Sporulation in *Bacillus subtilis* 168

COMPARISON OF ALKALINE PHOSPHATASE FROM SPORULATING AND VEGETATIVE CELLS

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1. The purification of the 'vegetative' alkaline phosphatase of *Bacillus subtilis* 168 was simplified by ionic elution of the enzyme from intact cells. 2. The enzyme has a molecular weight of about 70000 and treatment of the enzyme with 10mM-hydrochloric acid or 6.0M-guanidine hydrochloride, β -mercaptoethanol (0.1M) gives rise to enzymically inactive subunits. 3. The amino acid composition of the enzyme was determined. The N-terminal residue determined by the DNS chloride method is glycine. 4. The properties of this enzyme were compared with the 'sporulation' alkaline phosphatase of the same strain. 5. Although the 'sporulation' enzyme differs from the 'vegetative' enzyme in its physiology of appearance and apparent mRNA stability, an examination of properties of the enzymes revealed no differences. 6. The enzyme from both cell forms is bound to the particulate fraction of cell extracts, but can be solubilized by high concentrations of magnesium chloride; removal of the magnesium chloride, by dialysis, results in precipitation of both enzymes. Both enzymes can be removed from intact cells by ionic elution. 7. The 'vegetative' and 'sporulation' enzymes have identical pH optima, K_m and K_t values and electrophoretic mobilities in cellulose acetate. 8. Their half-life is 28min at 65°C and their Q_{10} is 1.25. 9. The molecular size determined by gel filtration on Sephadex G-100 is about 69000. 10. 'Vegetative' and 'sporulation' forms gave precipitin lines that were continuous and non-spurred when tested against antiserum prepared against the 'vegetative' enzyme. 11. The 'sporulation' alkaline phosphatase appears to be associated with stage II of sporulation and appears to be induced by something specifically concerned in sporulation and not by phosphate starvation.

The alkaline phosphatases of several bacterial species have been investigated and, although they differ one from another in certain respects, they seem in general to share the property of being repressed by P_i in *Escherichia coli* (Horiuchi, Horiuchi & Mizuno, 1959; Torriani, 1960; Garen & Levinthal, 1960), *Bacillus subtilis* (Anagnostopoulos, 1960; Demain & Hendlin, 1967; Takeda & Tsugita, 1967), *Pseudomonas fluorescens* (Friedberg & Avigad, 1967) and *Staphylococcus aureus* (Shah & Bloebel, 1967). However, Kuo & Blumenthal (1961) reported that phosphate-repressible alkaline phosphatases were absent from some strains of *S. aureus* and *E. coli*. Although the enzyme has been shown to have a surface localization in some strains of *B. subtilis* (Ghosh, Wouters & Lampen, 1970; Wood, 1968; Wood & Tristram, 1970) the latter workers have shown that it is not released by osmotic shock or by protoplast formation as is the enzyme from *E. coli* (Neu & Heppel, 1964, 1965; Malamy & Horecker, 1964). Takeda & Tsugita

(1967) reported the purification of the enzyme which they had solubilized by adding magnesium acetate in high concentrations to crude cell extracts of *B. subtilis* SB15.

However, an alkaline phosphatase is produced during sporulation of *B. subtilis* 168, even in the presence of concentrations of P_i that completely repress the 'vegetative' alkaline phosphatase (Warren, 1968). During work on asporogenous mutants blocked morphologically at various stages of sporulation Waites *et al.* (1970) found that there was a correlation between the appearance of alkaline phosphatase and the stage at which the mutants were blocked. This led them to conclude that the enzyme was specifically concerned in the sporulation process.

For these reasons we thought it likely that the 'sporulation' phosphatase and the 'vegetative' phosphatase were different enzymes. This supposition was strengthened by the fact that experiments with actinomycin D had suggested that the mRNA

for the 'sporulation' enzyme was long-lived (Sterlini & Mandelstam, 1969) whereas similar experiments with 'vegetative' enzyme indicated the opposite (Fan, 1966; Moses, 1967).

The present work was undertaken to compare the enzyme found during sporulation of *B. subtilis* 168 with the phosphate-repressible enzyme formed by the same strain in the vegetative state. The results suggest that the enzymic activities are, in all probability, due to a single protein species and that the differences in behaviour already referred to are the result of a complex regulation system.

MATERIALS AND METHODS

Reagents. *p*-Nitrophenyl phosphate was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Fast Blue RR Salt and α -naphthyl acid phosphate were from Sigma Chemical Co., St Louis, Mo., U.S.A. Other chemicals were A.R. grade where these were available. Deoxy-ribonuclease was from Worthington Biochemical Corp., Freehold, N.J., U.S.A., ribonuclease from Sigma and bovine serum albumin from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.

Organism. *B. subtilis* (Marburg 168) an indole or tryptophan auxotroph that sporulates normally was used. Asporogenous mutants E22 (blocked at stage 0) and E21 (blocked at stage II) were derived from it, and are described by Mandelstam & Waites (1968) and Waites *et al.* (1970).

Culture and procedure for sporulation. Cells were grown in a hydrolysed casein medium, and sporulation was induced by resuspending in a medium containing glutamate, tryptophan and inorganic ions (see Sterlini & Mandelstam, 1969). The phosphate concentration of the medium was 0.5 mM unless otherwise stated. Samples were taken at hourly intervals for measurement of E_{600} , alkaline phosphatase activity and refractility.

De-repression of the 'vegetative' alkaline phosphatase. Cells were grown in the glucose-glutamate-salts medium of Donnellan, Nags & Levinson (1964) containing L-tryptophan (0.02 g/l) and 1.0 mM- P_i with tris buffer, pH 7.4 (0.01 M), with shaking at 37°C to a density of 0.2 mg dry wt./ml. The cells were harvested by centrifugation and resuspended in a similar medium with phosphate omitted. The cultures were then incubated with shaking at 37°C and samples taken at intervals for determination of E_{600} and assay of enzyme activity.

Large-scale growth of bacteria. Cells of *B. subtilis* 168 were grown in a 400-litre fermenter in a medium containing the following (g/l): Bactopeptone (Difco), 10.0; glucose, 5.0; L-tryptophan, 0.05; tris, 1.21; $MgCl_2$, 2.03; NH_4Cl , 2.0; Na_2SO_4 , 1.0; $CaCl_2$, 0.5; NH_4NO_3 , 0.5; KCl, 1.0. The pH was adjusted to 7.4, but fell considerably during the growth of the culture and was 4.9 when the cells were harvested. Growth in this medium was limited by phosphate and cultures grown to the stationary phase contained large amounts of alkaline phosphatase. We are grateful to Dr H. J. Rogers and Mr J. Lee of the National Institute for Medical Research, London N.W.7, U.K., and to Dr K. Sargeant of the Microbiological Research Establishment, Porton Down, Wilts., U.K., for providing us with large-scale cultures.

Enzyme assay. Enzyme was added to *p*-nitrophenyl phosphate (1.0 mg/ml) in 1.0 M-diethanolamine-HCl, pH 10.0, at 30°C. After development of the colour, 1.0 ml of 2 M-NaOH was added to stop the reaction and the extinction at 410 nm measured. In some of the earlier experiments the buffer used was 1.0 M-tris-HCl, pH 8.0. In diethanolamine-HCl buffer enzyme activities were uniformly higher, but comparisons were not otherwise affected (see the Results section). Units are expressed as μ mol of *p*-nitrophenol released/min at 30°C. Specific activity is expressed as units/min per mg of protein or of bacterial dry weight.

Protein determination. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard, or by the method of Warburg & Christian (1941).

Bacterial density. This was measured spectrophotometrically by using a calibration curve relating E_{600} to dry weight.

Measurement of spore incidence. Refractile spores were counted in the phase-contrast microscope.

Sedimentation velocity. Solutions of the enzyme were centrifuged in a Spinco model E analytical ultracentrifuge at 20°C at 59780 rev./min. Sedimentation equilibrium was obtained at 15220 rev./min after 22 h.

Amino acid composition. Acid hydrolysis was performed as described by Spackman, Stein & Moore (1958). Performic acid oxidation was by the method of Hirs (1956). Tryptophan was determined spectrophotometrically by using the tyrosine/tryptophan molar ratio; the value for tyrosine was obtained directly from the amino acid analysis as described by Beaven & Holliday (1952).

Identification of the N-terminal residue. N-Terminal determination was performed by using DNS chloride (Gray & Hartley, 1963). Protein (0.5 mg) was dissolved in 0.1 ml of saturated $NaHCO_3$ plus 0.35 ml of 8 M-urea, which had been deionized by passage through a mixed-bed resin, and then 0.5 ml of DNS chloride (15 mg/ml) in acetone was added. Incubation at 37°C was carried out for 3 h and then the protein was precipitated with 10% (w/v) trichloroacetic acid, washed with 10% (w/v) trichloroacetic acid and acetone, hydrolysed with 6 M-HCl for 24 h and, after drying, the residue was taken up in 1 M- NH_3 . The dansyl amino acids were chromatographed on polyamide sheets (Cheng-Chin Trading Co. Ltd., Taipei, Taiwan) in a covered jar by ascending solvent flow with the following solvents: 1, water-90% formic acid (200:3, v/v); 2, benzene-acetic acid (9:1, v/v) (Woods & Wang, 1967); 3, ethyl acetate-methanol-acetic acid (20:1:1, by vol.) (Crowsham, Jessup & Ramwell, 1967). The spots were detected by u.v. light.

Cellulose acetate electrophoresis. Electrophoresis was carried out at pH 8.9, 100 V, 1.0 mA/strip for 2.5 h with a tris-veronal buffer (sodium veronal-veronal-tris, 10.3:1.84:7.2, g/l) or a tris-borate buffer, pH 8.9 (tris, 60.5 g + boric acid, 4.6 g/l) (Aronsson & Gronwall (1958), or in a β -alanine-acetate acid buffer, pH 5.0 (β -alanine, 15.6 g + acetic acid, 4.0 ml; pH adjusted to 5.0 with 1.0 M-NaOH) at 100 V and 0.7-1.0 mA per strip for various times. Protein was stained with Coomassie Blue and alkaline phosphatase activity was detected by using α -naphthyl acid phosphate and Fast Blue RR Salt in 0.6% borax (Gomori, 1951).

Preparation of cell-free extracts. Cell pellets were

suspended in 50 mM-tris-HCl buffer, pH 7.1 containing MgSO_4 , 1.0 mM; CaCl_2 , 1.0 mM; ZnSO_4 , 0.1 mM. The cell suspension (10 ml) was transferred to a Pyrex bottle containing Ballotini no. 12 beads (7 ml) and shaken in a Braun MSK cell homogenizer (Shandon Scientific Co. Ltd., London N.W.10, U.K.) for 5 min at 2000 oscillations/min. The extract was then filtered through sintered glass to remove the beads.

Sodium dodecyl sulphate polyacrylamide-gel electrophoresis. The conditions used were as described by Weber & Osborn (1969).

Sephadex chromatography. Columns of Sephadex G-100 were prepared by the method of Andrews (1964). A sintered-glass disc at the base of the gel was protected by Ballotini no. 12 beads and the surface of the gel by Sephadex G-10. Columns were run at 2–4°C.

Preparation of antiserum. Partially purified enzyme (1.0 mg) was mixed with Freund's adjuvant and injected into the foot pads of male rabbits. After 6 weeks a further 0.5 mg of the enzyme was injected. After a further fortnight the rabbits were bled and the serum tested for antiphosphatase activity. When tested by double diffusion against crude extracts of phosphate-starved *B. subtilis* 168 a major precipitin line and a minor precipitin line were seen. The major precipitin line stained positively for alkaline phosphatase. Double-diffusion experiments were conducted as described by Ouchterlony (1958) except that 0.2 M- MgCl_2 was added to the agar.

Buffer solutions. Unless stated otherwise tris-HCl buffer solutions were 10 mM and the pH was 7.4.

RESULTS

Induction of 'vegetative' alkaline phosphatase

When cells of *B. subtilis* 168 were transferred to a phosphate-free medium alkaline phosphatase synthesis began after 30–45 min. The lag period was probably due to utilization of phosphate carried over from the previous medium. A control culture in a medium containing 0.5 mM- P_i showed no increase in enzyme activity and grew normally. The addition of P_i to cells that had begun to produce enzyme resulted in prompt cessation of enzyme synthesis. Since Dawes & Mandelstam (1970) have shown that phosphate starvation of *B. subtilis* 168 does not lead to sporulation all the enzyme produced can be regarded as 'vegetative' enzyme.

Elution of enzyme by magnesium chloride

During their work on the alkaline phosphatase from *B. subtilis* SB15 Takeda & Tsugita (1967) found that the enzyme was soluble in high concentrations of magnesium acetate. We carried out preliminary centrifugation studies of phosphate-starved bacteria that showed that in *B. subtilis* 168 the enzyme was 90–96% cell-bound. In subsequent experiments any enzyme that might have been in the culture supernatant was neglected.

Cells were grown for 14 h, i.e. to stationary phase,

Fig. 1. Elution of the alkaline phosphatase from intact cells of *B. subtilis* 168 by MgCl_2 in tris-HCl buffer. Cells were cultured in phosphate-limited bacto-peptone-salts medium overnight to stationary phase, harvested by centrifugation and portions washed with tris buffer containing MgCl_2 at several different concentrations. The suspensions were then centrifuged at 60000g for 30 min and the supernatant fraction was assayed for alkaline phosphatase activity.

in the phosphate-limited bacto-peptone-salts medium, harvested by centrifugation and the pellets were washed with tris-HCl buffer containing various concentrations of magnesium chloride. The suspensions were centrifuged at 60000g for 30 min and the supernatants were assayed for enzyme activity. About half of the enzyme was eluted between 0.1 and 0.25 M-magnesium chloride; further increases in magnesium chloride concentration up to 1 M produced smaller increases in the proportion of enzyme removed (Fig. 1). This effect is not restricted to magnesium chloride, but has been repeated with varying efficiency with other salts. Magnesium sulphate, sodium sulphate and potassium sulphate and magnesium chloride and potassium chloride at a concentration of 1.0 M in tris-HCl buffer eluted 60–80% of the bound enzyme. Sodium chloride and sodium acetate and

potassium acetate were less effective. Wood & Tristram (1970) have obtained similar results with *B. subtilis* strains A and 60-009.

Ionic elution was subsequently employed to simplify the purification of the 'vegetative' enzyme.

Purification of 'vegetative' alkaline phosphatase

Step 1. Elution of the enzyme. Cells from the 400-litre fermentation (1550g wet wt.) were washed with 5 litres of 1.0M-magnesium chloride in tris-HCl buffer and centrifuged.

Step 2. Dialysis. The 5 litres of washings were dialysed against tris buffer. During dialysis the enzyme precipitated with other proteins and was collected by centrifugation at 22000g for 20min. The pellet fraction was taken up in 500ml of 0.5M-magnesium chloride in tris buffer and the solution clarified by centrifugation at 22000g for 30min.

Step 3. Dialysis. Soluble enzyme in 0.5M-magnesium chloride in tris buffer was dialysed against tris buffer for 24h and the resulting precipitate was collected by centrifugation at 22000g for 60min. The pellet fraction was dissolved in 30ml of tris buffer containing 0.2M-magnesium chloride. At this stage the E_{280}/E_{260} ratio was 0.68 and the suspension was very viscous.

Step 4. Deoxyribonuclease and ribonuclease treatment. To remove the nucleic acids deoxyribonuclease and ribonuclease each at a final concentration of 10 µg/ml were added to the enzyme suspension in 0.2M-magnesium chloride and incubated for 5.5h at 37°C. The material was dialysed against 0.2M-magnesium chloride in tris buffer with several buffer changes. After 24h the E_{280}/E_{260} ratio was 0.91 and after 48h 1.32.

Step 5. First fractionation with Sephadex G-100. One-third of the nuclease-treated material (10ml) was concentrated by dialysis against solid sucrose and layered on to a Sephadex G-100 column

(78cm×3.5cm). Protein was eluted with 0.2M-magnesium chloride in tris buffer. Fractions of 10ml were collected and the peak fractions of phosphatase activity were pooled.

Step 6. Second fractionation with Sephadex G-100. The pooled fractions (30ml) from the first cycle were concentrated against solid sucrose and re-cycled through the Sephadex G-100 column. The peak fractions of phosphatase activity were again pooled giving a solution of enzyme purified over 100-fold (Table 1).

Properties of 'vegetative' alkaline phosphatase

Alkaline phosphatase showed an absorption maximum at 279nm and an inflexion at 285nm at pH 7.4. The E_{280}/E_{260} ratio of purified material was 2.0, showing that the preparation was essentially free of nucleic acid contamination. Cellulose acetate electrophoresis at pH 8.9 gave a single protein band moving towards the cathode which corresponded with the alkaline phosphatase band.

Sedimentation velocity. In the analytical ultracentrifuge the enzyme (5.3mg/ml) in 0.2M-Mg²⁺ gave a single peak with a sedimentation coefficient of 5.4S.

Treatment of the enzyme at 2–4°C overnight with 10mM-hydrochloric acid or 10mM-hydrochloric acid+0.2M-magnesium chloride resulted in the formation of material which sedimented at 2.4S and was enzymically inactive. Extended dialysis of this material against 0.2M-magnesium chloride in tris buffer at pH 7.4 failed to bring about any significant reactivation of the enzyme (less than 0.01%) although the S value was 3.7. The appearance of the 2.4S material suggested the formation of subunits. Accordingly, enzyme (5.3mg/ml) was dialysed against 6M-guanidine hydrochloride with β-mercaptoethanol (0.1M) for 4–5 days and then centrifuged. The s_{20} was 0.56S corresponding to an

Table 1. *Summary of the purification of alkaline phosphatase*

Bacterial cells were grown in a phosphate-limited bactopeptone-glucose salts medium, harvested by centrifugation and the intact cells washed with 1.0M-MgCl₂ in tris-HCl buffer to elute the enzyme. The enzyme in the supernatant was then purified as described in the text.

Stage	Vol. (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg of protein)	Purifica- tion
Intact cells	—	—	—	1.26	1.0
1.0M-MgCl ₂ extract of intact cells	5000	4.2	21000	13.3	10.6
Dialysed material in 0.5M-MgCl ₂	500	5.5	2750	62.2	49.0
Dialysed material in 0.2M-MgCl ₂	30	90.0	2700	67.5	53.5
Ribonuclease-deoxyribonuclease-treated material dialysed against 0.2M-MgCl ₂	30	48.6	1458	75.0	59.5
First Sephadex G-100 fractionation	10	30.6	306	119.0	94.4
Second Sephadex G-100 fractionation	30	5.3	159	135.0	107.1

Table 2. *Formation of subunits by treatment of alkaline phosphatase by denaturing agents*

Enzyme (5.3 mg/ml) was dialysed against the denaturing agent at 2–4°C overnight except that treatment with 6.0M-guanidine hydrochloride and β -mercaptoethanol was carried out at room temperature for 4–5 days. The enzyme solutions were assayed after treatment for residual enzyme activity and centrifuged at 59780 rev./min in a Spinco model E analytical ultracentrifuge equipped with schlieren optics.

Treatment	Sedimentation coefficient	Specific activity (units/mg of protein)
0.2M-Mg ²⁺	5.4	135
0.5M-Mg ²⁺	4.7	—
8.0M-urea	4.9	0.248
6.0M-guanidine hydrochloride + 0.1M- β -mercaptoethanol	0.56	0.0
0.01M-HCl	2.4	0.0
0.01M-HCl+0.2M-Mg ²⁺	2.4	0.0
0.01M-HCl+0.2M-Mg ²⁺ neutralized to pH 7.4 by extensive dialysis	3.7	0.007

Table 3. *Amino acid analysis of alkaline phosphatase*

Duplicate samples (1.1 mg) were hydrolysed for 24 h and 72 h and examined on the amino acid analyser. Performic acid oxidation was used to determine cysteine. Tryptophan was determined by the tyrosine/tryptophan molar absorption ratio (Beavan & Holliday, 1952). The number of residues/molecule was calculated by using a molecular weight of 69000. The following corrections for destruction by acid hydrolysis were used: serine 10%, histidine 6% and threonine 4%.

Amino acid	Residues/molecule
Lysine	72
Histidine	12
Arginine	11
Aspartic acid	98
Threonine	40
Serine	32
Glutamic acid	50
Proline	15
Glycine	47
Alanine	59
Valine	32
Methionine	14
Isoleucine	39
Leucine	30
Tyrosine	21
Phenylalanine	21
Tryptophan	5
Cysteic acid	3
Hexosamine	0

apparent molecular weight of about 34 000 (Tanford, Kawahara & Lapanje, 1967). Enzyme (5.3 mg/ml) treated with 8M-urea gave a sedimentation coefficient of 4.9S (Table 2).

Treatment of the native enzyme with 10M-hydrochloric acid appears to result in an irreversible loss of enzyme activity, though this may only mean that the correct conditions for renaturation have not been found. Schlesinger & Barrett (1965) found that the enzyme from *E. coli* can be reversibly denatured by acid. The enzyme from *Bacillus licheniformis* is split into two subunits (mol.wt. 56000) by acid treatment (Cowling & Campbell, 1969) and another species (mol.wt. 26000) is reported on dialysis at pH 7.3.

Molecular-weight determination. Sedimentation equilibrium of the native enzyme (5.3 mg/ml) at 15220 rev./min for 22 h gave an apparent molecular weight of 75500. The graph of $1/r \cdot dc/dr$ against Δc was linear. Electrophoresis of the enzyme on sodium dodecyl sulphate polyacrylamide gels gave a major band of mol.wt. 35000–40000 with two minor bands of lower molecular weight.

Amino acid composition. Table 3 shows the amino acid composition based on duplicate 24 and 72 h acid hydrolyses. No half-cystine was detected on straightforward acid hydrolysis though a small amount (three residues of cysteic acid/molecule of enzyme) was detected in a performic acid-oxidized sample. The analysis revealed no hexosamine, which indicates that the enzyme is probably free from carbohydrate. The partial specific volume

from the amino acid composition is 0.734, with the method of Cohn & Edsall (1943). The N-terminal analysis with the dansyl chloride method revealed only dansyl-glycine, ϵ -dansyl-lysine and O-dansyl-tyrosine. This indicates that glycine is the N-terminal amino acid.

The N-terminal group reported by Takeda & Tsugita (1967) for the enzyme from *B. subtilis* SB15 was glutamate, whereas our enzyme appears to have glycine instead. This probably reflects a minor strain difference because the overall amino acid composition of the enzyme from strains SB15 and 168 is similar. One unusual feature found in the analysis is the high isoleucine content (39 mol/mol compared with only 30 mol of leucine/mol).

Production of 'sporulation' alkaline phosphatase and comparison of its properties with those of the 'vegetative' enzyme

Appearance of the 'sporulation' alkaline phosphatase. In the resuspension system described by Sterlini & Mandelstam (1969) alkaline phosphatase activity began to increase 1.5–2.0 h after resuspension, reached a maximum at 4.0–6.0 h, and thereafter decreased. Its appearance preceded that

of refractile spores by about 3h. There was considerable day to day variation in the final enzyme activity attained and this was more marked in the asporogenous mutants. Strain E22, a stage 0 mutant, failed to produce enzyme, whereas strain E21, blocked at stage II, did form the enzyme (see also Waites *et al.* 1970). When starved of phosphate, vegetative cultures of both mutants produced normal amounts of phosphatase.

Increased concentrations of P_i (up to 10mM) failed to prevent the increase of alkaline phosphatase activity during sporulation of the wild type. This was in marked contrast with the 'vegetative' enzyme which was completely repressed by similar phosphate concentrations. A constant feature to note about the 'sporulation' enzyme was that its activity was only about 5% of that found in derepressed vegetative cells.

Centrifugation studies. These experiments were done to determine whether there were any differences in the binding of the two types of activity to particulate cell fractions. In both de-repressed and sporulating cells only about 5–10% of the total enzyme was released into the medium: this was therefore disregarded. The cell-bound activity was examined as follows. Cell-free extracts of vegetative and sporulating cells were prepared, centrifuged at 100000g for 3h and the pellet and supernatant fractions were assayed for enzyme activity. About 80–90% of the total activity was found in the pellet fraction. Treatment of the pellet fractions with 1.0M-magnesium chloride in 10mM-tris-HCl buffer, pH 7.4, and further centrifugation at 100000g for 3h resulted in 70–80% of the total activity appearing in the supernatant fraction. Dialysis of the solubilized 'vegetative' and 'sporulation' alkaline phosphatase against 10mM-tris-HCl buffer, pH 7.4, resulted in the precipitation of the enzyme.

Preparation of 'sporulation' alkaline phosphatase. The release of the 'vegetative' phosphatase from intact cells by treatment with 1.0M-magnesium chloride has been described above. The enzyme from sporulating cells was found to be eluted similarly from intact cells. This provided a convenient way of obtaining the 'sporulation' enzyme in a partially purified form, and most of the experiments on the 'sporulation' enzyme were carried out with enzyme derived in this manner. Crude extracts of sporulating cells and phosphatase extracted from pellets with 1.0M-magnesium chloride gave similar results in the characterization tests.

pH optimum. In 1.0M-diethanolamine-HCl the maximum activity for the 'vegetative' and 'sporulation' enzyme was recorded at pH 10.0. In glycine (1.0M)-NaOH buffer the optimum pH value for both enzymes was 9.8 (Fig. 2). The activities were compared in 1.0M-tris-HCl buffer and in 1.0M-ammediol-HCl buffer (2-aminopropane-1,3-diol-

Fig. 2. Comparison of the alkaline phosphatase activities from vegetative and sporulating cells: effect of pH. The results are expressed as a percentage of the maximum activity recorded in 1.0M-diethanolamine-HCl buffer, pH 10.0. ○, 'Vegetative' enzyme in 1.0M-diethanolamine-HCl buffer; ●, 'sporulation' enzyme in 1.0M-diethanolamine-HCl buffer; △, 'vegetative' enzyme in 1.0M-glycine-NaOH buffer; ▲, 'sporulation' enzyme in 1.0M-glycine-NaOH buffer.

HCl) and the maximum activity was observed at the upper limit of both buffers, i.e. pH 9.1 and 9.6 respectively. The activity of both forms of the enzyme in tris, ammediol or glycine-NaOH buffers was lower than in 1.0M-diethanolamine-HCl over a wide range of pH values, e.g. at pH 10.0 in a glycine-NaOH buffer the activity of both enzymes was only 55% of that in 1.0M-diethanolamine-HCl at the same pH value (see Bodansky, 1946, for similar effects in preparations of rat liver alkaline phosphatase). Enzyme activity of both enzymes was higher by about 30% in 1.0M- than in 0.1M-diethanolamine-HCl buffer.

K_m and K_i values. The Michaelis constants of both enzymes were determined with *p*-nitrophenyl phosphate as substrate (over the concentration range 0.1–1.0mM) in 1.0M-diethanolamine-HCl buffer, pH 10.0. The K_m for the 'vegetative' enzyme was 0.27mM compared with the 0.22mM for the 'sporulation' enzyme. The K_i values with sodium phosphate as inhibitor were also determined and were 0.83mM and 0.80mM for the 'vegetative' and 'sporulation' enzyme respectively.

Heat stability. The heat stability of the enzymes was compared at 65°C. The protein concentration was adjusted to 5.0mg/ml with bovine plasma albumin. All the heat denaturation studies were

carried out in the presence of 0.2M-Mg^{2+} , which is known to stabilize some phosphatases (Garen & Levinthal, 1960). The half-life of the 'sporulation' enzyme was 28 min and that of the 'vegetative' enzyme was not measurably different.

Effects of temperature. The temperature-activity relationship of the 'vegetative' and the 'sporulation' enzyme was examined over the range $18-47^{\circ}\text{C}$ in 1.0M -diethanolamine-HCl buffer, pH 10.0, containing *p*-nitrophenyl phosphate (1.0mg/ml). The Q_{10} over the temperature range $27-37^{\circ}\text{C}$ was 1.25 for both enzymes.

Electrophoretic properties. Attempts at polyacrylamide-gel electrophoresis with various pore size gels failed because the enzymes remained at the origin. This lack of mobility was probably a reflection of the insolubility at low ionic strengths. Electrophoresis on cellulose acetate strips showed that at pH 8.9 and at pH 5.0 the enzymes had identical mobilities.

Gel filtration. The molecular size of the phosphatases was compared by gel filtration on Sephadex G-100 by using cytochrome *c*, soya-bean trypsin inhibitor, ovalbumin, bovine plasma albumin and γ -globulin (Cohn fraction) as protein standards. The void volume of the column ($78\text{cm} \times 3.5\text{cm}$) was

determined by the elution of Blue Dextran G-2000 measured at 610nm and was 160ml . Both the 'vegetative' and the 'sporulation' enzymes co-eluted with bovine plasma albumin indicating a molecular weight of about $69\,000$ (Fig. 3).

Immunochemical tests. Antiserum prepared against the purified 'vegetative' alkaline phosphatase was used in Ouchterlony (1958) tests. Double diffusion against antiserum was carried out with extracts obtained by treating vegetative and sporulating cells with 1.0M -magnesium chloride. The resulting precipitin lines were continuous, were without spurs and stained positively for alkaline phosphatase. In these experiments it was important, however, to load equivalent amounts of enzyme activity into the diffusion wells otherwise concentration differences resulted in the appearance of a spur after several days at 37°C . Double diffusion of the native vegetative enzyme against antiserum prepared against the partially purified material gave a single precipitin line. The subunits of the vegetative enzyme were also examined for cross reaction with the serum prepared against the native enzyme. Acidified and guanidine-treated material ($160\mu\text{g}$) failed to give any precipitin line, whereas samples treated with 8.0M -urea or 1% sodium dodecyl sulphate or with acid and then neutralized gave slight precipitin lines at this concentration.

Fig. 3. Estimation of the molecular weight of the 'vegetative' and 'sporulation' alkaline phosphatases on Sephadex G-100. The column ($78\text{cm} \times 3.5\text{cm}$) was calibrated with proteins of known molecular weight: 1, cytochrome *c*, 2, soya-bean trypsin inhibitor, 3, ovalbumin, 4, bovine plasma albumin, and 5, γ -globulin (Cohn fraction). The void volume was determined by the passage of Blue Dextran was 160ml . Proteins were eluted with MgCl_2 in tris-HCl buffer. Reference proteins were detected by E_{280} readings and alkaline phosphatase activity (eluted at the position of bovine serum albumin) by enzyme assay. The results are expressed in the form of elution volume plotted against the log (molecular weight) (Andrews, 1964).

DISCUSSION

There appears to be a marked strain variation in the amount of alkaline phosphatase that is cell-bound in *B. subtilis* and the values reported vary from about 7% in *B. subtilis* 60-009 (Cashel & Freese, 1964) to more than 99% in strain 15244 (Chesbro & Lampen, 1968). In our strain, however, 90-95% of the enzyme, under the conditions of growth used, was cell-bound.

Ionic elution of the enzyme from intact vegetative cells results in a tenfold purification and simplifies the initial stages of the purification of the enzyme. Similar salt elutions have been described for the solubilization of enzymes from *Saccharomyces* spp. by Weimberg & Orton (1965, 1966) and for the elution of the acid phosphatase from *Staphylococcus aureus* (Malveaux & San Clemente, 1967, 1969). Cowling (1969) and Cowling & Campbell (1969, 1970) found that the alkaline phosphatase from a thermophilic strain of *Bacillus licheniformis* was solubilized by using 1M -magnesium acetate. However, on removal of the salt the enzyme remained in solution, unlike the enzyme from *B. subtilis*.

The following criteria indicate that the final product we obtained is homogeneous or nearly so: there was a single peak on ultracentrifugation of native enzyme or guanidine-formed subunits; the

graph of $1/r \cdot dc/dr$ against Δc was linear; there was a single protein peak coincident with the alkaline phosphatase activity on Sephadex G-100 gel filtration; double-diffusion tests on the purified enzyme gave only a single precipitin line, whereas double diffusion of crude cell extracts of phosphate starved cells gave a second line; a single protein band after electrophoresis at pH 8.9 and a single N-terminal residue by the sensitive DNS chloride method. However, electrophoresis of the enzyme on sodium dodecyl sulphate polyacrylamide gels showed two minor bands migrating faster than the major band, but it is not known whether these represent contaminating material or degradation products. Klee (1970) has attributed a minor contaminating band seen after electrophoresis of histidine ammonia-lyase to cleavage of a particularly sensitive bond in the protein. Attempts at gel electrophoresis in a non-denaturing system have proved unsuccessful because of the solubility properties of the enzyme.

Comparison of 'vegetative' and 'sporulation' enzymes. Because the 'sporulation' enzyme occurs in much smaller amounts (0.03 unit/mg dry wt. as compared with 0.45 unit/mg dry wt. or higher for the vegetative enzyme) a parallel purification, though desirable, was impracticable. However, the purification procedures did not alter the kinetic properties of the 'vegetative' enzyme and there is no reason to expect that they would have altered those of the 'sporulation' enzyme either.

The pH response of the alkaline phosphatases from the two cell forms was similar with a maximum activity occurring in diethanolamine-HCl buffer, pH 10.0, and at pH 9.8 in glycine buffer. This value is similar to that recorded by Anagnostopoulos (1960) and Takeda & Tsugita (1967) for the *B. subtilis* enzyme but is higher than the values recorded (pH 8.0-8.6) for the enzymes of *E. coli* (Garen & Levinthal, 1960), *Ps. fluorescens* (Friedberg & Avigad, 1967) and *B. licheniformis* (Cowling, 1969). The Michaelis constant for the 'vegetative' and 'sporulation' alkaline phosphatases was 0.2 mM-*p*-nitrophenyl phosphate, a value tenfold higher than that reported by Takeda & Tsugita (1967) for the alkaline phosphatase of *B. subtilis* SB 15. This may reflect a strain difference or the conditions used to measure the Michaelis constant. Heppel, Harkness & Hilmoe (1962) found a 30-fold difference in the K_m value for the *E. coli* alkaline phosphatase depending on whether a very-high or a very-low concentration of substrate was employed. The enzymes from vegetative and sporulating cells of *B. subtilis* were fairly heat resistant with an apparently identical half-life of 27-28 min at 65°C.

The molecular weight of the enzyme from both cell forms estimated by gel filtration was about 69 000. Molecular-weight measurements for the

'vegetative' enzyme obtained by sedimentation studies are in reasonable agreement with this value. The corresponding value for the *E. coli* enzyme is 80 000 (Garen & Levinthal, 1960) and that for the *B. licheniformis* enzyme is 121 000 (Cowling & Campbell, 1970).

In summary the two activities appear to have the same localization, solubility characteristics, kinetic properties, heat stability, temperature coefficient, electrophoretic mobility on cellulose acetate, molecular size and immunochemical determinants (Table 4). It seems probable therefore that the 'vegetative' and the 'sporulation' alkaline phosphatase activities are due to a single protein species.

This finding is in general agreement with the work of Kornberg and his colleagues on other enzymes in vegetative cells and spores of *Bacillus* spp. (Falaschi & Kornberg, 1966; Spudich & Kornberg, 1969; Gardner & Kornberg, 1967; Tono & Kornberg, 1967*a,b*). However, differences between the enzymes in vegetative cells and spores have been reported by other workers (e.g. Rowley & Newcomb, 1964; Baillie & Norris, 1964; Green & Sadoff, 1965). Sadoff, Hitchins & Celikkol (1969) have presented evidence that the spore fructose 1,6-diphosphate aldolase of *B. cereus* is derived from the vegetative cell by proteolytic cleavage mediated by the sporulation specific protease. Sadoff & Celikkol (1969) have demonstrated the conversion *in vitro* of the vegetative aldolase into the spore aldolase by sporulation protease in *B. megaterium*, *B. cereus* and *B. subtilis*. The possibility of such a modification of the 'vegetative' alkaline phosphatase to give the 'sporulation' enzyme cannot be eliminated at present, although there seems to be no gross decrease in molecular size as there is for the *B. cereus* spore aldolase.

Even though it may be tentatively suggested that the 'vegetative' and 'sporulation' forms of the phosphatase are due to the same protein species, the differences (see the introduction and Table 4) in the physiology of regulation require some comment. First, the 'vegetative' enzyme is repressible and is produced only when the cells are starved of P_i , whereas the 'sporulation' enzyme appears even though there is an excess of P_i in the medium. Secondly, production of the 'vegetative' form of the enzyme is sensitive to inhibition by actinomycin D, whereas the 'sporulation' form is relatively insensitive to the drug at the same concentration. These apparent differences in regulation and in mRNA stability will be considered separately.

There are two possible explanations to account for the appearance of the alkaline phosphatase during sporulation. The first is that the cell becomes phosphate-starved, or perhaps more specifically that the spore protoplast becomes impermeable to P_i and that this results in de-repression of the

Table 4. *Comparison of properties of alkaline phosphatase from vegetative and sporulating cultures*

	'Sporulation' enzyme	'Vegetative' enzyme
Physiology of appearance	Appears during sporulation; not repressed by P_i	Appears when cells are starved of P_i ; formation repressed by P_i
Stability of mRNA	mRNA for enzyme apparently long-lived	mRNA apparently short-lived
Localization and solubility properties	Localized around the cytoplasmic membrane of the mother cell and membrane of the forespore; soluble only in solutions of high ionic strength	Localized around the cytoplasmic membrane of vegetative cells; soluble only in solutions of high ionic strength
pH	Maximum activity in 1.0M-diethanolamine-HCl buffer, pH 10.0; marked inhibitory effect of glycine-NaOH buffer (optimum at pH 9.8)	Maximum activity in 1.0M-diethanolamine-HCl buffer, pH 10.0; marked inhibitory effect of 1.0M-glycine-NaOH buffer (optimum at pH 9.8)
K_m	0.22 mM- <i>p</i> -nitrophenyl phosphate	0.27 mM- <i>p</i> -nitrophenyl phosphate
K_i	0.80 mM-sodium phosphate	0.83 mM-sodium phosphate
Molecular size on Sephadex G-100	69 000	69 000
Mobility on cellulose acetate electrophoresis	Identical mobilities	Identical mobilities
Immunochemical determinants	Cross-reaction continuous and non-spurred in Ouchterlony double-diffusion tests	Cross-reaction continuous and non-spurred in Ouchterlony double-diffusion tests
Half-life for the thermal inactivation at 65°C	28 min	28 min
Heat activation Q_{10} (27–37°C)	1.25	1.25

enzyme. The alternative explanation is that there is another mechanism for regulating the structural gene for the enzyme which is distinct from phosphate starvation and which is activated during sporulation.

Now, the alkaline phosphatase appears to be associated with stage II during the sporulation of *B. subtilis* 168. Thus, it has been shown that some stage II mutants, e.g. E21 (see the Results section), produce the enzyme whereas others do not as shown by Waites *et al.* (1970). These workers also showed that none of the mutants blocked earlier produced enzyme whereas all those that reached stage III did. Nelson & Kornberg (1970) have reported that at this stage of sporulation *B. subtilis* is freely permeable to P_i and remains so until just before the development of refractility. It is thus unlikely that phosphatase synthesis is initiated by the occurrence of phosphate starvation within the developing spore protoplast.

An equally compelling reason for rejecting phosphate starvation as the explanation for the appearance of the 'sporulation' enzyme is to be found in the behaviour of mutants that are phenotypically phosphatase-negative, i.e. mutants in which the vegetative cells have lost their ability to produce the enzyme when starved of P_i . These mutants nevertheless retain the ability to produce alkaline phosphatase when they are sporulating (A. R. Glenn & J. Mandelstam, unpublished work). The

implication is that something other than phosphate starvation is able to activate the regulation mechanism for phosphatase. The experiments with actinomycin D (Sterlini & Mandelstam, 1969) suggested that the 'sporulation' alkaline phosphatase mRNA is long-lived whereas the mRNA for the 'vegetative' enzyme is not. If we are correct in the assumption that both forms of activity are due to the same protein species it follows that the mRNA must also be the same, and we are left with the problem of explaining why its stability varies in this way. At present we can do no more than suggest tentatively that there might be some physical compartmentation of the mRNA species produced in sporulation so that they are protected from the action of ribonuclease. An obvious but unsubstantiated suggestion is that the developing spore protoplast has less, or no, ribonuclease and that any mRNA produced in this part of the cell will be stable in comparison with mRNA in the cytoplasm of the mother cell.

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