BBA 65634

EFFECT OF INORGANIC PHOSPHATE UPON *SALMONELLA TYPHIMURIUM* PHOSPHATASE ACTIVITIES: NON-REPRESSIBLE ALKALINE PHOSPHATASE AND NON-INHIBITED ACID PHOSPHATASE

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(Received April 21st, 1967)

SUMMARY

1. Three different phosphatase (orthophosphoric monoester phosphohydrolase) activities were found in cell-free extracts of *Salmonella typhimurium*. The criteria used for their differentiation were: pH optimum for their activity, differences in the inhibitory action of P_i and fluoride, variations in the rate of hydrolysis of several phosphorylated compounds, and their presence or absence in the cell extracts of the strains used.

2. Two of the phosphatases show maximal activity at acid pH values: 4.0 and 5.5, respectively. The third shows its peak activity at pH 9.0.

3. The pH 4.0 activity closely resembles the "acid phosphatase" (EC 3.1.3.2) of *Escherichia coli*: its presence is independent of the concentration of P_i in the medium and dependent on the carbon source used (low in carbohydrate medium and high in non-carbohydrate medium), *in vitro* it is partially inhibited both by P_i and by NaF.

4. The pH 5.5 activity is similar to the pH 4.0 activity except that it is not inhibited by P_i *in vitro*.

5. The alkaline activity differs from that of *E. coli* in being: (a) non-repressible in the presence of high concentrations of P_i in the culture medium; (b) dependent on the carbon source used for growing the cells; and (c) not stable to heating. It resembles the alkaline phosphatase (EC 3.1.3.1) activity of *E. coli* inasmuch as it is inhibited by P_i but not by NaF *in vitro*.

INTRODUCTION

Recent studies in this laboratory[1] demonstrated that cell-free extracts of *Sal-*

Abbreviations: bio⁻ nic⁻ str^r (pyk^-), phenotype of mutant strains requiring biotin, nicotinic acid, resistant to streptomycin, and, tentatively, pyruvate kinaseless.

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monella typhimurium mutants, tentatively classified as pyruvate kinaseless, manifested a rapid hydrolysis of phosphoenolpyruvate at several pH values.

This unusual activity and the fact that little is known about the phosphatase (orthophosphoric monoester phosphohydrolase) activities of *S. typhimurium*[2,3], as compared with those of *Escherichia coli*[4–13], prompted us to make the present study. The results obtained reveal several differences from those reported for the acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) of *E. coli*.

MATERIALS AND METHODS

Bacterial strains

S. typhimurium LT-2 (wild type) was kindly provided by the late Dr. M. DEMEREC. Mutant strains S-33-5 and S-14-A were obtained from the LT-2 strain by one-step selection with streptomycin and characterized as being auxotrophs, with an impairment in the utilization of carbohydrates, *i.e.*, they use only lactate or pyruvate as carbon and energy sources. These mutants have been tentatively classified as pyruvate kinaseless (pyk$^-$). A detailed biochemical and genetic characterization will be published elsewhere. The corresponding phenotypes are: *S. typhimurium* S-33-5 bio$^-$ str^r (pyk$^-$); *S. typhimurium* S-14-A bio$^-$ nic$^-$ str^r (pyk$^-$). All strains were kept on enriched lactate–agar medium.

Culture conditions

The minimal medium used was that of DAVIS AND MINGIOLI[14], citrate omitted, supplemented with biotin (25 mμg/ml) and nicotinic acid (5 μg/ml). According to the experiment carried out, either sodium lactate or glucose, both at 0.4% (w/v), were used as carbon and energy sources. The enriched lactate–agar medium contained, instead of biotin and nicotinic acid, yeast extract (Difco), 0.1% (w/v); acid-hydrolyzed casein (casamino acids, Difco), 0.1% (w/v); agar (Difco), 1.5% (w/v); and sodium lactate, 0.4% (w/v).

In order to study the derepression of the alkaline phosphatase synthesis, strain LT-2 was grown in TORRIANI's low-phosphate medium[11], supplemented with biotin and nicotinic acid at the above-mentioned concentrations.

Preparation of extracts

Cells were grown aerobically in minimal-lactate or minimal-glucose liquid media, in 2-l batches, for 18 h at 37°. Cells were harvested by centrifugation in the cold, washed once with cold distilled water, resuspended in cold distilled water and disrupted by sonication (MSE ultrasonic power unit, 0.8 A, 4–5 min). Intact cells and debris were separated by centrifugation of the sonicate for 40 min, at 4°, at 10 000 $\times$ *g*, in an IEC International centrifuge. The clear supernatant was dialyzed in the cold (4°) for 8 h against 4 l of a 1 mM solution of EDTA (pH adjusted to 7.5). The dialyzed extracts were used as such and were kept in the cold (4°). Under these conditions the phosphatase activities remained practically unchanged for about a week.

Determination of enzyme activities

Various authors[4–12,15] have used different assay conditions for the determina-

tion of microbial phosphatase activities. After several trials, the following procedures were adopted:

Acid phosphatase. The buffers used were: glycine–HCl (pH 2.5–4.0), acetic acid–sodium acetate (pH 3.6–5.5), and succinic acid–NaOH (pH 4.0–6.5). The incubation mixture contained, in 1 ml: 125 μmoles buffer; 10 μmoles *p*-nitrophenylphosphate; and extract (approx. 200–400 mg of protein). The buffer and the extract were incubated at 37° for 5 min to allow equilibration and then the *p*-nitrophenylphosphate was added. After 4 min incubation at 37°, to each ml of the incubation mixture, 4 ml of 0.1 M NaOH were added. The *p*-nitrophenol liberated was determined by reading the absorbance at 400 mμ using a Beckman DB spectrophotometer.

Alkaline phosphatase. The buffers used were: Tris–HCl (pH 6.5–9.0) and glycine–NaOH (pH 9.0–10.0). The composition of the incubation mixture, the conditions of the assay, and the determination of the *p*-nitrophenol liberated were as stated for the acid phosphatase assay.

Hydrolysis of phosphorylated compounds. The rate of hydrolysis of various phosphorylated compounds was determined at the pH optimum for each of the different phosphatase activities. The incubation conditions were as mentioned above, the substrate concentration was 10 mM of incubation mixture. The P_i liberated was determined by the method of FISKE AND SUBBAROW[16]. The corresponding controls (incubation mixture *minus* extract) were carried out with each assay.

Protein determination. Protein present in the extracts was determined according to LOWRY *et al.*[17].

RESULTS

Phosphatase activities of S. typhimurium LT-2 grown in glucose or lactate media, and glucose medium deficient in P_i

As the mutant strains do not use glucose as carbon and energy source, it was thought of interest to compare the phosphatase activities of the parental strain when grown in glucose with the activities of cells grown in lactate. The effect of variable concentrations of P_i on the derepression of the alkaline phosphatase activity was also studied. The results of these experiments are shown in Fig. 1.

There is a significant difference both in the pattern and in the specific activity of the phosphatases of cells grown in glucose and those grown in lactate (Curves A and B, Fig. 1). In the first case there is a plateau of activity between pH values of 4.0 and 8.0; below or above them the activity is very low. Cells grown in lactate present a sharp increase in their activities at all pH values and two distinct peaks appear, one at pH 4.0 and the other at pH 5.5–6.0. The alkaline activity also increases, but it does not show any isolated peak (see also Curve A, Fig. 2).

The pH 4.0 activity of the parental strain is markedly influenced by the buffer used. With acetate there is a doubling of the specific activity and a displacement of the pH optimum to 4.5 (Fig. 1, Curve A).

The phosphatase activities of cells grown in a P_i-deficient medium follow closely those present in cells grown in a P_i-rich medium (Curves A and C, Fig. 1). The phenomenon of derepression of alkaline phosphatase activity by growing the cells at a low concentration of P_i, present in *E. coli*[10–12], is not observed in *S. typhimurium* (Curve C, Fig. 1).

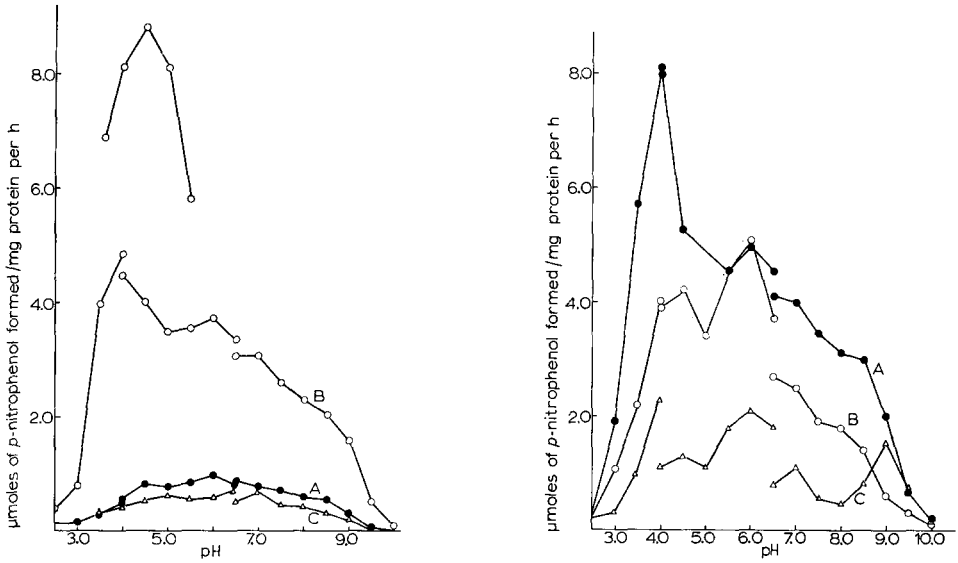


Fig. 1. Phosphatase activities (with *p*-nitrophenylphosphate as substrate) of extracts of *S. typhimurium* LT-2. A, cells grown in glucose medium; B, cells grown in lactate; C, cells grown in glucose P_i-deficient medium. Buffers used: glycine–HCl (pH 2.5–4.0), sodium acetate–acetic acid (pH 3.6–5.5), succinic acid–NaOH (pH 4.0–6.5), Tris–HCl (pH 6.5–9.0), glycine–NaOH (pH 9.0–10.0).

Fig. 2. Phosphatase activities (with *p*-nitrophenylphosphate as substrate) of extracts of lactate-grown cells of *S. typhimurium* LT-2. A, no inhibitor present; B, with P_i (87.5 mM); C, with NaF (10 mM). Buffers used as in Fig. 1.

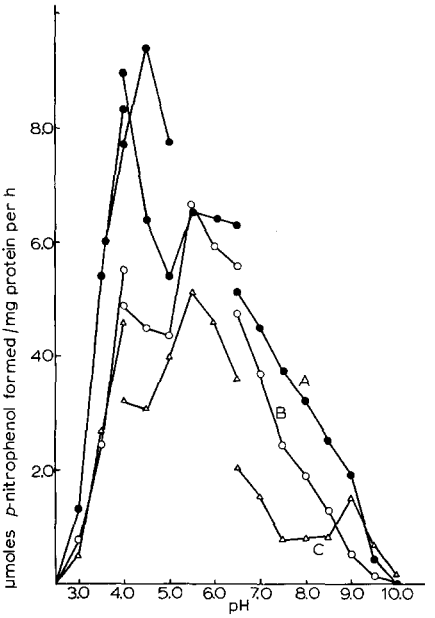
The phosphatase activities of strain S-14-A resemble very closely those of the LT-2 strain (Curve A, Fig. 3).

The extracts of strain S-33-5 show a different pattern of activities (Curve A, Fig. 4). There is no isolated peak of activity at pH 4.0; the activity increases rapidly from pH 3.0 up to a maximum at pH 5.0; after a small decrease the activity remains constant up to pH 8.0 and then it decreases sharply. The specific activities of strain S-33-5 were always greater than those of the other strains. In some experiments (not shown), they reached values four times greater than those exhibited by strains LT-2 and S-14-A.

The pH 4.0 phosphatases of both S-33-5 and S-14-A strains are not activated by acetate buffer but the shift to pH 4.5 is observed (Curve A, Figs. 3 and 4).

Inhibition by P_i

TORRIANI[11] reported that, at a concentration of 87.5 μmoles of P_i per ml, the acid (pH 4.0) and alkaline (pH 8.8) phosphatase activities of *E. coli* were inhibited approx. 60% and 100%, respectively. The inhibitory effect of this concentration of P_i on the phosphatase activities of strains LT-2, S-14-A, and S-33-5, is shown in Curve B of Figs. 2–4, respectively. In the inhibition experiments, each ml of the incubation mixture contained 87.5 μmoles of P_i buffer and 125 μmoles of the corresponding assay buffer, both of equal pH; change in the pH of the sample was thus avoided. There was no difference in the results when using either sodium or potassium phosphate.



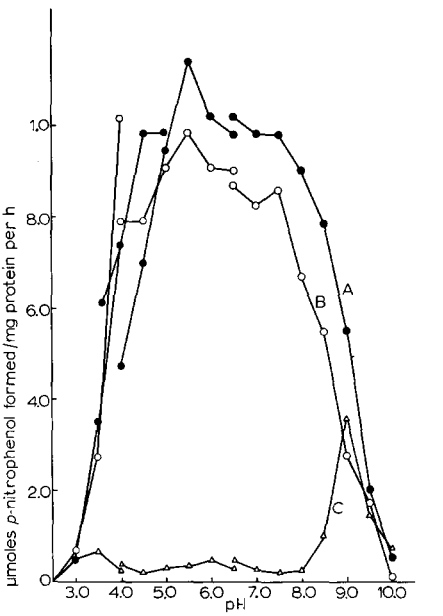


Fig. 3. Phosphatase activities (with *p*-nitrophenylphosphate as substrate) of extracts of lactate-grown cells of *S. typhimurium*, mutant strain S-14-A. A, no inhibitor present; B, with P_i; C, with NaF. Inhibitor concentrations and buffers used as in Fig. 2.

Fig. 4. Phosphatase activities (with *p*-nitrophenylphosphate as substrate) of extracts of lactate-grown cells of *S. typhimurium*, mutant strain S-33-5. A, no inhibitor present; B, with P_i; C, with NaF. Inhibitor concentrations and buffers used as in Fig. 2.

The phosphate inhibition pattern of strains LT-2 and S-14-A are similar. The activity at pH 4.0 was inhibited, in both cases, by approx. 50%; at pH 9.0 the inhibition was more marked, near 70%. The pH 5.5 activity was, however, either non-inhibited or slightly enhanced.

In the case of the phosphatases of mutant S-33-5 there is no inhibition by P_i at pH 4.0; at pH 5.5 there is a slight inhibition (near 20%); and the inhibition at pH 9.0 is close to 50%.

The phosphate inhibition of the pH 4.0 and pH 9.0 activities resembles the inhibition of the corresponding *E. coli* phosphatases[11], although it is less marked. The non-inhibition (and sometimes activation) of the pH 5.5 activity is interesting; it appears as if the reaction were independent of the concentration of P_i.

Inhibition by fluoride

It is known[11] that the acid phosphatase activity of *E. coli* is strongly inhibited by 0.01 M NaF while the alkaline activity is not affected by the same concentration of inhibitor.

The effect of 0.01 M NaF upon the phosphatase activities of the *S. typhimurium* strains LT-2, S-14-A, and S-33-5 is shown in Curve C of Figs. 2–4, respectively.

The fluoride inhibition patterns of the acid activities (pH 4.0 and 5.5) of strains LT-2 and S-14-A are very similar. Both acid activities of strain LT-2 are, however,

more strongly inhibited than those of strain S-14-A. In contrast, all the phosphatase activities of strain S-33-5, up to pH 8.0, are practically 100% inhibited by fluoride.

The alkaline phosphatase activity of the three strains is not affected by fluoride, at the concentration used. With this inhibitor it is possible to demonstrate the presence of a defined alkaline phosphatase activity between pH 8.5 and 9.5.

Stability to heating

It is known[11,12] that the alkaline phosphatase of *E. coli* is stable to heating (5 min at 100° destroys only 50% of the activity).

All the phosphatase activities in the three strains of *S. typhimurium* used were destroyed by heating the extracts for 10 min at 85°.

Specificity

Table I shows the results of the hydrolysis of different phosphorylated compounds by the phosphatases of the three strains, tested at their pH optima. In the case of the LT-2 strain, both extracts of cells grown in glucose and lactate were used. All values are given as relative reaction velocities taking, for each case, the hydrolysis of *p*-nitrophenylphosphate as unity.

The extracts of strain LT-2, from cells grown either in glucose or lactate, hydrolyze the phosphorylated compounds tested at approximately the same rate. There are, however, two exceptions: one in the hydrolysis of sugar phosphates and the other in the hydrolysis of phosphoenolpyruvate.

The level of "acid hexose-phosphatase" activity increases, both in *E. coli* and *S. typhimurium*, when the cells are grown in a non-carbohydrate medium[5,2,3]. This is found in the present study; the rate of hydrolysis of glucose 6-phosphate, ribose 5-phosphate and fructose 1,6-diphosphate increases in the extracts of lactate-grown cells, especially at pH 5.5.

The finding that the extracts of glucose-grown cells presented a greater activity in the hydrolysis of phosphoenolpyruvate than those of lactate-grown cells was rather unexpected. At present we do not know of any valid physiological reason to explain this result.

There is a close similarity in the rates of hydrolysis of the different phosphorylated compounds by the extracts of strains LT-2 (lactate-grown) and S-14-A, both at pH 4.0 and 5.5. This is no longer true for the pH 9.0 activity; in this case the S-14-A extracts failed to show any hydrolytic activity toward glucose 6-phosphate, fructose 1,6-diphosphate and phosphoenolpyruvate.

The hydrolysis of the phosphorylated compounds by the S-33-5 extracts follows a different pattern from those of the other strains. At pH 4.0 and 5.5 all the substrates are hydrolyzed at approximately the same rate. At pH 9.0 there is, in contrast to the LT-2 and S-14-A strains, an increased hexose-phosphate phosphatase activity and no hydrolysis of pyrophosphate.

It should also be noticed that in all the cases studied the highest ATPase-like activity is present in the alkaline range. In regard to this the reports about the ATPase-like activity of the alkaline phosphatase of *E. coli* are conflicting; TORRIANI[11] and GAREN AND LEVINTHAL[12] found no activity while HEPPEL, HARKNESS AND HILMOE[13] reported an elevated level.

TABLE I

HYDROLYSIS OF PHOSPHORYLATED COMPOUNDS BY THE DIFFERENT PHOSPHATASE ACTIVITIES

The reaction mixture contained 0.01 M substrate buffered by 0.125 M acetate (pH 4.0), succinate (pH 5.5), or Tris (pH 9.0) buffers. Enzyme activity was measured by the release of P_i. Values are given as relative reaction velocities taking, for each case, the hydrolysis of *p*-nitrophenylphosphate as unity.

Activity tested at pH	*4.0*				*5.5*				*9.0*			
Extract of strain	*LT-2*		*14*	*33*	*LT-2*		*14*	*33*	*LT-2*		*14*	*33*
Cells grown in / *Compounds tested as substrates*	*Glucose*	*Lactate*	*Lactate*	*Lactate*	*Glucose*	*Lactate*	*Lactate*	*Lactate*	*Glucose*	*Lactate*	*Lactate*	*Lactate*
p-Nitrophenylphosphate	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ADP	1.48	0.65	0.78	1.80	2.13	0.51	0.85	0.76	2.13	9.23	8.93	4.11
ATP	1.72	1.40	1.81	3.04	1.49	3.00	2.56	1.90	13.53	16.61	15.63	5.48
PP_i	1.23	0.86	0.78	1.82	1.92	1.52	1.28	1.90	2.13	1.85	2.23	0.00
Phosphoenolpyruvate	0.24	0.86	1.30	1.22	1.28	1.00	0.85	1.14	18.51	7.38	0.00	2.74
Glucose 6-phosphate	2.46	3.00	2.85	0.61	2.77	9.60	6.00	1.90	2.84	1.85	0.00	4.11
Ribose 5-phosphate	1.23	1.50	1.55	1.22	1.06	2.00	0.85	1.14	4.27	3.70	4.46	2.74
Fructose 1,6-diphosphate	2.71	4.30	3.88	2.43	1.49	6.06	4.27	1.14	2.84	5.54	0.00	8.23
TMP	0.49	0.20	0.26	1.22	0.85	0.50	0.43	1.52	0.71	0.00	0.00	0.00
Phosphoserine	0.24	0.20	0.26	1.22	0.64	1.00	0.43	0.38	3.56	1.85	0.00	1.37

DISCUSSION

Both in *S. typhimurium* and *E. coli* the level of the acid phosphatase activity varies with the carbon source used for growing the cells[2,3,5–7,11]. This activity is low in hexose-grown cells and high when they are grown in either acetate, succinate, or glycerol. The acid phosphatase of *E. coli* is insensitive to the phosphate concentration of the culture medium[11] and, is apparently, repressed by glucose[5]. The pH optimum for its activity is between 4.0 and 4.7, and it is markedly inhibited by fluoride and much less by P_i (ref. 11).

In this paper we report the presence, in *S. typhimurium* extracts of wild and mutant strains, of two acid phosphatase activities that in some aspects resemble, and in others differ from, the acid phosphatase of *E. coli*. One has a pH optimum of 4.0, the other of 5.5. Both show the "glucose effect" and their presence is insensitive to the P_i concentration in the culture medium. There are, however, differences in these activities: the pH 4.0 activity is inhibited *in vitro* by P_i and by fluoride, while the pH 5.5 activity is inhibited by fluoride but not by P_i.

The acid phosphatase activities of mutant S-33-5 present some marked differences from those of the LT-2 and S-14-A strains. There is no isolated peak of activity at pH 4.0 and P_i has no inhibitory effect at this pH. At the same time, the inhibition by fluoride of all the acid phosphatase activities of this strain is drastic. From the differences that its acid phosphatase activities show with respect to those of the other strains, it looks as if mutant S-33-5 has a genetic lesion, not present in mutant S-14-A, which would account for this behaviour.

With regard to the alkaline phosphatase activity, the now classical work of HORIUCHI, HORIUCHI AND MIZUNO[10], TORRIANI[11], and GAREN AND LEVINTHAL[12] demonstrated that, in *E. coli*, this activity was independent of the carbon source used for growing the cells, non-inhibited by fluoride and stable to heat. Moreover, it had a broad pH optimum for its activity (between 8.0 and 10.0), and was repressed and inhibited by P_i. Recently, however, DEALY[18], using a different strain, *E. coli* 15 TAU, reported that its alkaline phosphatase appears to be non-repressible by P_i; the activity is high even when the cells are grown in a high-P_i medium.

The alkaline phosphatase activity of the strains of *S. typhimurium* used in this work is similar to that of *E. coli* in being inhibited, *in vitro*, by P_i, but not inhibited by fluoride. It differs in not being independent of the carbon source used for growing the cells (low in glucose, high in lactate), in having a narrow pH optimum for its activity (between 8.5 and 9.5), in being destroyed by heat, and in that its formation is not repressed by P_i.

It appears that the manifestation of the repressor effect of P_i upon the synthesis of alkaline phosphatase depends on the bacterial strain tested. The phenomenon is very clear in the *E. coli* strain used by HORIUCHI, HORIUCHI AND MIZUNO[10], TORRIANI[11], and GAREN AND LEVINTHAL[12], but no repression is observed in the *E. coli* strain used by DEALY[18] nor in those of *S. typhimurium* used in the present work.

The different phosphatase activities of the strains studied manifest a rather broad specificity in the hydrolysis of several phosphorylated compounds. Probably we are dealing with several specific phosphatases that have in common the same pH optimum for their activity. This is not an improbable situation: ROGERS AND REITHEL[4], HOFSTEN AND PORATH[6], and NEU AND HEPPEL[8] have separated several

specific phosphatase activities from the "acid phosphatase" of *E. coli*. Recently, FRAENKEL AND HORECKER[9] separated the fructose-1,6-diphosphate phosphatase from the "acid phosphatase" of *E. coli*, and NYC, KADNER AND CROCKEN[19] have demonstrated the presence of two different alkaline phosphatases, both with the same pH optimum, in *Neurospora crassa*.

ACKNOWLEDGEMENTS

We thank Mr. JOSE CHAVEZ for his technical assistance.

G.C.-C. is a Predoctoral Fellow, Instituto Nacional de la Investigación Científica, México (1963–1964), and Fundación Sears Roebuck de México (1965–1966).

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