Phosphatase Synthesis in *Klebsiella (Aerobacter) aerogenes* Growing in Continuous Culture

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(Received 27 October 1971)

1. Phosphatase synthesis was studied in *Klebsiella aerogenes* grown in a wide range of continuous-culture systems. 2. Maximum acid phosphatase synthesis was associated with nutrient-limited, particularly carbohydrate-limited, growth at a relatively low rate, glucose-limited cells exhibiting the highest activity. Compared with glucose as the carbon-limiting growth material, other sugars not only altered the activity but also changed the pH-activity profile of the enzyme(s). 3. The affinity of the acid phosphatase in glucose-limited cells towards *p*-nitrophenyl phosphate (K_m 0.25–0.43 mM) was similar to that of staphylococcal acid phosphatase but was ten times greater than that of the *Escherichia coli* enzyme. 4. PO_4^{3-} -limitation derepressed alkaline phosphatase synthesis but the amounts of activity were largely independent of the carbon source used for growth. 5. The enzymes were further differentiated by the effect of adding inhibitors (F^- , PO_4^{3-}) and sugars to the reaction mixture during the assays. In particular, it was shown that adding glucose, but not other sugars, stimulated the rate of hydrolysis of *p*-nitrophenyl phosphate by the acid phosphatase in carbohydrate-limited cells at low pH values (<4.6) but inhibited it at high pH values (>4.6). Alkaline phosphatase activity was unaffected. 6. The function of phosphatases in general is discussed and possible mechanisms for the glucose effect are outlined.

Little information exists about acid phosphatase synthesis in *Klebsiella (Aerobacter) aerogenes*. Pett & Wynne (1938) first reported the presence of the enzyme in an unspecified strain, and Misawa *et al.* (1964) found a high activity in strain P14, a xanthosine-accumulating mutant. McCarthy & Hinshelwood (1959) studied acid phosphatase synthesis in *K. aerogenes* N.C.I.B. 418 growing in batch culture and concluded that the enzyme was especially required at an early stage of the growth cycle, since the enzyme activity of the cells passed through a maximum value at the beginning of the logarithmic phase and then declined rapidly. We observed that when the same strain of *K. aerogenes* was grown in chemostat culture the pH-dependence of the enzyme activity was influenced by the composition of the growth medium. In particular, there was a relationship between enzyme activity and the carbon source which suggested that the cells possessed several phosphatases whose relative activities varied according to its metabolic state. These results are reported in this paper. The cells were grown at a range of growth (dilution) rates (D) in various nutrient-limited chemostats and at the optimum rate in the turbidostat. In PO_4^{3-} -limited conditions alkaline phosphatase is also synthesized (Levinthal, 1959; Horiuchi, 1959*a,b*; Torriani, 1960). This has been investigated but in less detail. The broad-spectrum substrate *p*-nitrophenyl phosphate was used through-

out the assays and the effect of adding glucose and inhibitors to the reaction mixture evaluated.

Experimental

Organism

K. (Aerobacter) aerogenes N.C.I.B. 418 was used throughout these experiments. It was maintained by daily subculture in glucose-salts medium at 40°C under aerobic conditions, but before use in continuous-culture systems involving any other medium was always given at least 20 daily subcultures in that medium. Continuous culture was also carried out at 40°C and air at 1 litre/min passed through the culture vessel (300 ml working volume). Except where otherwise stated the pH of the culture was maintained at 7.10 ± 0.05 by means of a Pye-Ingold glass electrode connected to a Pye-Dynacap pH-meter and auto-titrator assembly. The continuous-culture apparatus, the general techniques for its operation, and the method for determining bacterial dry weight are described by Dean & Rogers (1967).

Solutions

The standard glucose-salts medium contained per litre: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.039 g; $(\text{NH}_4)_2\text{SO}_4$, 0.96 g; KH_2PO_4 , 1.14 g; Na_2HPO_4 ,

12 H₂O, 6.13 g; glucose, 5.0 g; the pH was 7.1. This medium was used for turbidostat culture, the glucose being replaced by other sugars at the same concentration when necessary. For chemostat operation the concentration of the rate-limiting nutrient in the medium was decreased to a value that gave a steady-state cell mass in the culture vessel of 0.12 mg dry wt./ml over as wide a range of dilution rates as possible. In C-limited conditions this was 400 mg/litre when sugars and related substances were the carbon sources, and 1 g/litre when intermediates of the tricarboxylic acid cycle were used. NH₄⁺-, K⁺-, Mg²⁺-, SO₄²⁻- and PO₄³⁻-limited systems contained respectively 27.3, 2.0, 0.35, 1.4 and 10.0 mg-ions/litre, the concentrations of ions other than that limiting the rate of growth being maintained at that in the standard medium. Sodium acetate-acetic acid buffer (0.2 M) was used in assays in the pH range 3.0–6.0 and tris-HCl buffer (0.05 M) for the pH range 7.0–9.0. Phosphate buffer (66 mM, pH 7.1) contained per litre: KH₂PO₄, 2.96 g; Na₂HPO₄, 12 H₂O, 16.0 g.

Enzyme assay

The method used was based on the procedure of Ohmori (1937) and Bessey *et al.* (1946). The cells were separated from the growth medium by centrifugation for 5 min at 8000g, washed by resuspension in NaCl (0.154 M), separated again and finally resuspended in the appropriate buffer at a cell density equivalent to 0.18–0.24 mg dry wt./ml. A 10 ml cell suspension was added to a T-tube immersed in a thermostatically controlled water bath maintained at 30°C and fitted with a shaker arm, and when thermal equilibrium had been reached the reaction was started by adding 2 ml of an aqueous solution of disodium *p*-nitrophenyl phosphate (12 mg/ml). At intervals 2 ml samples were withdrawn, added to 4 ml of NaOH (0.2 M) and the extinction was read at 400–410 nm in a Unicam SP.600 spectrophotometer against a blank withdrawn at zero time. Phosphatase specific activity was determined from the initial rate of the reaction and expressed as nmol of *p*-nitrophenol liberated/min per mg dry wt. of cells. When necessary, the cells were disrupted by ultrasonic treatment at 0°C in an MSE-Mullard ultrasonic disintegrator or by shaking in buffer saturated with toluene at 30°C for various times (see the text).

Results and Discussion

Phosphatase activities in carbon-limited conditions

Preliminary experiments showed that the cells in 24 h glucose-limited batch cultures possessed appreciable acid (pH 4.6) phosphatase activity. On inoculation into a fresh supply of the same medium the

activity decreased rapidly as growth proceeded, reaching a minimum value late in the exponential phase of the growth cycle and then increased again as glucose exhaustion began to terminate growth. This determined the choice of carbon-limited chemostat conditions as the best method for studying the effect of using different carbon sources in the growth medium on the enzyme activity. Accordingly, the phosphatase specific activities of cells that had reached the steady state of growth at $D = 0.4 \text{ h}^{-1}$ in various carbon-limited chemostats, containing an excess of PO₄³⁻ and maintained at pH 7.1, were assayed at a range of pH values. Cells utilizing acetate, fumarate, malate or succinate had negligible activity at any assay pH, whereas those utilizing sugars and related substances possessed appreciable phosphomonoesterase activity. The results obtained with substances in the latter category are shown in

Fig. 1. pH profiles of the phosphatase activity of cells grown at $D = 0.4 \text{ h}^{-1}$ in chemostats on various carbon sources

The following sole carbon sources were used: ○, glucose; ●, sucrose; ◐, maltose; △, lactose; ▲, glycerol; □, cellobiose; ■, melibiose. (a) and (b), C-limited conditions; (c), SO₄²⁻-limited conditions. The substrate was *p*-nitrophenyl phosphate.

Fig. 1. Glucose-grown cells had the highest activity, sucrose and lactose-grown cells being about 75% as active and maltose-grown cells slightly less. Glycerol, melibiose and cellobiose-grown cells had still lower activities and, in addition, the optimum was shifted from pH 4.6 to 5.6. Fig. 1 also shows that changing the carbon source in the medium affects the pH-activity relationships of the enzyme(s). These ranged from relatively small changes in activity over a fairly wide pH range as seen, for example, in the behaviour of lactose-grown cells to the marked decline in activity shown by maltose and sucrose-grown cells as the assay pH was moved from the optimum.

Other experiments in which assays were carried out at pH 4.6 and 7.1 only showed that cells grown on glucose 6-phosphate and fructose have activities identical with glucose-grown cells (i.e. 100 units at pH 4.6 and 49 units at pH 7.1), whereas those of mannitol- and raffinose-grown cells were 68 and 57 units respectively at pH 4.6 and 30 and 27 units at pH 7.1. In sharp contrast the specific activity of 82 units found in gluconate-grown cells when assayed at pH 7.1 was 50% higher than that at pH 4.6.

Preliminary experiments also determined the choice of $D = 0.4 \text{ h}^{-1}$. In general, the steady-state acid (pH 4.6) phosphatase specific activity increased steadily as D was progressively decreased below 1.0 h^{-1} . In sucrose-limited conditions this increase continued until $D = 0.1 \text{ h}^{-1}$, the lowest rate used, was reached but in glucose-, lactose- and maltose-limited systems maximum values occurred at $D = 0.4 \text{ h}^{-1}$. Other carbon-limited systems were not investigated, but on the evidence available $D = 0.4 \text{ h}^{-1}$ seemed a reasonable compromise.

More detailed studies with some of these carbon sources, particularly glucose, are reported below. In them assays were carried out at pH 4.6 and 7.1 only, since from the results already reported this seemed a sufficient characterization of the pH-activity profiles.

Nutrient-limitation other than C-limitation

Table 1 shows the steady-state phosphatase specific activity in cells grown at $D = 0.4$, 0.7 and 1.0 h^{-1} in NH_4^+ -, K^+ -, Mg^{2+} - and SO_4^{2-} -limited chemostats containing glucose as the sole carbon source. (PO_4^{3-} -limitation is dealt with separately in connexion with alkaline phosphatase synthesis.) As in C-limitation decreasing D again increased the optimum (pH 4.6) activity, but the values varied from one nutrient-limited system to another, the order $\text{C} > \text{NH}_4^+ > \text{K}^+ > \text{Mg}^{2+} > \text{SO}_4^{2-}$ being preserved at $D = 0.4$ and 0.7 h^{-1} but not at 1.0 h^{-1} . Nor was this order observed in the activities at pH 7.1 of cells grown at any dilution rate, which implies that changing the rate-limiting nutrient in the medium also changes the pH-activity profile of the phosphatase. Essentially similar results were obtained when carbon sources other than glucose were used. For example, the steady-state phosphatase specific activities at pH 4.6 in cells utilizing maltose, sucrose, lactose or glycerol at $D = 0.4 \text{ h}^{-1}$ were 35, 53, 33 and 4 units respectively in NH_4^+ -limited conditions, compared with 69, 77, 72 and 57 units respectively in C-limitation. The very low activity of glycerol-utilizing cells when grown in NH_4^+ -limited conditions is particularly striking, and when assayed at pH 7.1 they exhibited no phosphatase activity at all.

Changing the rate-limiting nutrient in a glycerol-salts medium from glycerol to Mg^{2+} changed the composition of the cell wall of *K. aerogenes* growing in chemostat culture (Ellwood & Tempest, 1967). Such effects might conceivably alter the rate at which *p*-nitrophenyl phosphate comes into contact with the phosphatase(s) in the cells grown in the various nutrient-limited conditions described above. However, cellular disruption, particularly by ultrasonication, would be expected to unmask cryptic effects of this sort, and it will be shown later that, irrespective of their source, the percentage changes in the activity of intact cells brought about by disruption were very

Table 1. Effect of dilution rate (D) on acid (pH 4.6) and neutral (pH 7.1) phosphatase specific activities of intact cells

Glucose was used as the sole carbon source throughout and the cells were grown in chemostat culture in the given nutrient-limited conditions until steady-state conditions were achieved.

$D \text{ (h}^{-1}\text{)}$	Assay pH	Limiting nutrient ...	Phosphatase specific activity				
			C	NH_4^+	K^+	Mg^{2+}	SO_4^{2-}
0.4	4.6		100	88	75	60	38
	7.1		50	14	46	52	58
0.7	4.6		76	69	54	43	30
	7.1		30	19	38	39	55
1.0	4.6		35	47	36	31	26
	7.1		11	15	21	22	35

similar. On the other hand, a possible connexion between cell-wall synthesis and phosphatase activity emerges from the finding (Ellwood & Tempest, 1967) that, like the activity at pH 4.6, the wall content in both the C- and Mg^{2+} -limited cells increased progressively as the dilution rate was decreased. Marked dissimilarities were, however, observed in the quantitative changes (with D) in the total phosphorus and carbohydrate contents of the walls from the two types of organisms.

Table 1 also shows that increasing the assay pH increased the phosphatase activity only of SO_4^{2-} -limited cells, an effect that was also more pronounced at low dilution rates. Gluconate-limited cells have been shown above to behave similarly, but further investigation revealed that in SO_4^{2-} -limited conditions (glucose as C-source) the behaviour was more complex than a mere change in the pH optimum, since the pH-activity profile had two maxima, one at pH 4.6 and the other at pH 7.1 (Fig. 1c). Aryl-sulphatase activity has been detected in *K. aerogenes* (Rammner *et al.*, 1964a,b). Decreasing the SO_4^{2-} content of the medium derepresses the enzyme, and although purified aryl sulphatase preparations show little activity towards aryl phosphates, arylesterases can hydrolyse *p*-nitrophenyl phosphate (Augustinson, 1961). The participation of a non-specific sulphatase in the apparent high phosphatase activity of SO_4^{2-} -limited cells at pH 7.1 cannot therefore be discounted.

Growing *K. aerogenes* in turbidostat conditions should eliminate these varied consequences of nutrient-limitation, since all the ingredients in the medium are present in excess of requirement. This has been confirmed in systems containing glucose, maltose, sucrose, lactose or gluconate as the sole carbon source. The acid (pH 4.6) and neutral (pH 7.1) phosphatase specific activities remained constant at 26 ± 1 and 5 ± 1 units respectively, which shows that any influence of the carbon source, as observed in chemostat culture, has also been eliminated. The growth rates, which were the maximum possible, ranged from 1.13 to $1.24 h^{-1}$ and were higher than those used in the corresponding chemostats, and the enzyme activities were lower.

Clearly, acid phosphatase synthesis in *K. aerogenes* varies with the growth rate and the composition of the growth medium, the highest activity occurring in carbohydrate- (particularly glucose-) limited conditions. Hofsten & Porath (1961) observed a similar growth-rate effect with *Escherichia coli*, but in sharp contrast with our results obtained marked enzyme synthesis in succinate-limited conditions. On the other hand, Englesberg & Watson (1961) found that the high acid phosphatase activity of 'glucose-resistant' mutants of *Salmonella typhimurium* was insensitive to changes in the growth medium or in the physiological state of the cells. Apparently the

regulation of the enzyme varies from organism to organism, which is further borne out by the finding that even various sub-strains of *E. coli* displayed large differences in phosphatase-synthesizing ability (Neu & Heppel, 1965).

The underlying control mechanisms are also obscure. The role of catabolite repression, in the usual sense of the term, is difficult to assess. Since acid phosphatase is not (apparently) a catabolic enzyme, catabolite repression may not be as important as, for example, in the case of β -galactosidase (Mandelstam, 1962; Silver & Mateles, 1969) or amidase (Clarke *et al.*, 1968) synthesis. Admittedly, in the chemostat systems, an excess of carbon source, as occurs in all nutrient-limited systems other than C-limitation, depressed the phosphatase activity, but glucose was less effective than other sugars and glycerol was very effective indeed. The pH-activity profiles introduce further complications. Their narrowness in some examples suggests the presence of a single enzyme, but the appreciable activity occurring over a relatively wide pH range in others is more characteristic of a range of enzymes. Multiple forms of acid phosphatases of animal origin have been reported (Moore & Angeletti, 1961). For example, that in rat liver has been separated into three fractions, which differed in their affinity for *p*-nitrophenyl phosphate, pH optima and degree of inhibition by fluoride (see below). Moreover, in *E. coli* the term non-specific acid phosphatase is thought to describe a number of enzymes (Rogers & Reithel, 1960).

Non-steady states

These occurred during the transition between batch culture and the steady state of continuous culture and persisted for about ten generations of growth in the turbidostat and in C- and NH_4^+ -limited chemostats, compared with about 15 generations in the other nutrient-limited systems. The acid (pH 4.6) and neutral (pH 7.1) phosphatase specific activities of the cells changed steadily throughout and without oscillation to the new values, and once steady-state conditions had been achieved at one dilution rate further changes in D were accommodated rapidly.

Effect of cell disruption

Ultrasonication or toluene treatment, which were equally effective, increased the acid phosphatase specific activity of C-limited cells suspended in 0.05M-tris-HCl buffer, pH 7.1, by 10–30% (Fig. 2). In contrast, treatment at pH 4.6 led to a rapid fall in activity. Cells grown in other nutrient-limited conditions behaved similarly. For example, after disruption at pH 4.6, 5.6, 6.4 and 7.1 the specific activities of NH_4^+ -limited cells (glucose as C-source)

Fig. 2. Effect of cellular disruption on phosphatase specific activity

C-limited cells grown at $D = 0.4 \text{ h}^{-1}$ were washed (see the Experimental section) and then disrupted in acetate buffer (pH 4.6) or tris buffer (pH 7.1) by ultrasonication or toluene treatment. ● and ▲, Glucose-limited cells ultrasonicated at pH 7.1 and 4.6 respectively; ○, gluconate-limited cells ultrasonicated at pH 7.1; △, maltose- and □, lactose-limited cells treated with toluene at pH 4.6.

were 37, 70, 75 and 117% respectively of the value in intact cells.

Compared with other enzymes, such as β -galactosidase, the increase in activity after disruption at neutral pH is small, suggesting that no well-defined permeability barrier separates enzyme and substrate and that the acid phosphatase is located at or near to the cell surface. Studies with *E. coli* (Torriani, 1960; Hofsten & Porath, 1961; Neu & Heppel, 1964, 1965; Nossal & Heppel, 1966), *Neisseria meningitidis* (Lee & Sowokinos, 1967) and some strains of *Staphylococcus aureus* (Malveaux & San Clemente, 1969a,b) led to similar conclusions.

Enzyme-substrate affinity

Michaelis-Menten kinetics were obeyed when intact cells that had reached the steady state of growth at $D = 0.4 \text{ h}^{-1}$ in a glucose-limited chemostat were assayed at various pH values and substrate concentrations (Fig. 3). The K_m values were not significantly affected by pH changes in the range in

Fig. 3. Effect of substrate concentration on the rate of *p*-nitrophenyl phosphate hydrolysis

The following pH values were used: △, pH 3.6; ▲, pH 4.6; ●, pH 5.6; ○, pH 7.1. s is the *p*-nitrophenyl phosphate concentration (mM) and v is the phosphatase specific activity. The K_m values at pH 3.6, 4.6, 5.6 and 7.1 are 0.29, 0.38, 0.25 and 0.43 mM respectively.

which the hydrolysis rate (activity) varies considerably (compare Fig. 1a), showing that the decline in activity with increasing assay pH is not due to large changes in enzyme-substrate affinity. Neu & Heppel (1964) using *E. coli*, and Barnes & Morris (1957) and Malveaux & San Clemente (1969a,b) using *S. aureus*, showed that K_m values measured with intact cells and cell-free extracts are the same. Our results are about one-tenth of those obtained with *E. coli* but of the same order as those found for the staphylococcal enzyme (0.2–0.4 mM).

Effect of adding inhibitors during the assay

Glucose-limited cells in the steady state of growth were again used, since they display appreciable activity over a wide pH range (Fig. 1a).

Fluoride. Inhibition by F^- appears to be a common property of acid phosphatases whether of microbial or animal origin (Torriani, 1960). Addition of sodium fluoride to the assay mixture immediately inhibited *p*-nitrophenyl phosphate hydrolysis by our strain. Disrupting the cells or preincubating intact cells with F^- for periods up to 10 min before adding the substrate did not increase the effect, which shows

Fig. 4. Effect of adding F^- or PO_4^{3-} during the assay of glucose-limited cells grown at $D = 0.4h^{-1}$ in medium containing excess (25mM) of orthophosphate (a) $\circ$, 10mM- F^- ; $\bullet$, 50mM- F^- . (b) $\circ$, Control; $\bullet$, 25mM- PO_4^{3-} .

that the anion has immediate access to the enzyme sites and that it does not block the uptake of substrate by intact cells. However, the degree of inhibition varied with the assay pH, being greatest at pH 7–8 and least between pH 4.5 and 5.6. The results obtained with 10 and 50mM-sodium fluoride are shown in Fig. 4. Up to pH 7.0 the higher concentration was less active but at higher pH values this anomalous effect disappeared. Compared with the acid phosphatase of *E. coli* the enzyme(s) in *K. aerogenes* are less susceptible. At pH 4.7 Torriani (1960) observed 85% inhibition, by 10mM F^- , compared with our value of 40–50%.

It is tempting to infer the existence of two regions of phosphatase activity, an acid phosphatase with low sensitivity to fluoride and a neutral phosphatase three or four times more susceptible. In this respect there is some similarity to staphylococcal phosphatase (Tirunarayanan, 1968). Reiner *et al.* (1955) envisage a competition between fluoride or polymers of fluoride, substrate and protective ions for cationic sites on the enzyme. This could account for our results, since the equilibrium established between the three species will depend on pH and concentration, and in acid solution the enzyme may be protected

from fluoride or the active inhibiting species may be present in low concentration. However, the exact mechanism is not clear.

The pattern of inhibition observed with glucose-limited cells was generally true for cells grown in other carbohydrate-limited systems. Gluconate-limited cells were an exception; 50mM-sodium fluoride increased the phosphatase specific activity by 50% at pH 7.1. That gluconate-limited cells showed maximum 'acid' phosphatase activity at pH 7.1 rather than at acid pH values has been pointed out above.

Phosphate. The growth medium contains P_i in excess of requirement. Addition of the same concentration (25mM) during the assay decreased the phosphatase specific activity of intact cells by 25–30% (Fig. 4) but unlike fluoride inhibition the effect was not influenced by pH or increased at higher concentrations. Indeed, the activity of cells suspended in 66mM-phosphate buffer, pH 7.1, was 70% of that obtained with 50mM-tris-HCl buffer at the same pH.

Formaldehyde. Formaldehyde strongly inhibits permeases (Koch, 1963, 1964). Its presence at 0.13M concentration in the assay mixture had no effect on the rate of hydrolysis of *p*-nitrophenyl phosphate by intact cells, reinforcing our other evidence that substrate transport is not a rate-limiting step.

Glucose stimulation and inhibition of *p*-nitrophenyl phosphate hydrolysis

Addition of glucose to the reaction mixture stimulated the hydrolysis of *p*-nitrophenyl phosphate at low pH (<4.6) by intact glucose-limited cells, but inhibited it at higher pH (>4.6). The effects are conveniently discussed separately.

Stimulation. Increasing the glucose concentration increased the degree of stimulation (Fig. 5). The effect appears to be specific for glucose. 2-Deoxy-glucose and α -methylglucoside were about one-sixth as active as glucose; fructose, galactose, mannitol and glycerol were inactive, and glucose 6-phosphate and gluconate were slightly inhibiting. The specific activities of the phosphatase of cells grown in other carbohydrate-limited chemostats were similarly stimulated by glucose (Table 2) but the carbon sources used for growth were irrelevant or produced only a small response (~10%) even when they contained a glucose residue. The simultaneous addition of 50mM-sodium fluoride abolished glucose stimulation.

Inhibition. In contrast with stimulation, maximum inhibition occurred at the low value of 3mM-glucose (Fig. 5). As in stimulatory conditions, however, sugars other than glucose had a negligible effect. For example, 33mM-galactose decreased the activity of glucose-grown cells by only 8%, compared with the

Fig. 5. Glucose stimulation and inhibition of *p*-nitrophenyl phosphate hydrolysis at various assay pH values
○, pH3.6; ●, pH4.6; △, pH5.6; ▲, pH7.1.

Table 2. Effect of adding sugars to the reaction mixture during the phosphatase assay of intact cells

The cells were grown in C-limited chemostats at $D = 0.4\text{h}^{-1}$ until steady-state conditions prevailed. Enzyme activity is expressed as a percentage of the control value (no additions). The additions were made at 11 mM.

Carbon source in growth medium	Relative enzyme activity after the addition	
	Glucose	Growth substrate
Glucose	145	145
Maltose	163	114
Lactose	150	100
Sucrose	165	105
Cellobiose	200	100
Glycerol	160	110

50% decrease brought about by 3 mM-glucose, and 33 mM-fructose had no action at all. Again cells grown in other carbohydrate-limited chemostats were also susceptible, the addition of 3 mM-glucose decreasing the activity of glycerol-, lactose- and cellobiose-limited cells by about 40% at pH5.6 and by about 50% at pH7.1. Maltose- and sucrose-limited cells behaved similarly at pH5.6 but could not be tested at pH7.1, since their activity is so low at that pH in

normal circumstances (Fig. 1a). Gluconate-limited cells again behaved atypically, 11 mM-glucose decreasing their (maximum) phosphatase specific activity by only 10%, compared with the 50% decrease generally observed.

Stimulation and inhibition were immediate responses. Disruption of the cells or preincubation with glucose for periods of up to 1 h before adding the enzyme substrate did not increase the effects. Moreover, they were characteristic of carbohydrate-limited cells. Addition of glucose during the assays on cells that had utilized any of the carbon sources mentioned above in nutrient-limited conditions other than C-limitation had no effect, even when the test procedure included preincubation or disruption.

Possible mechanisms of glucose stimulation and inhibition

The marked specificity of glucose in these responses might be a consequence of the previous history of the cells. Apart from short periods in nutrient broth the strain has been maintained as a routine over the last 30 years by daily subculture in glucose-salts medium so that a modified (constitutive) glucose metabolism may have been impressed on the cells.

In principle, stimulation of *p*-nitrophenyl phosphate hydrolysis could result from a direct interaction between enzyme, glucose and *p*-nitrophenyl phosphate, or the situation may be more complex and involve other metabolic pathways. The immediate nature of the effect and the irrelevance of preincubation procedures, which show that a metabolic transformation of glucose is not a necessary prerequisite, argue against the latter. Moreover, marked stimulation occurred at pH3.6 and it is unlikely that any of the glucose-metabolizing enzymes in *K. aerogenes* are active at this low growth-inhibiting value. Indeed in *E. coli* glucose phosphate isomerase does not act below pH5.0 (Klungsoyr & Endressen, 1964).

Scheme 1 shows two possible mechanisms. In normal circumstances *p*-nitrophenol is liberated from an enzyme-substrate complex, a process in which a phosphoenzyme complex may be a distinct or a transient species. In model 1 the direct binding of glucose to the enzyme is assumed, the active species being a glucosyl-enzyme, and the subsequent breakdown of the glucosyl-enzyme-substrate complex to yield *p*-nitrophenol is faster than that of the normal complex. The intermediate, glucosyl-enzyme-phosphate, may regenerate free enzyme by yielding hexose phosphate. According to model 2 direct binding of glucose to free enzyme does not occur. Glucose acts at the level of enzyme-substrate or 'phosphoenzyme' complex, accelerating the breakdown of the complex in the former case and assisting in the regeneration of free enzyme in the latter. This involves glucose acting as a phosphate acceptor and

(a) Normal mechanism of substrate hydrolysis

(b) Glucose addition. Model 1

(c) Glucose addition. Model 2

Scheme 1. Possible schemes for glucose stimulation of acid phosphatase

Nph-P, *p*-nitrophenyl phosphate; enz, free enzyme (phosphatase); (Nph-P-enz), enzyme-substrate complex; (P-enz), phosphoenzyme intermediate; Glc, glucose; (Glc-enz), glucosyl-enzyme complex; (Glc-enz-P-Nph), glucosyl-enzyme-substrate complex; Glc-P, glucose 1-(or 6-)phosphate; P_i, inorganic phosphate.

the overall process may be considered as a transfer of phosphate from *p*-nitrophenyl phosphate to glucose mediated by a phosphatase possessing phosphotransferase activity.

Although the present evidence does not permit a definite choice between the two models some pertinent comments can be made. Phenyl phosphates are effective phosphate donors in several enzyme-catalysed reactions: in the formation of nucleotides (Chargaff & Tunis, 1956), in the phosphorylation of numerous biological alcohols by human acid phosphatase (Meyerhof & Green, 1952) and in the formation of glucose 6-phosphate by a similar acid phosphatase preparation (Vescia, 1959). However, phosphatase-catalysed phosphotransferase reactions generally require high concentrations (1–2M) of non-specific hydroxylic acceptors (Morton, 1953; Nordlie & Arion, 1964; Fujimoto *et al.*, 1965). Our effect is unusual in its pH-dependence and in the low concentrations of glucose necessary to promote it, and on the whole the formation of a glucosyl-enzyme complex, as in model 1, is more compatible with the high specificity of the process than the alternative mechanism of model 2. Doudoroff (1961) postulated a similar complex to explain the function of disaccharide phosphorylases in the metabolism of sucrose, maltose and cellobiose in *Pseudomonas saccharophila* in terms of a transfer of the glucosyl moiety to phosphate to yield α - or β -glucose 1-

phosphate. Moreover, a direct binding of glucose to enzyme can also account for the inhibition of glucose stimulation of phosphatase activity on addition of fluoride if, as postulated for human prostate acid phosphatase (Vescia & Chance, 1958), distinct and separate binding sites exist for water (or acceptor) and glucose. The binding of fluoride to both, but a more effective competition for the glucose sites, could lead to a complete blockage in the uptake of glucose but not of water, resulting in the abolition of glucose stimulation but only a decrease in phosphatase activity. This has been observed.

The change from stimulation to inhibition by glucose as the assay pH is increased can also be explained in terms of models 1 and 2 if a change in the binding sites or in the rate-limiting step is assumed. The involvement of products of other enzyme reactions is more feasible than in stimulatory conditions, since marked inhibition occurred at pH 7.1. However, the rapidity of the process and the irrelevance of preincubation procedures again favour a direct interaction between enzyme, glucose and *p*-nitrophenyl phosphate. Moreover, fructose, which is oxidized as rapidly as glucose by *K. aerogenes* (Dean & Rodgers, 1969), was inactive and gluconate, the first oxidation product of glucose, was only slightly inhibitory.

Phosphatase synthesis in PO₄³⁻-limitation

As in other bacteria, in general, PO₄³⁻-limitation depressed alkaline phosphatase synthesis in *K. aerogenes*, the maximum activity now occurring at assay pH values between 8 and 9 (Table 3), and the activities at acid pH values were generally lower than in C-limited conditions (cf. Fig. 1). Moreover, in contrast to acid phosphatase(s), the alkaline phosphatase content of the cells was largely independent of the carbon source in the growth medium. This decline in acid phosphatase activity after the change-over from C- to PO₄³⁻-limitation contrasts with the behaviour observed in *E. coli* (Torriani, 1960) and *Saccharomyces cerevisiae* (Schurr & Yagil, 1971). In the former the activity approximately doubled and in the latter increased about 12-fold when glucose was the carbon source, and these changes were accompanied by increases of about 25–30-fold and threefold in the alkaline phosphatase activities of *E. coli* and *S. cerevisiae* respectively, compared with an increase of about fourfold in *K. aerogenes*.

Decreasing the pH of the culture medium did not lead to an increase in the activity assayed at pH > 8.0, as might be expected if the acid environment impaired the efficiency of the alkaline phosphatase, and more enzyme was synthesized as a compensatory adjustment. Instead there was a shift in the maximum activity to lower pH values, which suggests the preferential synthesis of different phosphatases,

Table 3. *Phosphatase specific activities of intact PO₄³⁻-limited cells assayed at various pH values*

The cells were grown in PO₄³⁻-limited chemostat culture at $D = 0.4\text{h}^{-1}$ until steady-state conditions were achieved.

Carbon source in growth medium	Growth pH	Phosphatase activity at the assay pH					
		3.6	4.5	5.6	7.1	8.0	8.8
Glucose	5.0	130	164	188	130	80	59
	6.0	61	57	53	74	57	55
	7.1	37	29	29	74	94	103
Glycerol	7.1	16	13	20	94	123	107
Acetate*	7.1	16	13	25	131	147	140
Malate	7.1	45	37	33	82	98	92
Lactose	7.1	16	25	25	86	94	94

* $D = 0.2\text{h}^{-1}$.

active at low pH, in response to the changed growth conditions. At pH 5.0 these were becoming very unfavourable, 'clumping' was often evident, and any further decrease in pH led to the dilution out of the culture at the growth rate (0.4h^{-1}) used.

The synthesis of alkaline phosphatase when acetate or malate was the carbon source is particularly noteworthy, since PO₄³⁻-limitation was the only condition in which cells utilizing these compounds exhibited appreciable activity at all. The activity at low assay pH values probably represents some residual activity of the phosphate-repressible enzyme rather than the synthesis of a defined acid phosphatase.

Disrupting PO₄³⁻-limited cells (glucose as C-source for growth) by toluene treatment for 10 min at pH 8.0 increased their activity at pH 8.0 by 85%, which contrasts with the small increase observed when glucose-limited cells were disrupted at neutral pH values (Fig. 2). Addition of glucose or inhibitors revealed further differences. Glucose (11 mM) had no effect, and 20 mM-sodium fluoride, which had inhibited acid phosphatase considerably at pH 8.0 (Fig. 4), stimulated the rate of *p*-nitrophenyl phosphate hydrolysis by alkaline phosphatase by 40% (cf. Heppel *et al.*, 1962). On the other hand 25 mM-PO₄³⁻ decreased the phosphatase activity of PO₄³⁻-limited cells by 80%, which is three to four times greater than the inhibition observed with acid phosphatase. Further, cells rich in alkaline phosphatase readily utilized β -glycerophosphate as sole phosphorus source, but when the enzyme was repressed by excess of PO₄³⁻ in the growth medium, as in glucose-limited conditions, glycerophosphate could not be utilized as the sole carbon source even though the acid phosphatase content of the cells was high. Presumably, since a specific transport system for β -glycerophosphate is not thought to exist, the alkaline phosphatase in the PO₄³⁻-limited cells splits the substrate at the cell membrane, the phos-

phate moiety then being transferred into the cell. In contrast, the inability of C-limited cells to utilize β -glycerophosphate, despite their high phosphatase activity, indicates that the function of the enzyme synthesized in these conditions does not facilitate the accumulation of phosphorylated substrates. This would require dephosphorylation and transport of the glycerol residue into the cell, and glucose-limited cells readily metabolize glycerol.

These various properties identify the enzyme synthesized in PO₄³⁻-limited cells with the alkaline phosphatases described by others. On the same basis, the apparent 'acid' or 'neutral' phosphatase in gluconate-limited cells could be similarly classified, since it also possessed these properties in fair measure but was not repressed by excess of PO₄³⁻ in the growth medium. However, phosphate repression of alkaline phosphatase, although widespread, is not a general effect. The sensitivity to exogenous PO₄³⁻ varied in different strains of *E. coli* and in one strain the presence of PO₄³⁻ actually stimulated its synthesis (Kuo & Blumenthal, 1961).

Function of phosphatase activity

Compared with alkaline phosphatase the function of non-specific acid phosphatase is not clear. The present results suggest an association with carbohydrate metabolism, but since none of the growth substrates was supplied in the phosphorylated form a primary catabolic function of the enzyme cannot be envisaged. Phosphatase activity may exert a regulating effect on carbohydrate metabolism by 'short-circuiting' at the hexose phosphate stage, i.e. regulating the size of cellular hexose and hexose phosphate pools. In this capacity the phosphatases may be exhibiting phosphotransferase activity. Phosphatase activity may also be linked to permeation processes. This function has been inferred since the demonstration of a phosphorylated sugar transport mechanism,

widespread in many bacteria, particularly *K. aerogenes* (Tanaka & Lin, 1967). In such systems phosphatases may function by releasing the free substrate into the cell after transport as a phosphorylated intermediate.

We are indebted to the Medical Research Council for the provision of a scholarship for training in research methods to P. G. B.

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