prodigiosin has yet been established, we suggest that a possible mechanism whereby P_i inhibits pigmentation is that of specifically interfering with the formation or activity of such enzymes.

ACKNOWLEDGMENTS

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