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Degradative Acetolactate Synthase of *Bacillus subtilis*: Purification and Properties

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A degradative acetolactate synthase (acetolactate pyruvate-lyase [carboxylating], EC 4.1.3.18) from *Bacillus subtilis* has been partially purified and characterized. The synthesis of the enzyme was induced by growth of cells in minimal medium plus isobutyrate or acetate. The enzyme was partially purified by ammonium sulfate fractionation, gel filtration, and hydroxyapatite chromatography. The pH optimum of the purified enzyme was 7.0 in phosphate buffer. When assayed in phosphate buffer (pH 7.0), activity was stimulated by acetate and inhibited by sulfate. When assayed in acetate buffer (pH 5.8), activity was inhibited both by sulfate and phosphate. Michaelis-Menten kinetics was observed when the enzyme was assayed in phosphate buffer (pH 6.0 or 7.0), and inhibition by sulfate was competitive and activation by acetate was noncompetitive. When assayed in acetate buffer (pH 5.8), nonlinear Lineweaver-Burk plots were obtained; inhibition by phosphate appeared to be competitive and that by sulfate was of the mixed type. The approximate molecular weight of the purified enzyme was 250,000 as determined by gel filtration.

It has long been known that certain species of bacteria can form acetoin (acetylmethylcarbinol, 3-hydroxy-2-butanone) as a fermentation product of glucose (3). However, little progress was achieved in elucidating the mechanism of acetoin formation until the study of Silverman and Werkman (14) in 1941. They found that crude extracts of *Aerobacter aerogenes* could form acetoin from pyruvate. Some 10 years later, Juni (8) resolved the crude acetoin-forming system of *A. aerogenes* into two distinct activities. The first activity condensed two molecules of pyruvate to give α-acetolactate, and the second activity decarboxylated the acetolactate to produce acetoin. Halpern and Umbarger (2) subsequently showed that the acetolactate-forming activity participating in acetoin formation was distinct from a second acetolactate-forming enzyme involved in the biosynthesis of valine and isoleucine. The degradative acetolactate synthase of *A. aerogenes* has been purified and studied by Störmer (16-18).

In a previous publication (5), evidence was presented suggesting that *Bacillus subtilis* has an acetolactate synthase probably analogous to the degradative enzyme present in *A. aerogenes*. The purification and properties of this enzyme are the subject of this report.

MATERIALS AND METHODS

Organism. Wild-type *B. subtilis* 746 was used throughout this study.

Medium. The standard minimal medium (pH 7.0) was that described previously (1). Medium of pH 6.0 was prepared by changing the proportions of the monopotassium and dipotassium phosphates.

Growth conditions. Routine liquid cultures of *B. subtilis* were grown as described previously (1). Cells used for enzyme purification were grown for at least two generations in minimal medium (pH 6.0) plus 0.2% potassium acetate at 37 C with moderate agitation. Cells were harvested by centrifugation in the cold, washed once with cold 0.05 M potassium phosphate (pH 7.0) plus 0.5 mM $MgCl_2$ and 0.01 mM $MnCl_2$, and stored frozen until use.

Preparation of crude extracts. Crude extracts were prepared by lysing the cells for 30 min at 37 C in a solution of the following: potassium phosphate (pH 7.0), 0.05 M; KCl, 0.05 M; $MgCl_2$, 5 mM; diphosphothiamine, 80 μg/ml; lysozyme, 1 mg/ml; and deoxyribonuclease, 10 μg/ml for 30 min at 37 C. Cell debris was removed by centrifugation at 27,000 $\times$ *g* for 30 min at 0 to 5 C, and the supernatant fluid was retained as a crude extract.

Enzyme assay. Two versions of the acetolactate synthase assay were used. For assay I the reaction mixture contained in 1.0 ml: potassium phosphate (pH 7.0), 100 μmol; potassium pyruvate, 20 μmol; $MgCl_2$, 10 μmol; diphosphothiamine, 80 μg. The reaction was initiated with enzyme and terminated after 5 to 10 min at 37 C with 0.1 ml of 18 N H_2SO_4.

For controls, enzyme was added after the acid. Precipitated protein was removed by centrifugation. The supernatant fluid was incubated for 30 min at 37 C, and then 0.2 ml was transferred to 0.8 ml of 0.45 N NaOH plus 1 ml of creatine reagent (20) and 1 ml of α-naphthol reagent (20). Color was developed for 15 min at 37 C and the absorbance was read at 530 nm on a Coleman 139 spectrophotometer. An absorbance of 0.4 equals 60 nmol of acetolactate (acetoin) in a final volume of 3.0 ml. This method was used with crude extracts. For assay II the reaction mixture (0.5 ml) contained one-half the amount of each component in assay I, except that $MgCl_2$ was reduced to 2 μmol. The reaction was terminated with 0.1 ml of 9 N H_2SO_4. After 35 min at 37 C, 0.5 ml of 2 N NaOH, 1 ml of creatine reagent and 1 ml of α-naphthol reagent were added, and color was developed as in assay I. This assay was used with column fractions and the purified enzyme. If only acetoin formation was being determined, the reaction was terminated by placing the tubes in an ice bath, 0.1 ml of water replaced the acid and color was developed immediately. One enzyme unit equals the formation of 1 nmol of acetolactate per min. Specific activity equals units per milligram of protein. Protein was determined by the method of Lowry et al. (13), with crystalline bovine serum albumin used as a standard.

Enzyme purification. The procedures used for ammonium sulfate fractionation, gel filtration, and hydroxyapatite chromatography were similar to those described previously (5, 6). All purification procedures were carried out at 0 to 5 C. A crude extract was prepared from cells grown in minimal medium (pH 6.0) plus 0.2% acetate. The extract was fractionated with solid ammonium sulfate added to yield the following saturations: 0 to 25%, 25 to 50%, and 50 to 75%. The 50 to 75% fraction, which contained most of the activity, was redissolved in the extraction buffer and then placed on a column (1.7 by 60 cm) of Sephadex G-200 equilibrated with the extraction buffer and eluted with the same buffer. The fractions containing most of the activity were combined, and the protein was precipitated with ammonium sulfate (0 to 50% and 50 to 80% saturation). The precipitate of the 50 to 80% fraction was suspended in 0.05 M potassium phosphate (pH 6.9) plus 2 mM $MgCl_2$ and 80 μg of diphosphothiamine per ml and applied to a column (0.9 by 27 cm) of hydroxyapatite equilibrated with the same buffer. Activity was eluted with 200 ml of a 0.05 to 0.25 M linear potassium phosphate gradient (pH 6.9) containing diphosphothiamine and $MgCl_2$. The fractions containing most of the activity were combined, and the protein was precipitated with ammonium sulfate (0 to 50% and 50 to 80% saturations). The precipitate of the 50 to 80% fraction was resuspended in the initial extraction buffer.

Molecular weight determination. Dry Sephadex G-200 was swelled for 2 weeks in distilled water. After deaeration and removal of fines, it was poured into a column 1.7 cm in diameter to a height of 60 cm. The column was washed through with 0.05 M potassium phosphate (pH 7.0) plus 0.05 M KCl at 0 to 5 C for 4 to 5 days before use (the flow rate was 10 ml/h). The void volume of the column was determined by using blue dextran (average molecular weight 2×10^6), and the marker proteins were: ovalbumin (molecular weight 43,000), bovine serum albumin (molecular weight 70,000), yeast alcohol dehydrogenase (molecular weight 150,000), and pyruvate kinase (molecular weight 237,000). Fractions of 1.8 ml were collected by drop counter. The absorbance of blue dextran was read at 620 nm and that of the proteins was read at 280 nm. Acetolactate synthase activity was determined as described above. Identical procedures were used for each elution run.

Chemicals. The following is a list of chemicals and their sources. Potassium pyruvate, Calbiochem; diphosphothiamine, bovine serum albumin, ovalbumin (grade V), yeast alcohol dehydrogenase, and rabbit muscle pyruvate kinase (type II), Sigma Chemical Co.; Sephadex G-200, Pharmacia; acetaldehyde, Eastman Organic Chemicals.

RESULTS

Induction of degradative acetolactate synthase by fatty acids. It was reported previously that acetate and propionate induced synthesis of the degradative acetolactate synthase of *B. subtilis* (5). In a subsequent study the relative effectiveness of most short-chain fatty acids at inducing enzyme synthesis was examined. Wild-type cells were grown for one generation in minimal medium (pH 7.0) plus fatty acids (as the potassium salts) at a concentration of 27 mM. This is the same final concentration as the glucose and lower than that used in the previous experiment. Crude extracts were examined for the pH optimum and specific activity of acetolactate synthase (Table 1). The most effective inducer of the enzyme was isobutyrate, and it was about twice as effective as acetate. Propionate, at this lower concentration, had only a weak inductive effect.

Enzyme purification. The degradative enzyme was purified from wild-type cells grown in minimal medium (pH 6.0) plus acetate for at least two generations. Cells grown under these conditions possess no detectable biosynthetic acetolactate synthase and show a high degree of induction for the degradative enzyme. (When a crude extract of cells grown under these conditions is chromatographed on hydroxyapatite under conditions identical to those described in reference 5, the enzyme elution profile reveals only a single acetolactate synthase peak, and it is the degradative enzyme [peak A].) A representative purification is presented in Table 2. The final preparation showed a 12.6-fold purification over the crude extract. However, the specific activity of the crude extract was about 18-fold higher than that of crude extracts of cells grown in pH 7.0 minimal medium. Although the enzyme present in these cells, of

TABLE 1. *Effectiveness of various fatty acids at inducing the degradative acetolactate synthase*[a]

Medium supplement	Sp act of ALS	Relative activity	pH optimum
None	34.8	1.00	7.5-8.0
Acetate	91.2	2.62	6.5-7.0
Propionate	44.0	1.26	6.5-7.0
Butyrate	40.5	1.16	6.5-7.0
Isobutyrate	175.6	5.05	6.5-7.0
Valerate	25.0	0.72	[b]
Isovalerate	32.0	0.92	[b]
DL-2-Methyl-butyrate	44.0	1.26	7.5-8.0

[a] Wild-type cells were grown in minimal medium until the Klett reading reached 100 (red filter), and were diluted to give a Klett reading of 50 with fresh medium. The cultures were then supplemented with 27 mM (final concentration) fatty acids (as the potassium salts). Growth was allowed to continue until the Klett readings reached 100 at which time the cultures were harvested. Acetolactate synthase (ALS) was assayed in crude extracts. Specific activities are expressed as nanomoles of acetolactate formed per minute per milligram of protein. Specific activities were calculated by using the activity obtained at the pH that gave the highest activity (pH 7.5 for the minimal only and the 2-methylbutyrate extracts, and pH 7.0 for the others).

[b] Little change in activity with pH.

course, is the biosynthetic acetolactate synthase, its specific activity in crude extracts is useful as a reference value from which to measure the extent of induction of the degradative enzyme. On this basis, the purified enzyme showed a 225-fold increase in specific activity over that of the biosynthetic enzyme in crude extracts of minimal-grown cells.

Properties of the purified enzyme. The pH optimum of the purified enzyme was determined in both phosphate and acetate buffers (Fig. 1). In phosphate buffer the optimum was at 7.0. In acetate buffer there was little activity below pH 4.8, but higher pH values gave a sharp increase in activity. At pH 5.8 slightly greater activity was observed than in phosphate buffer at pH 7.0.

When assayed in phosphate buffer (pH 7.0) by method II, the enzyme formed no significant amount of acetoin directly from pyruvate (about 1.0%). If an equivalent amount of acetaldehyde replaced pyruvate as the substrate, no appreciable acetolactate or acetoin was formed (<1.0%). If equivalent amounts of pyruvate and acetaldehyde were present in the reaction mixture, no significant amount of acetoin was synthesized (<1.0%), and the amount of acetolactate formed was similar to that formed from pyruvate alone.

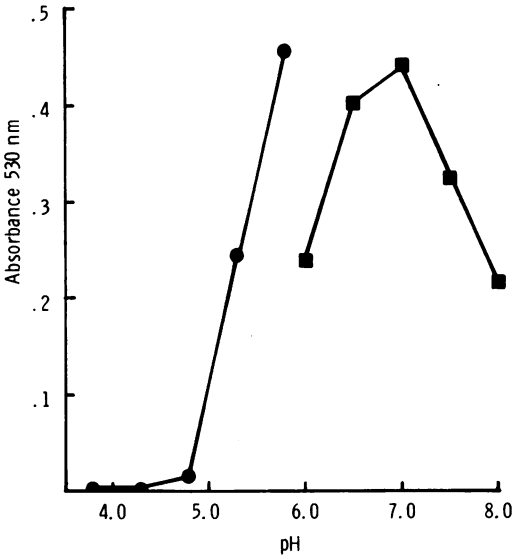

FIG. 1. *Activity of the purified acetolactate synthase as a function of pH in phosphate and acetate buffers. The 12.6-fold-purified ammonium sulfate III fraction (Table 2) was used as a source of enzyme. The enzyme was diluted 1:200 in either 0.05 M potassium phosphate (pH 7.0) or 0.05 M potassium acetate (pH 5.8), and 0.1 ml was used in the assay (assay II). The assay buffer was either 50 μmol of potassium phosphate or 50 μmol of potassium acetate. Symbols: ■, phosphate buffer; ●, acetate buffer.*

TABLE 2. *Purification scheme for the degradative acetolactate synthase*[a]

Fraction	Total activity (U)	Total protein (mg)	Sp act (U/mg)	% Yield	Purification
Crude extract	182,892	278.8	656.0	100.0	1.0
$(NH_4)_2SO_4$ I (45-75%)	155,555	152.6	1,019.1	85.0	1.6
Sephadex G-200	108,108	36.4	2,970.0	59.1	4.5
$(NH_4)_2SO_4$ II (50-80%)	113,204	29.8	3,803.9	61.9	5.8
Hydroxyapatite	56,882	17.5	3,250.4	31.1	5.0
$(NH_4)_2SO_4$ III (50-80%)	64,127	7.7	8,280.9	35.1	12.6

[a] Enzyme units are expressed as nanomoles of acetolactate formed per minute. Specific activity equals units per milligram of protein.

The effect of various compounds on activity was examined by using assay II. When assayed in phosphate buffer (pH 6.5), 0.01 M isoleucine, valine, or leucine had no significant effect on activity. In addition, no stimulatory effect by 10 μg of flavin adenine dinucleotide was observed. On the other hand, activity was inhibited by sulfate and stimulated by acetate (Table 3). Activation by acetate was greatest at pH 7.0 and negligible at pH 6.0. Conversely, inhibition by sulfate was more pronounced at the lower pH. When the enzyme was assayed in acetate buffer (pH 5.8), both sulfate and phosphate inhibited activity (Table 3).

Kinetic studies were performed by varying the pyruvate concentration in the presence and absence of either sulfate, phosphate, or acetate depending on which buffer was used. The pyruvate concentrations utilized were those that gave a linear response of activity with time at both pH 7.0 and 5.8. Lineweaver-Burk plots of results showing the effect of acetate and sulfate on kinetics when the assay was carried out in phosphate buffer (pH 7.0) are presented in Fig. 2. Inhibition by sulfate was competitive, whereas activation by acetate appeared to be noncompetitive. With the substrate concentrations utilized, no deviations from linearity were observed. The K_m for pyruvate was about 1.3×10^{-2} M. Somewhat similar results were obtained in phosphate buffer (pH 6.0), except that at this pH acetate had no stimulatory effect (Fig. 3). In addition the K_m for pyruvate showed an increase to about 4×10^{-2} M. Figure 4 presents data showing the effect of phosphate and sulfate on kinetics when the assay was performed in acetate buffer (pH 5.8). In the absence of either effector some deviation from linearity in the double-reciprocal plot was observed. Inhibition by phosphate appeared to be competitive; that of sulfate appeared to be of the mixed type.

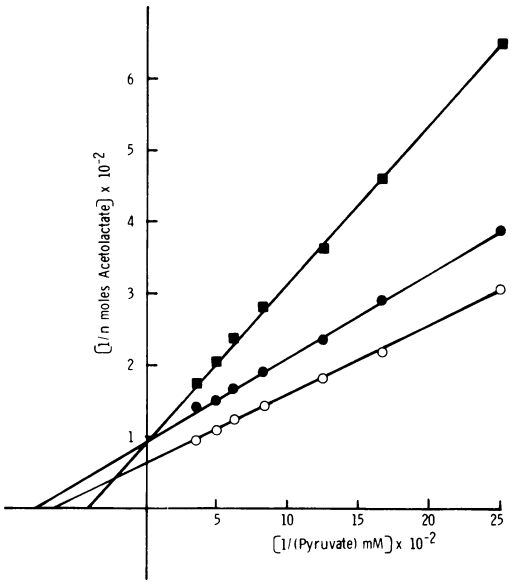

FIG. 2. *Lineweaver-Burk plots of the activity of the purified acetolactate synthase versus pyruvate concentration: effect of acetate and sulfate in pH 7.0 phosphate buffer. The 12.6-fold-purified ammonium sulfate III fraction (Table 2) was used as a source of enzyme. The reaction mixture contained: 50 μmol of potassium phosphate (pH 7.0), potassium pyruvate, 2 μmol of $MgCl_2$, 80 μg of diphosphothiamine, and enzyme (0.1 ml of a 1:200 dilution in 0.05 M potassium phosphate, pH 7.0). Assay II was used. Symbols: ●, no additions; ○, plus 25 μmol of acetate; ■, plus 20 μmol of sulfate.*

TABLE 3. *Effect of acetate, sulfate, and phosphate on activity of the purified degradative acetolactate synthase*[a]

Assay buffer	Buffer pH	Effector (50 mM)	AL formed (nmol)	Relative activity
Phosphate	6.0		49.6	1.00
		Acetate	46.0	0.92
		Sulfate	13.5	0.27
Phosphate	7.0		39.1	1.00
		Acetate	55.6	1.42
		Sulfate	22.9	0.58
Acetate	5.8		30.1	1.00
		Phosphate	13.9	0.46
		Sulfate	2.2	0.07

[a] The 12.6-fold purified ammonium sulfate III fraction (Table 2) was used as a source of enzyme. Activity was measured by assay II. Phosphate, sulfate, and acetate were present as the potassium salts. AL, Acetolactate.

The approximate molecular weight of the purified enzyme was determined by gel filtration through a calibrated column of Sephadex G-200. The enzyme elution profile revealed a single symmetrical peak having a V_e/V_o ratio of 1.31. This would indicate a molecular weight of about 250,000 (Fig. 5).

DISCUSSION

This work presents some properties of an acetolactate synthase from *B. subtilis* that is distinct from the biosynthetic enzyme. Because of the existence in some organisms of several other enzymes that can form acetolactate and/or acetoin from pyruvate as side reactions, it is worth pointing out properties of the *B. subtilis* enzyme which indicate that it is, in fact, an acetolactate synthase. Several investigators (8, 9, 15) have shown that pyruvate decarboxyl-

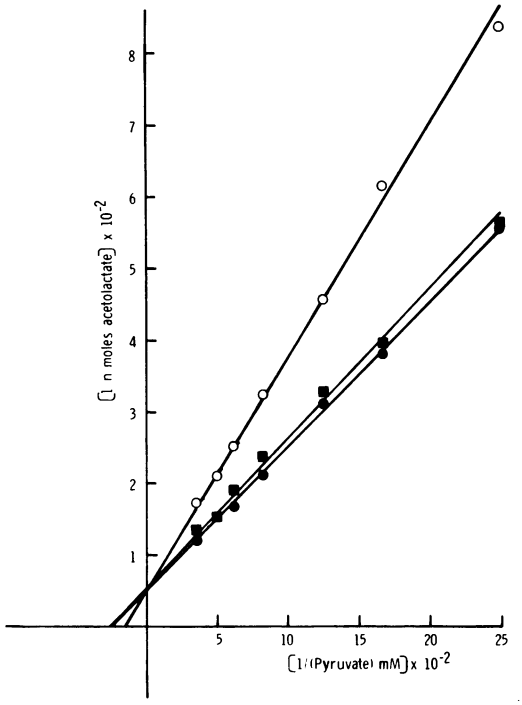

FIG. 3. *Lineweaver-Burk plots of the activity of the purified acetolactate synthase versus pyruvate concentration: effect of acetate and sulfate in pH 6.0 phosphate buffer. Procedures were the same as described in the legend to Fig. 2, except that the enzyme was diluted with pH 6.0 phosphate buffer and pH 6.0 phosphate buffer was used in the assay. Symbols: ●, no additions; ■, plus 20 μmol of acetate; and ○, plus 4 μmol of sulfate.*

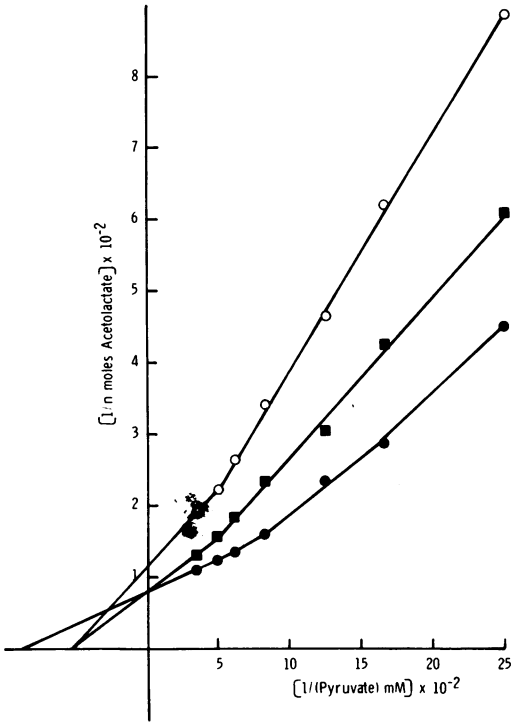

FIG. 4. *Lineweaver-Burk plots of the activity of the purified acetolactate synthase versus pyruvate concentration: effect of phosphate and sulfate in pH 5.8 acetate buffer. Procedures were the same as described in the legend to Fig. 2, except that the enzyme was diluted in pH 5.8 acetate buffer and pH 5.8 acetate buffer was used in the assay. Symbols: ●, no additions; ■, plus 6 μmol of phosphate; and ○, plus 1.5 μmol of sulfate.*

ase can synthesize acetoin directly from pyruvate. The purified *B. subtilis* enzyme, however, formed no significant amount of acetoin directly, which suggests that it is not pyruvate decarboxylase. It has been reported (7, 10) that pyruvate dehydrogenase can synthesize acetolactate and acetoin from pyruvate. Kuwana et al. (12) purified from *Neurospora crassa* an enzyme that they originally called an acetohydroxyacid synthetase (acetolactate synthase). It had a pH optimum of 6.0 and could form large amounts of acetoin from pyruvate plus acetaldehyde. In a subsequent publication (4), this enzyme was identified as pyruvate dehydrogenase. The enzyme examined in this work did not form any appreciable acetoin from pyruvate plus acetaldehyde. Furthermore, its molecular weight of about 250,000 is substantially below the molecular weight of 4.8×10^6 reported for the pyruvate dehydrogenase complex of *Escherichia coli* (11). Also of significance are several properties that the *B. subtilis* enzyme shares

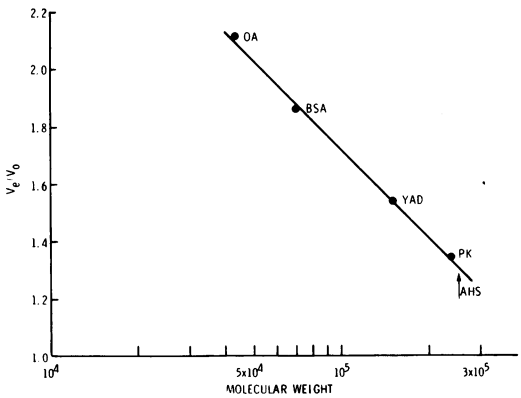

FIG. 5. *Molecular weight determination of the purified acetolactate synthase using Sephadex G-200. The 12.6-fold-purified ammonium sulfate III fraction (Table 2) was used as the source of enzyme. OA, Ovalbumin; BSA, bovine serum albumin; YAD, yeast alcohol dehydrogenase; PK, pyruvate kinase; AHS, acetolactate synthase.*

with the degradative acetolactate synthase of *A. aerogenes*: inhibition by sulfate and phosphate and activation by acetate (17), and induction of synthesis by acetate (19). However, the two enzymes do exhibit a number of differences. First, the most effective inducer of the *A. aerogenes* enzyme is acetate; isobutyrate has a much weaker inductive effect (19). The *B. subtilis* enzyme, on the other hand, is more effectively induced by isobutyrate. Second, the purified enzyme of *A. aerogenes* exhibits a pH optimum of 5.4 in phosphate buffer (17), whereas the *B. subtilis* enzyme shows an optimal pH of 7.0 in phosphate buffer. Third, the *A. aerogenes* enzyme obeys Michaelis-Menten kinetics only when assayed in acetate buffer; in phosphate buffer nonlinearity of Lineweaver-Burk plots is observed (17). By contrast, Michaelis-Menten kinetics is obtained with the *B. subtilis* enzyme when assayed in both pH 6.0 and 7.0 phosphate buffer. In pH 5.8 acetate buffer, on the other hand, some nonlinearity of double-reciprocal plots is observed.

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