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# Properties of Fructose 1,6-Diphosphate Aldolases from Spores and Vegetative Cells of *Bacillus cereus*[1]

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Fructose 1,6-diphosphate aldolase from cells of *Bacillus cereus* appears to be typical Class II aldolase as judged by its functional and physical properties. Spore and vegetative cell aldolase had similar enzymatic, immunochemical, and heat resistance properties in the absence of calcium, but they differed in their thermal stabilities in the presence of calcium, their Stokes' radii, their mobility in acrylamide gel electrophoresis, and their molecular weights. The *p*H optimum for both enzymes was 8.5, and their $K_m$ with respect to substrate was $2 \times 10^{-3}$ M. Highly purified spore and vegetative cell aldolases were both heat labile with half-lives of 4 min at 53 C and *p*H 6.4. In the presence of $3 \times 10^{-2}$ M solution of calcium ions, the stability of the spore protein increased 12-fold but the vegetative form became more heat labile. The enhanced stability of the spore aldolase was not diminished by dialysis or gel filtration but was lost after chromatography on diethylaminoethyl cellulose at *p*H 7.4. Aldolase from vegetative cells exists in an equilibrium mixture of two molecular weights, 115,000 and 79,000 in the approximate ratio of 1:4, respectively. The molecular weight of spore aldolase is 44,000. Spore aldolase was more mobile during electrophoresis than its vegetative cell counterpart because of its smaller size.

Bacterial spores have a great number of enzymatic capabilities in common with vegetative cells from which they are derived. It is this fact, rather than readily apparent differences in their enzyme complements, which is of major importance and which must be considered in approaching an understanding of spore morphogenesis and physiology. The very close similarity of the enzyme array and in vitro properties of enzymes from each cell type suggests that the genomic units which direct the synthesis of the vegetative cell proteins also direct synthesis of corresponding proteins in spores. This proposition has been substantiated by the use of mutants for alanine dehydrogenase in cells and spores of *Bacillus subtilis* (10) and for purine nucleoside phosphorylase in *B. cereus* (11). Therefore, the cell and spore forms of each of these enzymes presumably have the same primary structure. Halvorson (16), Kornberg et al. (21), and Murrell (26) have reviewed spore metabolism and list 60 enzymes present in both cells and spores of *Bacilliaceae*. Of these, the cell and spore forms of alanine dehydrogenase (24, 27, 43, 44), deoxyribonucleic acid polymerase (8, 9), purine nucleoside phosphorylase (11), and inorganic pyrophosphatase (40, 41) have been highly purified and subjected to functional and physicochemical comparisons. For each enzyme, the proteins from cells and spores were very similar but some differences could be noted in one or more aspects of their catalytic properties. We have attempted to study another spore-cell enzyme set in detail in order to discern the basis for the minor differences in properties noted between cell and spore enzymes. This effort is part of our research into the mechanism of sporulation and the molecular basis of heat resistance. Fructose 1,6-diphosphate aldolase was selected for the investigation because it is the characteristic enzyme of the Embden-Meyerhof pathway which is functional in cells and spores of *B. cereus* (13). Furthermore, aldolase is very widespread in nature, and the enzyme is of interest as a model of biochemical evolution (31). The enzymes from cells and spores were purified and direct comparisons were then made, under identical conditions, of their catalytic properties, their *p*H and heat stabilities, their immunochemical, chromatographic, and gel filtration properties, and their molecular weights.

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## MATERIALS AND METHODS

Vegetative cells and spores of *B. cereus* were grown in modified G-medium (17, 38) at 30 C with aeration in a 100-liter pilot plant fermentor (Stainless and Steel Products Co., St. Paul, Minn.). The preparation of active inocula has been described elsewhere (32). Vegetative cells and spores were harvested after 8 and 24 hr of growth, respectively, in a Sharples continuous flow centrifuge (The Sharples Corp., Philadelphia, Pa.) and stored as a frozen paste at −20 C. The progress of growth and sporulation was followed by phase contrast microscopy. Sporulation and dissolution of the sporangium occurred in more than 95% of all cells.

**Preparation of spore and vegetative cell extracts.** Spores or vegetative cells in 500-g (wet weight) amounts were thawed and suspended in 1,200 ml of 0.05 M phosphate buffer (*p*H 6.5). The spore suspensions were heated at 70 C for 30 min to denature any extrasporal aldolase because thermal inactivation studies showed that the vegetative enzyme was labile under these conditions. The spores and vegetative cells were disrupted after 20 min at 5 C in an Eppenbach colloid mill (Gifford-Wood Co., Hudson, N.Y.) to which 800 g of no. 110 Superbrite beads (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) had been added. The cell or spore extracts and washings of the beads were pooled and spun at 15,000 × *g* for 20 min, and the supernatant solutions were retained for purification.

**Aldolase assay and localization.** Aldolase assay procedures which are applicable to pure enzyme preparations are usually inapplicable for the assay of crude preparations. Therefore, aldolase activity was determined by either of two procedures, depending on the purity of the enzyme. The colorimetric method of Sibley and Lehninger (34) is suited to the assay of crude extracts and utilizes fructose 1,6-diphosphate (FDP) as substrate, hydrazine as the trapping agent for the resulting trioses, and 2,4-dinitrophenylhydrazine as the color reagent. The assays were carried out in 0.004 M Tris buffer (*p*H 8.6). The extent of the reaction over 15 min was proportional to the amount of enzyme used. One unit of aldolase cleaved 1 μmole FDP/min at 25 C and produced a change in absorbance of 2.0 at 540 nm in 15 min.

The activities of purified aldolase were determined spectrophotometrically (5) in a coupled assay of a 3-ml volume by employing 17 mM FDP, 17 mM arsenate, 0.17 mM nicotinamide adenine dinucleotide (NAD), 20 mM cysteine, and an excess (0.25 mg) of crystalline D-glyceraldehyde-3-phosphate dehydrogenase (Calbiochem, Los Angeles, Calif.) in 27 mM glycine buffer (*p*H 7.6) at 25 C. The unit of aldolase activity was 1 μmole of FDP cleaved/min, which was equivalent to the reduction of 1 μmole of NAD per min in the absence of triose phosphate isomerase. Specific activity was expressed as micromoles of FDP cleaved per minute per milligram of protein. The location of aldolase in gels employed for immunodiffusion or gel electrophoresis was achieved by coupling substrate cleavage to the reduction of Nitro Blue Tetrazolium. Developing solution (25 ml), adjusted to *p*H 7.6, contained the following: FDP, 500 μmoles; sodium arsenate, 500 μmoles; glycine, 800 μmoles; NAD, 5 μmoles; D-glyceraldehyde-3-phosphate dehydrogenase, 2 mg; phenazine methosulfate, 0.6 mg; and *p*-Nitro Blue Tetrazolium chloride, 25 mg. The gels were immersed in solution, incubated at 4 C for 1 hr and then at 37 C until discernible color developed (usually 30 min).

**Protein determination.** Protein concentrations were estimated by the spectrophotometric method of Warburg and Christian (42) or by the procedures of Lowry et al. (23).

**Disc electrophoresis.** The purity of cell and spore aldolases and their relative mobilities were tested by acrylamide gel electrophoresis at *p*H 8.3 and 4 C by the method of Davis (6). Tris(hydroxymethyl)aminomethane (Tris)-glycine, 0.01 M, was the buffer and up to 0.2 mg of protein was applied to each gel. The columns of 5% gel (0.5 by 5.0 cm) were maintained at a constant current of 5 ma each and bromophenol blue was used as an anionic marker. The duration of each run was approximately 2 hr during which time the dye migrated to approximately 0.5 cm from the anodic end of the gel. Gels were stained for protein with 1% amido schwarz in 7% acetic acid and then decolorized electrolytically in 7% acetic acid. The tetrazolium reduction reaction was used for localization of the aldolases.

**Immunochemical procedures.** Purified vegetative cell and spore aldolases, specific activities of 57.9 and 6.38 units per mg, respectively, were each emulsified in equal volumes of Freund's complete adjuvant and used for multiple subcutaneous injections of rabbits. A total of 0.03 mg of protein in 1 ml was introduced into the axillary and inguinal regions of two animals for each aldolase. A second injection into the animals of the same amount of protein was made after 10 days with Freund's incomplete adjuvant, and subsequent injections of protein in the incomplete adjuvant were made at 3-week intervals. Blood was collected from the animals by ear vein incision, and serum was separated after storage of the clots for 16 hr at 4 C. The antialdolase activity of each serum was tested by reacting twofold serial dilutions of the serum with the appropriate aldolase in gel diffusion plates. The antibody-antigen lines which formed were specifically stained for aldolase. The crude sera contained variable amounts of aldolase activity which was inactivated by heating at 56 C for 30 min or removed during the purification of the gamma globulin fraction. The antibody preparations used for Ouchterlony (28) and Cohn and Torriani (4) tests were gamma globulin fractions which precipitated at 37% saturation with ammonium sulfate. The globulin was resuspended in a volume of 0.85% NaCl equal to the initial serum volume. Immunodiffusion and precipitation reactions were incubated at 4 C in order to preserve the enzymatic activity of the aldolases. Cohn and Torriani tests were run in polypropylene centrifuge tubes (13 by 100 mm) on 0.28 mg of anti-spore aldolase, 0.75 mg of anti-vegetative cell aldolase, and 0.75 mg of normal gamma globulin per tube. Graded amounts of cell or spore aldolase, 0 to 0.45 units per tube, were added, and the volume was adjusted to 2.5 ml with buffered saline (*p*H 7.0). The reaction tubes were

incubated at 4 C for 40 hr, the precipitates were removed by centrifugation, and the supernatant solutions were assayed for aldolase activity.

**Sedimentation studies.** Sedimentation constants for purified vegetative cell and spore aldolases were determined by the procedures of Martin and Ames (25). The sucrose density gradients, 5 to 20%, were prepared in 0.05 M phosphate buffer (*p*H 6.5). Crystalline human hemoglobin (Nutritional Biochemicals Corp., Cleveland, Ohio) 4.2*S* and fluorescein-labeled gamma globulin 7*S* were used as standards. Portions (0.1 ml) of spore or vegetative aldolase, 0.2 or 0.17 units, respectively, were layered with 0.05 ml of the standards on top of the sucrose gradients. The tubes were spun at 39,000 rev/min for 8 to 16 hr in a SW 39 rotor of a Spinco Model L centrifuge. At the end of the tests the tubes were sampled in the usual manner by puncturing and collecting 2-drop fractions.

## RESULTS

**Purification of spore and vegetative cell aldolases.** The enzyme in crude spore extracts was precipitated by ammonium sulfate in the concentration range of 60 to 80% saturation and 7.7-fold purification was obtained upon resuspension of the precipitate in 0.05 M phosphate buffer (*p*H 6.5). Two negative adsorptions were performed with calcium phosphate gel (Sigma Chemical Co., St. Louis, Mo.) by utilizing 0.65 mg (dry wt) of gel per mg of protein. Approximately 70% of the protein in solution was adsorbed by the gel yielding a 2.5-fold purification of the enzyme for the treatment or an overall 19.6-fold purification. This preparation was stable when stored at −20 C. The aldolase was dialyzed versus 0.01 M phosphate buffer (*p*H 7.4) and chromatographed on a diethylaminoethyl (DEAE) cellulose column (2.5 by 40 cm) which was equilibrated with the same buffer. The spore enzyme was eluted by means of a linear sodium chloride gradient in the range of 0.38 to 0.42 M. A 3.6-fold purification of the spore aldolase was obtained in this process resulting in a 71-fold overall purification. The specific activity was 6.38 units/mg as is shown in Table 1.

Aldolase in extracts of vegetative cells was concentrated by precipitation with ammonium sulfate in the range of 30 to 80% saturation. The enzyme was taken up in 0.05 M phosphate buffer (*p*H 6.5) and reprecipitated with ammonium sulfate in the range 60 to 70% saturation. After resuspending the precipitate in 0.01 M phosphate buffer (*p*H 7.4), an 11-fold purification was noted. This fraction was subjected to negative adsorption with aluminum hydroxide gel [5 mg (wet wt) of gel per mg of protein] with a resultant 2.9-fold purification and then chromatographed on DEAE(cellulose) at conditions identical to those used to purify the spore aldolase. The enzyme was eluted from the column in the concentration range of 0.38 to 0.42 M NaCl, and the purest fraction had a specific activity of 57.9 units/mg at 8% yield. Chromatography resulted in a 5.3-fold purification with an overall value of 170-fold as indicated in Table 2. The vegetative cell aldolase became unstable on purification, but its activity could be maintained by the addition of $10^{-4}$ moles of dithiothreitol per liter and $10^{-3}$ moles of magnesium ions per liter. Upon acrylamide gel electrophoresis, only one protein or enzyme staining band could be noted corresponding to the purest preparation of vegetative cell aldolase. The purest spore enzyme preparation consisted of one major component, shown to be aldolase, and three minor components which had no detectable aldolase activity.

**Electrophoretic mobilities.** Fig. 1 is a photo-

TABLE 1. *Purification of spore FDP aldolase from B. cereus*

| Fraction | Total activity units[a] | Total protein | Specific activity[b] | Purification | Yield |
|---|---|---|---|---|---|
| | | *mg* | | | % |
| Crude extracts | 990 | 10,900 | 0.09 | 1.0 | 100 |
| $(NH_4)_2SO_4$ precipitate | 781 | 1,130 | 0.69 | 7.7 | 79 |
| Calcium phosphate gel | 616 | 350 | 1.76 | 19.6 | 62 |
| DEAE (cellulose) chromatography | 376 | 59 | 6.38 | 71.0 | 38 |

[a] One unit of aldolase cleaves 1 μmole of fructose 1,6-diphosphate per min at 25 C.
[b] Expressed in units per milligram.

TABLE 2. *Purification of vegetative cell FDP aldolase from B. cereus*

| Fraction | Total activity units[a] | Total protein | Specific activity[b] | Purification | Yield |
|---|---|---|---|---|---|
| | | *mg* | | | % |
| Crude extract | 4,800 | 14,000 | .34 | 1.0 | 100 |
| $(NH_4)_2SO_4$ precipitation | | | | | |
| 30–80% | 4,360 | 6,900 | .63 | 1.9 | 91 |
| 60–70% | 3,470 | 917 | 3.80 | 11.0 | 72 |
| $Al(OH)_3$ gel treatment | 3,350 | 310 | 10.80 | 32.0 | 70 |
| DEAE (cellulose) chromatography | 382 | 6.6 | 57.90 | 170.0 | 8 |

[a] See Table 1.
[b] Expressed in units per milligram.

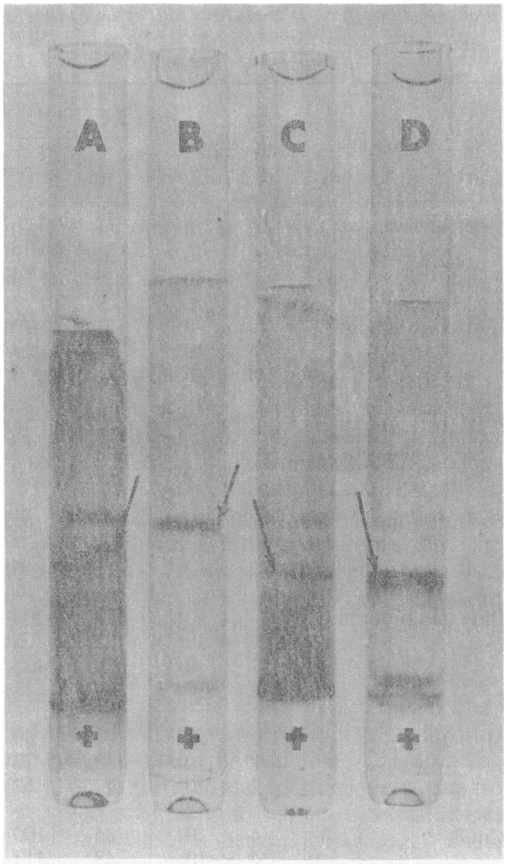


FIG. 1. *Acrylamide gel electrophoresis patterns of vegetative cell and spore aldolases at pH 8.3 in which the anodic ends are indicated (+). The gel columns (0.5 by 5.5 cm), topped by 0.5 cm of spacer gel, were loaded with 100 to 150 μg of protein and run at 5 ma/column. Tubes A and B are vegetative cell aldolase, 10.8 units/mg, stained with amido schwartz and enzyme specific stain, respectively. The aldolase band, indicated by the arrow, had a relative mobility of 0.59 to 0.60. Tubes C and D are spore aldolase, 6.38 units/mg, stained with amido schwartz and enzyme stain, respectively. The spore aldolase band, indicated by the arrows, had a relative mobility of 0.71 to 0.72.*

graph of gel electrophoresis tubes showing the mobility, relative to the anionic marker, of vegetative cell and spore aldolases. Paired tubes were stained for protein and enzymatic activity. The protein band corresponding to aldolase was identified by the *R*m (protein distance/anionic dye distance) obtained in tubes stained by the tetrazolium reduction system. All vegetative cell aldolase preparations had an *R*m of 0.59 to 0.60 regardless of the state of purity. The *R*m of spore aldolase was 0.71 to 0.72.

**Kinetic and inhibitor studies.** Spore and vegetative cell FDP aldolases, specific activities of 1.76 and 10.8 units per mg, respectively, were assayed colorimetrically. As seen in Fig. 2, their *p*H optimum was 8.5 and the activities of both enzymes varied in an identical manner over the *p*H range 6.6 to 9.7. The effect of substrate concentration on each enzyme is shown in Fig. 3 in which the *K*m of both spore and vegetative cell aldolases was 2 mM FDP. The fractions of cell and spore aldolases derived from DEAE cellulose chromatography were dialyzed and found to have properties in common with Class II aldolases (30). Neither vegetative cell nor spore aldolases were appreciably inhibited on incubation at *p*H 6.4 with sodium borohydride in the presence or

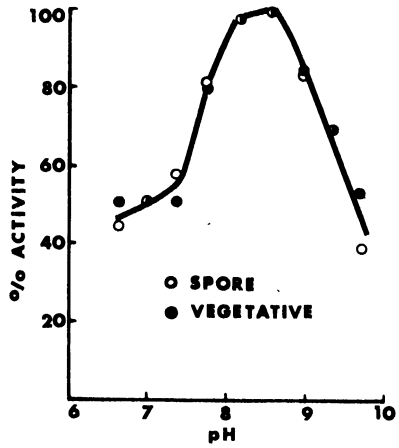


FIG. 2. *Effect of hydrogen ion concentration on the activity of partially purified vegetative cell and spore aldolases of B. cereus at 25 C. The enzymes were assayed by the colorimetric procedure.*

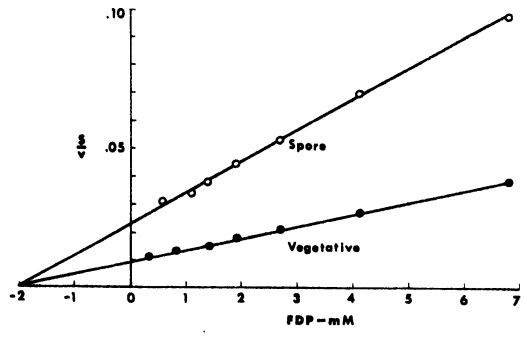


FIG. 3. *Reciprocal velocity versus fructose 1,6-diphosphate concentration at pH 8.5 for 0.20 units of vegetative cell and 0.072 units of spore aldolase at 25 C over the substrate concentration range 2.5 × $10^{-4}$ to 7 × $10^{-3}$ M.*

absence of substrate (14, 15, 22; Table 3). These data are presumptive evidence that the *B. cereus* enzymes are Class II aldolases. Table 4 shows the effects of a variety of reagents on the activity of cell and spore aldolases. They were inhibited by the chelators, ethylenediamine tetraacetic acid (EDTA), and dipicolinic acid and were reactivated by the addition of certain divalent metals. Both enzymes were stimulated by potassium ions but to significantly different extents. The activity of the vegetative-cell enzyme was enhanced 1.7-fold at 0.1 M potassium acetate but inhibited at 0.2 M concentration of the salt. The spore enzyme activity increased to 1.35-fold at 0.1 M and 1.48-fold at 0.2 M potassium acetate. An enhancement of activity by potassium salts up to 0.1 M and inhibition of activity at higher salt levels has been reported for yeast aldolase (29). The relationship of iron, calcium, and magnesium to the activity of the two aldolases is of particular interest. Calcium and ferrous ions stimulated the activity of the spore enzyme but inhibited the vegetative-cell aldolase. Iron functioned at 0.01 the concentration of calcium. Conversely, magnesium stimulated vegetative cell aldolase and inhibited the enzyme derived from spores. Zinc and manganese inhibited both aldolases of *B. cereus*.

**Immunochemical tests.** A higher antibody response was noted in those animals receiving injections of spore-derived preparations than in

TABLE 3. *The effect of borohydride reduction on cell and spore aldolases of Bacillus cereus*[a]

| Expt | Additions | Reduction with $NaBH_4$ | Aldolase recovery units |
|---|---|---|---|
| 1 | Veg aldolase + FDP | − | 0.16 |
| | Veg aldolase | + | 0.14 |
| | Veg aldolase + FDP | + | 0.15 |
| 2 | Spore aldolase + FDP | − | 0.078 |
| | Spore aldolase | + | 0.070 |
| | Spore aldolase + FDP | + | 0.071 |

[a] The reaction mixture (1 ml) was incubated at 0 C and contained either 0.18 units of vegetative (veg) or 0.09 units of spore aldolase, 0.2 M phosphate buffer (*p*H 6.4), 10 μmole FDP, and 100 μmole $NaBH_4$ added in increments. The *p*H was maintained by the addition of 25% $H_3PO_4$ with a microsyringe. After 30 min of incubation, the enzymes in the reaction mixtures were precipitated by the addition of 4 ml of saturated ammonium sulfate solution. The proteins were resuspended in 1 ml of 0.05 M Tris buffer (*p*H 8.5) and assayed. The recovery of the vegetative and spore aldolase controls (nonreduced) was 89 and 87%, respectively.

TABLE 4. *The effect of chelators, sulfhydryl reagents, and various cations on the activity of FDP aldolases of B. cereus*[a]

| Reagent | Concn[b] | Percentage activity | |
|---|---|---|---|
| | | Vegetative cell | Spore |
| EDTA | $2 \times 10^{-4}$ | 36 | 0 |
| | $2 \times 10^{-3}$ | 12 | 0 |
| | $2 \times 10^{-2}$ | 8 | 0 |
| Dipicolinic acid | $4 \times 10^{-5}$ | 84 | 70 |
| | $4 \times 10^{-4}$ | 40 | 9 |
| | $4 \times 10^{-3}$ | 0 | 0 |
| *p*-Hydroxymercuribenzoic acid | $2 \times 10^{-5}$ | 72 | 56 |
| | $2 \times 10^{-4}$ | 11 | 12 |
| | $2 \times 10^{-2}$ | 0 | 0 |
| Sodium iodoacetate | $2 \times 10^{-4}$ | 97 | 69 |
| | $2 \times 10^{-3}$ | 93 | 37 |
| | $2 \times 10^{-2}$ | 75 | 16 |
| Potassium acetate | $4 \times 10^{-3}$ | 113 | 100 |
| | $2 \times 10^{-2}$ | 121 | 105 |
| | $1 \times 10^{-1}$ | 170 | 135 |
| | $2 \times 10^{-1}$ | 137 | 148 |
| $MgSO_4$ | $2 \times 10^{-4}$ | 100 | 80 |
| | $2 \times 10^{-3}$ | 113 | 77 |
| | $2 \times 10^{-2}$ | 117 | 67 |
| $CaCl_2$ | $4 \times 10^{-4}$ | 85 | 100 |
| | $4 \times 10^{-3}$ | 48 | 113 |
| | $4 \times 10^{-2}$ | 25 | 150 |
| $FeSO_4$ | $4 \times 10^{-6}$ | 93 | 104 |
| | $4 \times 10^{-5}$ | 66 | 112 |
| | $4 \times 10^{-4}$ | 66 | 150 |
| $MnCl_2$ | $10^{-4}$ | 63 | 100 |
| | $10^{-3}$ | 30 | 61 |
| | $10^{-2}$ | 16 | 50 |
| $ZnCl_2$ | $2 \times 10^{-4}$ | 95 | 40 |
| | $2 \times 10^{-3}$ | 90 | 30 |
| | $2 \times 10^{-2}$ | 5 | 25 |
| NaCl | 0.17 | 89 | 91 |
| | 0.52 | 65 | 66 |
| | 1.0 | 45 | 46 |

[a] These data were obtained by the colorimetric assay to eliminate possible effects of the reagents or cations on the glyceraldehyde-3-phosphate dehydrogenase. The assay system (2.5 ml) at *p*H 8.5 contained 0.1 units of cell or spore aldolase (57.9 and 6.38 units/mg, respectively), 10 μmoles of Tris, 140 μmoles of hydrazine, and 25 μmoles of FDP. The enzyme, Tris-hydrazine buffer, and reactants were preincubated for 10 min at 25 C, and the reaction was initiated by the addition of FDP.

those receiving enzymes from vegetative cells. The vegetative cell and spore aldolases cross-reacted with heterologous antienzyme sera prepared in rabbits. Typical Ouchterlony (28) tests were run in gels containing 1% agar and the enzyme-antibody precipitation lines in the agar were identified by specific staining. They were continuous and nonspurred. Cross-reactivity between enzymes and heterologous sera in precipitation studies was determined by utilizing the Cohn and Torriani (4) procedure (Fig. 4). The serum directed against vegetative cell aldolase precipitated either cell or spore aldolase. However, whereas the serum directed against spore aldolase precipitated either enzyme, it appeared to be slightly more effective in precipitating (or inactivating) the spore aldolase (right-hand portion of Fig. 4); recovery of activity of the spore aldolase in the antigen excess portion of the curve was less than that of the vegetative cell aldolase.

**Heat resistance.** The stability of the aldolases of *B. cereus* at 37 and 45 C varied over the *p*H range 5.6 to 8.6 with maximal stability occurring at *p*H 6.4. All thermal inactivation studies were therefore carried out at this hydrogen ion concentration. Inactivation tests at 53 C demonstrated that the spore enzyme in crude preparations was more heat resistant than the vegetative aldolase. Its inactivation was biphasic, with 40% of the enzyme possessing a half-life very similar to that of the vegetative cell enzyme. The respective half-lives of the crude vegetative cell aldolase and the stable fraction of the spore protein were 3 min and 30 min at 53 C and *p*H 6.4. The stability of the cell aldolase increased slightly on purification to a half-life of 4 min. Spore aldolase became heat labile after ammonium sulfate fractionation but was stable once more after negative adsorption with calcium phosphate gel. The purified spore enzyme which was eluted from DEAE cellulose columns was heat labile with a half-life identical to that of vegetative cell aldolase. Apparently, purification procedures which could strip calcium from the spore aldolase reduced its heat stability. In order to test this hypothesis, cell and spore aldolases of specific activity 57.8 and 6.38 units /mg, were inactivated at 53 C in the presence of $CaCl_2$ the concentration of which ranged from 3 $\times 10^{-4}$ to 3 $\times 10^{-2}$ M. An enhancement in the heat resistance of spore aldolase occurred with increasing calcium concentration, whereas the vegetative cell enzyme became labile (Fig. 5.) At the highest calcium concentration, the half-life of the spore enzyme was 50 min, whereas that of the vegetative aldolase was 1.5 min.

**Gel filtration.** Cell and spore aldolase, 57.9 and 6.38 units/mg, respectively, were subjected to gel filtration in a series of calibrated Sephadex G-200 columns (2.5 by 30 cm). These were prepared in 0.01 M phosphate buffer (*p*H 6.5) and calibrated by the procedures of Ackers (1) with dextran and $^{32}PO_4$ for determination of void volume, $V_0$, and internal volume, $V_i$, respectively. The crystalline protein standards employed were cytochrome *c*, human hemoglobin, and rabbit muscle aldolase for which the Stokes' radii were 1.42, 3.08, and 4.65 nm, respectively. The average pore radius of

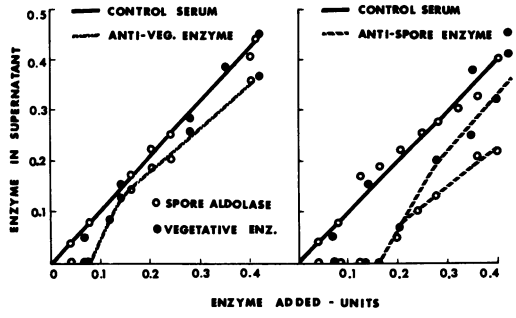


FIG. 4. *Precipitation of vegetative cell and spore aldolases of B. cereus by specific antibodies produced in rabbits. The reaction system in phosphate buffered saline, pH 7, contained either 0.15 ml (0.75 mg) of antivegetative aldolase globulin or 0.07 ml (0.29 mg) of antispore globulin in a total volume of 2.5 ml. The reaction mixtures were incubated at 4 C for 40 hr and the precipitates were removed by centrifugation prior to assay.*

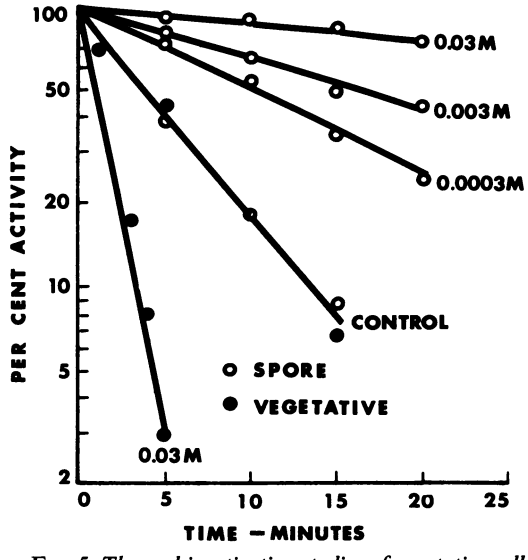


FIG. 5. *Thermal inactivation studies of vegetative cell and spore aldolase at 53 C in 0.05 M phosphate buffer (pH 6.4) containing various concentrations of $CaCl_2$ as indicated on each curve. The spore aldolase was stabilized by calcium while that derived from vegetative cells became labile.*

the gels in 10 experiments was 18.8 ± 0.8 nm which is in agreement with published values (1). In two filtration experiments on a single column, the characteristics of vegetative cell and spore aldolase were determined (Fig. 6). The existence of two forms of the vegetative aldolase is readily apparent. The larger component (that eluting first) constitutes approximately 20% of the total activity. Also noteworthy is the lower molecular size of the spore protein. The Strokes' radii and

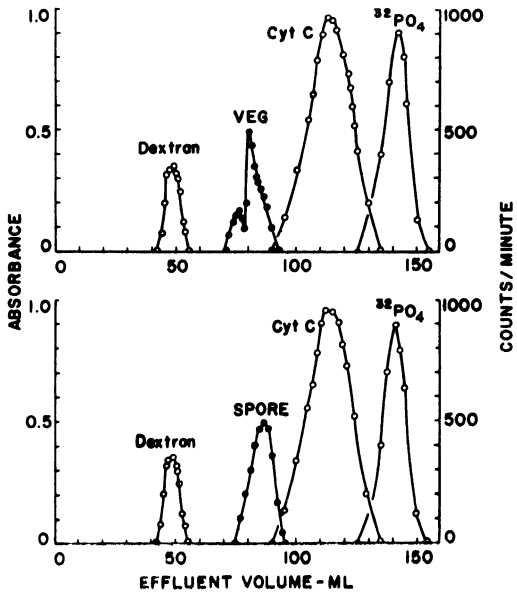


FIG. 6. *Gel filtration of vegetative cell and spore aldolases of Bacillus cereus on Sephadex G-200 columns (2.5 by 30 cm) calibrated with Blue dextran 2000, cytochrome c and $^{32}PO_4$ by the method of Ackers (1). The void volume, $V_0$, was 49 ml; the internal volume, $V_i$, was 92 ml; and the elution volume of the cytochrome c solution, $V_e$, was 114 ml. The aldolases were assayed colorimetrically at a wave length of 540 nm, the dextran at 600 nm and the cytochrome c at 425 nm. The Stokes' radii calculated for $veg_1$, $veg_2$, and spore aldolase were 4.2, 3.9, and 3.2 nm, respectively.*

diffusion constants for cell and spore aldolase were calculated from the results of several gel filtration experiments. The cell enzymes designated $veg_1$ and $veg_2$, in order of their elution from the columns, had average Stokes' radii of 4.4 and 4.0 nm, respectively, corresponding to diffusion constants ($D_{20,w}$) of 4.9 and 5.4 × $10^{-7}$ $cm^2$ per sec. The corresponding values for the spore enzyme were 3.3 nm and 6.5 × $10^{-7}$ $cm^2$ per sec. Statistical analysis of the data in Table 5 was carried out by the student "*t*" test (36) which showed that the Stokes' radii were all significantly different at the 95% confidence interval.

The difference in heat resistance due to calcium was utilized to distinguish between the spore and vegetative aldolases when they were co-chromatographed on dextran gel columns. Control experiments showed that 100% recovery of spore aldolase was obtained after negative adsorption of the enzyme with calcium phosphate gel and heating at 50 C for 15 min at *p*H 6.5. This level of heat resistance remained even after gel filtration. The same conditions completely inactivated vegetative cell aldolase. Spore aldolase was treated with calcium phosphate gel and then mixed with an equal activity of vegetative aldolase. Of the enzyme mixture, 2 ml was applied to a Sephadex G-200 column (2.5 by 30 cm) and subsequently eluted with the 0.1 M buffer (*p*H 6.5). The fractions were assayed for aldolase activity, heated to inactivate vegetative aldolase, and reassayed. The elution profile of the labile vegetative aldolase which precedes the more stable spore protein down the column was calculated. The asymmetry of the elution profile for vegetative aldolase reaffirms that this enzyme exists in more than one molecular size (12). The slight skewing of the elution profile for the spore aldolase was only observed in co-chromatography experiments. These results might indicate an interaction between spore and vegetative cell aldolases.

**Sedimentation.** The sedimentation constants of

TABLE 5. *Physical parameters of partially purified FDP aldolases from cells and spores of Bacillus cereus*[a]

| Aldolase | Stokes' radius | $D_{20,w}$ ($10^{-7}$)[b] | $S_{20,w}$ ($10^{-13}$) | Mol wt[c] |
|---|---|---|---|---|
| | *nm* | | *sec* | |
| $Veg_1$ | 4.4 ± 0.24 (6) | 4.9 | 6.5 ± 0.70 (7) | 115,000 |
| $Veg_2$ | 4.0 ± 0.17 (6) | 5.4 | 4.9 ± 0.31 (7) | 79,000 |
| Spore | 3.3 ± 0.27 (6) | 6.5 | 3.3 ± 0.30 (10) | 44,000 |

[a] Standard deviations between the samples and the number of observations (in parentheses) are presented.

[b] Expressed in square centimeters per second.

[c] Expressed in grams per mole. Values are based on an estimated, partial, specific volume of 0.72 $cm^3/g$.

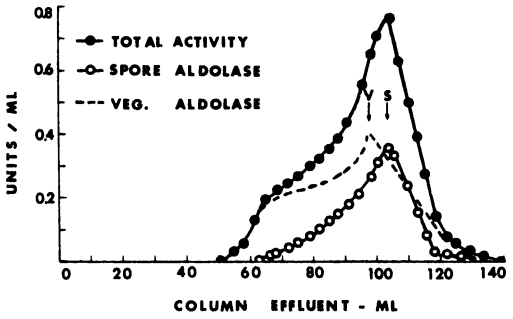


FIG. 7. *Co-chromatography of vegetative cell and calcium phosphate gel-treated spore aldolase of Bacillus cereus on a Sephadex G-200 gel column (2.5 by 30 cm). The fractions were collected, assayed colorimetrically, heated at 50 C for 15 min, and reassayed for aldolase. The difference between the two assays corresponds to the labile vegetative cell enzyme. The peaks marked V and S correspond to elution volumes for vegetative cell and spore aldolases, respectively.*

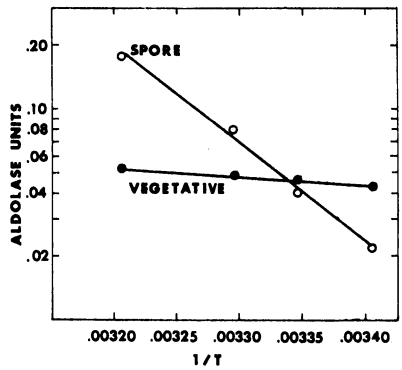


FIG. 8. *Arrhenius plot of the effect of temperature over the range 20 to 38 C on the activities of 0.045 units of vegetative cell and 0.04 units of spore aldolase of Bacillus cereus. The activities of the aldolases are expressed as equivalent units (25 C).*

the aldolases are listed in Table 5 where some spread can be seen in the data. Nevertheless, statistical analysis shows that these sedimentation values are significantly different at the 95% confidence interval. Molecular weights were calculated by the usual relationship between the sedimentation and diffusion constants assuming that the partial specific volume of each protein was 0.72 $cm^3/g$. The molecular weights of $veg_1$, $veg_2$, and spore aldolase were, respectively, 115,000, 79,000, and 44,000.

**Temperature-activity relationships.** The activities of cell and spore aldolase, 57.9 and 6.38 units per mg, respectively, were tested as a function of temperature over the range 20 to 38 C. The colorimetric assay was used in order to minimize complications which could arise due to the effects of temperature on the glyceraldehyde-3-phosphate dehydrogenase employed in the coupled assay. Clearly, the two aldolases can be differentiated on the basis of temperature-activity response (Fig. 8). The calculated Arrhenius activation energies were 2,770 and 21,600 calories, respectively, for the cell and spore aldolases.

## DISCUSSION

At least three possibilities exist with regard to the structural relationships between vegetative cell and spore aldolase. The proteins could be (i) identical, (ii) similar and presumably structurally related, or (iii) uniquely different. Research already cited (8, 9, 10, 11, 24, 27, 40, 41, 43, 44) suggests that most spore enzymes are the products of genes which produce corresponding enzymes in cells. This hypothesis was based on the facts that cells and spores of a given organism have similar enzyme complements, corresponding enzymes from cells and spores have similar properties when tested in vitro, and mutant cells which are deficient in a given enzyme produce spores which are also deficient in the same enzyme. Kornberg et al. (21) point out the "biological economy" of such a system which would produce structurally identical enzymes in cells and spores.

The synthesis of a protease (3) is obligatory in the sporulation of a number of *Bacillus* species (37; (J. Mandelstam, W. W. Waites, S. C. Warren, and J. M. Sterlini, Proc. Biochem. Soc. 477th meeting, p. 4, 1967)). Spore proteins might arise by the action of this sporulation-specific protease on enzymes which are the normal complement of vegetative cells. The products of such action would indeed be structurally related and could be quite similar in properties to the vegetative cell proteins. Recent studies of protein conversions indicate the nature of the changes which could occur. These include the tryptic digestion of cytochrome *b* which yields a stable, functional "core protein" (39), modifications of the catalytic properties of a variety of enzymes (7, 33; E. Juni and G. A. Heym, *in press*); and modifications of the quaternary structure of yeast hexokinase (19).

The third possibility listed above centers about enzymes the synthesis of which is repressed in cells but derepressed in the course of sporulation. At least 27 such enzymes are known (21) including enzymes of diacetyl oxidation, the citric acid cycle, alkaline phosphatase, glucose dehydrogenase, and sporulation-specific protease. If spore aldolase synthesis is directed by a gene different from that directing synthesis of the enzyme in cells, its structure could be different from that of the vegetative cell aldolase.

This research has attempted to resolve the rela-

tionships between the cellular and spore aldolases. The experimental approach was to investigate a variety of properties of the purified cell and spore aldolases under identical conditions with the assumption that the parameters selected were reflections of the structure of the enzymes. As part of the overall comparison, tests were made to determine the classification (30) of the aldolases. Class I enzymes are derived from animal or plant tissue (including protozoan and algal) and are characterized by their broad *p*H response, insensitivity to chelating agents and divalent cations, their lack of requirements for added sulfhydryl compounds and molecular weights in the range 150,000 to 160,000. These enzymes form an azomethine between an $\epsilon$-$NH_2$ group at their active center and dihydroxyacetone phosphate and thus can be inhibited by borohydride reduction in the presence of substrate (14, 15, 22). Class II aldolases, which are found in bacteria, fungi, and blue-green algae, are inhibited by chelating agents and sulfhydryl reagents, are not inhibited by borohydride reduction in the presence of substrate, have enhanced activity in the presence of divalent cations and potassium ions, have a sharp *p*H optimum, and have molecular weights corresponding to sedimentation constants in the range 5 to 5.4 (31). The most prevalent form of aldolase from cells of *B. cereus* has all these properties and therefore is presumably a Class II aldolase. Spore aldolase also corresponds to a Class II aldolase with the exception of its sedimentation constant, 3.3*S*. The enzyme from cells or spores was inhibited by EDTA in the concentration range which inhibits other Class II aldolases (30, 31). Dipicolinic acid, which comprises 8 to 15% of the dry weight of spores and which is lost from spores on germination or extended heat shock, is a chelating agent to which the *B. cereus* enzymes were also sensitive. It is significant that the spore aldolase was more inhibited by this compound (or EDTA) than the vegetative cell enzyme, and thus chelation may be part of the mechanism of metabolic dormancy of spores.

The *p*H response of the aldolases from the two cell forms was identical with optimal activity occurring at *p*H 8.5. This value is higher than the 7.2 to 7.4 for yeast aldolase (29), 7.5 for clostridial aldolase (2), or 7.2 to 7.6 for the enzyme from *Aspergillus niger* (18) and may be due to differences in assay procedures employed (30). The Michaelis constant for either cell or spore aldolase was $2 \times 10^{-3}$ M FDP which corresponds to values for *A. niger* (18) and *Clostridium perfringens* (2) but is 10-fold higher than that of yeast aldolase (29). The aldolases were tested in two kinds of immunochemical procedures and found to be cross-reacting with sera prepared in rabbits against either purified cell or spore enzyme. During chromatography, the cell and spore aldolases eluted from DEAE cellulose columns at the same NaCl concentration. This indicated that the net charge on each aldolase was similar. The heat resistances of the two aldolases were identical after chromatography on DEAE cellulose. By itself, any one parameter tested would be insufficient to distinguish between identical, similar, or uniquely different aldolases. However, on the basis of the near identity of the cell and spore aldolases in five types of tests, we excluded the possibility that the aldolases were uniquely different and concluded that they were either identical or quite similar proteins.

The aldolases from cells and spores were of different molecular size. This conclusion was drawn from the results of gel filtration and sedimentation studies. Ackers (1) had shown that restricted diffusion was the principal molecular separation mechanism in loosely cross-linked dextran gels such as Sephadex G-200 and that diffusion constants could be calculated from data derived from calibrated gel columns. The results of gel filtration analysis indicated that the vegetative enzyme consisted of a major and minor enzymatically active component whose concentrations were in the approximate ratio of 4:1. The Stokes' radius of the major aldolase component was significantly different from the minor component, and its standard deviation was 4.3% of the calculated value. This is the same order of accuracy reported by Ackers. The Stokes' radius of the spore aldolase was significantly different at the 95% confidence interval from those of the vegetative enzymes.

The minor component of vegetative aldolase noted in gel filtration was also observed in sucrose density sedimentation studies. Once again, the sedimentation constants for cell and spore aldolases were significantly different at the 95% confidence interval.

The molecular weights calculated from these data (35) were based on an estimated partial specific volume of 0.72. These molecular weights are therefore subject to some error but are convenient to judge the relative size and turnover numbers of cell and spore aldolases. The spore enzyme is approximately 50% of the molecular weight of the vegetative aldolase if the specific volumes of the proteins are the same. The greater mobility of spore aldolase in gel electrophoresis was therefore due, in part, to its smaller size. A principal kinetic difference between the two aldolases was in their respective turnover numbers. The enzyme from vegetative cells cleaved

5,500 moles of FDP per min per mole of enzyme at 25 C. This value was calculated by the use of an average molecular weight of 96,000 and is similar to the turnover number for aldolase from cells of *C. perfringens* (31). The turnover number of the spore enzyme was 420 moles of FDP cleaved per min per mole based on an estimated purity of 65% for the best spore preparations. These striking differences in activity are reminiscent of the results of Drechsler et al. (7) who observed a 20-fold reduction in the activity of aldolase upon degradation by carboxypeptidase.

The cell and spore aldolases could also be readily differentiated by the effect of cations on their activity and thermal stability. The cellular aldolase of *B. cereus* was activated 1.7-fold by 0.1 M potassium acetate which was similar to the effect on the enzyme from *B. megaterium* but slightly lower than other bacteria with respect to the magnitude of the activation (31). The inhibition of the vegetative aldolase by 0.2 M potassium acetate may have been due to high ionic strength. The spore enzyme was also activated by potassium ions but to a lesser extent than the vegetative enzyme. Manganous and zinc ions inhibited the enzymes but are known to enhance activity in other Class II aldolases (18, 20). Ferrous ion, which increased activity of the clostridial aldolase (2), also enhanced the activity of spore aldolase but inhibited the enzyme derived from vegetative cells of *B. cereus* at two orders of magnitude less concentration than calcium. Ferrous ion may be the functional divalent cation which promotes the activity of aldolase in the early outgrowth stages prior to the synthesis of vegetative cell aldolase since calcium is lost from spores on germination. The addition of calcium to the spore enzyme resulted in increased activity and stability but calcium inhibited vegetative aldolase. Conversely, magnesium ions enhanced the activity of the vegetative enzyme but inhibited the spore aldolase.

The Arrhenius activation energies of the cell and spore aldolase were strikingly different. The significance of the difference is unknown but the parameter provides yet another means of differentiating between the two aldolases.

Based on differences in molecular size, turnover numbers, mobility in gel electrophoresis, cation effects, heat stability in the presence of calcium, and activation energies for the cleavage of FDP, we conclude that the aldolases from cells and spores of *B. cereus* are not identical. However, the aldolases were very similar in their catalytic and immunological properties, their heat resistance in the absence of calcium, and their net charge. These results have lead us to believe that cell and spore aldolases of *B. cereus* are structurally related. The smaller size of the spore enzyme is consistent with the hypothesis that the spore enzyme is derived from the vegetative aldolase. This hypothesis has been tested in vitro (H. L. Sadoff and E. Celikkol. Bacteriol. Proc., p. 25, 1968) and a proteolysis product which was indistinguishable from spore enzyme has been derived from vegetative cell aldolase after treatment of the protein with sporulation-specific protease. The details of the conversion of cell to spore aldolase will be presented in a forthcoming communication.

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